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# Inflammasomes and the Innate Immune Response Against Yersinia Pestis: A Dissertation

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# INFLAMMASOMES AND THE INNATE IMMUNE RESPONSE AGAINST *YERSINIA PESTIS*

A Dissertation Presented

By

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Interdisciplinary Graduate Program
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*Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.*

*-Louis Pasteur*
*As quoted in Free Lance of Science (1960), René Dubos*

# DEDICATION

For my Grandfather; a monarch in his own right.

Who taught me the importance of knowledge, education, and hard work.

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# ABSTRACT

*Yersinia pestis*, the causative agent of plague, is estimated to have claimed the lives of 30-50% of the European population in five years. Although it can now be controlled through antibiotics, there are still lurking dangers of outbreaks from biowarfare and bioterrorism; therefore, ongoing research to further our understanding of its strong virulence factors is necessary for development of new vaccines. Many Gram-negative bacteria, including *Y. pseudotuberculosis*, the evolutionary ancestor of *Y. pestis*, produce a hexa-acylated lipid A/LPS which can strongly trigger innate immune responses via activation of Toll-like receptor 4 (TLR4)-MD2. In contrast, *Y. pestis* grown at 37$^{o}$C generates a tetra-acylated lipid A/LPS that poorly induces TLR4-mediated immune activation. We have reported that expression of *E. coli lpxL* in *Y. pestis*, which lacks a homologue of this gene, forces the biosynthesis of a hexa-acylated LPS, and that this single modification dramatically reduces virulence in wild type mice, but not in mice lacking a functional TLR4. This emphasizes that avoiding activation of innate immunity is important for *Y. pestis* virulence. It also provides a model in which survival is strongly dependent on innate immune defenses, presenting a unique opportunity for evaluating the relative importance of innate immunity in protection against bacterial infection. TLR signaling is critical for the sensing of pathogens, and one implication of TLR4 engagement is the induction of the pro-forms of the potent inflammatory cytokines IL-1β and IL-18. Therefore *Y. pestis* is able to suppress production of these which are generated through caspase-1-activating nucleotide-binding domain and leucine-rich repeat (NLR)-containing inflammasomes. For my thesis, I sought to elucidate the role of NLRs and IL-18/IL-1β during bubonic and pneumonic

plague infection. Mice lacking IL-18 signaling led to increased susceptibility to wild type *Y. pestis*, and an attenuated strain producing a *Y. pseudotuberculosis*-like hexa-acylated lipid A. I found that the NLRP12, NLRP3 and NLRC4 inflammasomes were important protein complexes in maturing IL-18 and IL-1β during *Y. pestis* infection, and mice deficient in each of these NLRs were more susceptible to bacterial challenge. NLRC4 and NLRP12 also directed interferon-gamma production via induction of IL-18 against plague, and minimizing inflammasome activation may have been a central factor in evolution of the high virulence of *Y. pestis*. This is also the first study that elucidated a pro-inflammatory role for NLRP12 during bacterial infection.

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# PREFACE to CHAPTER 1

This section includes segments of a review accepted for publication in Current Opinions in Microbiology, January 2013: Inflammasomes and host defenses against bacterial infections.

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G.I.V. created all of the figures as outlines from published work, and was the principal writer and coordinator of the manuscript text.

Co-authors contributed the following data:
R.M.R. created Table 1.1 and collected information and wrote sections on bacterial recognition by NLRP3. D.W. wrote the section on cell death after inflammasome activation. S.G. and E.L. assisted in editing and organization of the manuscript.

# CHAPTER 1: Introduction

## The Innate Immune System

The germline-encoded innate immune system is the first line of defense against invading pathogens. Innate immunity is comprised of a number of pattern recognition receptors (PRRs) recognizing conserved pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). PRRs sense extracellular and intracellular pathogens and are specific for various classes of molecules including proteins, lipids, carbohydrates and nucleic acids (Janeway and Medzhitov, 2002; Ye and Ting, 2008; Kawai and Akira, 2010). PAMPs are molecular patterns specific to invading pathogens while DAMPs are host cell-danger signals elicited by pathogen assault. PRRs are largely divided into four types based on structural homology: Toll-like receptors (TLRs), RIG (retinoic acid-inducible)-I-like receptors (RLRs), and NOD (nucleotide oligomerization domain)-like receptors (NLRs), and an emerging family that broadly encompasses intracellular DNA sensors (Kawasaki et al., 2011; Keating et al., 2011). Ligand binding by these receptors leads to the activation a battery of immune effector mechanisms which facilitate the eradication of the pathogen (Janeway and Medzhitov, 2002). These include signaling pathways that culminate in the induction of type-one interferons (IFNs) or pro-inflammatory responses mediated by various cytokines and chemokines. This cytokine and chemokine response leads to local tissue inflammation at the site of the PAMP or DAMP, which includes the production of antimicrobial peptides, reactive oxygen species (ROS), and further cellular recruitment. The reaction elicited during the innate immune response directs inflammatory cell recruitment and elicits activation of humoral immunity (Vallabhapurapu and Karin, 2009; Kawasaki et al., 2011).

An important class of cytosolic immune sensors is the inflammasome: multi-protein complexes comprised of a subset of NLRs, activated after detection of specific PAMPs and DAMPs. Upon activation, they form higher-order structures that, with or without an adapter protein ASC (apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain)), recruits the zymogen pro-caspase-1 (Franchi et al., 2009). Upon recruitment, pro-caspase-1 is cleaved into active caspase-1 which can catalytically activate the potent pro-inflammatory cytokines pro-IL (interleukin)-1β and pro-IL-18, whose expression is upregulated prior to inflammasome activation in an NF-κB dependent manner (Stutz et al., 2009).

Mounting evidence indicates that inflammatory responses can be a double-edged-sword as innate receptors can also be activated in the absence of infection by self-molecules that appear during disease conditions. “Sterile inflammation” is thought to underlie the symptoms associated with inflammatory responses in crystal-based diseases, e.g. gout caused by crystals of uric acid (Martinon et al., 2006). Thus, our understanding of these systems will not only assist in fighting disease, but also allow us to combat inflammatory syndromes from self-processes gone rogue.

It is the culmination of this multi-pathway based innate immune response the host depends on to yield pathogen spread until the adaptive immune system can be initiated.

**Toll-like Receptors (TLRs)**

The TLR family is an extensively studied innate immune PRR and its members are believed to be the system’s primary sensor (Janeway and Medzhitov, 2002). The Toll receptor was originally discovered in flies; its importance embryonic patterning, anti-bacterial, and anti-

fungal responses lead to the discovery of its mammalian homologues (Lemaitre et al., 1996). TLRs recognize different conserved PAMPs and activate innate immune function through a variety of pathways (Kawai and Akira, 2007; 2010). TLRs are type-I transmembrane proteins with ectodomains that are localized either to the plasma membrane (TLRs 1, 2, 4, 5, and 6) or the endolysosome (TLR 3, 7, and 9) (**Figure 1.1**) (Takeda and Akira, 2004). Each consists of a C-terminal leucine-rich repeat domain (LRRs), which is tasked with PAMP-recognition, transmembrane domains, and intracellular Toll-interleukin receptor (TIR) domains required for downstream signaling (Gazzinelli et al., 2004; Kawai and Akira, 2007; 2010). Crystal structures and mechanistic studies have provided some insight into the TLRs in their active and inactive forms - a conserved “m-shape” when bound with specific ligand (two TLRs together), or a “horseshoe-like” shape when unbound (one TLR).

So far, 12 functional TLRs have been identified in mice and 10 in humans; TLR1-TLR9 are conserved between each species, while murine TLR10 is an inactive pseudogene caused by a retrovirus insertion and TLR11-TLR13 have been lost from the human genome (Guan et al., 2010; Kawai and Akira, 2010). Studies of single TLR knockout (KO) mice have shown that each TLR is responsible for recognizing specific PAMPs dependent on their LRR structure or binding partners. The diverse group of bacterial and viral PAMPs which are recognized include lipoproteins, lipids, proteins and multiple nucleic acid species. The TLRs recognizing nucleic acid are localized to the endolysosome while the others are localized to the outer membrane (Takeda and Akira, 2004); their locations define the two general categories.

***Extracellular TLRs: ligands, structure, and function***

The extracellular TLR group is comprised of TLRs1, 2, 4, 5, and 6. These cell surface-expressed TLRs recognize proteins, lipoproteins, and lipids, mostly from bacteria. They are thought to sample the extracellular space for possible PAMPs (Takeda and Akira, 2004).

*TLR4*

TLR4 is the most extensively studied TLR, and has been identified as the receptor for bacterial lipopolysaccharide (LPS) (**Figure 1.1**) (Nagai et al., 2002), an extracellular lipid which decorates the cell-wall of Gram-negative bacteria, and the cause of septic shock (Battafaraono et al., 1995; Poltorak et al., 2000). In the presence of the lipid A component of LPS, TLR4 forms a tetrameric complex on the cell surface, consisting of two TLR4s, along with two molecules of the co-adapter, MD2. (**Figure 1.1**) (Shimazu et al., 1999; Park et al., 2009). In the absence of lipid A, a single TLR4 and MD2 can associate, but the tetrameric complex cannot form.

In the TLR4-MD2 dimer, TLR4 forms hydrophobic and hydrophilic interactions with the LPS and with Phe126 and Leu87 of MD2. A crystal structure of the TLR4-MD2 in complex with hexa-acylated lipid A shows that five of the acyl-chains are positioned inside the β-cup fold structure of the MD2 hydrophobic pocket and the remaining is exposed to the MD2 surface that is hydrophobically bound with TLR4 (Park et al., 2009). The six acyl-chains push the negatively charged phosphate group on the glucosamine-backbone up, to ionically interact with positively charged TLR4/MD2 clusters (Park et al., 2009). The resulting complex that forms initiates signal transduction intracellularly by recruiting adapter molecules to the TIR domain (Kawai and Akira, 2007). Eritoran, a drug used to counteract extreme sepsis in patients (Shahzad G Raja, 2007), is a TLR4 antagonist (Shimamoto et al., 2006), as is lipid IVa, a lipid A precursor (Saitoh S, 2004). Eritoran and lipid IVa contain the same glucosamine-backbone with charged phosphate groups at

positions 1 and 4; however, only four acyl chains are present and therefore the structure falls too deep within the MD2 binding pocket (Park et al., 2009). Therefore, the charged phosphate groups do not interact with either TLR4 due to a ~5Å shift and a almost 180$^{o}$ rotation of the ligand and this prevents downstream signaling (Park et al., 2009). Interestingly, Park *et al.* showed that Phe126 of MD2 is exposed to the solvent, instead of the TLR4 binding partner, when an antagonist was bound; thus it is this structural change that is necessary for dimerization and subsequent downstream signaling. Point mutation studies of the Phe126 loop also support this finding (Motshwene et al., 2009).

To note, lipid IVa does activate mild pro-inflammatory responses via rodent TLR4, but not by mammalian TLR4 (Meng et al., 2010). This is due to an interaction between the negatively charged 4' phosphate on lipid IVa with two positively charged residues on the mouse TLR4; these residues are absent from human TLR4 (Meng et al., 2010).

Many bacterial pathogens, such as *Yersinia pestis* and *Francisella tularensis,* limit TLR4 signaling by expressing lipid A/LPS that is antagonist (i.e. tetra-acylated) to TLR4 (Montminy et al., 2006; Schilling B, 2007). This is described in detail in the *Y. pestis* section.

Other proteins that play a role in LPS recognition including LPS-binding protein (LBP), a soluble protein which binds LPS (Tobias et al., 1997) and CD14, a glycosylphosphatidylinositol (GPI)-linked LRR-containing receptor which binds LBP and delivers it to the TLR4-MD2 complex (Wright et al., 1990; Becker et al., 2000) (**Figure 1.1**).

*TLR2*

TLR2 is unique as it recognizes a wide range of PAMPs via heterodimerization with two TLR binding partners (TLR1 and TLR6) (Jin et al., 2007; Kawai and Akira, 2010) (**Figure 1.1**). These PAMPs include bacterial lipopeptides, Gram-positive specific peptidoglycan and

lipoteichoic acid, lipoarabinomannan from mycobacteria, zymosan from fungi, and others (Akira et al., 2003; Kang et al., 2009). Major recognized ligands for the TLR2-TLR1 heterodimer are triacylated lipopeptides (Pam3Cys4) from Gram-negative bacteria and mycoplasma, and the TLR2-TLR6 heterodimer recognizes diacylated lipopeptides (Pam2Cys4) from Gram-positive bacteria (Kawai and Akira, 2010) (**Figure 1.1**).

In the TLR2-TLR1-Pam3Cys4 crystal structure, two of the three lipid chains (the ester bound lipids) locate inside the TLR2 pocket while the third (amide bound lipid) binds inside the hydrophobic channel of TLR1 (Kang et al., 2009). The hydrophilic binding channel of TLR6 is half as long as TLR1 due to the bulky side-chains of Phe343 and Phe365, therefore the TLR2-TLR6 complex discriminates against triacylated lipopeptides, and recognizes diacylated lipopeptides based on this feature (Kang et al., 2009).

*TLR5 and TLR11*

TLR5 recognizes flagellin, a protein component of the bacterial flagellum (Hayashi et al., 2001) (**Figure 1.1**). Flagellin is highly conserved among motile bacteria and is readily accessible by the innate immune system, thus serving as the perfect target for innate immune activation (Hayashi et al., 2001). TLR11, a relative of TLR5, is also suspected of recognizing bacterial components and profilin-like molecular patterns (Yarovinsky et al., 2006; Kawasaki et al., 2011).

***Intracellular TLRs: ligands, structure, and function***

Intracellular TLRs are expressed mainly in the endocytic compartments and primarily recognize nucleic acid PAMPs (ss/dsRNA or ss/dsDNA) derived from various viruses and bacteria (**Figure 1.1**).

*TLR3*

TLR3 recognizes dsRNA and was discovered by its ability to bind a synthetic ligand, polyinosinic-polycytidylic acid (poly(I:C)) (Alexopoulou et al., 2001) (**Figure 1.1**). Poly(I:C) can induce prototypical antiviral immune responses including type-I interferons and pro-inflammatory cytokine production. The crystal structure of the human TLR3 ectodomain has been described(Choe et al., 2005). It is the binding of dsRNA to TLR3 that induces dimerization and downstream signaling. TLR3 mainly recognizes the genomic RNA of retroviruses, and the dsRNA produced during the replication of ssRNA viruses such as respiratory syncytial virus and West Nile Virus (Akira et al., 2003; Kalali et al., 2008).

*TLR7 and TLR8*

TLR7, originally identified as recognizing the anti-viral, immune modulating compound, resiquimond (R-848) (Jurk et al., 2002), activates innate immune responses after detection of ssRNA from RNA viruses such as influenza A and human immunodeficiency virus (HIV) (**Figure 1.1**) (Heil et al., 2004; Barchet et al., 2005). Recognition of ssRNA viruses by TLR7 occurs in a replication-independent manner after internalization and recruitment of the pathogen to the endolysosome where anti-viral immune responses are triggered. TLR7 induction can also occur when virus replication intermediates are internalized via autophagy, the processes of self-degradation of cellular proteins (Manuse et al., 2010).

Human TLR8, which also recognizes ssRNA and R-848, is phylogenetically most similar to TLR7, though mice deficient in TLR8 respond normally to these ligands(Jurk M, 2002; Jurk et al., 2002).

*TLR9*

Unmethylated 2’-deoxyribo(cytidine-phosphate-guanosine) (CpG) are small DNA motifs that are frequently present in bacteria and viruses but are rare in mammalian cells. These are

recognized by TLR9 to elicit an IFN-based immune response (**Figure 1.1**) (Bauer et al., 2001; Hornung and Latz, 2010). The 2' deoxyribose backbone of the CpG determines the TLR9 activating ability (Haas et al., 2008). These CpGs can directly activate dendritic cells (DCs) and macrophages, which then drive a strong Th1 response (Hornung and Latz, 2010).

TLR9 is localized to the endoplasmic reticulum (ER) of DCs and macrophages under resting conditions, and relocates to early endosomes that contain CpG DNA (Latz et al., 2004). Though the crystal structure of TLR9 has yet to be elucidated, the mechanism of downstream pathway activation via CpG DNA binding to the receptor has been worked out. Binding of CpG changes the TLR9 ectodomain conformation and decreases spacial separation of the TIR domain of TLR9 homodimers (Latz et al., 2007). The TLR9 dimer is not sufficient for activation and requires ligand induced conformational changes (Latz et al., 2007).

TLR9 recognizes DNA viruses such as murine cytomegalovirus (MCMV), HSV-1, and HSV-2 (Lund J, 2003; Kumar et al., 2011). TLR7 and TLR9 have been linked to autoimmune disorders such as systemic lupus erythematous (SLE), since self-nucleic acid can, in unusual circumstances, act as immune-stimulating ligands (Celhar et al., 2012).

### *TLR sub-cellular localization and trafficking*

Nucleic acid-sensing TLRs localize to various intracellular compartments such as the ER, endosome, and endolysosome; and blocking of endosome acidification prevents TLR9-induced signaling due to a failure of TLR9 prototypic cleavage (Park et al., 2008). This suggests that intracellular delivery of internalized nucleic acids to the TLR-containing compartments is pivotal for their interaction. TLR3, 7, an 9 are sequestered to the ER via retention signals and are rapidly

trafficked to endolysosomes by the ER-localization protein UNC93B, a 12-membrane-spanning protein (Latz et al., 2004; Kim et al., 2008). Mice deficient in UNC93B (3d mice) cannot signal though TLR3, 7, or 9. The intracellular localization of these TLRs is thought help prevent activation by self-DNA as stated above.

It is not only the intracellular TLRs which traffic, but signaling via TLR4 is also dependent on movement, and cellular responsiveness to LPS is partly regulated by the physical amount of TLR4 present on the cell surface (Kalis et al., 2003) (**Figure 1.1**). In resting human monocytes, TLR4 is located at the golgi, and LPS-bound MD2 is necessary for correct intracellular distribution of the TLR4 from the golgi to the plasma membrane as well as TLR4 activation (Latz E, 2002; Husebye et al., 2006). It has been demonstrated that the TLR4-MD2-CD14 complex, when engaged with LPS, rapidly traffics to and from the plasma membrane and the golgi in a process that is distinct from downstream signal transduction (Latz et al., 2002). Additionally, after LPS engagement of TLR4-MD2, the complex is recruited into the endosome (Tanimura et al., 2008) (**Figure 1.1**). This relocation of the complex has been linked to downstream signaling via separate adapter proteins, discussed below, then utilized at the surface membrane. The movement of the LPS-detection complex has been linked to CD14 signaling, which is required for, and mediates the endocytosis (Zanoni et al., 2011).

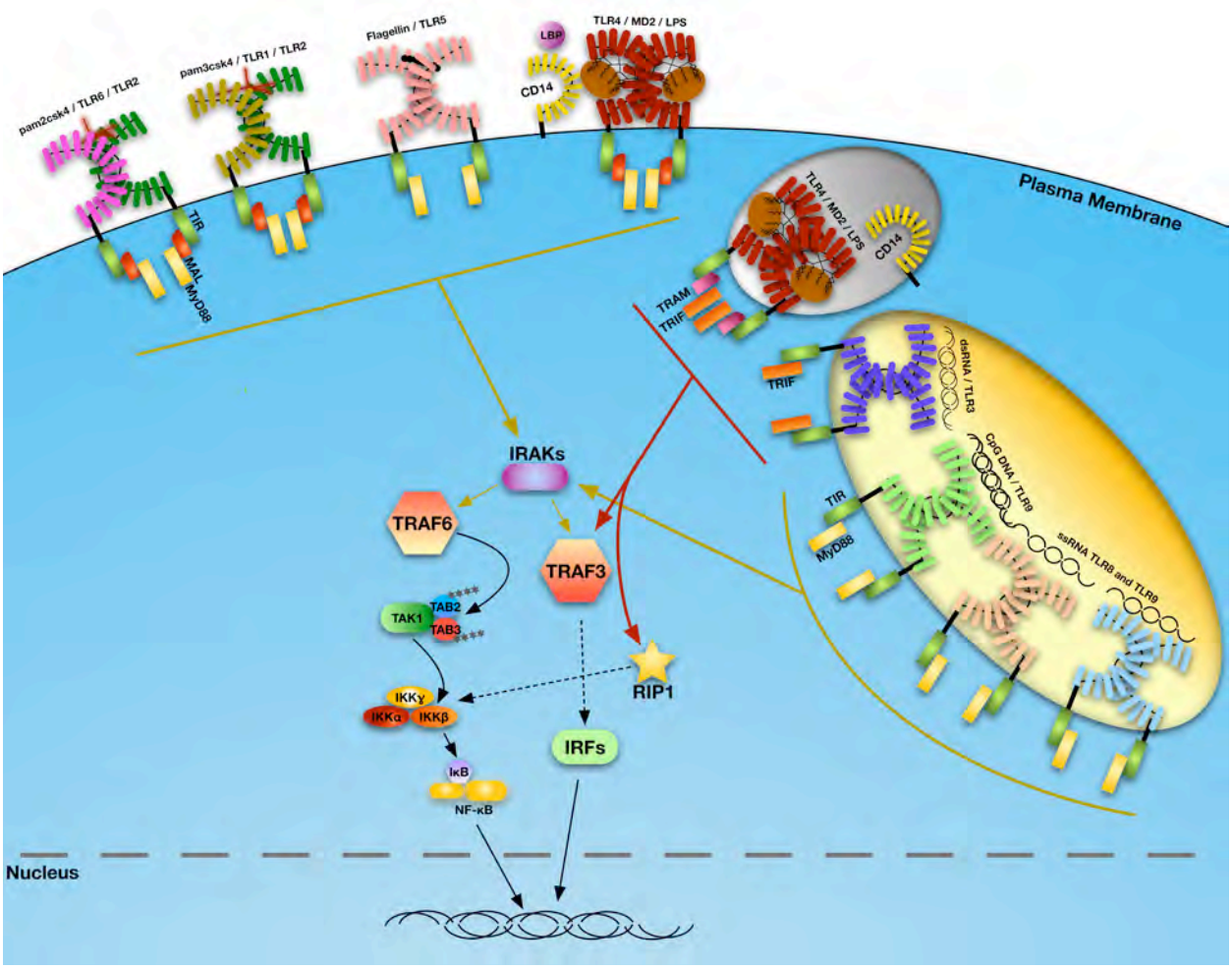


**Figure 1.1: Surface and endolysosome localized TLRs and their cognate antigens with simplified signaling pathways**

TLRs 1, 2, 4, 5, and 6 are mainly localized to the cell surface, while TLRs 3, 7, 8, and 9 are mainly localized in the endolysosome. CD14 and LBP assist in TLR4 signaling. The signaling pathways of TLRs are mediated through the adapter proteins MAL/MyD88 (yellow lines) or TRAM/TRIF (red lines) to downstream signaling cascades. The culmination of these pathways results in the activation of the transcription factors NF-κB and IRFs which promote the expression of type-I IFNs and pro-inflammatory cytokines. Solid arrows depict direct pathways while dotted lines depict simplified pathways.

***TLR adapter pathways - Downstream responses***

After TLR-ligand engagement, specific biological processes, determined by the adapters recruited to the intracellular TIR domains of the activate TLR, are triggered. These TIR-containing adapter proteins include MyD88, Mal, TRIF and TRAM; adapter usage determines downstream effects (**Figure 1.1**) (Wesche et al., 1997; Fitzgerald et al., 2001; Yamamoto M, 2003; Yamamoto et al., 2003b). TLR3 is the only TLR that solely uses TRIF and TRAM whereas TLR4 is the only TLR able to utilize all 4 adapters for signaling (Akira et al., 2003). TLR4 initially recruits Mal and MyD88 at the plasma membrane (Kagan and Medzhitov, 2006) and activates early NF-κB genes (Andrade et al., 2010). Activated TLR4 then undergoes dynamin-dependent endocytosis and is trafficked to the endosome (as described above) where it associates with TRIF and TRAM to activate late NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) genes as well as IRF-dependent type I IFNs (Fitzgerald Ka, 2003; Yamamoto et al., 2003b; Rowe et al., 2006). Therefore, TLR4 has a two-tiered activation mechanism of innate immunity, which could explain why LPS is such a potent innate immune activator.

*MyD88-dependent pathway*

Once a TLR, with the exception of TLR3, recognizes its PAMP, MyD88 is recruited to the intracellular TIR, which then engages members of the IRAK family (IL-1 receptor-associated kinase) (Akira et al., 2003). IRAK4 is activated first and leads to the subsequent interaction with TRAF6 (tumor necrosis factor receptor - associate factor), an E3 ligase responsible for downstream signaling (Cao et al., 1996). TRAF6 catalyzes the addition of polyubiquitin K63 (Lys63)-linked chains on target proteins with assistance from E2 ubiquitin-conjugating enzymes(Xia et al., 2009). K63-linked chains are added to TAB2 and TAB3, the regulatory components of the kinase TAK1 (transforming growth factor-β-activated kinase 1); activation of

IKKɣ (also called NEMO (NF-κB essential modulator)), a component of IKK (IκB (inhibitor of NF-κB) kinase) is also necessary for activation. Activated TAK1 subsequently phosphorylates IKKβ, another component of the IKK complex, which leads to the ubiquitination and degradation of IκB and finally NF-κB activation and nuclear localization (Takaesu et al., 2000; Vallabhapurapu and Karin, 2009; Fan et al., 2010).

NF-κB is an essential, and highly-regulated, pro-inflammatory transcription factor, its activation leads to the expression of a number of antimicrobial defense systems and cytokines such as IL-6 and TNF (tumor necrosis factor) which control downstream immune effects (**Figure 1.1**) (Li and Verma, 2002; Kawai and Akira, 2007).

*TRIF-dependent pathway*

The TRIF/TRAM-dependent pathway is downstream of TLR3 (located at the endolysosome) and late-phase TLR4 activation (Fitzgerald et al., 2003; Yamamoto et al., 2003a), this activates IRF3 (interferon regulatory factors) and NF-κB-based immune responses. Internal TIR domains of TLR3 and TLR4 draft TRAM and TRIF, which leads to the recruitment of TRAF6 and the subsequent K63-ubiquitination of TAK1 for NF-κB activation. TRIF also recruits RIP1, which is required for NF-κB activation downstream of TLR3 (Tatematsu et al., 2010). TRIF-dependent pathways also lead to phosphorylation, and subsequent activation, of IRFs, and type-1 IFN production via TRAF3 (**Figure 1.1**) (Sato S, 2003).

*TLR signaling summary*

Tremendous research has been performed to elucidate the roles and importance of TLRs in pathogen recognition during infection. TLRs, after detection of their cognate antigen, initiate downstream pathways that culminate in the activation of IRFs, NF-κB, and MAPK (mitogen-activated protein kinase) transcription factors to elicit a potent pro-inflammatory milieu. The

known crystal structures of a handful of TLRs have elucidated the exact mechanisms of ligand interactions, though more work must be done to solve all structures. It is the understanding of these complex pathways which further our knowledge in order to create better anti-microbial compounds and therapies.

**Cytosolic Receptors**

Extracellular and endolysosome TLRs are absolutely necessary for tissue surveillance and pro-inflammatory responses, however, pathogens have developed ways to subvert TLR signaling and hide in the cell cytosol. During the past decade, a large focus of innate immune research has been focused on families of cytosolic innate-immune receptors and their importance in host defense signaling. From DAMP sensing, such as crystal-associated inflammation (Duewell et al., 2010), PAMPs such as bacterial secretion systems, toxins, flagellin, LPS, peptidoglycan (PGN) (Averette et al., 2009; Miao et al., 2010), and pathogen nucleic acid (Ablasser et al., 2009; Hornung et al., 2009; Poeck et al., 2009; Sander et al., 2011), cytosolic receptors have been described to recognize diverse ligands which initiate strong pro-inflammatory pathways. Cytosolic surveillance is compulsory for cellular and host survival as many bacterial and viral pathogens replicate in the relatively safe niche of this cellular area. Once a pathogen has escaped lysosomal degradation, or has been phago- or pinocytosed, it would have unlimited access to the host cell machinery for replication. The cytosolic receptors fall into multiple classes: RIG (retinoic acid inducible gene)-like receptors (RLRs), which detect RNA, and NOD (nucleotide-binding domain and leucine-rich repeat containing)-like receptors (NLRs) which respond to various PAMPs and DAMPs, as further explained below.

***NOD-Like Receptors and the Inflammasome***

The NLR family of proteins has emerged as important and necessary receptors in pathogen detection and innate immune activation, and much evidence has been provided to indicate roles for the NLR family members in host inflammatory disorders. NLRs have been shown to complement and synergize with TLR signals arising from pathogen recognition, and are not necessarily separate pathways. Evolutionarily, NLR orthologs can be found in plants (R-genes / R-proteins), and are required for anti-pathogen responses (Ronald and Beutler, 2010). The detection of avirulence (avr) proteins delivered by pathogenic bacteria trigger rapid host defenses in plants. The detection of avr proteins by R-proteins can occur by direct binding or by detection of altered cellular function caused by the avr proteins (guardian hypothesis vs receptor-ligand model) (Ronald and Beutler, 2010).

The NLR family can be divided into two groups, one group which activates NF-κB- and MAPK-dependent responses, and the other group which signals through pro-inflammatory caspases (such as caspase-1) to activate idle pro-inflammatory cytokines. The latter group is termed the inflammasome-forming NLRs. To date, the NLR family is comprised of 23 human genes, and 34 in the mouse - and although these are primarily expressed in immune cells, a few are expressed in other tissues, such as epithelial cells (Shaw et al., 2008).

NLRs are comprised of, and characterized by, three domains: the first, located at the N-terminus, is a pyrin domain (PYD), CARD, or a baculovirus inhibitor of apoptosis protein repeat (BIR) (Stutz et al., 2009) (**Figure 1.2**). The CARD and PYD domains are part of the death domain superfamily of proteins, classified by their fold: 6 to 7 tightly packed α-helices. The BIR belongs to the zink-finger domain family. The central domain is a NBD (nucleotide-binding

domain) responsible for binding ribonucleotides and possibly involved in NLR-oligomerization(Duncan et al., 2007). Finally, NLRs contain an LRR domain at the C-terminus which is believed to play a role in the recognition of DAMPs and PAMPs (Stutz et al., 2009).

NLR proteins are sub-classified by the differences in their varying N-terminal domains. The largest group shares an N-terminal PYD, termed NLRP, and contains proteins such as hNLRP1, NLRP3, NLRP6, NLRP7, and NLRP12 (**Figure 1.2**). Another group expresses an N-terminal CARD domain and is termed NLRC. The NLRC group contains the proteins NOD1 and NOD2 as well as NLRC4 (IPAF). Other NLR families have emerged containing a BIR domain such as NAIP5 (apoptosis inhibitory protein 5).

*NOD1 and NOD2*

NOD1 (also called CARD4) and NOD2 (CARD15) have been demonstrated as important cytosolic receptors for microbial pathogens(Chamaillard et al., 2003; Girardin et al., 2003a; Meinzer et al., 2007), and activate downstream NF-κB and MAPK responses (Shaw et al., 2008). NOD1 is ubiquitously expressed in all cells while NOD2 is expressed only in monocytes, macrophages, dendritic cells and some epithelial cells. It has been shown that NOD1 and NOD2 recognize different structures of core motifs from various PGN (peptidoglycan) structures, a component of bacterial cell walls. NOD1 is activated by y-D-glutamyl-meso-diaminopimelic acid (meso-DAP) (Chamaillard et al., 2003; Girardin et al., 2003a), which is unique to PGN structures from all Gram-negative and some Gram-positive bacteria. NOD2 is activated by MDP (muramyl dipeptide), which is present in all Gram-positive and -negative PGN (Girardin et al., 2003b).

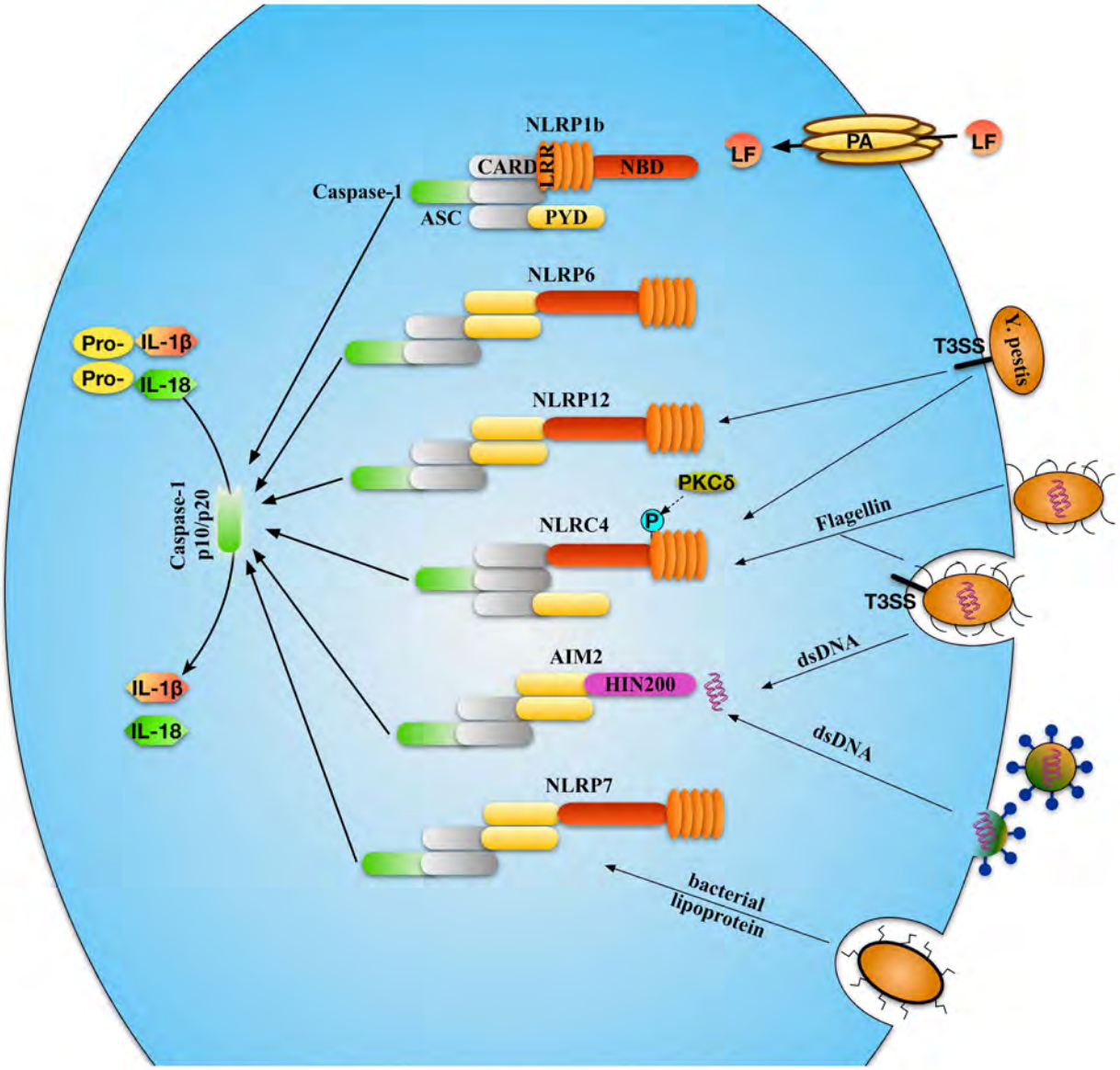
NLRP1b
CARD
LRR
NBD
Caspase-1
ASC
PYD
LF
PA
LF
NLRP6
Pro-
IL-1β
Pro-
IL-18
T3SS
Y. pestis
NLRP12
PKCδ
P
NLRC4
Flagellin
Caspase-1
p10/p20
T3SS
AIM2
HIN200
dsDNA
IL-1β
IL-18
dsDNA
NLRP7
bacterial
lipoprotein

**Figure 1.2: Inflammasome design and activators**

Inflammasome design and activators. Inflammasome-forming NLRs share the same general features, although mechanisms for ligand recognition may differ. NLRP3, NLRP6, NLRP7 and NLRP12 all share a C-terminal leucine-rich-repeat (LRR) region, an internal nucleotide-binding-domain (NBD), and an N-terminal Pyrin domain (PYD), which recruits the adapter protein ASC – a caspase recruitment and activation domain (CARD) and PYD containing protein which links the NLR or AIM2 to caspase-1. The ASC adapter is believed to be an integral part of NLRP3, NLRP6, NLRP7 and NLRP12 inflammasomes. NLRC4 contains an N-terminal CARD domain which can recruit caspase-1 directly, though ASC involvement may increase caspase-1 processing activity. NLRP1b, like NLRC4, has a N-terminal CARD domain, but has an internal LRR and a C-terminal NBD domain. Lastly, AIM2 has a HIN200 DNA binding domain and a PYD for ASC recruitment. Different types of stimuli signal inflammasome activation via the various NLRs or AIM2, some of which are depicted.

***The Inflammasome***

A relatively new and interesting PRR-containing complex in innate immunity is the inflammasome, a multi-protein complex that acts as a platform for the activation of the pro-inflammatory caspase-1 (originally called ICE (IL-1-converting enzyme))(Martinon et al., 2002). Upon activation, caspase-1 proteolytically cleaves the cytosolic-sequestering leader sequence from pro-IL-1β, and pro-IL-18 (Wang et al., 1998; Davis et al., 2011b; Franchi et al., 2012b) to generate mature cytokines which are released from the cell to mediate downstream inflammatory effects (**Figure 1.2**). Pro-IL-1β and pro-IL-18 are upregulated downstream of TLR stimulation and NF-κB activation (Watari et al., 2000). The mature IL-1β is a potent pyrogen with pleiotropic function including inciting tissue inflammation by activating epithelial cells (Dinarello, 2009). IL-18 can induce IFNɣ, a type-II IFN expressed by T-cells and NK (natural killer) cells, and other pro-inflammatory cytokines (Okamura et al., 1995).

Inflammasomes are constructed of pro-caspase-1 and proteins in the cytosolic NLR family, or AIM2; some require the adapter protein ASC that mediates interaction between the NLR and pro-caspase-1 (Stutz et al., 2009). NLR composition is outlined in **Figure 1.2**. The LRR is thought to be responsible for the recognition of the specific activating signal (Stutz et al., 2009). Inflammasome-mediated cytokine release follows a multi-step activation pathway: first an NF-κB-dependent upregulation of the inactive pro-forms of IL-1β and IL-18, and also of some NLRs, like NLRP3 (Bauernfeind et al., 2009), and second, activation of the NLR itself resulting in inflammasome formation. Recently, a 3-step activation pathway has been described for some

Gram-negative bacteria that involves caspase-11 and TLR4/TRIF (Rathinam et al., 2012b) (**Figure 1.3**). It should be noted that some cells may have a simpler activation process due to higher basal levels of pro-forms of caspase-1 and/or pro-cytokines (Netea et al., 2009).

Some NLRs have been well characterized and shown to form inflammasomes (NLRC4, NLRP3, AIM2), whereas details are emerging for others (NLRP1, NLRP6, NLRP7, NLRP12). Great amounts of evidence have shown the importance of the inflammasome in pathogen clearance, however, the inflammasome is also responsible for many host disorders that lead to detrimental effects (Dinarello, 2009). Inflammasome activation has also been linked to cell death pathways (e.g., pyroptosis), which may be a novel anti-pathogen pathway (Miao et al., 2011).

*NLRP3 Inflammasome*

The NLRP3 inflammasome is involved in host responses to a wide variety of pathogenic microorganisms (**Table 1.1**), is expressed by myeloid cells, and is upregulated in response to TLR stimulation (Bauernfeind et al., 2009). NLRP3 contains a C-terminal LRR, internal NBD, and a N-terminal PYD domain (**Figure 1.3**). NLRP3 activation and subsequent inflammatory damage has also been linked to the pathogenesis of diseases characterized by crystal-mediated sterile inflammation, e.g., atherosclerosis caused by the deposition of cholesterol crystals (Duewell et al., 2010). Other examples of exogenous NLRP3 activators include silica and asbestos, leading to silicosis and asbestosis (Cassel et al., 2008; Hornung et al., 2008).

| Pathogen | Putative Bacterial Activator | Inflammasome | Proposed Mechanism | Role in host defense in vivo | References |
|---|---|---|---|---|---|
| *Bacillus anthracis* | Lethal toxin | NALP1b/NLRP1b | phospho-PKR, ATP leakage/K+ efflux, pyroptosis | Yes | 19, 40, 58, 59 |
| *Burkholderia pseudomallei* | BsaK | NLRP3, NLRC4 | nd | Yes | 23, 60 |
| *Burkholderia thailandensis* | BsaK | NLRC4/NAIP2 | Ligand binding to NAIP | nd | 22 |
| *Chlamydia pneumoniae* | nd | NLRP3 | oxidized mtDNA, K+ efflux, lysosomal acidification, cathepsin B release | nd | 16, 61 |
| *Chromobacterium violaceum* | CprI | NLRC4/human NAIP | Ligand binding to NAIP | nd | 22 |
| *Citrobacter rodentium* | mRNA | NLRP3 | TLR4/TRIF, Caspase-11 | nd | 5 |
| *Escherichia coli* | mRNA | NLRP3 | TLR4/TRIF, Caspase-11, lysosomal rupture | Yes | 5, 13 |
| | nd | NLRP3 | phospo-PKR | Yes | 19 |
| | EprJ, Escl | NLRC4/NAIP5, NLRC4, | nd | Yes | 23, 30 |
| *Franciscella tularensis* | DNA | AIM2 | K+ efflux, lysosomal acidification | yes | 35, 36 |
| *Group B Streptococcus* | β-hemolysin | NLRP3 | K+ efflux | Yes | 12 |
| *Legionella pneumophila* | Flagellin | NLRC4/NAIP5 | Ligand binding to NAIP, cPLA2, eicosanoid release, caspase-7 activation | Yes | 22, 27, 31 |
| *Listeria monocytogenes* | nd | NLRP3 | GBP5 | Yes | 18 |
| | LLO, DNA? | NLRP3, AIM2 | nd | nd | 57 |
| *Mycobacterium tuberculosis* | DNA | AIM2 | nd | Yes | 62 |
| | ESX-1, ESAT-6 | NLRP3 | pore formation | nd | 63 |
| | | ASC/Caspase-1 | dependent in vitro, not in vivo | yes | 66 |
| *Pseudomonas aeruginosa* | PscL | NLRC4 | nd | nd | 23 |

| | | | | | |
|---|---|---|---|---|---|
| | Flagellin | NLRC4/NAIP5 | Ligand binding to NAIP | nd | 22 |
| *Salmonella enterica* serovar Typhimurium | nd | NLRP3, NLRC4 | GBP5, phospo-PKR | nd | 18, 19 |
| | PrgJ | NLRC4, NLRC4/ NAIP2 | Ligand binding to NAIP | Yes | 23, 22, 26 |
| | T3SS, Flagellin | NLRP3, NLRC4 | phospho-Ser533, PKCδ, TLR4/TRIF, Caspase-11 | Yes | 28, 30, 33, 4 |
| *Shigella flexneri* | Mxil | NLRC4 | nd | nd | 23 |
| *Staphylococcus aureus* | α-hemolysin | NLRP3 | K+ efflux | Yes | 64 |
| *Streptococcus pneumoniae* | pneumolysin | NLRP3 | K+ efflux | Yes | 65 |
| *Treponema denticola* | Td92 | NLRP3 | ATP leakage/K+ efflux | nd | 67 |
| *Vibrio cholera* | cholera toxin B | NLRP3 | Caspase-11 dependent | nd | 53 |
| *Yersinia pestis* | T3SS | NLRP3, NLRC4 | ND | Yes | 24 |
| | YopJ | NLRP3 | K+ efflux | nd | 68 |
| | T3SS, YopJ | NLRP12, NLRP3 | nd | Yes | 46 |

**Table 1.1: Bacterial pathogens, ligands, and their known signaling inflammasomes.**
The table contains upstream, and in some cases, downstream inflammasome effects after pathogen activation.

Three models of NLRP3 activation in response to microbial ligands have been proposed (Davis et al., 2011b). The channel model suggests that extracellular ATP (adenosine triphosphate) from microbial pathogens activates the $P_2X_7$ receptor ($P_2X_7R$) and allows the efflux of intracellular potassium ion ($K^+$) resulting in NLRP3 activation (**Figure 1.3**) (Franchi et al., 2007; Arlehamn et al., 2010). A number of bacterial pore-forming toxins (e.g. Group B Streptococcus β-hemolysin (Costa et al., 2012)) can also cause cellular ion dysregulation and subsequent NLRP3 activation (**Table 1.1**). As well LRR-mediated binding of regulatory proteins, such as ubiquitin ligase–associated protein SGT1 and heat shock protein 90 (HSP90) may assist in controlling NLRP3 activity before activation (Mayor et al., 2007).

Escape from the lysosome after phagocytosis is an important step during the movement of many pathogens, toxins, and cholesterol-dependent cytolysins. The lysosomal rupture model for NLRP3 activation posits that the release of lysosomal enzymes, such as cathepsin B, into the cell cytoplasm during lysosomal destabilization leads to NLRP3 activation (**Figure 1.3**) (Davis et al., 2011b). Recent studies have shown that prokaryotic mRNA (messenger RNA) released from the lysosome into the cytosol during degradation of phagocytosed live bacteria can activate NLRP3 (Sander et al., 2011), thus, bacterial RNA may be a key trigger of the NLRP3 inflammasome during many infections, either by direct binding or though an adapter.

Reactive oxygen species (ROS) released from the mitochondria are considered to be a cellular stress-induced alarms and may trigger NLRP3 inflammasomes. The ROS model is based on observations showing that NLRP3 is activated upon mitochondrial damage and release of ROS (Zhou et al., 2011). Oxidized mitochondrial DNA (mtDNA) from mitochondria damaged by bacterial infection or other means was suggested to bind and activate NLRP3 (Shimada et al.,

2012) (**Figure 1.3**). This phenomenon was negatively regulated by the anti-apoptotic protein Bcl2, suggesting a link between apoptosis and inflammasome activation. The idea that the NLRP3 inflammasome senses mitochondrial dysfunction could also help in understanding the prior observations suggesting an association of mitochondrial damage with inflammatory diseases (Beal, 2003).

Recently, GBP5 (guanylate-binding protein 5) and PKR (dsRNA-dependent protein kinase) have been proposed to play integral roles in NLRP3 activation (**Figure 1.3**). GBP5 interacts with the pyrin domain of NLRP3 and aids in the oligomerization of the inflammasome complex (Shenoy et al., 2012). Importantly, GBP5 promotes a role in NLRP3 activation by live bacteria, but not during sterile inflammation *in vitro*. Infection of GBP5-deficient mice had higher uncontrolled infection and faster disease progression in comparison to wild type mice.

PKR has also been shown to autophosphorylate upon macrophage stimulation with NLRP3 ligands, and active PKR can bind NLRP3 (as well as NLRP1b, NLRC4 and AIM2) (Lu et al., 2012). This study proposes that PKR can directly activate the NLRP3 inflammasome and mediate the release of pro-inflammatory cytokines when stimulated with many ligands including bacterial pathogens. It is, however, unclear how the PKR phosphorylation leading to NLRP3 inflammasome activation is triggered during infection.

Since many of the NLRP3 stimuli lead to inhibition of protein synthesis, it has also been found that direct blocking of ribosomal function leads to inflammasome activation (Vyleta et al., 2012).

Methods of controlling inflammasome function are also now just being discovered: NLRP3 inflammasome activation triggers autophagosome formation via the G protein RalB

which targets ubiquitinated inflammasome for lysosome degradation (Shi et al., 2012). Moreover, blocking autophagy increased inflammasome activity and inducing autophagy limited it. The post-translational modifications of NLRP3 may also control activation as the deubiquitination of NLRP3 by BCRR3 regulates inflammasome activity (Py et al., 2012).

Although multiple ways may lead to NLRP3 activation, the array of ligands which trigger the NLRP3 inflammasome indicates that common denominators exist.

The NLRP3 inflammasome can also be activated by host/self molecules, such as ATP, monosodium urate (MSU), cholesterol crystals, and amyloid-β - these are associated with cell death, tissue damage, or danger (Martinon et al., 2006; Halle et al., 2008; Duewell et al., 2010). These have been important findings in human health and disease. For instance, cholesterol crystals deposited within arteries are the cause, not the result of, inflammation in an NLRP3 dependent matter and therefore provide new insights into the pathogenesis of atherosclerosis and may indicate targets for therapy (Duewell et al., 2010). Moreover, MSU deposits within joints is indicative of the inflammatory disorder gout, which is also linked to NLRP3-dependent activity (Martinon et al., 2006).

Genetic predisposition for auto-activation of the NLRP3 inflammasome is associated with many inflammatory disorders called cryopyrin-associated periodic fever syndromes (CAPS) (Ozkurede and Franchi, 2012). These disorders include familial cold-induced auto-inflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal onset multi-system inflammatory disorder (NOMID); all of these present with varying disease severity and symptoms include reoccurring fevers, rashes, chills and arthralgia. NOMID, the most severe, can cause neurological problems including aseptic meningitis and deafness (Posch et al., 2012).

Patients with CAPS usually have mutations within the NBD domain of NLRP3, and a small percentage within the LRR (Hoffman and Brydges, 2011). Mice with FCAS and MWS NLRP3 mutation knock-ins mimicked clinical manifestations and treatment of these animals with anti-IL-1β partially yielded the disease progression (Brydges et al., 2009). However, Brydges *et al.* found that cells from these animals did not spontaneously secrete IL-1β but did respond the lower concentrations of PAMPs then WT.

The crystal structure of the NLRP3 PYD domain has been solved and shows 6 α-helicases that form the canonical anti-parallel folds that are constant though the PYD domain family (Bae and Park, 2011). The protein contains two hydrophobic regions, one in the center which the helicases are packed around, and a second which is essential for stabilization. Both of these patches are necessary for interaction with ASC (Bae and Park, 2011).

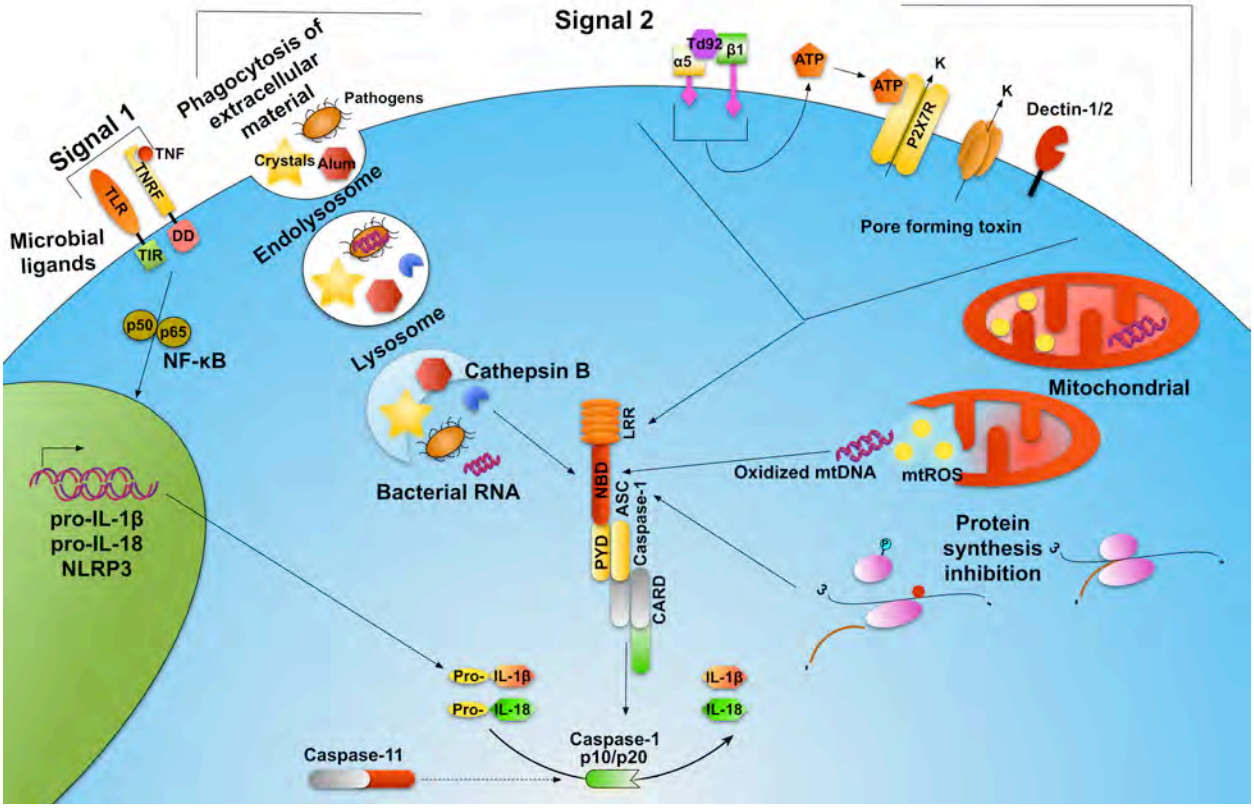


**Figure 1.3: Model of NLRP3 activation**

Model of NLRP3 activation. Activation of caspase-1 by the NLRP3 inflammasome is a multi-signal process. Signal 1 occurs when TNF or a TLR ligand binds its cognate receptor resulting in the translocation of NF-κB into the nucleus where expression of NLRP3 and the immature (pro-) forms of IL-1β and IL-18 are induced. Signal 2 is the activation of NLRP3 resulting in recruitment and cleavage of pro-caspase-1 to its active form leading to cleavage of the immature inflammatory cytokines. At least three distinct NLRP3 activation pathways have been identified. Phagocytosis of extracellular particulates and pathogens results in lysosomal destabilization and release of cathepsin B and bacterial mRNA which trigger NLRP3 activation. A decrease in intracellular K+ has been shown to result in activation of NLRP3. K+ efflux occurs by engagement of extracellular ATP with the P2X7R or directly through bacterial pore-forming toxins. ROS generated during mitochondrial damage and oxidized mitochondrial DNA (mtDNA) produced during apoptosis lead to activation of NLRP3. Td92, a surface protein of *Treponema denticola*, can interact with the α5β1 integrin resulting in ATP release and K+ efflux. Inhibition of ribosomal function and protein synthesis can also direct NLRP3 activation, and this mechanism may involve lysosomal destabilization, K+ efflux and ROS. Caspase-11 has been defined upstream of caspase-1 during NLRP3 inflammasome activation.

NLRC4 Inflammasome

NLRC4 is expressed mainly in macrophages and hematopoietic tissues and cells, and its expression is not believed to be upregulated upon TLR activation. Instead of the N-terminal PYD domain which is necessary for ASC recruitment, NLRC4 contains a CARD domain that can interact directly with pro-caspase-1 (**Figure 1.2**). A number of bacteria, including *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Listeria monocytogenes*, are thought to signal caspase-1 activation and IL-1β/IL-18 maturation via NLRC4 (**Table 1.1**) (Miao et al., 2008; Warren et al., 2008; Brodsky et al., 2010; Miao et al., 2010; Zhao et al., 2011). NLRC4 is specifically activated by a functional bacterial type-three or -four secretion system (T3SS/T4SS) or flagellin (Miao et al., 2010; Zhao et al., 2011) (**Figure 1.2**). *S. typhimurium* was one of the first bacterial species shown to activate caspase-1 via the NLRC4-inflammasome; and mutants of *S. typhimurium* lacking genes that encode flagellin monomers are unable to induce caspase-1 (Miao et al., 2010).

Although the mechanism is still unclear, the monomers are thought to interact with distinct members of the NAIP (neuronal apoptosis inhibitor protein) family that act activate NLRC4 (Lightfield et al., 2008). NAIP5, like NLRC4, contains a NBR and LRR but it exchanges the C-terminal CARD for a BIR domain and can form hetero-oligomers with NLRC4 (Zamboni et al., 2006). NAIP2 and 6 (with 5) are involved in NLRC4 activation, providing an adapter service for flagellin and T3SS proteins, respectively (Kofoed and Vance, 2011). Although this model of ligand binding and differentiation by NAIPs is appealing, it should be pointed out that there is only one human NAIP protein and this has only been found to recognize the *Chromobacterium violaceum* T3SS needle protein CprI (Zhao et al., 2011).

Interestingly, NLRC4/NAIP5 inflammasome activation has been directly tied to eicosanoid release from resident murine peritoneal macrophages (Moltke et al., 2012). Activation of the NLRC4 inflammasome by flagellin also causes a rapid “eicosanoid storm”, defined as a release of signaling lipids (prostaglandins and leukotrienes) which rapidly cause inflammation and vascular fluid loss in mice (Moltke et al., 2012). This response was initiated within minutes of flagellin detection, however, these *in vivo* responses were not confirmed during infection and the relationship to antimicrobial responses is unknown.

NLRC4 has been implicated in a pro-inflammatory defense mechanism in the intestine against foreign, but not commensal, bacteria (Franchi et al., 2012a). Intestinal mononuclear phagocytes (iMPs) are hyporesponsive to TLR stimulation however, they contain high basal levels of pro-IL-1β and NLRC4 which are activated by foreign bacteria presenting a T3SS or T4SS (Franchi et al., 2012a). It has been determined that NLRC4 is activated upon phosphorylation at Ser533 by PKCδ, which provides some insight into general NLR activation (**Figure 1.2**)(Qu et al., 2012).

*NLRP12 Inflammasome*

NLRP12 was the first NLR shown in biochemical assays to interact with the adaptor protein ASC to form an active IL-1β-maturing inflammasome (**Figure 1.2**) (Wang et al., 2002). The role of NLRP12 in innate immunity has remained unclear as both inflammatory and inhibitory functions have been suggested, as has a role in hypersensitivity - though there have been disagreements from different groups, and from within the same group, regarding expression and activity of NLRP12. In 2003, Williams *et al.* reported NLRP12 expression in human DCs, monocytes, and neutrophils (Williams et al., 2003). In this study, an overexpression HeLa cell-

line and siRNA knockdown in monocytes were used to suggest that NLRP12 controlled MHC (major histocompatibility complex) I expression (Williams et al., 2003). The same group showed NLRP12 expression is downregulated in human primary PBMCs (peripheral blood mononuclear cells) or granulocytes after exposure to high levels of TLR stimulants or *M. tuberculosis* (Williams et al., 2005). In the latter study, they used overexpression and luciferase systems to show that a NLRP12 NBD domain associates with IRAK upon TLR stimulation resulting in impaired NF-κB expression downstream from MyD88 (Williams et al., 2005). Separately, another overexpression system in THP-1 cells was used to show that the NBD and LRR domains of NLPR12 associate with NIK (NF-κB inducing kinase). NIK is a kinase upstream of non-cononical NF-κB activation resulting in the expression of pro-inflammatory cytokines which support ongoing immune responses, downstream from CD40 (Coope, 2002). The association of NLRP12 and NIK induces NIK degradation in a proteasome-dependent fashion (Lich et al., 2007). The degradation may involve HSP90, and ATP binding (Arthur et al., 2007; Ye et al., 2008), as in NLRP3 signaling (Mayor et al., 2007). These studies of NLRP12 describe a negative regulation function, but have used only overexpression and siRNA knockdown systems, which have sometimes been unreliable.

The NLRP12 KO animal was first utilized in 2010 in an allergic dermatitis model to show reduced swelling and cellular influx at the site of elicitation. However, this is not due to reduced amounts of IL-1β or other cytokines, such as TNF, as in NLRP3-deficient animals in the same model (Watanabe et al., 2007; Arthur et al., 2010). NLRP12-deficient animal responses are abrogated due to a reduction in DC and myeloid cell migration; in addition, the DCs, as well as neutrophils, from these animals fail to respond to chemokines *in vitro* (Arthur et al., 2010).

However, these studies do not correlate with studies from the same group as levels of NF-κB-induced cytokines or chemokines are not increased, nor is MHCI expression altered. Moreover, NLRP12 expression in monocytes was not consistent with those observed in four other publications nor did they publish caspase-1 western blots for this model. Up to this point, the NLRP12 animal had not been used for any infectious disease studies, and the role of the NLRP12 inflammasome in infections was unknown.

Interestingly, as with NLRP3, mutations in NLRP12 are linked to hereditary inflammatory disease (Jéru et al., 2008), and mutations appear to lead to increased ASC speckle formation and caspase-1 activity after a conserved missense-mutation (Jéru et al., 2011b), as well as increased NF-κB activation after a nonsense-mutation (Jéru et al., 2011a). These results mimicked previous observations from NLRP3 mutations (Jéru et al., 2010). It has been reported that patients carrying NLRP12 mutations associated with increased inflammasome activation have been successfully treated with anti-IL-1 therapy, similar to patients containing mutations in NLRP3 (Hawkins and Lachmann, 2003; Lachmann et al., 2009; Jéru et al., 2011a).

*NLRP1 Inflammasome*

The original description of the inflammasome complex involved human NLRP1(Martinon et al., 2002), which has been shown to be expressed in most cells including adaptive immune cells and non-hematopoietic tissues. Human NLRP1 has a N-terminal PYD, central NBD, LRR, and FIIND (domain with function to find) domains, and a C-terminal CARD. Studies in THP1 cells showed that NLRP1 forms a complex with CARD8, ASC, caspase-5, and caspase-1 to subsequently process IL-1β (Martinon et al., 2002). Mice, unlike humans who have a single NLRP1 gene, have 3 paralogs expressing NLRP1a, NLRP1b, and NLRP1c-ps. NLRP1b,

the major focus of immune studies, contains a C-terminal NBD, an internal LRR, and a N-terminal CARD domain (**Figure 1.2**).

Mouse NLRP1b has also been linked to IL-1β production by muramyl-dipeptide (MDP) (Bruey et al., 2007). Moreover, it has been described as a receptor for lethal toxin from *Bacillus anthracis* in the host cytosol and participates in caspase-1-mediated IL-1β production and pyroptosis, *in vivo* and *in vitro* (**Figure 1.2**) (Terra et al., 2009). The detection of lethal toxin has also been linked to induction of the eicosanoid response as previously described for NLRC4 (Moltke et al., 2012).

Studies have determined that human NLRP1 is activated in a "two-step process": first MDP binds directly or indirectly to NLRP1 and results in a conformational change that allows NTP (nucleotide triphosphate) to bind. This then leads to the oligamerization of NLRP1 and recruitment of pro-caspase-1 (Faustin et al., 2007).

*NLRP6, NLRP7, and NLRC5*

NLRP6 (**Figure 1.2**), expressed in the gastrointestinal tract, kidney, liver, and epithelial cells (Elinav et al., 2011), has been reported to be involved in obesity, intestinal inflammation and tumorigenesis, the regulation of commensal microflora, and most recently, bacterial recognition (Chen et al., 2011; Elinav et al., 2011; Anand et al., 2012; Henao-Mejia et al., 2012).

A pro-inflammatory role for NLRP6 has been proposed since NLRP6-deficient mice showed altered gut microbiota (increase in normally suppressed communities) and a predisposition to colitis as a result of decreased levels of IL-18 secretion by intestinal epithelial cells (Elinav et al., 2011). However, Anand *et al*. presented a novel function for NLRP6 during certain bacterial infections as a negative regulator of innate immunity; mice deficient for NLRP6 were resistant to infection with *L. monocytogenes*, *Salmonella*, and *E. coli* (Anand et al., 2012).

Infection of the NLRP6 KO animal resulted in increased monocyte and neutrophil populations in the blood and increased NF-κB signaling (Anand et al., 2012), thereby providing evidence of NLRP6 in dampening inflammatory signaling, although the mechanism remains elusive.

NLRP7 is not expressed in mice, but hNLRP7 has been linked to inflammasome function in response to bacterial lipopeptides (TLR2 ligands) (**Figure 1.2**) using a siRNA knockdown system (Khare et al., 2012). The NLRP7 PYD 3D structure has been solved by NMR and also has a six α-helical bundle fold, as in other NLR PYDs around a conserved hydrophobic core (Pinheiro et al., 2010).

The main function of another family member, NLRC5, appears to be in regulation of MHC class I genes (Meissner et al., 2010), although knock-down data in human cells suggest NLRC5 may participate in inflammasome activation during infection (Davis et al., 2011a).

*AIM2 Inflammasome*

AIM2 (Absent in Melanoma 2) is a cytosolic receptor for double-stranded DNA and is known to form an inflammasome and activate caspase-1 in the presence of bacteria and viruses (**Figure 1.2**) (Hornung et al., 2009; Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). It contains an N-terminal pyrin domain and a C-terminal DNA-binding HIN200 domain and is the only known HIN200 domain-containing protein with the ability to mature IL-1β and IL-18 via interactions with ASC and caspase-1 (Hornung et al., 2009). Macrophages from AIM2 KO mice are deficient in caspase-1, pro-IL1β, and pro-IL-18 processing after infection with *L. monocytogenes* and the *F. tularensis* LVS strain (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010), thus defining the importance of inflammasome activation against bacteria that replicate intracellularly. This anti-bacterial phenotype was recapitulated *in vivo* as AIM2-deficient mice were more susceptible to subcutaneous infection with *F. tularensis* LVS and resulted in reduced

serum IL-18 levels (Fernandes-Alnemri et al., 2010). Moreover, AIM2 plays a major role in the detection of cytosolic DNA viruses such as murine cytomegalovirus (MCMV). After MCMV infection, the IL-18 and the NK cell-dependent IFNɣ secretion were decreased and correlated to increased viral titers in mice deficient in AIM2 (Rathinam et al., 2010).

*Inflammasome and Caspases*

Recent studies have shown that mouse caspase-11 contributes to caspase-1-independent cell death in response to a number of pathogens (Kayagaki et al., 2011; Broz et al., 2012; Rathinam et al., 2012b). To the great surprise of the inflammasome field, it was revealed that that the widely used caspase-1-deficient mice, generated on a 129 background, also lack a functional allele of caspase-11, and are therefore functionally caspase-1/caspase-11 double knockouts (Kayagaki et al., 2011).

Caspase-11 was found to be a key molecule in inflammasome activation by cholera toxin, *E. coli*, *Vibrio cholera* and *Citrobacter rodentium*, as well as a central mediator of LPS-induced lethal shock (Kayagaki et al., 2011), although caspase-11 works upstream of caspase-1 for IL-1β processing and independently of caspase-1 for the induction of cell death. Rathinam *et al.* subsequently found that TLR4/TRIF-dependent type I IFN production is crucial for caspase-11 activation in response to various Gram-negative bacteria, and this licenses NLRP3-inflammasome-induced caspase-1 processing, thus providing another link between TLR and NLR signaling (Rathinam et al., 2012b). Broz *et al.* supported this role for TRIF, and it appears that in the absence of caspase-1, lysis of macrophages and the release of intracellular *Salmonella* can be detrimental to the host in a caspase-11-dependent manner (Broz et al., 2012).

### *Inflammasome role in intestinal homeostasis*

#### *NLRP3 and NLRC4*

At the mucosal intestinal surface, the immune system must limit immune responses against commensal flora, while protecting the barrier against foreign pathogens. The NLRP3 inflammasome has emerged as a crucial regulator of intestinal homeostasis, and has been linked to inflammatory bowel disease (Zaki et al., 2011a). NLRP3-deficient animals were shown to be more susceptible to developing DSS (dextran sodium sulfate)-induced colitis (Zaki et al., 2010). The defective inflammasome activity led to loss of epithelial structure, resulting in systemic spread of commensal bacteria, massive leukocyte infiltration, and increased chemokine production in the colon (Zaki et al., 2010). This study determined that basel level IL-18 production by the NLRP3 inflammasome in colonic epithelial cells is a critical mediator of the mucosal protection against DSS-induced colitis. IL-18 is involved in proliferation and repair of epithelial cells, which may protect against IBD and the DSS-induced colitis (Takagi et al., 2003; Reuter and Pizarro, 2004). However, there have been conflicting reports that NLRP3-deficient animals are resistant to DSS-induced colitis (Bauer et al., 2010). The study by Bauer *et al.* determined that IL-1β and IL-18 mediated inflammation in the pathogenesis of IBD, therefore the inability to produce of these cytokines protects the NLRP3-deficient animal.

Basel level inflammasome activity of NLRC4 in regulating responses against commensal bacteria in the intestines is described above (Franchi et al., 2012a).

#### *NLRP6*

NLRP6 has also been linked to IBD syndromes as NLRP6-deficient mice are more susceptible to DSS-induced colitis, just like the NLRP3 mice (Chen et al., 2011), and another linked the absence of NLRP6 to reduced IL-18 levels and a deregulation of gut flora (Elinav et

al., 2011). It was speculated that NLRP6 in the latter model provides a steady-state level of IL-18 production, and the reduction of this basal level provides opportunity for suppressed microbiota to grow.

*NLRP12*

Two recent papers have highlighted the role of NLRP12 in intestinal homeostasis. NLRP12-deficient mice were highly susceptible, like other NLR-deficient mice, to DSS-induced colitis due to an increase in NF-κB activation resulting increased chemokine production (Allen et al., 2012). Moreover, this study found that NLRP12-deficient mice developed colitis-induced colon cancer presenting with an increase of target cancer genes (Allen et al., 2012). Allen *et al.* proposed a mechanism that deregulated NF-κB activation, due to NLRP12-deficiency, lead to these findings. This study was followed up by Zaki *et al.* who also found that NLRP12-deficient animals were more susceptible to DSS-induced colitis and colorectal tumorigenesis (Zaki et al., 2011b). Zaki *et al.* noted an increase in cellular recruitment to the spleen, colon and MLN (mesenteric lymph nodes) in the NLRP12-deficient animals during DSS diet, as well as an increase in some pro-inflammatory cytokines in the colon of these animals, however, neither IL-1β nor caspase-1 activity was analyzed. This study also showed that macrophages lacking NLRP12 have an increase in NF-κB activation pathways, though only at later time points (Zaki et al., 2011b). Taken together, these studies show that mice lacking NLRP12 develop DSS-induced colitis faster then WT mice, which mimmic NLRP3-deficient animals, however not through a inflammasome / caspase-1 mediated pathway. The latter of the two studies does notice a reduction of caspase-1 activity in macrophages from the NLRP12-deficient animal stimulated with bacteria and synthetic ligands.

### *Summary of Inflammasomes in Innate Immunity*

Inflammasome activation is a key event in the response to pathogens. However, this is a double-edged sword as dysfunction and dysregulation can drive human inflammatory diseases, and there is a need for balance between resolution of infection and excessive inflammation. Thus, we will likely see further studies of regulators of inflammasome activity and infections (Rathinam et al., 2012a). It is interesting to note the relationship of the different inflammasome-forming NLRs during the protection against colitis and bowel-inflammation versus anti-pathogenic processes. Many of the NLRs described utilize and are involved in common activation and regulatory pathways. Moreover, some pathogens are capable of activating multiple inflammasomes, such as *Y. pestis*, *Salmonella*, and *L. monocytogenes* (Broz et al., 2010; Kim et al., 2010; Vladimer et al., 2012); and cooperation and synergism between multiple NLRs or AIM2 and specific caspases during infections is likely to be the focus of future research. Total overview of NLR activation by bacterial pathogens is in **Table 1.1**. Continuing investigation into inflammasome activation mechanisms, including proposed upstream activators such as cathepsins, ROS, GBP5, PKR and PKC will drive our understanding of inflammation and hopefully elucidate novel drug targets for both antimicrobial and anti-inflammatory uses.

### *Cytosolic nucleic acid recognition / RIG-Like Receptors (RLRs)*

#### *Cytosolic RNA detection*

Foreign nucleic acid within the cytosol is an extremely potent PAMP, and elicits a strong innate immune response. Viruses depend on the host machinery for nucleic acid replication, and moreover, bacterial and viral lysis causes the release of a variety of nucleic acid species (Hornung and Latz, 2010; Barbalat et al., 2011). Both double and single stranded DNA and

RNA, as well as some tertiary structures have been shown to be innate immune activators (Kato et al., 2006; Poeck et al., 2009; Rathinam et al., 2010; Keating et al., 2011; Sharma et al., 2011).

The RLR family contains two major receptors: RIG-I and MDA5 (Barbalat et al., 2011), which are both known to detect poly(I:C) in the cell cytoplasm (Kato et al., 2008). Longer polymers of poly(I:C) are preferentially recognized by MDA5, whereas smaller polymers are recognized by RIG-I (Kato et al., 2008). RIG-I has also been implicated in the cytosolic detection of short, uncapped 5'-triphosphate ssRNA (Hornung et al., 2006). Therefore, RIG-I can discriminate between self/nonself based on the absence of the 7-methyal-guanosine cap group on viral mRNA.

RIG-I and MDA5 signal IRF3 activation though the adapter protein MAVS (mitochondrial antiviral signaling) (Kawai et al., 2005). MAVS contains an N-terminal CARD domain that mediates the RLR/IRF3 activation axis, and is located in the mitochondrial outer membrane (Kawai et al., 2005). RIG-I also must also be ubiquinated by the E3 ligase TRIM25 (tripartite motif-containing 25), in order for recruitment of MAVS and subsequent signaling (Gack et al., 2007). The ubiquitination of RIG-I may "prime" the receptor before binding viral RNA, which may work as a control mechanism.

As TRIM25 is upstream of RIG-I signaling, STING (stimulator of IFN gene) may be downstream (Ishikawa and Barber, 2008). STING is a transmembrane protein implicated in RIG-I signaling via MAVS but not for MDA5 (Ishikawa and Barber, 2008), although the exact role for STING still remains controversial (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Nakhaei et al., 2009; Barbalat et al., 2011).

*Cytosolic DNA detection*

To date, knowledge of DNA detection, excluding the discovery of AIM2 described above, in the cytosol is still limited. The transfection of poly(dA:dT) (poly(deoxyAdenylic-Thymidylic)) DNA into cells activates IRF3, independently of TLRs (Stetson and Medzhitov, 2006), but though an unknown receptor. It is known that transfected poly(dA:dT) can be transcribed by RNA polymerase III (PolIII) to generate 5‘cap-deficient RNAs for signaling though RIG-I (Hornung et al., 2006). DAI (DNA-dependent activator of IRF) has also been shown to bind DNA and activate IRF3 for downstream type 1 IFN production in L929 cells (Takaoka et al., 2007). However, DAI-deficient animals do not present a phenotype (Ishii et al., 2008), possibly due to a redundancy of cytosolic DNA receptors.

## *Yersinia pestis*

### Overview

*Yersinia pestis*, the Gram-negative causative agent of plague, is estimated to have killed 200 million people over three pandemics (Perry and Fetherston, 1997). Though plague outbreaks are rare and infections can be treated through the early use of antibiotic therapy, there is still great concern over the possibility of weaponized *Y. pestis* as a bioterrorism agent (Butler, 2009). *Y. pestis* infects via two routes: bubonic, and pneumonic, which both lead to septicemic spread (Perry and Fetherston, 1997); all are treatable if intervention is started within 24 hours after the onset of symptoms. Bubonic plague is transmitted between mammalian hosts when a flea takes a blood meal from an infected rodent and then another (Perry and Fetherston, 1997). The bacterium is then trafficked by macrophages to the draining lymph node where it escapes and replicates rapidly, thereby causing buboes (enlarged lymph nodes), necroses, and eventually death of the host (Perry and Fetherston, 1997; Guinet et al., 2008).

The development, and continued spread, of plague requires that *Y. pestis* establish a systemic infection characterized by very high bacteremia, exceeding $10^8$ per ml of blood; this is after transfer of only a few bacteria into the intradermal skin layer delivered by a flea bite (Perry and Fetherston, 1997; Hinnebusch, 2005). Large foci of plague infection are usually devoid of inflammatory cells, therefore, plague can cause bacteremia while avoiding innate immune activation. Once *Y. pestis* has become systemic, it can colonize the lung and can spread person-to-person via aerosol droplets, which defines Pneumonic plague (Perry and Fetherston, 1997).

To evade and quell local inflammation, *Y. pestis* uses several mechanisms that are necessary for virulence and the factors are encoded on three “virulence plasmids”. These factors

include a type III secretion system (T3SS), which delivers multiple effector proteins to the cytoplasm of host cells in contact with the bacteria, and Pla, an outer membrane protease with plasminogen activator activity (Sodeinde et al., 1992; Cornelis, 2002b). Extracellular release of the T3SS-associated protein LcrV has also been suggested to contribute to immune suppression (Pouliot et al., 2007).

Many Gram-negative bacteria, including *Y. pseudotuberculosis*, a very close ancestor of *Y. pestis*, produce a hexa-acylated lipid A and LPS, which has the potential of strongly triggering innate immunity via TLR4-MD2 signaling (Therisod et al., 2002; Rebeil et al., 2004; Raetz Cr, 2007; Munford, 2008). In contrast, *Y. pestis* generates a tetra-acylated lipid A/LPS that poorly induces TLR4-mediated cellular activation (Kawahara K, 2002; Knirel et al., 2005; Montminy et al., 2006; Rebeil et al., 2006). We have reported that expression of *E. coli lpxL* in *Y. pestis*, which lacks a homolog of this gene, forces the biosynthesis of a hexa-acylated lipid A/LPS and that this single modification dramatically reduces virulence in wild-type mice, but not in mice lacking a functional TLR4 (Montminy et al., 2006). This emphasizes that avoiding activation of innate immunity is important for *Y. pestis* virulence.

**Evolution of highly pathogenic *Yersinia pestis***

The genus *Yersinia* contains three pathogenic species: *Y. pestis*, the causative agent of plague, and the enteric food- and water-borne pathogens *Y. pseudotuberculosis* and *Y. enterocolitica*, which cause self-limiting gastroenteritis. Plague is responsible for the deaths of approximately 200 million people in Europe and North Africa during two pandemics termed Justinian's plague (541–767 A.D.) and the Black Death (1346–19th century) (Achtman et al.,

2004). *Y. pestis* is classified into three biovars. Orientals, Medievalis, (estimated to be responsible for the Black Death), and Antiqua (DEVIGNAT, 1951; Achtman et al., 2004). The biovars are have distinct IS100 insertion elements, DNA islands, and multilocus variable number of tandem repeats (Achtman et al., 1999; Deng et al., 2002; Morelli et al., 2010).

*Y. pestis* evolutionarily emerged from *Y. pseudotuberculosis* approximately 2,603–28,646 years ago, and shares strong sequence homology, making exact dating difficult (Achtman et al., 1999; Morelli et al., 2010). Though these pathogens are so closely related, the clinical manifestations are dramatically different (Perry and Fetherston, 1997). There have been reports of sequence comparisons of *Y. pseudotuberculosis* to *Y. pestis* which identified 32 *Y. pestis* chromosomal genes and two *Y. pestis* specific virulence plasmids that represent the only new genetic material that *Y. pestis* acquired since the divergence (Chain, 2004). In contrast, 317 genes, and 149 pseudogenes, from *Y. pseudotuberculosis* are absent from *Y. pestis*, indicating that nearly 13% of genes no longer function in *Y. pestis* (Chain, 2004). Chain *et al.* also reported many genomic rearrangements and reductive genetic evolution through gene loss, which resulted in elimination and modification of gene expression pathways. This appeared to be more important for plague virulence than acquisition of genes.

Necessary to the virulence of all *Yersinia* is the shared requirement of a virulence plasmid which encodes a type III secretion system which is responsible for injecting a number of effectors termed Yops (Yersinia outer proteins) into host cells that inhibit bacterial phagocytosis and pro-inflammatory pathways (Michiels et al., 1990; Perry Rd, 1998).

Two additional plasmids unique to *Y. pestis* are necessary during tissue invasion and capsule formation, as well as infection of the plague flea vector(Cornelis, 2002a; Butler, 2009).

However, the presence of these unique plasmids by themselves cannot account for the remarkable increase in virulence observed in *Y. pestis* (Butler, 2009).

Phylogenetic analysis shows that *Y. pestis* evolved in or near China and spread to Europe, South America, Africa, and Southeast Asia, leading to country-specific lineages with unique SNPs (single nucleotide polymorphisms) (Morelli et al., 2010). Moreover, only slight genetic drift in important *Y. pestis* virulence factors have been detected in the 660 years of evolution as a ruthless human pathogen, suggesting that the previous increase in virulence may not only be due to single molecular changes, but is one component of a number of factors including population, living conditions, and vector dynamics which caused the major outbreaks (Bos et al., 2011).

## Virulence factors of *Yersinia pestis*

The success of *Yersinia pestis* as a human pathogen comes from its ability to promote bacterial survival and transmission while disrupting normal cellular function and thwarting host defense responses. Plague has three virulence plasmids, as previously mentioned, and a number of virulence factors within the genome that all play a role in pathogenesis, (outlined in **Table 1.2**) (Perry and Fetherston, 1997; Kraushaar et al., 2011).

### *Genome encoded virulence factors*

#### *Lipopolysaccharide and TLR4 evasion*

LPS, which decorates the outer membrane of Gram-negative bacteria and is anchored to the membrane by lipid A (endotoxin) (Raetz Cr, 2007), is an extremely potent immune activating PAMP. Lipid A is a di-glucosamine molecule covalently modified with fatty acid chains and phosphate groups; it is synthesized by eight enzymes encoded in the genome of Gram-negative bacteria (Brozek and Raetz, 1990).

The eight to nine responsible enzymes are highly-conserved among Gram-negative bacteria, however, the composition of the acyl-chains can vary widely between different, and the same, bacterial species. The number of, and modifications to, acyl-chains can be effected by many environmental factors (Raetz Cr, 2007). The majority of Gram-negative bacteria are not viable when lacking early enzymes in the lipid A biosynthesis pathway, however, bacteria lacking the late-acyl-transferases are generally able to recover (Clementz et al., 1996; Zhou Z, 1999; Rebeil et al., 2006).

The late-acyl-transferases add the secondary chains to the tetra-acylated lipid A precursor lipid $IV_A$ (Rebeil et al., 2006). All closely related bacterial pathogens to *Y. pestis* express a hexa-acylated lipid A, which has 6 fatty acid chains. This hexa-acylated lipid A signals strongly through the TLR4/MD2 complex as defined above (Lien et al., 2000; Park et al., 2009).

*Y. pestis* has two variations of lipid A depending on the growth conditions (Kawahara et al., 2002); when grown at 26$^{o}$C, *Y. pestis* expresses a hexa-acylated lipid A, just as its other relatives, above (Montminy et al., 2006). However, when grown at 37$^{o}$C, *Y. pestis* has a tetra-acylated lipid A, which is lacking the two secondary acyl-chains (Kawahara et al., 2002; Montminy et al., 2006). These two temperatures are important to the plague life-cycle as they are the major growth temperatures of the two hosts: flea and mammal. The lipid A that is expressed at 37$^{o}$C is not TLR4 stimulatory, and this was described by Montminy *et al.* as a major virulence factor (Montminy et al., 2006). Moreover, purified LPS from *Y. pestis* grown at 37$^{o}$C inhibits TLR4 activation by TLR4-activating LPS (Montminy et al., 2006). This lipid A phenotype is due to the evolutionary loss of a homolog of the *E. coli* LpxL, a late-acyl transferase, from *Y. pestis*. *Y. pestis* can express hexa-acylated lipid A at 26$^{o}$C as it upregulates expression and activity of a

cold-shock-induced late-acyl-tranferase, LpxP. LpxL and LpxP add the second to last secondary acyl-chain to Lipid $IV_A$, the last chain is added by a conserved LpxM which has temperature-independent activity(Montminy et al., 2006; Feodorova et al., 2009). This LPS expression model will be referred to in the following chapters.

*Iron Assimilation*

Also expressed within the genome is the ability for *Y. pestis* to sequester and store iron as a nutrient, allowing it to grow in iron-deficient environments (Perry and Fetherston, 1997). *Y. pestis*, for biosafety is classified into two groups: pigmented ($Pgm^+$) and non-pigmented ($Pgm^-$), the latter has a spontaneous 102kb deletion from its genome responsible for iron assimilation. The term pigmentation comes from the absorption of exogenous heme, causing pigmented colonies when growing on particular media (Perry and Fetherston, 1997). The $Pgm^-$ strains are avirulent unless hemin, ferrous sulfate, or ferric chloride is injected with the pathogen. However, in 2009 a naive researcher became infected and later died from bubonic plague following contact with a $Pgm^-$ strain due to his possible hemochromatosis-induced iron overload which was able to supplement the lack of virulence (Centers for Disease Control and Prevention (CDC), 2011).

Within the Pgm locus are the genes for hemin storage (Hms), as well as genes for iron uptake. $Hms^+$ bacteria store hemin within the outer membrane when grown at 26$^o$C and this has been shown to be essential for flea blockage, but its role in mammalian infection is unknown (Hinnebusch, 2005).

| Location | Gene / products | Function |
|---|---|---|
| Genome | | |
| | loss of LpxL | Synthesis of lipid-A unrecognized by TLR4. Assists in immune evasion |
| | Pigmentation locus | Iron assimilation and storage for growth in low iron environments |
| pPCP1 Plasmid | | |
| | Pesticin | Antibiotic |
| | Pesticin immunity protein | Blocks effect of pesticin |
| | Plasminogen activator | Coagulase and fibrinolytic activity |
| pCD1 Plasmid | | |
| | T3SS | Yop Delivery |
| | YopB / YopD | Pore forming |
| | LrcV | T3SS tip |
| | YopH | Tyrosine phosphatase - dephosphorylates focal adhesions |
| | YopE | GTPase-activating protein - antiphagocytic |
| | YopT | Cysteine protease - actin-depolymerization |
| | YpkA | Autophosphorylating serine/threonine kinase - actin-depolymerization |
| | YopJ | Serine/threonine acetyltransferase /de-ubiquitinase - anti-inflammatory |
| | YopK | Function unknown, assocates at tip of T3SS possibly regulating Yop delivery |
| | YopM | LRR containing Yop, possibly binds host factors |
| pMT1 Plasmid | | |
| | F1 capsular antigen | inhibition of phagocytosis |
| | Murine Toxin | β-adrenergic blocking and flea transmission |

**Table 1.2: Summary of Plague Virulence Factors**

*Y. pestis* contains numerous important virulence factors which are encoded in the genome or on three virulence plasmids. pCD1 is conserved among *Yersinia* family members and pMT1 and pPCP1 were obtained upon evolutionary divergence from *Y. pseudotuberculosis*.

### *Virulence Plasmids*

The *Y. pestis* derivative KIM (Kurdistan Iran Man; from where it was isolated) contains three virulence plasmids: pPCP1 (pesticin, coagulase, plasminogen activator), pCD1 (calcium dependence), and pMT1 (murine toxin) (Perry and Fetherston, 1997); only the pCD1 plasmid is conserved among other *Yersinia* species (called pYV1 in *Y. pseudotuberculosis*). The plasmids are described below from smallest to largest.

#### *pPCP1 Plasmid*

The 9.5kb pPCP1 plasmid encodes three virulence genes. Pesticin (*pst*), a secreted bacteriocin which kills related surrounding bacteria within the same niche by hydrolyzing peptidoglycan (Perry and Fetherston, 1997). *Y. pestis* pPCP1 also expresses a pesticin immunity protein (*pim)*, localized to the periplasm, which inactivates the bacteriocin thereby protecting itself from destruction (Pilsl et al., 1996).

Most importantly, pPCP1 contains the gene for the plasminogen activator, Pla, a surface protease and proven virulence determinant. Pla is responsible for coagulase and fibrinolytic activity during infection (Sodeinde et al., 1992; Perry and Fetherston, 1997). Pla-deficient pathogens were significantly less virulent upon subcutaneous or intranasal infection (Sodeinde et al., 1992; Lathem et al., 2007), but had comparable virulence when injected intravenously (Sodeinde et al., 1992). This lead to the hypothesis that Pla protease's function was to promote bacterial dissemination from the local tissue/site of infection.

#### *pCD1 Plasmid, secretion system, and Yops*

The calcium dependence plasmid encodes the low-calcium response stimulon (LCRS) which controls the expression of the T3SS that delivers secreted effector proteins termed Yops (*Yersinia* outer proteins) to the cytosol of host cells (Michiels et al., 1990; Cornelis, 2002a).

Expression of pCD1 is highest at 37$^{o}$C in the absence of millimolar concentrations of calcium, thereby expressing virulence factors at the highest level when in a mammal and close to host cells (Higuchi et al., 1959).

The Yops have multiple functions: YopB and YopD are secreted by the system and make up the pore in the host membrane (Neyt and Cornelis, 1999) while LcrV (V antigen) forms the tip of the needle apparatus (**Table 1.2**) (Price Sb, 1991). Seven secreted Yops have been identified: YopH, YopE, YopT, YpkA, YopJ, YopM, and YopK (activity outlined in **Table 1.2**) (Perry Rd, 1998).

Four of the Yops (YopH, YopE, YopT, and YpkA) limit phagocytosis by inhibiting dynamics of the cytoskeleton of macrophages and neutrophils (Perry and Fetherston, 1997; Andersson et al., 1999; Black et al., 2000). YopH is a tyrosine phosphatase which dephosphorylates focal adhesions thereby reducing phagocytosis, as well as lymphocyte proliferation (Black and Bliska, 1997). YopE, YopT and YpkA (Yersinia protein kinase A) act on monomeric GTPases of the Rho family which are anchored to the cytoplasmic face of the plasma membrane of host cells and control actin polymerization/depolymerization (Cornelis, 2002a). Specifically, YopE acts as a GTPase-activating protein which changes the GTPases on the inside of the plasma membrane ‘off’ by speeding up GTP hydrolysis yielding phagocytosis activity (Black and Bliska, 2000). YopT, a cysteine protease cleaves Rho GTPases and detaches them from the membrane causing actin-depolymerization (Iriarte and Cornelis, 1998; Zumbihl et al., 1999). Lastly, YpkA, an autophosphorylating serine/threonine kinase, becomes activated after actin interaction and assists in actin-depolymerization and changes in cytoskeleton dynamics (Håkansson et al., 1996; Juris et al., 2000).

YopJ can inhibit pro-inflammatory signal transduction by modifying various proteins upstream from the transcription factors MAPK and NF-κB, and blocking production of downstream cytokines (Boland and Cornelis, 1998; Orth K, 2000; Sweet et al., 2007; Paquette et al., 2012), though the exact mechanism is still under investigation. YopJ from *Y. pestis* has been described as a serine/threonine acetyltransferase that can modify critical residues in factors such as TAK1 and IKKβ upstream of MAPK and NF-κB activation (the latter in **Figure 1.4**) (Orth K, 2000; Paquette et al., 2012). YopP of *Y. pseudotuberculosis* (the homologue of YopJ) uses the TAK1 acetylation to subvert NOD2 activity in the gut, leading to intestinal barrier dysfunction (Meinzer et al., 2012). This YopJ-induced modification competes directly with the phosphorylation that occurs during normal activation. Therefore, YopJ also blocks pro-survival signals and is considered a pro-apoptotic virulence factor (Cornelis, 2000).

YopJ has also been described to have de-ubiquitinating function and interferes with TRAF6/3 signaling downstream of TLR activation, thereby blocking MAPK, NF-κB and IRF activation (**Figure 1.4**) (Sweet et al., 2007).

It is possible that YopJ contains both of these features and has multiple roles in halting pro-inflammatory cytokine translation downstream from transcription factor activation.

Multiple groups have also shown that YopJ activity is necessary for caspase-1 activation and downstream pro-IL-1β and pro-IL-18 processing (Brodsky et al., 2010; Zheng et al., 2011; 2012), although the direct mechanism has not been elucidated. It has been suggested that the inhibitory effect of YopJ on the NF-κB pathway leads to inflammasome activation in a NLRP3, ASC, and caspase-1 dependent manner, and does not necessarily activate the NLR directly (Zheng et al., 2011). YopJ activity was also necessary for the apoptosis of infected cells (Lilo et

al., 2008). The cell-death pathway activated seemed to depend on the activation state of the cells; activated cells died by the more pro-inflammatory pyroptosis pathway instead of apoptosis (Bergsbaken and Cookson, 2009; Brodsky, 2012). This YopJ-induced caspase-1 activation and cell death was shown to be independent of mitochondrial-directed apoptosis, but is involved in necrosis of infected cells (Zheng et al., 2012). YopJ has a strong role within cells *in vitro;* however, strains lacking YopJ have shown no changes in virulence during animal infections.

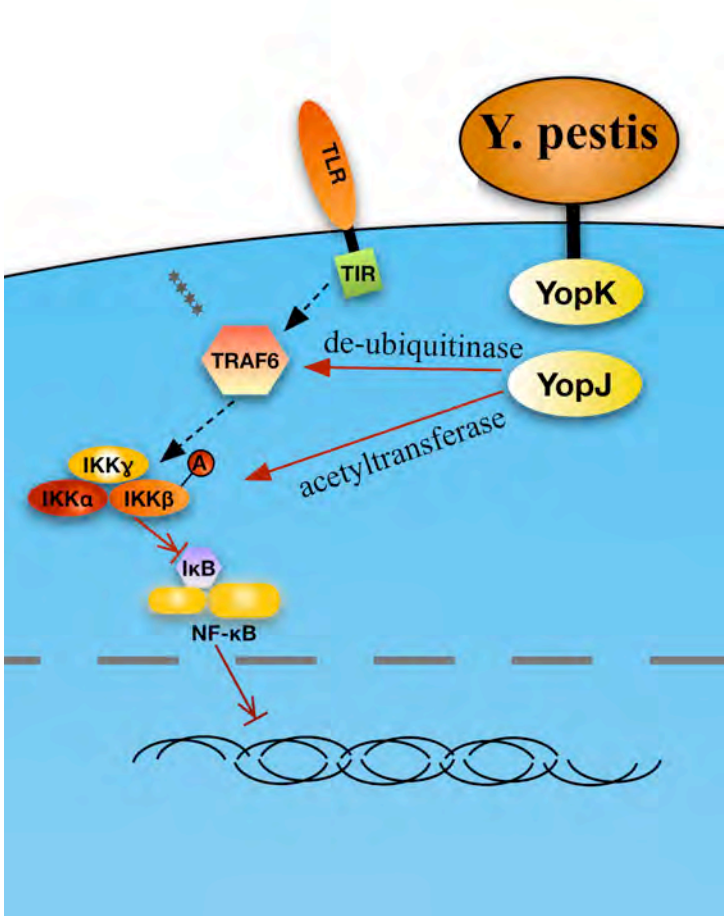


**Figure 1.4: Activity of *Y. pestis* YopJ and location of YopK**

YopJ plays a dual role inside cells during *Y. pestis* infection. As an acetyltransferase, YopJ acetylates activators, IKKβ here, of pro-inflammatory transcription factors (such as NF-κB). The acetyl group blocks the normal phosphorlation-dependent activation rendering the pathway off. YopJ has also been described to have a de-ubiquitinase function, removing the K-63 linked poly-ubiqutin chains from TRAF proteins upstream from pro-inflammatory transcription factors. Red lines with arrows are YopJ function, red lines with stop arrows are blocked pathway function, black dotted arrows are summarized pathways,'A' is the acetyl group, and the gray stars are K63-linked poly-Ub.

YopK, which shares no sequence identity with other known proteins, has been shown to play a role in Yop regulation and delivery, and possibly localizes to the tip of the T3SS with LcrV inside the host cell (Holmstrom et al., 1995; Holmström et al., 1997; Brodsky et al., 2010; Dewoody et al., 2011). YopK also has been linked to regulating inflammasome activation during macrophage infection with an engineered *Y. pseudotuberculosis* strain, as YopK-deficient strains cause an increased caspase-1 activity, however the mechanism of this is still unknown (Brodsky et al., 2010).

The role of YopM during plague infection also has yet to be determined fully; it contains an N-terminal LRR which makes up most of the protein and is shaped in a TLR-like horseshoe structure, and possibly binds eukaryotic proteins (Evdokimov et al., 2001). YopM, after injection, traffics to the nucleus of macrophages in a vesicle-dependent manner (Skrzypek et al., 1998). Bubonic infection of C57BL/6 mice with fully virulent plague versus a strain lacking YopM showed markedly decreased virulence, though this was not the case during pneumonic plague (Ye et al., 2009). Very recently, YopM was implicated in the binding of caspase-1 and therefore restricting inflammasome formation (LaRock and Cookson, 2012). However, only *Y. pseudotuberculosis* infections with YopM deficient strains were performed and the relation to plague infection us unknown.

*The pMT1 Plasmid*

pMT1 is the largest virulence plasmid, but only encodes two known virulence factors: murine toxin (Ymt) and the structural gene for fraction 1 (F1) protein capsule antigen (Perry and Fetherston, 1997). At 37$^{o}$C, the F1 protein is expressed and forms a gel-like capsule responsible for blocking phagocytosis by macrophages, possibly by limiting receptor interaction (Perry and Fetherston, 1997; Du et al., 2002). Ymt refers to a protein which has β-adrenergic blocking

ability, and when released from lysing bacteria during systemic plague infection, causes vascular collapse and hypotension (Montie, 1981). However, the expression of Ymt is higher at 26$^{o}$C, and was discovered that the expression is necessary for flea transmission (Hinnebusch et al., 2002).

*Y. pestis* replicates in the midgut of fleas and forms cohesive aggregates, eventually blocking normal feeding; the blockage is necessary for efficient transmission as blocked fleas feed more often (Perry and Fetherston, 1997). *Y. pestis* expressing a mutant Ymt could not cause flea blockage, and therefore, fleas infected with the mutant had decreased transmission rates (Hinnebusch et al., 2002).

**Plague Vaccines**

*Yersinia pestis* easily avoids innate host defenses, however, animals which survive initial infection effectively resist re-infection (Perry and Fetherston, 1997). The ability to resist infection suggests that an efficient vaccine that confers strong protection can be created. Formalin-killed whole bacteria were licensed and sold in the United States as a plague vaccine and given to members of the US military during the Vietnam War (Butler, 2009). While the vaccine protected against bubonic plague, it did not protect against pneumonic plague. Furthermore, the vaccine would cause adverse reactions after booster injections (Cavanaugh et al., 1974). This led to the understanding that killed whole-cell vaccines will probably not protect against weaponized plague.

Subunit vaccines targeting F1 protected mice and rats against bubonic and pneumonic plague (Feodorova and Corbel, 2009). However, using F1 alone does not protect against F1-negative *Y. pestis* strains (Worsham et al., 1995). Single subunit vaccines using recombinant LcrV work well to protect against pneumonic and bubonic plague, with or without F1 capsule

protein (Anderson et al., 1996). However, there is evidence that plague strains express LcrV variants, and therefore, this subunit vaccine may not confer cross-protective immunity (Roggenkamp et al., 1997). Putting these two subunit vaccines together and creating an F1/LcrV fusion may provide greater protection against animal challenge (Williamson et al., 1995; Heath et al., 1998).

Live attenuated plague vaccines have also been developed; the use of a live and virulent *Y. pestis* expressing a TLR4 stimulating lipid A (described above) can protect against pneumonic and bubonic plague, however the strains described were still considered controlled pathogens and could pose a threat (Montminy et al., 2006).

## Thesis Rationale, Objectives, and Summary

*Yersinia pestis* has a dark history as an exceptionally powerful pathogen causing widespread death while directing centuries of cultural changes. *Y. pestis* kills by evading innate immune system activation while multiplying to almost $10^8$ bacilli per milliliter of blood resulting in massive bacterial sepsis (Perry and Fetherston, 1997). *Y. pestis* effectors stop immune signaling while halting phagocytosis, dissolving clots to assist in dissemination, all while hiding itself from the host by expressing modified lipids on the its surface (Sodeinde et al., 1992; Cornelis, 2002a; Rebeil et al., 2004). The innate immune system can counteract most pathogens using TLR and NLR mediated surveillance to elicit an inflammatory response to assist in clearance, and the connection between plague, the cytosolic NLRs, and downstream pathways had yet to be elucidated.

We discovered that expressing a single gene, LpxL, resulted in a hexa-acylated lipid A on the surface of *Y. pestis*, and that this could protect mice from initial pneumonic and bubonic infection, as well as challenges in a TLR4-dependent manner (Montminy et al., 2006). Using this modified strain, which effectively activates the innate immune system, as well as wild type plague, we sought to elucidate the inherent control mechanism that plague can bypassed during infection. As an important cytosolic receptor, it was our hypothesis that the inflammasome was necessary for eliciting a strong inflammatory response during plague infection, and that an effector of *Y. pestis* either activated or controlled inflammasome function. Moreover, we worked to elucidate the evolutionary history of *Y. pestis* loss of LpxL and how this may have played a role in inflammasome signaling.

Overall, we determined that *Y. pestis*, either wild type or expressing a hexa-acylated lipid A, activates the NLRP3, NLRC4 and NLRP12 inflammasomes in macrophages in a T3SS / YopJ dependent manner. These inflammasomes are necessary for animal survival during bubonic plague infection with the LpxL modified strain, and during pneumonic plague when infected with WT plague. Moreover, the downstream cytokines IL-18, IL-1β, and IFNγ were extraordinarily important for protection against subcutaneous or respiratory infection. With this model we also determined a novel important pro-inflammatory role for the NLRP12 inflammasome, which had not been previously elucidated.

This work suggests that the inflammasome components are prime targets of *Y. pestis* ligands and strongly initiate important immune function for the clearance of plague. By studying how plague evades innate immune pathways, we can uncover more successful vaccine strategies and therapeutic targets, as well as novel roles for known receptors. As more and more drug

resistance bacterial strains emerge, the importance of having a broader range of understanding of how pathogens and the immune system cooperate will be more important then ever.

# PREFACE to CHAPTER 2

This section is a version of a manuscript currently being drafted:

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This chapter represents a thesis project of G.I.V., who generated the *in vitro* stimulation and *in vivo* infection data. Others gathered data indicated below. G.I.V. created the figures from primary data, and was the principal writer and coordinator of the manuscript text.

Co-authors contributed the following data:
S.W.M.P. and D.W. provided purified lipids and analyzed the data from K.L.S., A.S.B., R.J.C. MALDI-TOFF M/S in Figures 2.1 A-B, H-I, 2.2 A-C, 2.4, 2.5 C-D. S.W.M.P. provided the data in figure 2.1 D, 2.2 2.2 D-E, and 2.3. S.W.M.P. constructed the pYtbLpxL plasmid, M.M.P. constructed the IP2666ΔLpxL strain, D.W. constructed the pEcLpxL and pYtbLpxLE172A plasmids. E.L. assisted with *in vivo* cytokine and c.f.u. sample collection and analysis.

Note: Chapter 2 was published after Chapter 3 and therefore Chapter 3 is referenced multiple times in this work.

# CHAPTER 2

## Evolution of virulence in *Yersinia pestis* by loss of lipid A acyl-transferase LpxL: impact on NLRC4 inflammasome activation

***Abstract***

*Yersinia pestis* has several means of evading host defenses. We propose that its inability to synthesize TLR4-activating hexa-acylated LPS caused by loss of lipid A biosynthesis enzyme LpxL was one of the key determinants for increased virulence during the evolutionary split of *Y. pestis* from the closely related *Y. pseudotuberculosis*. Modified *Y. pestis* generating a *Y. pseudotuberculosis*- or a *Y. enterocolitica*-like hexa-acylated lipid A are largely attenuated. Here, we implicate the inflammasome molecule NLRC4 in host responses against *Y. pestis* (and *Y. pseudotuberculosis*). NLRC4-deficient animals produced less IL-18, IL-1β and IFNγ upon infection with *Y. pestis*, and survival of animals infected with *Y. pestis* was NLRC4 dependent. We suggest an evolutionary advantage in development of high virulence for *Y. pestis* by subverting detection via TLR4, with a subsequent reduction in activation of inflammasomes.

***Introduction***

The success of *Y. pestis* as a pathogen comes from its ability to evade immune detection early during infection utilizing several virulence factors. As a result, plague is characterized by very high bacterial numbers during septic infection (Perry and Fetherston, 1997). Key factors for immune evasion include a type III section system (T3SS), a membrane bound plasminogen activator (Pla), and a strong iron acquisition system (Cornelis, 2002a; Lathem et al., 2007). The Yersinia T3SS delivers modifying proteins into the host cell cytoplasm that disrupt signaling, induce apoptosis, and limit immune activation functions (Cornelis, 2002a).

Related Gram-negative bacteria, including *Y. pseudotuberculosis*, the direct evolutionary parent of *Y. pestis*, and *Y. enterocolitica*, produce a hexa-acylated lipid A and LPS which can strongly trigger innate immunity via Toll-like receptor 4 (TLR4)-MD-2 signaling (Therisod et al., 2002; Rebeil et al., 2004; Raetz Cr, 2007; Munford, 2008). In contrast, *Y. pestis* generates a tetra-acylated lipid A-LPS that poorly induces TLR4-mediated cellular activation (Kawahara et al., 2002; Knirel et al., 2005; Montminy et al., 2006; Rebeil et al., 2006). This is due to the lack of a late lipid A acyl transferase (LpxL, also called HtrB) by *Y. pestis* needed to produce a hexa-acylated LPS at mammalian body temperature. Previously we have reported that expression of *E. coli lpxL* in *Y. pestis* causes the biosynthesis of a hexa-acylated LPS (Montminy et al., 2006), and that this single addition reduces virulence of plague during bubonic and pneumonic infection in wild type mice, in a TLR4 dependent manner. This change in virulence highlights the importance of immune evasion during *Y. pestis* evolutionary divergence, and creates a model to study the immune pathways necessary for detection of bacterial pathogens.

The three pathogenic Yersiniae share many virulence factors and it is reported that *Y. pestis* may have diverged from *Y. pseudotuberculosis* between 1,500 and 20,000 years ago (Achtman et al., 2004; Chain, 2004; Morelli et al., 2010). This is much more recent than the divergence of *Y. pseudotuberculosis*/*Y. pestis* from *Y. enterocolitca* estimated at 41-186 million years ago (Kraushaar et al., 2011). Despite the recent divergence and close similarity of *Y. pestis* and *Y. pseudotuberculosis* these two pathogens cause very different clinical manifestations in humans. Plague often results in sepsis with high mortality whereas *Y. pseudotuberculosis* and *Y. enterocolitica* mostly cause self-limiting gastroenteritis and mesenteric lyphadenitis. While the later species are transmitted orally, *Y. pestis* is transmitted primarily by the bite of infected fleas or spread of aerosol droplets. Adaptation to this mode of transmission, which imposed requirements for survival in the flea gut and the ability to rapid establish system infection following inoculation into the skin, may have driven the development of high virulence in *Y. pestis* (Hinnebusch, 2005). The production of a tetra-acylated LPS may also be an important factor for better enabling infection via a flea bite at a peripheral site. In this regard, it may be relevant that a number of gastrointestinal Gram-negative pathogens do in fact produce a hexa-acylated LPS, whereas infection via other sites often can be associated with hypo-acylated lipid A (Munford et al., 1982).

TLR signaling is critical for the sensing of pathogens, and one implication of TLR4 engagement is the induction of the pro-forms of the central inflammatory cytokines IL-1β and IL-18 downstream from NF-κB activation. The cleavage of the pro-cytokines into active IL-1β and IL-18 is dependent upon activation of the inflammasome, a cytosolic multi-protein complex consisting of inactive pro-caspase-1 and members of the nucleotide-binding domain-leucine rich

repeat (NLR) family of immune system proteins(Stutz et al., 2009). The assembly of an inflammasome leads to proteolytic activation of caspase-1 which in turn cleaves pro-IL-1β and pro-IL-18 (Latz, 2010). The NLR family is evolutionary conserved and has more than 20 members. Many are proposed to play a role in innate immunity, such as, NOD1/2 (recognizing peptidoglycan fragments), NLRP1 (which senses anthrax lethal toxin), NLRP3 (which is activated by alterations in membrane potential and exposure to many crystal structures and pathogens), NLRC4 (which senses intracellular flagellin and bacterial type III secretion rod proteins), NLRP6 (both inflammasome activation and a potential negative regulator of inflammation), NLRP12 (promotes pro-inflammatory responses against *Y. pestis*) and Naip5 (which promotes *Legionella pneumophila* resistance) (Ye et al., 2008; Kanneganti, 2010; Chen et al., 2011; Horvath and Schrum, 2011; Allen et al., 2012; Anand et al., 2012; Lamkanfi and Kanneganti, 2012; Vladimer et al., 2012).

Here we provide evidence for the loss of LpxL to be a major event in the divergence of *Y. pestis* from *Y. pseudotuberculosis* and the development of differences in virulence between the two pathogens. The absence of LpxL minimizes the incorporation of secondary acyl chains in *Y. pestis* lipid A. The result is a decrease in activation of NLRC4 inflammasome, and reduced subsequent IL-18 and IL-1β release. Our results suggest that the action of NLRC4 is effective in limiting *Y. pestis* growth in vivo. Thus, we have shown that *Y. pestis* is able to activate multiple innflammasomes - NLRP3, NLRP12 and NLRC4 - and we propose that *Y. pestis* needs to minimize strong inflammasome activation in innate immunity by lipid A modification in order to induce lethal bubonic plague.

***Results***

*Y. pestis LPS structure and evolution of virulence.*

At 37°C Y. pestis produces a tetra-acylated LPS with poor stimulatory activity (Kawahara K, 2002; Rebeil et al., 2004; Montminy Sw, 2006). We have earlier expressed *E. coli* LpxL in *Y. pestis*, enabling the production of a TLR4-stimulating hexa-acylated LPS at 37°C, and found that this single modification greatly reduces virulence (Montminy et al., 2006). A comparison of *Y. pseudotuberculosis* and *Y. pestis* genomes shows that *lpxL* is part of a block of *Y. pseudotuberculosis* genes present in *Y. pseudotubercolosis* that has been deleted from *Y. pestis* (Chain, 2004). We cloned *lpxL* from *Y. pseudotuberculosis* IP2666 and expressed it *Y. pestis*, under its own promoter, to to determine its effects on virulence and on the stimulation of TLR4. We found that *Y. pestis* KIM5 expressing *Y. pseudotuberculosis* LpxL on pBR322 (KIM5-pYtbLpxL) produces a hexa-acylated lipid A, comparable to the lipid A from wild type (WT) *Y. pseudotuberculosis* IP2666 (referred to as IP2666) (**Figure 2.1 A,B**). Mass spectrometry analysis revealed that the lipid A composition in *Y. pestis*-pYtbLpxL and *Y. pseudotuberculosis* is strikingly similar, with some heterogeneity in the lipid A species present (**Figure 2.2 A,B,C**). Consequently, purified LPS from IP2666 and KIM5-pYtbLpxL have comparable abilities to induce cellular activation when added to human PBMC or HEK293/TLR4-MD-2 cells (**Figure 2.1 C,D**), and are much more active than LPS from parental *Y. pestis* KIM5. The LPS failed to activate HEK293/TLR2 or mock transfected cells (**Figure 2.2 D,E**).

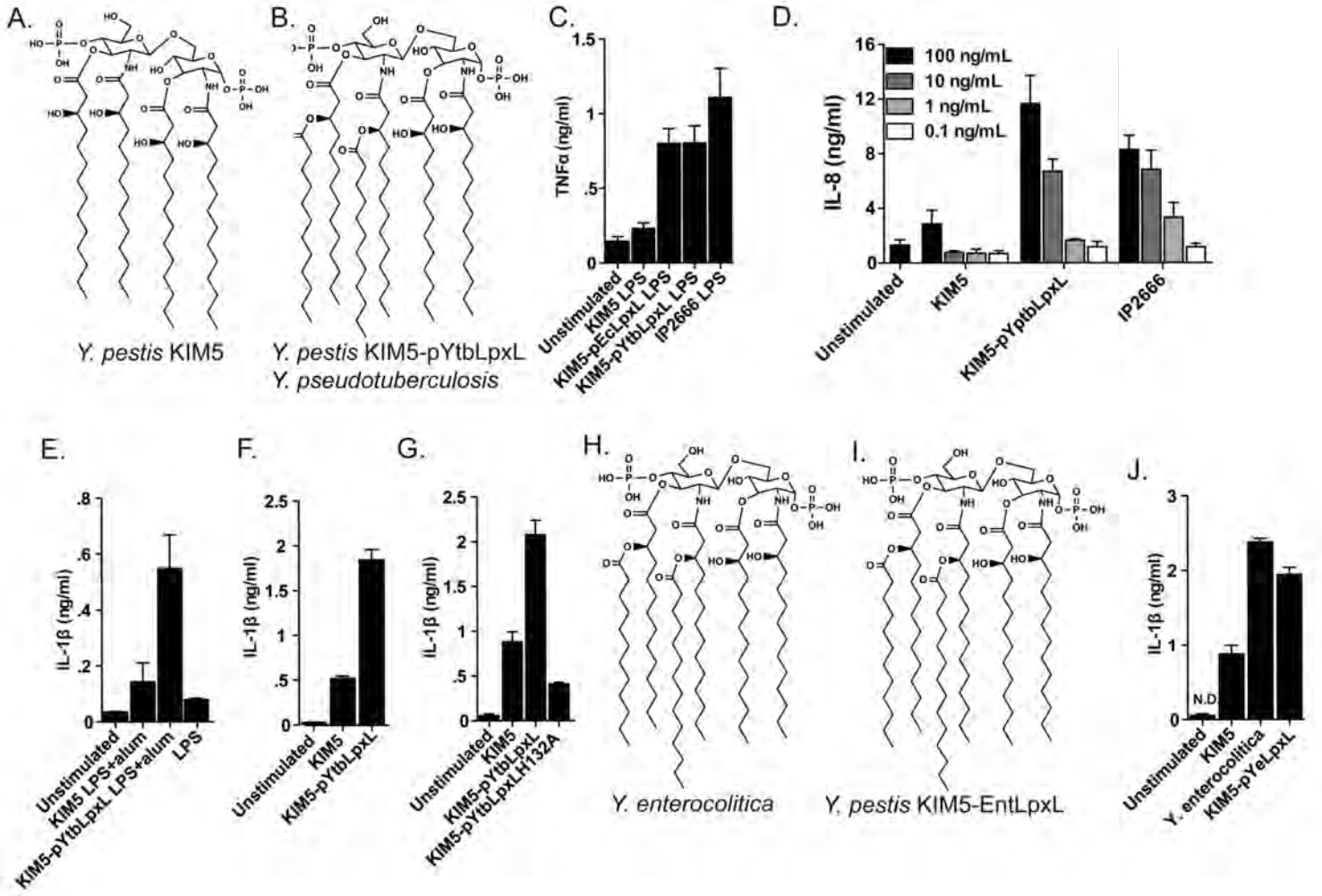
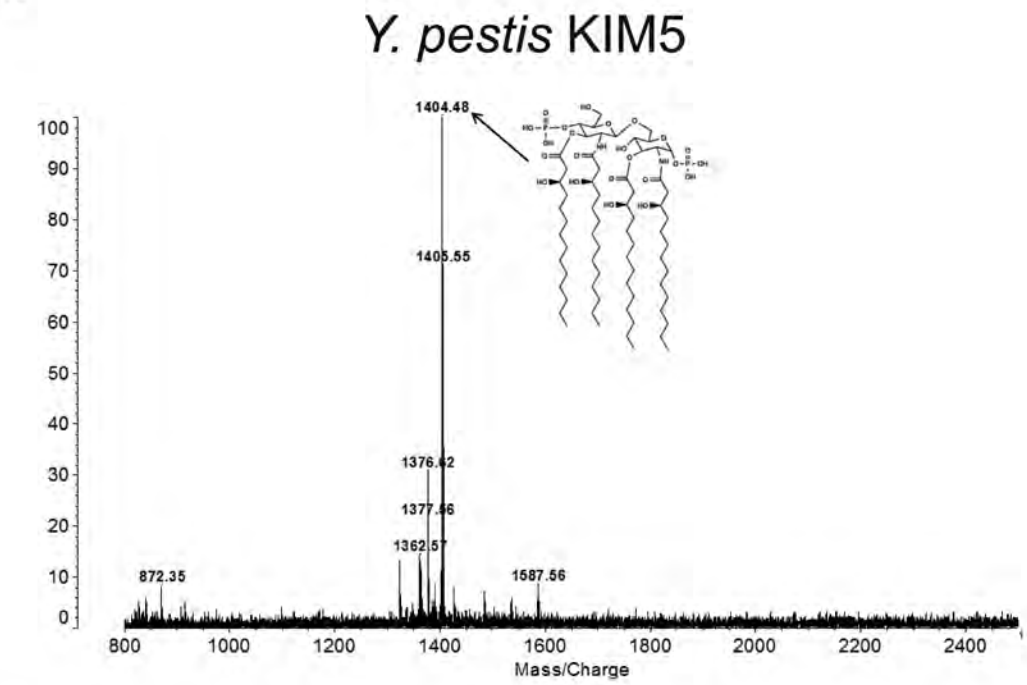

A.
B.
C.
D.
Y. pestis KIM5
Y. pestis KIM5-pYtbLpxL
Y. pseudotuberculosis
TNFα (ng/ml)
Unstimulated
KIM5 LPS
KIM5-pEcLpxL LPS
KIM5-pYtbLpxL LPS
IP2666 LPS
IL-8 (ng/ml)
100 ng/mL
10 ng/mL
1 ng/mL
0.1 ng/mL
Unstimulated
KIM5
KIM5-pYptbLpxL
IP2666
E.
F.
G.
H.
I.
J.
IL-1β (ng/ml)
Unstimulated
KIM5 LPS+alum
KIM5-pYtbLpxL LPS+alum
LPS
KIM5
KIM5-pYtbLpxL
KIM5-pYtbLpxLH132A
Y. enterocolitica
Y. pestis KIM5-EntLpxL
N.D
Y. enterocolitica
KIM5-pYeLpxL

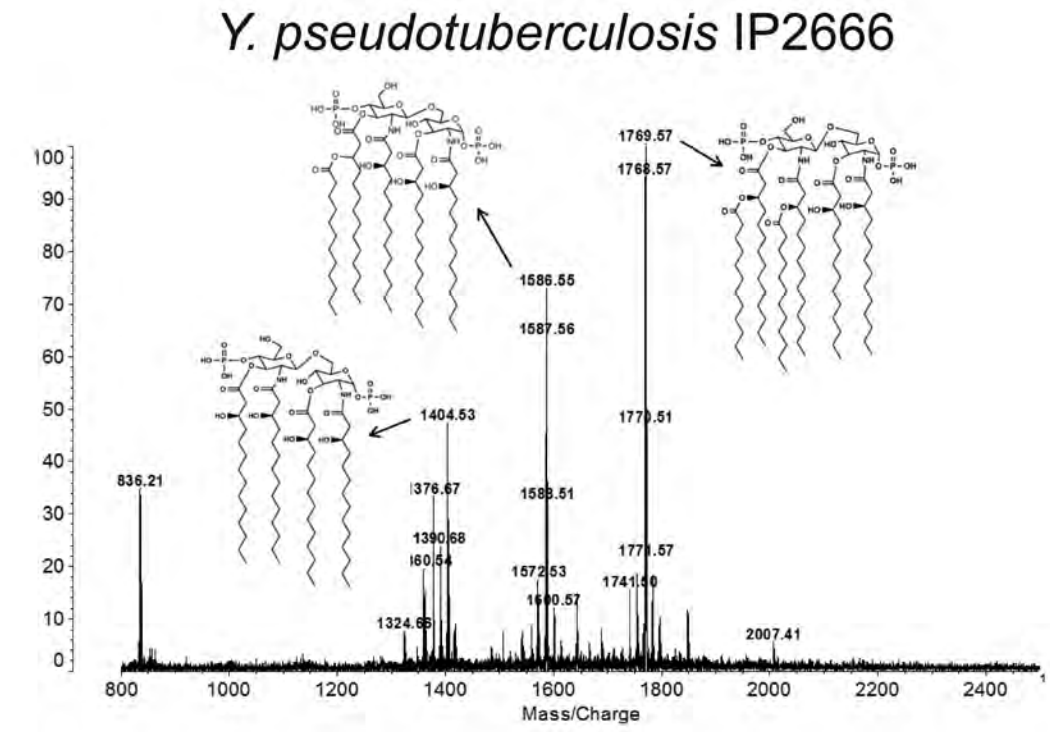
**Figure 2.1: The addition of a lost late-acyl transferase increases immune recognition of *Y. pestis***

(A-B, H-I) Major lipid A structures depicted from mass spectrometry analysis of (A) *Y. pestis* KIM5, (B) *Y. pestis* KIM5-pYtbLpxL and *Y. pseudotuberculosis*, (H) *Y. enterocolitica*, and (I) *Y. pestis* KIM5-EntLpxL all grown at 37$^{o}$C. (C-G,J) ELISA of supernatants after stimulation of (C) human PBMCs (D) HEK293 expressing mouse TLR4/MD2 (E-G, J) or BMDMs for (C) TNF, (D) IL-8, or (F-H, K) IL-1β. Cells were stimulated either overnight (C-D) or for 6 hours (F-H, K).

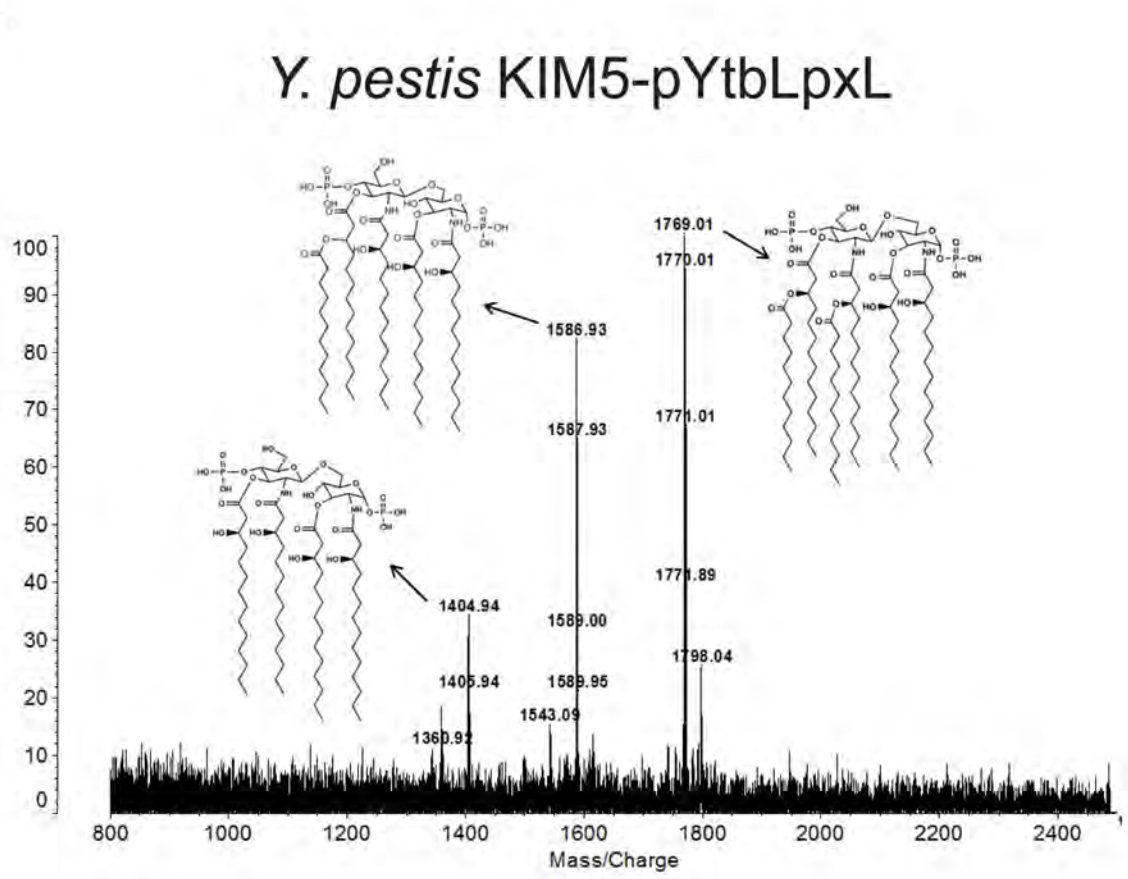
A.


Y. pestis KIM5
1404.48
1405.55
1376.62
1377.66
1362.57
872.35
1587.56
Mass/Charge

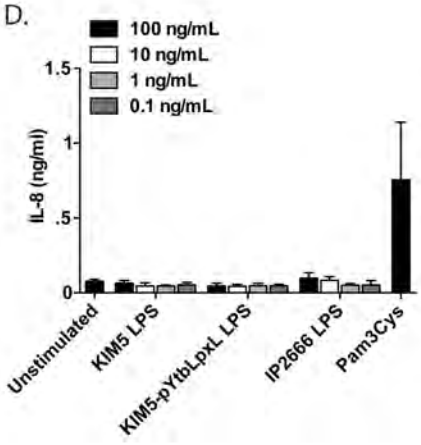
B.


Y. pseudotuberculosis IP2666
1769.57
1768.57
1586.55
1587.56
1404.53
1770.51
836.21
376.67
1588.51
1390.68
1360.54
1771.57
1572.53
1741.50
1600.57
1324.65
2007.41
Mass/Charge

C.


Y. pestis KIM5-pYtbLpxL
100
90
80
70
60
50
40
30
20
10
0
800
1000
1200
1400
1600
1800
2000
2200
2400
Mass/Charge
1586.93
1587.93
1769.01
1770.01
1771.01
1404.94
1589.00
1771.89
1798.04
1405.94
1589.95
1543.09
1360.92

D.


100 ng/mL
10 ng/mL
1 ng/mL
0.1 ng/mL
IL-8 (ng/ml)
1.5
1
.5
0
Unstimulated
KIM5 LPS
KIM5-pYtbLpxL LPS
IP2666 LPS
Pam3Cys

E.

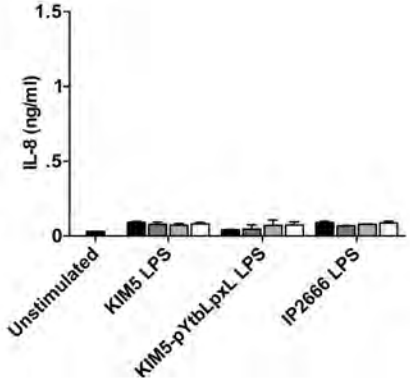

IL-8 (ng/ml)
1.5
1
.5
0
Unstimulated
KIM5 LPS
KIM5-pYtbLpxL LPS
IP2666 LPS

**Figure 2.2: Mass Spectrometry analysis of variations of *Yersinia* lipid A reveals the changes of the late-acyl transferase LpxL on structure**

*Y. pestis* KIM5, KIM5-pYptbLpxL, and IP2666 were grown at 37 ºC and the lipid A purified from whole bacteria by Bligh-Dyer two-phase organic extraction. The lipid was then further purified over a DE52 column and analyzed by MALDI-TOF mass spectrometry. The negative ion spectra shown here are representative of multiple extractions. (A) Negative mode MALDI-TOF analysis of Y. pestis KIM5. Tetra acyl lipid A (m/z 1405) with 4 C14:0 acyl groups is the predominant lipid A species found in this bacterium. (B) Negative mode MALDI-TOF analysis was also conducted on *Y. pseudotuberculosis* IP2666. Three predominant species present; tetra-acyl lipid A with four C14:0 acyl groups (m/z 1405), a penta-acyl lipid A with an additional secondary C12:0 acyl chain (m/z 1587), and a hexa-acyl lipid A with two additional C12:0 acyl chains (m/z 1769). (C) KIM5-pYptbLpxL expresses a very similar acyl chain pattern to IP2666. (D-E) ELISA of IL-8 in supernatants of HEK293 cells expressing (D) mouse TLR2 and (E) a control plasmid; stimulation with various ligands was overnight. D-E are control experiments for **Figure 2.1 D**.

Purified LPS alone is unable to induce release of IL-1β, and needs addition of an inflammasome specific stimulator such as alum (triggering NLRP3 activation) (**Figure 2.1 E**) for IL-1β secretion, suggesting that other bacterial factors are needed for activation of the inflammasome. We observed that *in vitro* infection of cells with KIM5 induced some release of IL-1β, but the response was markedly increased after infection with KIM5-pYtbLpxL (**Figure 2.1 F**). The experiments indicate that the increased immune response is due to the change in LPS structure as *Y. pestis* producing a hexa-acylated LPS at 37$^{o}$C has normal growth *in vitro* and i*n vivo*, preserved membrane stability, Pla activity, and type III secretion activity (data not shown). Thus, it appears that the presence of a potent LPS in KIM5-pYtbLpxL significantly enhanced the ability of the cells to produce IL-1β.

Analysis of known genome segments of various Yersiniae suggested that lpxL is present in all members of the genus except *Y. pestis* (**Figure 2.3 A-B**). As with *Y. pseudotuberculosis* LpxL, the expression of *Y. enterocolitica* LpxL in KIM5 (KIM5-pYeLpxL) enabled the production of a hexa-acylated lipid A (**Figure 2.1 H-I, 2.4 A-B**) and the secondary 3' C14 acyl chain in lipid A from KIM5-pYeLpxL, presumably added by *Y. entrocolitica* LpxL, resembled that of wild type *Y. enterocolitica* (Rebeil et al., 2004). The addition of LpxL and resulting modification resulted in the enhanced the ability of the bacteria to induce IL-1β in mouse cells, compared to KIM5 (**Figure 2.1 J**).

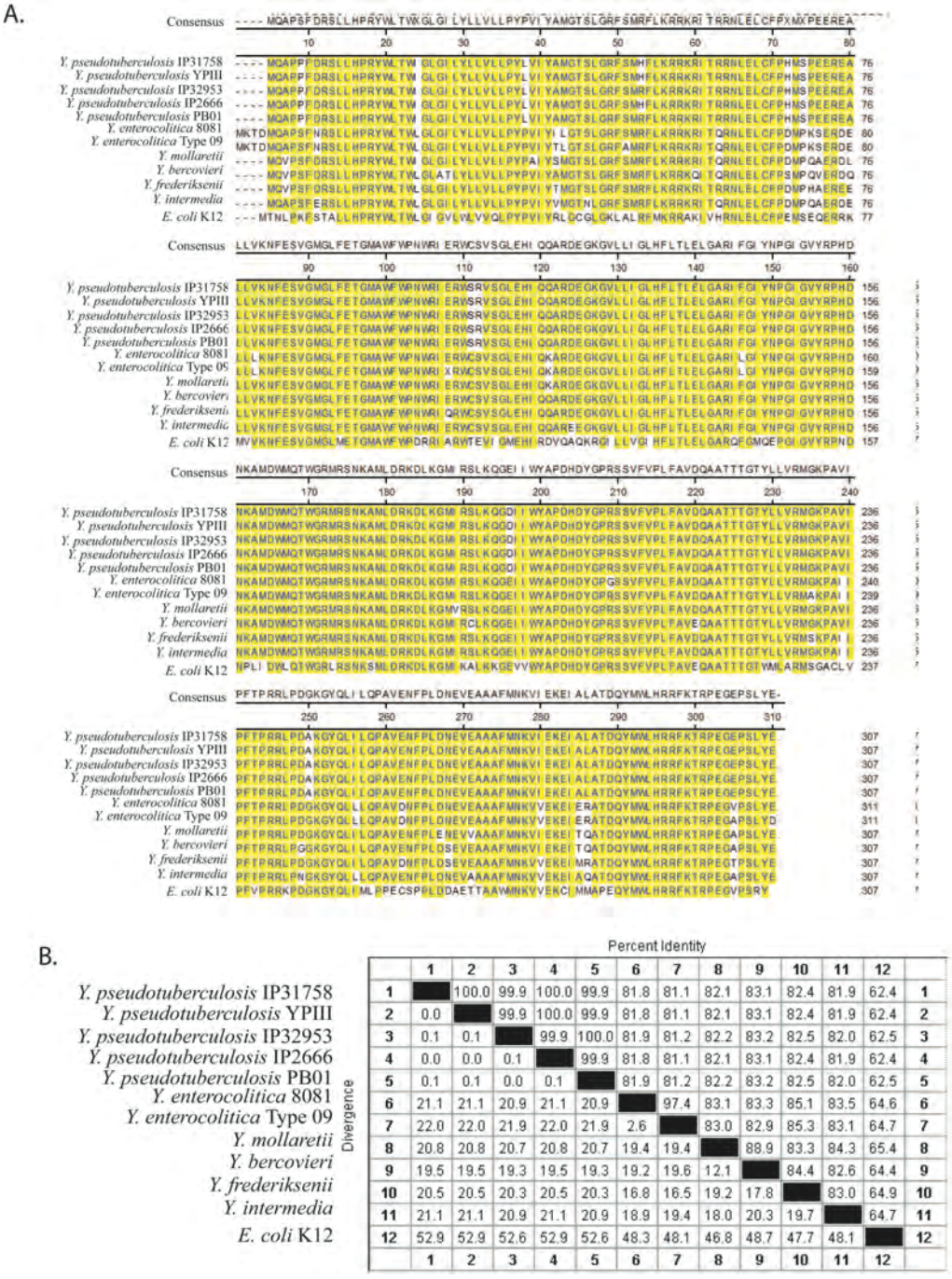
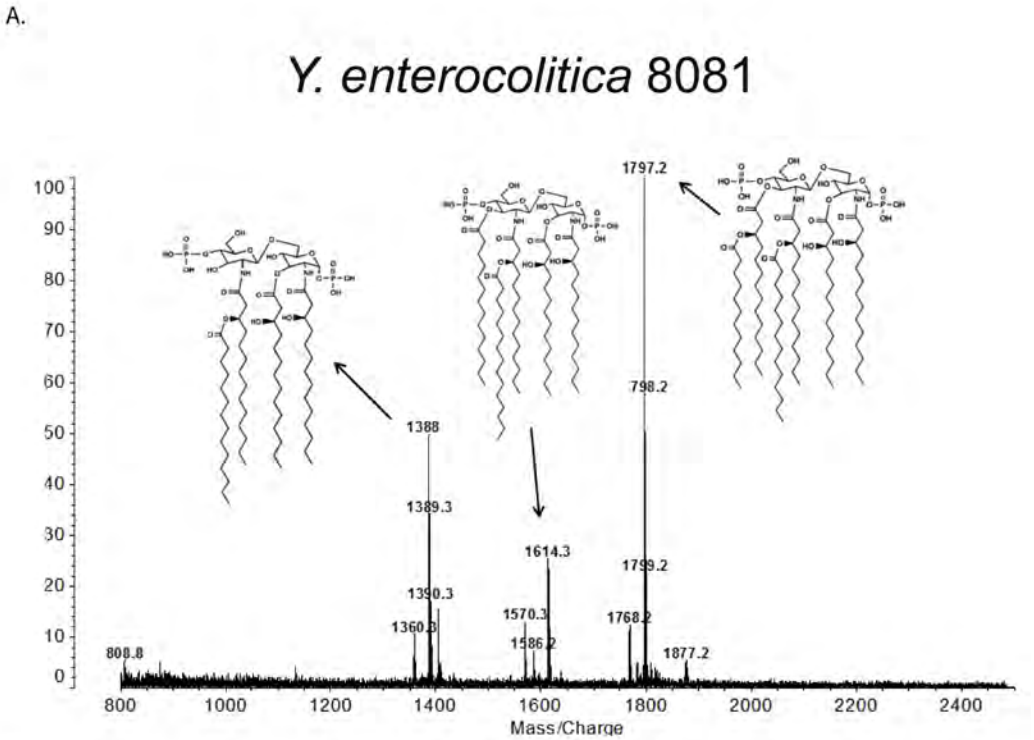



**Figure 2.3: *lpxL* in *Yersinia***

Punitive genes for *lpxL* were identified by BLAST analysis using *E. coli* K12 *lpxL* within sequenced *Yersinia* strains. Punitive *lpxL* genes were located in all sequenced *Yersinia* with the exception of *Y. pestis*. (A) Clustal-W alignment of punitive *lpxL* genes in sequenced Yersinia strains. Yellow shading denotes area of sequence homology, with the consensus sequence is listed above. (B) Percent identity and divergence of the *lpxL* sequences generated after clustal-W alignment.

A.


Y. enterocolitica 8081
1797.2
798.2
1388
1389.3
1614.3
1799.2
1390.3
1570.3
1768.2
1360.3
1586.2
1877.2
808.8
Mass/Charge

B.

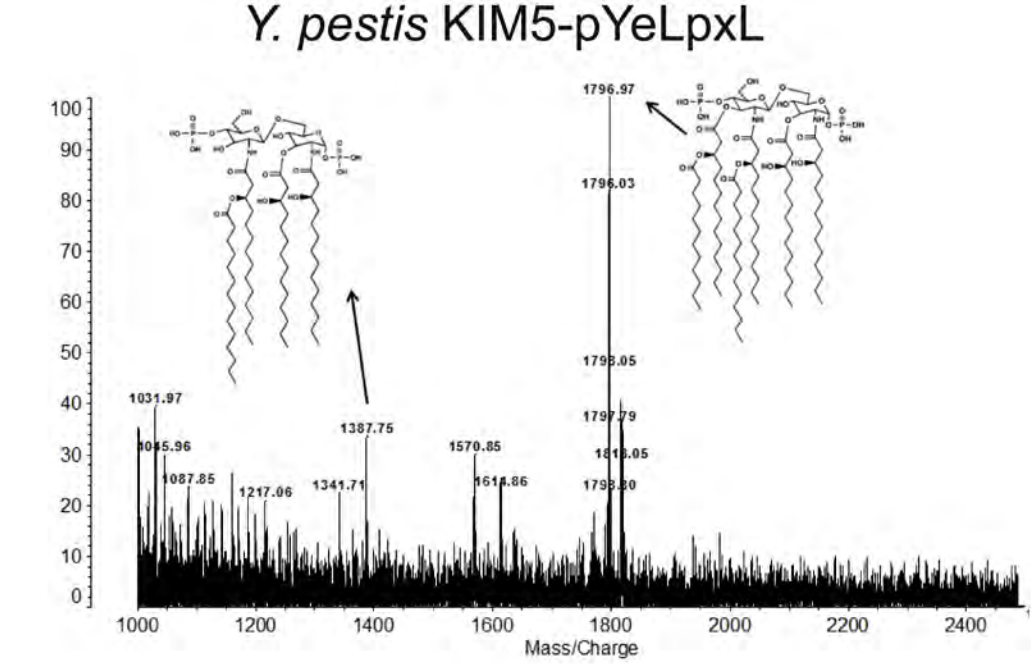

Y. pestis KIM5-pYeLpxL
1796.97
1796.03
1798.05
1797.79
1031.97
1387.75
1570.85
1087.85
1217.06
1341.71
1618.86
Mass/Charge

**Figure 2.4: *Yersinia enterocolitica* LpxL**
(A) Mass spectra analysis of *Y. enterocolitica* strain 8081. The predominant species present are typical tetra-acyl lipid A with three C14:0 acyl groups on the digucosomine backbone and one secondary C14:0 acyl chain, a penta-acyl lipid A with an additional primary C14:0 acyl chain, and a hexa-acyl lipid A with a secondary C12:0 acyl chains. (B) *Y. pestis* expressing *Y. enterocolitica* LpxL (KIM5-pYeLpxL) expresses a very similar acyl chain pattern to 8081.

To test pathogen virulence, mice were subjected subcutaneous (s.c.) infection with fully virulent *Y. pestis* KIM1001 or KIM1001-pYtbLpxL (or our previously published KIM1001-EcLpxL), and observed TLR4-dependent survival in animals infected with strains expressing potent lipid A, whereas all mice infected with KIM1001 died (**Figure 2.5 A**). Along the same lines, the expression of *Y. enterocolitica lpxL* in KIM1001 also protected mice from infection (**Figure 2.5 B**). We previously published that the survival of mice infected with KIM1001-pYtbLpxL correlated with the absence of bacteria in spleens, compared to KIM1001, indicating control of the pathogen before systemic spread (Vladimer et al., 2012). To further study the importance of *lpxL* expression on TLR4 engagement and cytokine production via hexa-acylated lipid A, we engineered a strain with a deleted lpxL gene from *Y. pseudotuberculosis*, generating IP2666ΔLpxL (**Figure 2.5 C-D**). HEK293 cells expressing TLR4/MD2 stimulated with the IP2666ΔLpxL had reduced IL-8 responses in comparison to stimulations with WT IP2666, this was regained after the addition of the pYtbLpxL plasmid used in KIM5 above (**Figure 2.5 E**). Although we studied present-day *lpxL* genes only, we propose that the loss of *lpxL* was a necessary step in the evolution of virulence from *Y. pseudotuberculosis* to *Y. pestis*, related to the production of a tetra-acylated LPS and the resulting evasion of TLR4 signaling.

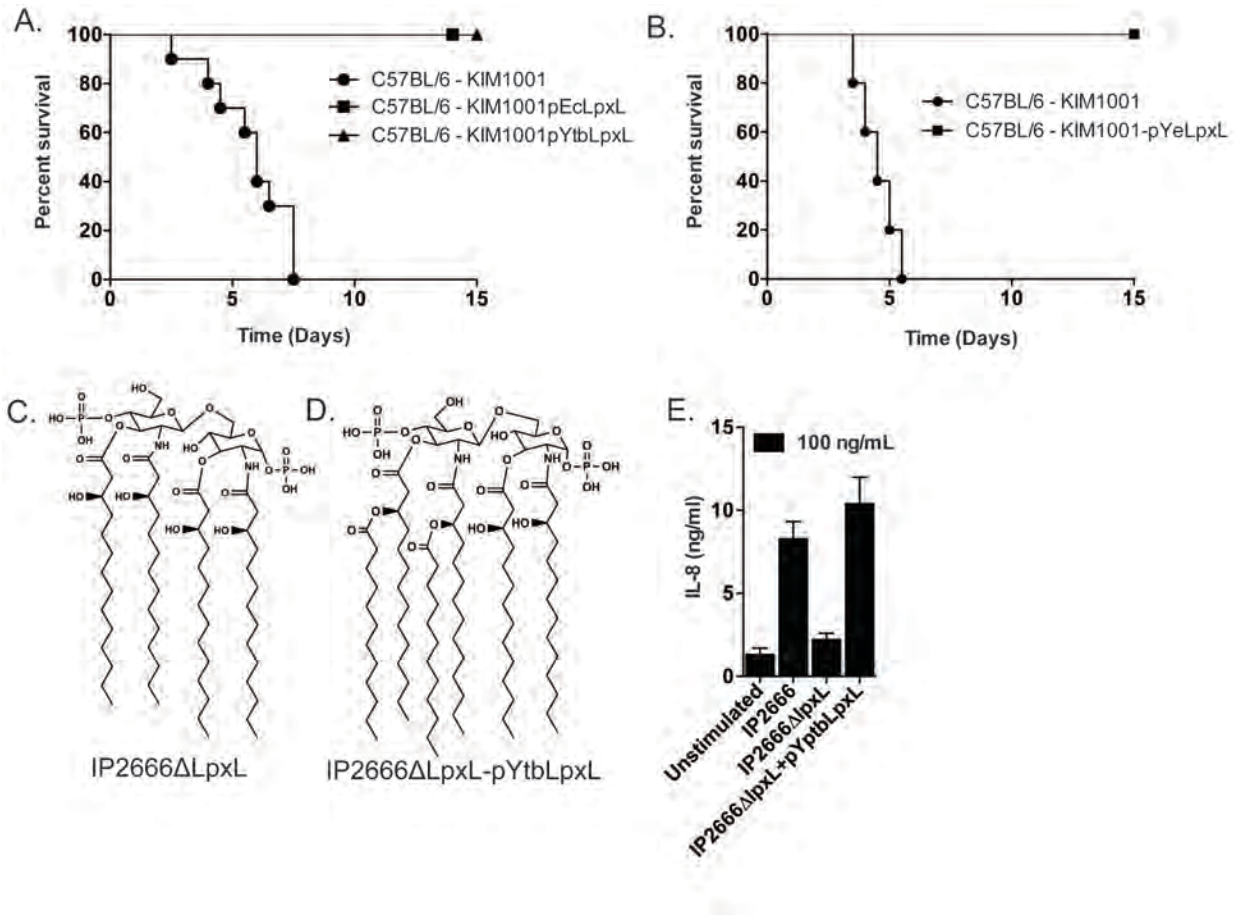



**Figure 2.5: The Addition of LpxL protects animals from subcutaneous infection with *Y. pestis***

(A-B) Survival of animals infected subcutaneously, to mimimc bubonic plague, with fully virulent (A) *Y. pestis* KIM1001, KIM1001-pEcLpxL, or KIM1001-pYtbLpxL and (B) *Y. pestis* KIM1001 or KIM1001-pYeLpxL. (C-D) The major lipid A structures of *Y. pseudotuberculosis* IP2666 lacking LpxL, or with an LpxL addback. (E) ELISAs of supernatant IL-8 from HEK293 cells expressing TLR4/MD2 after stimulation for 18 hours

*Inflammasome and macrophage response to wild type and modified strains of Y. pestis and Y. pseudotuberculosis*

Our *in vitro* experiments (**Figure 2.1**) verified that *Y. pestis* induced IL-1β release, and this release was markedly enhanced by the expression of *Y. pseudotuberculosis* or *Y. enterocolitica* LpxL. Previous experiments with *Y. pestis* expressing *E. coli* and *Y. pseudotuberculosis* LpxL suggested that IL-1R and IL-18R signaling could play a more important role in resistance to *Y. pestis* strains generating a hexa-acylated lipid A/LPS than signaling via type I IFN, IL-12 or TNFR I signaling (Montminy et al., 2006; Vladimer et al., 2012). We also showed that mice deficient in MyD88, an adaptor common to TLR, IL-1R and IL-18R signaling pathways, revealed that these mice were more susceptible to wild type *Y. pestis* KIM1001 than wild-type C57Bl/6 mice (Vladimer et al., 2012). The possible involvement of IL-1β and IL-18 led us to perform *in vitro* experiments to identify candidate inflammasome components that promote caspase-1 cleavage and IL-1β/IL-18 release following infection with *Y. pestis* modified strains. We have previously shown a role for NLRP12 and NLRP3 in innate immune responses to parental *Y. pestis* and strains producing a hexa-acylated LPS. *Y. pestis* harbors a type III secretion system, often associated with NLRC4 activation, and it has been proposed that NLRC4 participates in macrophage responses to attenuated *Y. pestis* strains (Brodsky et al., 2010). We found that NLRC4 participates in the release of IL-1β from macrophages infected with KIM5 and KIM5-YptbLpxL (**Figure 2.6 A-B**). NLRC4 deficient macrophages responded normally to alum/LPS but did not respond to *S. typhimurium* (Miao et al., 2010) (**Figure 2.6 C**). Moreover, we see the same strong IL-1β production from BMDM stimulated with WT IP2666 in a LpxL dependent manner (**Figure 2.6 D).** NLRC4 deficiency had no impact on TNF release when stimulated with various ligands (**Figure 2.6 E**).

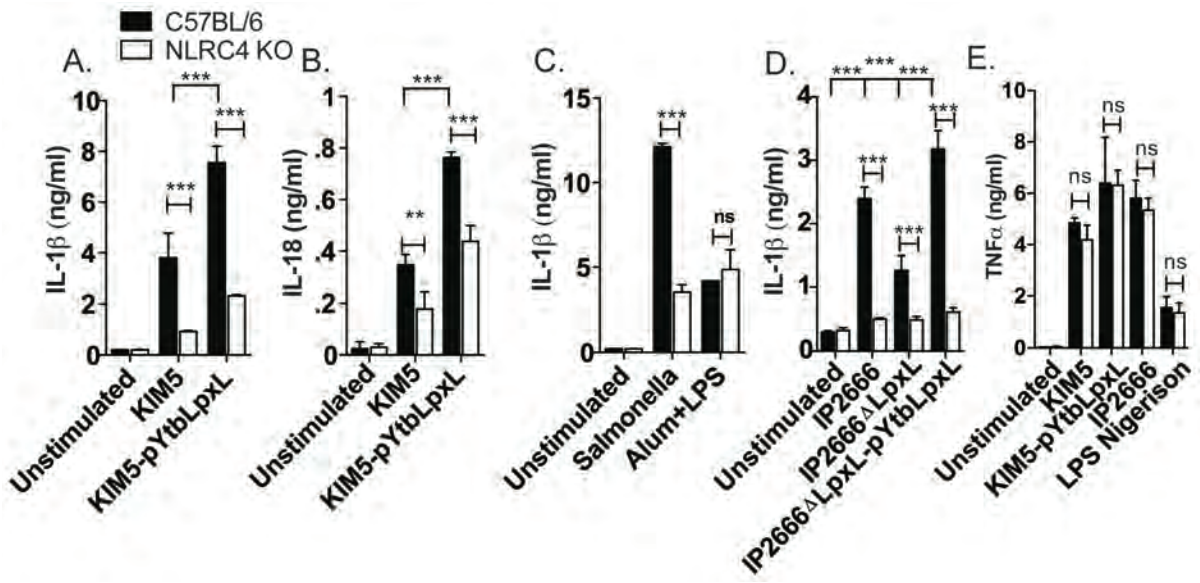



**Figure 2.6: *Y. pestis* activates the production of IL-1β in a NLRC4 dependent manner, upregulated by expression of LpxL**

(A-E) ELISAs of supernatant from stimulated BMDM stimulated with (A-B, E) KIM5, or KIM5-pYtbLpxL (C) Salmonella or ALUM+KIM5pYtbLpxL LPS (D-E) IP2666, IP2666ΔLpxL, or IP2666ΔLpxL-pYtbLpxL to detect (A, C-D) IL-1β, (B) IL-18, or (E) TNF. Stimulations all lasted 6 hours, with 50μg/ml gentamycin added after 3 hours. Bacterial stimulations used 10cfu.

We conclude that IL-1β and IL-18 release in macrophages involve multiple inflammasome components: caspase-1, ASC, NLRP3, NLRP12 (Vladimer et al., 2012) and NLRC4. Some other live pathogens are also reported to interact with different inflammasome NLRs, or NLRs plus AIM2 (Broz et al., 2010; Kim et al., 2010). It is at present unclear how these different inflammasome components cooperate in order to provide maximum protection of the host. Future studies may determine if multiple parallel signals generate different inflammasomes containing either NLRP3, NLRP12 or NLRC4, or if all signals converge into larger caspase-1 processing complexes simultaneously containing all these NLRs. It is of note that single ASC containing complexes are often observed by microscopic studies of macrophages infected with *S. typhimurium*.

*NLRC4 mediates in vivo host resistance to Y. pestis infection.*

Performing *in vivo* infections with *Y. pestis* strains expressing a potent LPS using mice deficient in key innate immunity molecules will clarify the basis for the advantage gained by *Y. pestis* via loss of *lpxL* during evolution. Moreover, the strains could be used as tools to probe novel innate immunity pathways, as we have previously shown (Vladimer et al., 2012). We subjected wild type and NLRC4 KO mice to s.c. infection with *Y. pestis* KIM1001-pYtbLpxL. Mice with defects NLRC4 showed greatly increased susceptibility to the bacteria, with approximately 70% of the mice succumbing the bacterial challenge **(Figure 2.7 A)**. The improved survival observed in animals infected with i.v. with 500 cfu of KIM1001-pYtbLpxL as compared to fully virulent KIM1001 at 48 hpi was correlated with significantly increased IL- 18 and IL-1β in sera and spleens of animals **(Figure 2.7 B-C)**. This increase was diminished in the NLRC4 KO mice, and their cytokine levels generally reduced **(Figure 2.7 B-C)**.

Intravenous infection revealed a significant reduction of spleen bacterial load when mice were infected with KIM1001-pYtbLpxL compared to wild-type KIM1001 (**Figure 2.7 D**), indicating beneficial host responses induced by the presence of the hexa-acylated LPS. However, these differences in systemic bacterial load between the two bacterial strains were absent in the NLRC4 KO mice. Furthermore, NLRC4 KO mice also had increased bacterial loads compared to wild type mice when infected with the virulent *Y. pestis* (**Figure 2.7 D**).

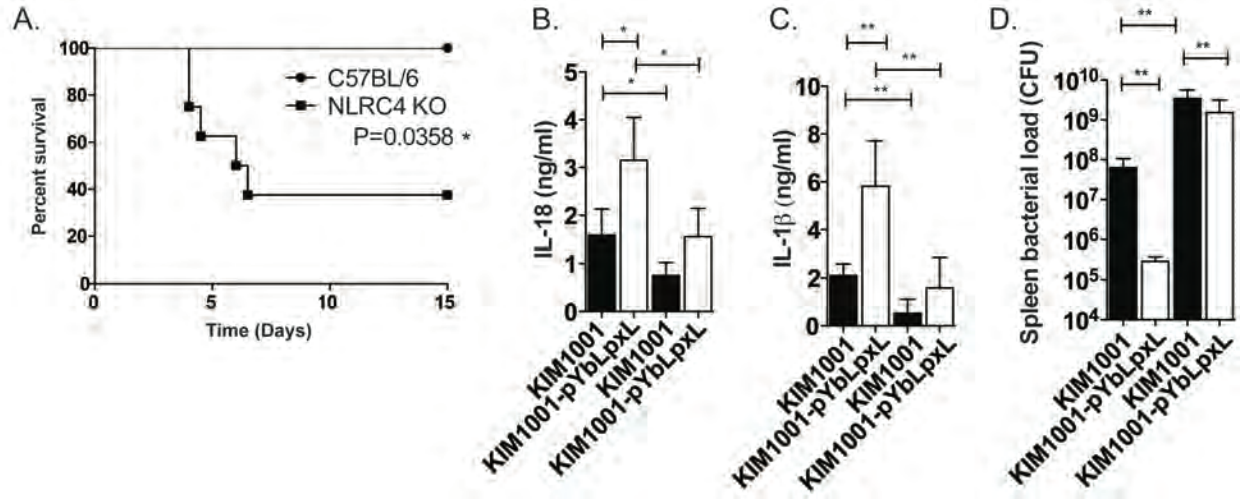



**Figure 2.7: *Y. pestis* activates the production of IL-1β in a NLRC4 dependent manner, upregulated by expression of LpxL**

(A-E) ELISAs of supernatant from stimulated BMDM stimulated with (A-B, E) KIM5, or KIM5-pYtbLpxL (C) Salmonella or ALUM+KIM5pYtbLpxL LPS (D-E) IP2666, IP2666ΔLpxL, or IP2666ΔLpxL-pYtbLpxL to detect (A, C-D) IL-1β, (B) IL-18, or (E) TNF. Stimulations all lasted 6 hours, with 50μg/ml gentamycin added after 3 hours. Bacterial stimulations used 10cfu. *$p < 0.05$; **$p < 0.001$.

**Discussion**

*Y. pestis* is considered the most recently evolved of the human-pathogenic *Yersiniae*. It is believed that the ancestors to present-day *Y. enterocolitica* and *Y. pseudotuberculosis* split many million years ago, whereas the split between *Y. pestis* and *Y. pseudotuberculosis* is much more recent – a few thousand years ago (Achtman et al., 2004; Chain, 2004; Zhou and Yang, 2009). Much interest is associated with finding critical factors that contributed to the greatly increased virulence in *Y. pestis* compared to its ancestors.

In this report we provide results consistent with the hypothesis that loss of LpxL, and the resulting inability to synthesize a potent TLR4-activating hexa-acylated LPS at 37$^{o}$C, was a major event in the the evolution of high virulence in *Y. pestis*. *Y. pestis* expressing LpxL from *Y. pseudotuberculosis* strain produces a lipid A which is similar to that of *Y. pseudotuberculosis*, more so that the *Y. pestis* containing *E. coli* LpxL (Montminy et al., 2006). Our current data indicate that the production of IL-1β and IL-18 are crucial components of LPS-induced protection in the context of *Y. pestis* infection. We propose that avoiding the effects of these inflammatory cytokines was a major selective force driving the divergence of *Y. pestis* from *Y. pseudotuberculosis*. We also conclude that inflammasome activation by the bacteria is an effective way of limiting spread of *Y. pestis* in the host, thus, evading these mechanisms is key in the strategy of *Y. pestis* to limit innate immunity.

We show that the expression of *Y. pseudotuberculosis* and *Y. entrocolitica* LpxL in *Y. pestis* enables the production of hexa-acylated LPS at 37$^{o}$C, increasing TLR4-dependent release of potent pro-inflammatory cytokines and greater activation of the NLRC4 inflammasome. This increased activity correlates with increased resistance to the modified pathogen. In fact, the

results indicate that a major consequence of producing LPS with low TLR4-activating potential could be lack of priming necessary for effective synthesis of active IL-1β and IL-18. Strains lacking LpxL, or containing LpxL with a point mutation at a residue associated with enzymatic activity, had reduced release of IL-1β and IL-18 in vitro and in vivo and strongly increased virulence.

Our data supports the thought that the cytosolic inflammasome complex is necessary for a strong host response against many pathogens. It is known that *Y. pestis* limits activation of NF-kB and MAPK pathways by acetylating upstream components via a type III secretion effector YopJ (Mittal et al., 2006; Sweet et al., 2007; Paquette et al., 2012). We, and others have demonstrated YopJ can promote IL-1β release and inflammasome activation (Vladimer et al., 2012; Zheng et al., 2012). Previous studies have shown that *Y. pestis* can activate the NLRP3, NLRP12 and now the NLRC4 inflammasome, and this suggests that NLRs may work together for optimal protection of the host. More work must be completed to resolve the association of all of the inflammatory NLRs which protect against pathogens. Greater knowledge of molecular relationships this will assist in determining better targeted therapeutics and vaccine candidates.

## ***Materials and Methods***

### *Bacterial Strains and Growth Conditions*

*Y. pestis* KIM is originally a clinical isolate from a Kurdistan Iran man (Perry and Fetherston, 1997). *Y. pestis* strains KIM5, KIM1001, KIM5-pYtbLpxL, and KIM1001-pYtbLpxL were as reported (Vladimer et al., 2012). *Y. pseudotuberculosis* IP2666 (containing a complementation of PhoP/PhoQ deficiency) and Y. enterocolitica 8081 were provided by J. Mecsas. Strains were

grown in tryptose-beef extract (TB) broth with 2.5 mM CaCl2 all by shaking at 37C. All strains containing plasmids above remained tetracycline sensitive. KIM1001 (pPCP1+, pCD1+, and pMT1+) is highly virulent (Perry and Fetherston, 1997), whereas KIM5 bears the chromosomal deletion ‘‘Δpgm,’’ which substantially attenuates virulence. The pgm locus contains no genes thought to affect LPS biosynthesis. For the generation of IP2666-ΔLpxL, the product was cloned in the allelic exchange vector pRE107 in *E.coli* K12 strain B2155 and transferred to IP2666 by conjugation; recombinants were selected on TB medium containing 100 mg/ml ampicillin but no diaminopimelic acid. After counter selection with 5% sucrose, deletion mutants were identified by PCR. For *in vitro* infections, bacteria were grown overnight at 37$^{o}$C in TB broth with or without ampicillin, diluted 1:4 in fresh media, and cultured for three more hours at 37$^{o}$C, then washed three times with PBS and resuspended in DMEM or RPMI. *S. enterica* serovar typhimurium strain SL1344 was provided by M. O’Riordan.

*Lipid preparations*

Pyrogen-free reagents and supplies were used as much as possible during isolation of lipids. Lipid A and LPS were prepared as described (Montminy et al., 2006) after suspension growth of strains overnight at 37$^{o}$C. Briefly, LPS was prepared by hot water-phenol extractions followed by two phenol re-extractions, and lipid A was prepared directly from whole bacteria using a Bligh-Dyer two-phase chloroform-methanol/water organic extraction.

*Cell Stimulations*

Mouse BMDMs were prepared by maturing fresh bone marrow cells for 5–7 days in the presence of M-CSF containing supernatant from L929 cells. Mouse BMDMs were plated at $10^5$ per well in 96-well plates for ELISA or $10^6$ per well in 12-well plates for immunoblotting. Stimulation

was for 6 hr and supernatants were collected for cytokine analysis. Three hours after bacterial infections, 50 mg/ml of gentamycin was added. Alum was from Pierce; nigericin and poly(dA:dT) was from Sigma. IL-1β p17 and Caspase-1 p10 immunoblots were conducted mainly as described (Hornung et al., 2009) with antibodies from Santa Cruz Biotechnology (caspase-1 p10) and R&D (IL-1β). The antibody against β-actin was from Sigma. ELISA kits for IL-1β, TNF-a, IL-8, CXCL12, IFN-ɣ (R&D), and IL-18 (MBL) were used for cytokine detection.

*Mice*

All experiments involving animals were approved by the Institutional Animal Care and Use Committee. ASC (PYCARD) and NLRC4 (IPAF) mice were generated by Millennium Pharmaceuticals and were backcrossed 8–11 generations to C57BL/6 background. Mice deficient in TLR4 (TLR4) were from S. Akira. Wild-type, C57BL/6 (from Jackson Laboratories or bred at UMass) and knockout mice were infected s.c. in the nape of the neck with *Y. pestis,* or orally with IP2666, and their survival was monitored twice a day for 30 days. For cytokine and CFU analysis, mice were infected i.v. and sacrificed at the indicated time points. Serum was generated by centrifugation in microtainer tubes (BD), and spleens were homogenized in 0.5 ml PBS with a closed system Miltenyi gentleMACS dissociator and c-tubes to preserve intact cells; subsequently cells/debris were removed by centrifugation. Samples for cytokine analysis were subjected to protease inhibitor (Roche) treatment. Hematoxylin and eosin (H&E) staining and microscopy were performed as published (Montminy et al., 2006).

# PREFACE to CHAPTER 3

This section is a version of a manuscript published in Immunity:

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This chapter represents a thesis project of G.I.V., who generated all data except that indicated below. G.I.V. created all of the figures from primary data, and was the principal writer and coordinator of the manuscript text.

Co-authors contributed the following data:
D.W., J.B., and E.L. assisted with *in vivo* sample collection, D.W. performed Figure 2.8 C. S.K.V. and V.R. performed experiments in Figures 3.7 H and 3.8 B. J.C. performed experiment in Figure 3.6 D, and M.M.P. created the KIM5ΔYopJ strain used for *in vitro* stimulations.

Note: Chapter 3 was published before Chapter 2

# CHAPTER 3:

## The NLRP12 and NLRP3 Inflammasomes Recognize *Yersinia pestis* During Bubonic Plague Infection

***Abstract***

*Yersinia pestis*, the causative agent of plague, is able to suppress production of inflammatory cytokines IL-18 and IL-1β, which are generated through caspase-1-activating NLR-containing inflammasomes. Here, we sought to elucidate the role of NLRs and IL-18 during bubonic plague. Lack of IL-18 signaling led to increased susceptibility to *Y. pestis* and *Y. pestis* expressing a modified lipid A / LPS. We found that the NLRP12 and NLRP3 inflammasomes were important regulators controlling IL-18 and IL-1β production after *Y. pestis* infection, and inflammasome-deficient mice were more susceptible to bacterial challenge. NLRP12 and NLRP3 also directed interferon-γ (IFN-γ) production via induction of IL-18, but NLRP12 deficiency had minimal effect on NF-κB signaling. These studies reveal a novel role for NLRP12 in host resistance against pathogens. Minimizing NLRP12, and other, inflammasome activations may have been a central factor in evolution of the high virulence of *Y. pestis*.

***Introduction***

The NLR family has more than 20 members, and their relative roles in promoting resistance to infection are in many instances unclear. There is evidence supporting a function in bacterial recognition for several NLRs, reviewed above. The role of NLRP12 in innate immunity, however, has remained unclear. Both inflammatory and inhibitory functions have been suggested, as has a role in hypersensitivity (Wang et al., 2002; Lich et al., 2007; Lich and Ting, 2007; Arthur et al., 2010; Zaki et al., 2011b). Interestingly, like for NLRP3, mutations in NLRP12 are linked to hereditary inflammatory disease (Jéru et al., 2008). It has been reported that patients carrying NLRP12 mutations associated with increased inflammasome activation have been successfully treated with anti-IL-1 therapy, similar to patients containing mutations in NLRP3 (Hawkins and Lachmann, 2003; Lachmann et al., 2009; Jéru et al., 2011a). No previous studies have addressed the role of NLRP12 in host resistance to infectious agents.

Evading innate immunity early in infection plays a key role in virulence of many microorganisms including the plague bacillus *Yersinia* pestis (Perry and Fetherston, 1997; Cornelis, 2000; Stenseth et al., 2008). This pathogen has several means of minimizing immune activation (Sodeinde et al., 1992; Monack et al., 1997; Zhou et al., 2005; Mukherjee et al., 2006; Lathem et al., 2007), with the effect that bacterial replication can proceed with minimal interference by the immune system. As a result, plague is often characterized by very high bacterial numbers in patient sera and organs (Perry and Fetherston, 1997). Major factors neutralizing host defenses by active means include a complex type III secretion system (T3SS) (Perry and Fetherston, 1997; Cornelis, 2002a), the plasminogen activator Pla (Sodeinde et al., 1992; Lathem et al., 2007), and a high-affinity iron acquisition system (Perry and Fetherston,

1997). The *Yersinia* T3SS delivers effector proteins, which disrupt signaling within the host cell to prevent phagocytosis, induce apoptosis, and evade the immune response (Cornelis, 2002a). Many Gram-negative bacteria, including *Y. pseudotuberculosis*, a very close ancestor of *Y. pestis*, produce a hexa-acylated lipid A-LPS which has the potential of strongly triggering innate immunity via Toll-like receptor 4 (TLR4)-MD-2 signaling (Therisod et al., 2002; Rebeil et al., 2004; Raetz Cr, 2007; Munford, 2008). In contrast, *Y. pestis* generates a tetra-acylated lipid A/ LPS that poorly induces TLR4-mediated cellular activation (Kawahara et al., 2002; Knirel et al., 2005; Montminy et al., 2006; Rebeil et al., 2006). We have reported that expression of *E. coli* LpxL in *Y. pestis*, which lacks a homologue of this gene, forces the biosynthesis of a hexa-acylated LPS (Montminy et al., 2006), and that this single modification dramatically reduces virulence in wild type mice, but not in mice lacking a functional TLR4. This emphasizes that avoiding activation of innate immunity is important for *Y. pestis* virulence. It also provides a model in which survival is strongly dependent on innate immune defenses, presenting a unique opportunity for evaluating relative importance of innate immunity signals in protection against bacterial infection.

One implication of TLR4 engagement is the induction of the immature forms of the central pro-inflammatory cytokines IL-1β and IL-18. TLR4 signaling can also promote expression of inflammasome components such as NLRP3 (Bauernfeind et al., 2009). This establishes links between TLR4 activation and the inflammasome pathways. In this study we have used wild type *Y. pestis* and attenuated strains expressing a strong TLR4-activating hexa-acylated LPS as a model system to investigate the involvement of NLRP12 in pathogen recognition and IL-18/IL-1β release.

Here we show that NLRP12 is an inflammasome component that is central in the recognition of *Y. pestis* and that IL-18 signaling substantially contributes to resistance against bacteria. Compared to wild type mice, NLRP12 deficient animals had higher mortality and increased bacterial loads following infection, correlated with lower levels of IL-18, IL-1β and IFNγ. We propose a role for NLRP12 in the sensing of microbial pathogens.

***Results***

*IL-18 signaling is essential for resistance to attenuated Y. pestis*

We have found that all members of the genus Yersinia other than *Y. pestis*, and including the very closely related *Y. pseudotuberculosis*, contain the lpxL gene (Chapter 2, unpublished). Absence of *lpxL* and the resulting production of a tetra-acylated LPS was proposed to be essential for *Y. pestis* virulence (Montminy et al., 2006). To study the evasion of TLR4 signaling in an evolutionary perspective, we cloned *lpxL* from the closely related *Y. pseudotuberculosis* and expressed it in *Y. pestis*, generating *Y. pestis*-pYtbLpxL, to determine its effects on virulence. *Y. pestis* grown at 37$^{o}$C has a tetra-acylated lipid A (**Figure 3.1 A**) (Montminy et al., 2006) whereas *Y. pseudotuberculosis* and *Y. pestis*-pYtbLpxL have a hexa-acylated lipid A (**Figure 2.1 B**). Mice infected s.c. (subcutaneously) with 500 c.f.u. (colony forming units) of highly virulent *Y. pestis* KIM1001 rapidly succumb to infection (**Figure 3.2 A**). All wild-type mice infected with KIM1001-pYtbLpxL expressing a hexa-acylated *Y. pseudotuberculosis*-like lipid A survived (**Figure 3.2 A**), and the animals were protected towards challenge with virulent KIM1001 (**Table 3.1**). Survival of mice was strongly TLR4 dependent (**Figure 3.2 A**). To determine the pathways responsible for *in vivo* clearance, mice from several strains deficient in inflammatory cytokines or cytokine receptors were infected s.c. with 500 c.f.u. of KIM1001-pYtbLpxL (**Figure 3.2 B**). Interestingly, 100% of the animals lacking IL-18 and IL-18R died, as did the TLR4 KO mice and

70% of the IL-1RI KO mice. Weaker effects were observed in animals lacking IFNαβR, TNFR1, or IL-12p40 (**Figure 3.2 B**).

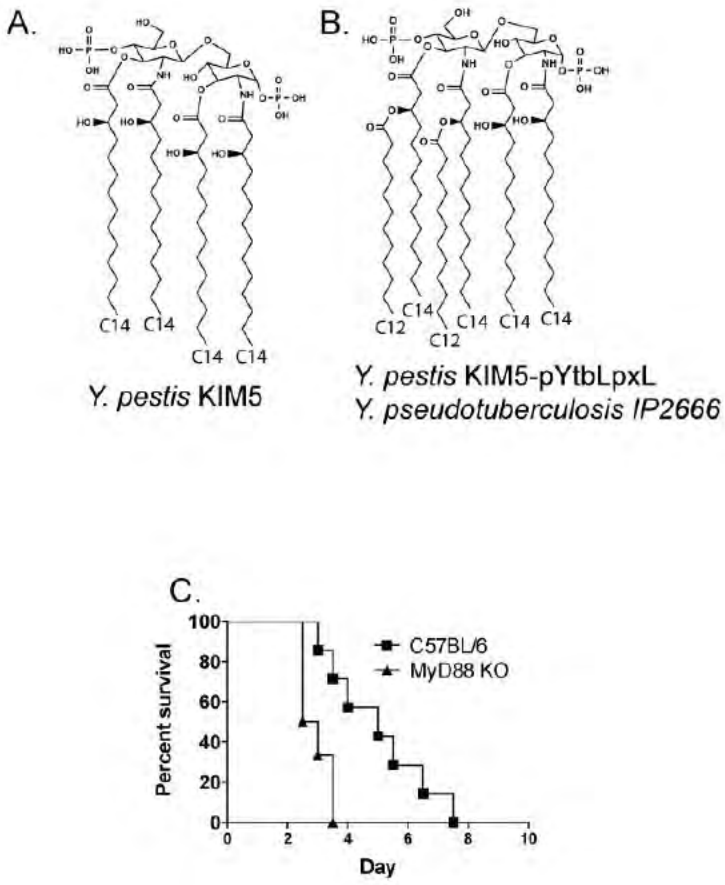


**Figure 3.1: Major Lipid A Structures of *Y. pestis* and *Y. pestis* expressing LpxL and The Importance of Innate Immune Signaling on Plague Infection**

(A-B) Significant lipid A structures of (A) *Y. pestis* KIM5 and (B) *Y. pestis* KIM5-pYtbLpxL and *Y. pseudotuberculosis* IP2666. (C) Survival curve of WT and MyD88 KO mice infected s.c. with 500 c.f.u. of *Y. pestis* KIM1001. WT vs MyD88: $p<0.001$

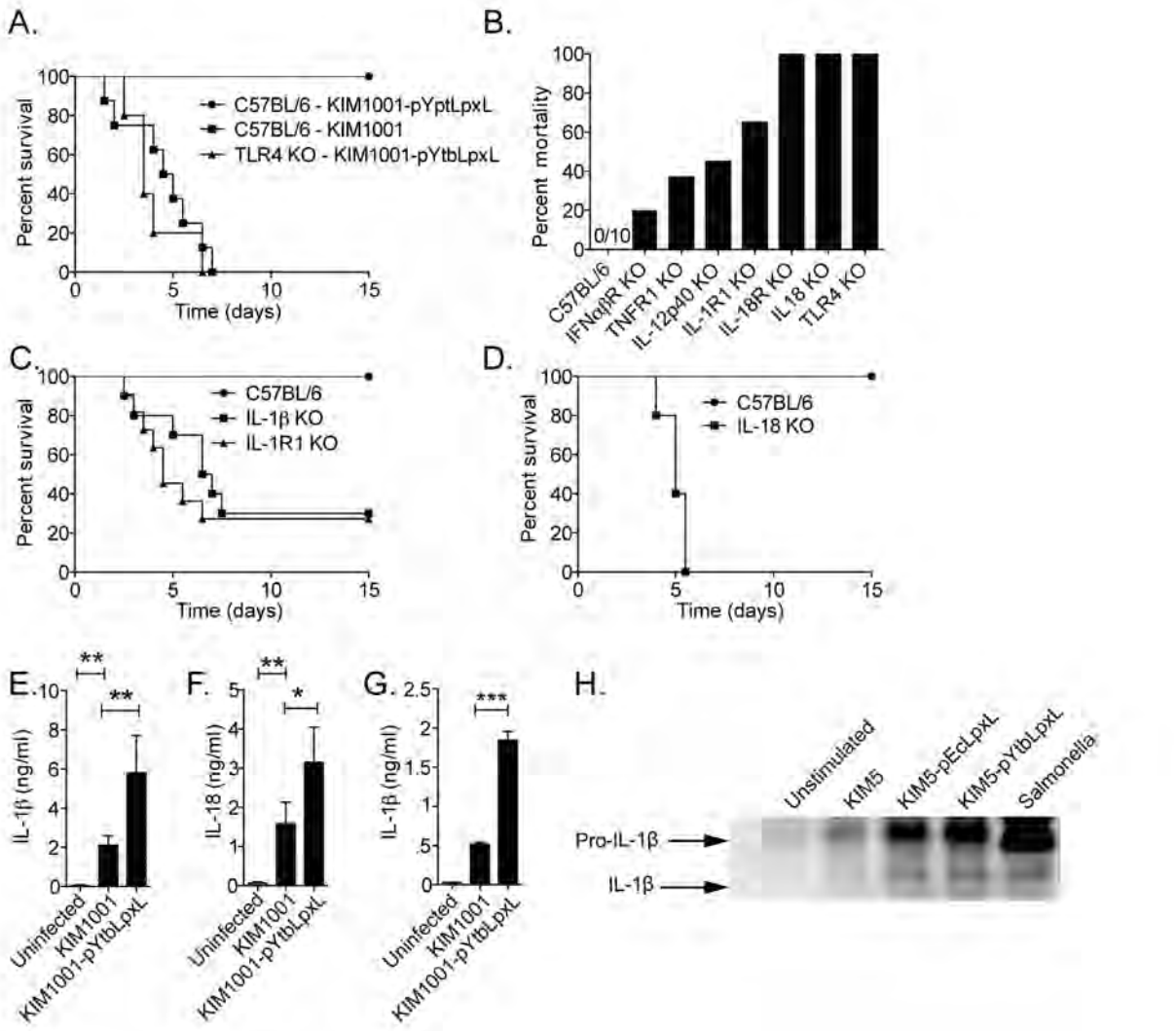
A.
Percent survival
Time (days)
C57BL/6 - KIM1001-pYptLpxL
C57BL/6 - KIM1001
TLR4 KO - KIM1001-pYtbLpxL
B.
Percent mortality
0/10
C57BL/6
IFNαβR KO
TNFR1 KO
IL-12p40 KO
IL-1R1 KO
IL-18R KO
IL18 KO
TLR4 KO
C.
Percent survival
Time (days)
C57BL/6
IL-1β KO
IL-1R1 KO
D.
Percent survival
Time (days)
C57BL/6
IL-18 KO
E.
IL-1β (ng/ml)
Uninfected
KIM1001
KIM1001-pYtbLpxL
F.
IL-18 (ng/ml)
Uninfected
KIM1001
KIM1001-pYtbLpxL
G.
IL-1β (ng/ml)
Uninfected
KIM1001
KIM1001-pYtbLpxL
H.
Unstimulated
KIM5
KIM5-pEcLpxL
KIM5-pYtbLpxL
Salmonella
Pro-IL-1β
IL-1β

**Figure 3.2: Infection of Mice with Y. pestis-pYtbLpxL Is Controlled by IL-18**
(A) Survival of mice infected s.c. with 500 CFUs of *Y. pestis* KIM1001 (C57Bl/6: n = 8) or KIM1001-pYtbLpxL (C57Bl/6 and Tlr4$^{-/-}$[TLR4 KO]: n = 10) bacteria. (B) Mortality of animals infected s.c. with 500 CFUs of KIM1001-pYtbLpxL (n = 7 for Ifnar$^{-/-}$[IFNabR KO], 8 for Il12b $^{-/-}$[IL-12p40 KO], 10 for C57Bl/6, Il1r1 $^{-/-}$[IL-1R1 KO], Il18r1 $^{-/-}$[IL-18R KO], Il18 $^{-/-}$[IL-18 KO], and TLR4 KO). Statistical differences in TLR4, IL-18, or IL-18R versus IL-1R and other strains: $p < 0.002$. Statistical differences in IL-12p40, IL-18, IL-18R, and TLR4 versus C57Bl/6: $p < 0.001$. (C and D) Survival of mice deficient in (C) IL-1β (Il1β$^{-/-}$, IL1β KO) and IL-1R and (D) IL-18 infected s.c. with 500 CFUs of KIM1001-pYtbLpxL (n = 10 of each genotype). (E and F) Concentrations of spleen IL-1β and IL-18 from C57BL/6 mice infected i.v. with 500 CFU of KIM1001 or KIM1001-pYtbLpxL for 44 hr.(G) IL-1β in supernatants from BMDM stimulated with 10 multiplicity of infection (m.o.i.) of KIM5 or KIM5-pYtbLpxL for 6 hr, 50 mg/ml of gentamicin was added to wells after 3 hr.p.i.; error bars represent the SD. (H) Immunoblot of IL-1β in the combined lysates and supernatants of BMDMs stimulated with 10 m.o.i. of *Y. pestis* KIM5, KIM5-pEcLpxL, and KIM5-pYtbLpxL and 1 m.o.i. of Salmonella typhimurium. Both pro-IL-1β (upper band) and mature IL-1β (lower band) are shown. Shown is representative of three to five performed experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

| Vaccination (KIM1001-pYptbLpxL) | Challenge (KIM1001) | Survival |
|---|---|---|
| None | 500 CFU | 0 of 10 |
| $1x10^3$ CFU | 500 CFU | 10 of 10 |

**Table 3.1: Mice immunized with *Y. pestis* KIM1001-pYtbLpxL are resistant to challenge with KIM1001**

Survival of wild type mice vaccinated subcutaneously with *Y. pestis* KIM1001-pYptbLpxL and naive mice (None) after subsequent subcutaneous challenge with *Y. pestis* KIM1001, presented as surviving mice of total mice. Data representative of two to three experiments.

Resistance to infection in IL-1β and IL-1R1 deficient animals was reduced to a similar degree, with approximately 30% of animals surviving (**Figure 3.2 C**). However, IL-18 was critically important for resistance to infection in this model, as IL-18 and IL-18R deficient mice developed all symptoms of bubonic plague, and rapidly succumbed to disease when infected with KIM1001-pYtbLpxL (**Figure 3.2 B and 3.2 D**). As inflammasomes are responsible for processing of IL-18 and IL-1β into mature forms, this result indicates that this infection model is well-suited for the study of inflammasome mechanisms and implications of IL-18 release. Mice deficient in MyD88, an adaptor molecule common to TLR, IL-1R and IL-18R signaling pathways were more susceptible to wild type *Y. pestis* KIM1001 than wild-type C57Bl/6 mice (**Figure 3.1 C**) and are also highly susceptible to strains expressing LpxL (Montminy et al., 2006). Intravenous (i.v.) infection causes systemic infection even when attenuated bacterial strains are used, hence the inflammatory capacity in tissues for various bacterial strains can better be compared using this route of delivery. We found elevated levels of spleen IL-1β and IL-18 during i.v. infection with *Y. pestis*, and fully virulent KIM1001 induced lower cytokine levels as compared to KIM1001-pYtbLpxL producing the potent LPS (**Figure 3.2 E-F**). A similar release pattern could also be seen in vitro using bone marrow derived macrophages (BMDM) (**Figure 3.2 G**) after stimulation with KIM5 (a pgm mutant attenuated strain used for *in vitro* experiments) or KIM5-pYtbLpxL. Western blot analysis (**Figure 3.2 H**) indicated that pro-IL-1β was indeed cleaved into mature IL-1β following infection with Y. pestis strains, a sign of inflammasome action. Infection with the *Y. pestis*-YtbLpxL strain markedly increased levels of pro- and cleaved IL-1β. These results indicate that minimizing inflammasome priming may

have been an important implication of *lpxL* loss during evolution of *Y. pestis* from *Y. pseudotuberculosis*.

*NLRP12 is involved in recognition of Y. pestis*

We next wanted to determine which NLRs were involved in resistance to *Y. pestis* strains and in IL-18 and IL-1β release. NLRP12 and NLRP3 have both been shown to interact with ASC in generating an IL-1β processing inflamammasome (Manji et al., 2002; Wang et al., 2002; Agostini et al., 2004), but little is known of the role of NLRP12 during infection. We infected NLRP3 KO and NLRP12 KO mice (**Figure 3.3 A-B**) s.c. with 500 c.f.u. of KIM1001-pYtbLpxL, and found that only 20% of NLRP12 KO and 50% of NLRP3 KO mice survived. This suggests that NLRP12 plays an important role in host defense against some bacterial pathogens. In contrast, NLRP12 KO mice were resistant to infection with *S. typhimurium*, whereas TLR4 KO mice all succumbed to the infectious challenge (**Figure 3.3 C**).

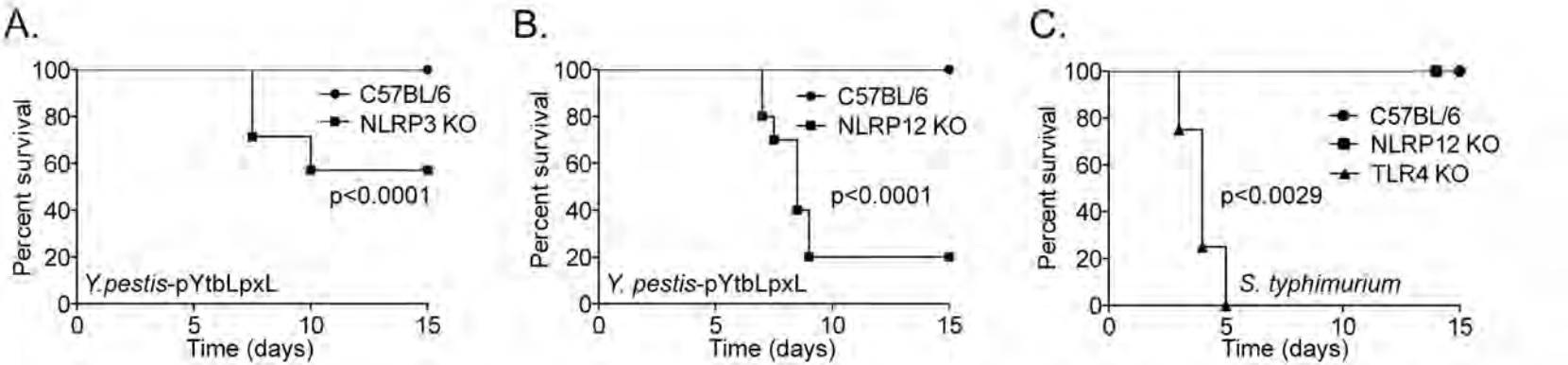
A.
Percent survival
100
80
60
40
20
0
0
5
10
15
Time (days)
C57BL/6
NLRP3 KO
p<0.0001
Y.pestis-pYtbLpxL
B.
Percent survival
Time (days)
C57BL/6
NLRP12 KO
p<0.0001
Y. pestis-pYtbLpxL
C.
Percent survival
Time (days)
C57BL/6
NLRP12 KO
TLR4 KO
p<0.0029
S. typhimurium

**Figure 3.3: NLRP12 Is Involved in Host Resistance to Attenuated *Y. pestis***
(A and B) Survival of C57Bl/6 (circles), (A) Nlrp3$^{-/-}$(NLRP3 KO, squares), and (B) Nlrp12 $^{-/-}$ (NLRP12 KO, squares) mice infected s.c. with 500s CFU of KIM1001-pYtbLpxL. (C) Survival of C57BL/6 (circles), NLRP12 KO (squares), or TLR4 KO (triangle) mice infected i.p. with 500 CFUs of *S. typhimurium*; $p < 0.003$ (NLRP12 KO or WT versus TLR4 KO).

This indicates that NLRP12 deficient animals are not universally more sensitive to infections. The function of NLRP12 is not well understood, but mRNA is detectable in several organs and immune cells (**Figure 3.4 A-B**), including macrophages, although prolonged macrophage maturation led to a decrease in NLRP12 levels (**Figure 3.4 C**). NLRP12 KO mice (**Figure 3.4 D**) had a normal composition of cell populations in spleen and bone marrow (**Figure 3.4 E**). The possible involvement of NLRP12 in maturation of IL-1β and IL-18 led us to perform *in vitro* experiments with mouse cells to study inflammasome components that promote caspase-1 cleavage and IL-1β/IL-18 release following infection with Y. pestis and modified strains. Neutrophils express more NLRP12 than macrophages (**Figure 3.4 B**), but the role of inflammasomes in pathogen-induced neutrophil release of IL-1β and IL-18 is not yet studied in detail for many microbes. We found that thioglycollate-elicited neutrophil-enriched peritoneal cells released IL-1β after *Y. pestis* infection (**Figure 3.5 A**). When compared to cells from wild-type mice, the amounts of IL-1β, but not TNF (**Figure 3.6 A**) released from the NLRP12 KO neutrophils were significantly reduced after stimulation with *Y.pestis* strains. Moreover, infected neutrophils from the caspase-1 KO mice lack IL-1β in the supernatant, suggesting that *Y. pestis*-induced neutrophil IL-1β release involves caspase-1 inflammasomes, although we cannot rule out a role for other neutrophil proteases (Netea et al., 2010). It is also unclear which role caspase-11 plays relative to caspase-1 in *Y. pestis*-induced inflammasome activation, as the caspase-1 deficient mice utilized in this study contain the same truncated and apparently non-functional caspase-11 as previously published (Kayagaki et al., 2011). Macrophages deficient in NLRP12 or NLRP3 also had a reduced ability to release both IL-18 and IL-1β after infection with parental *Y. pestis* and *Y.pestis*-pYtbLpxL (**Figure 3.5 B-C**).

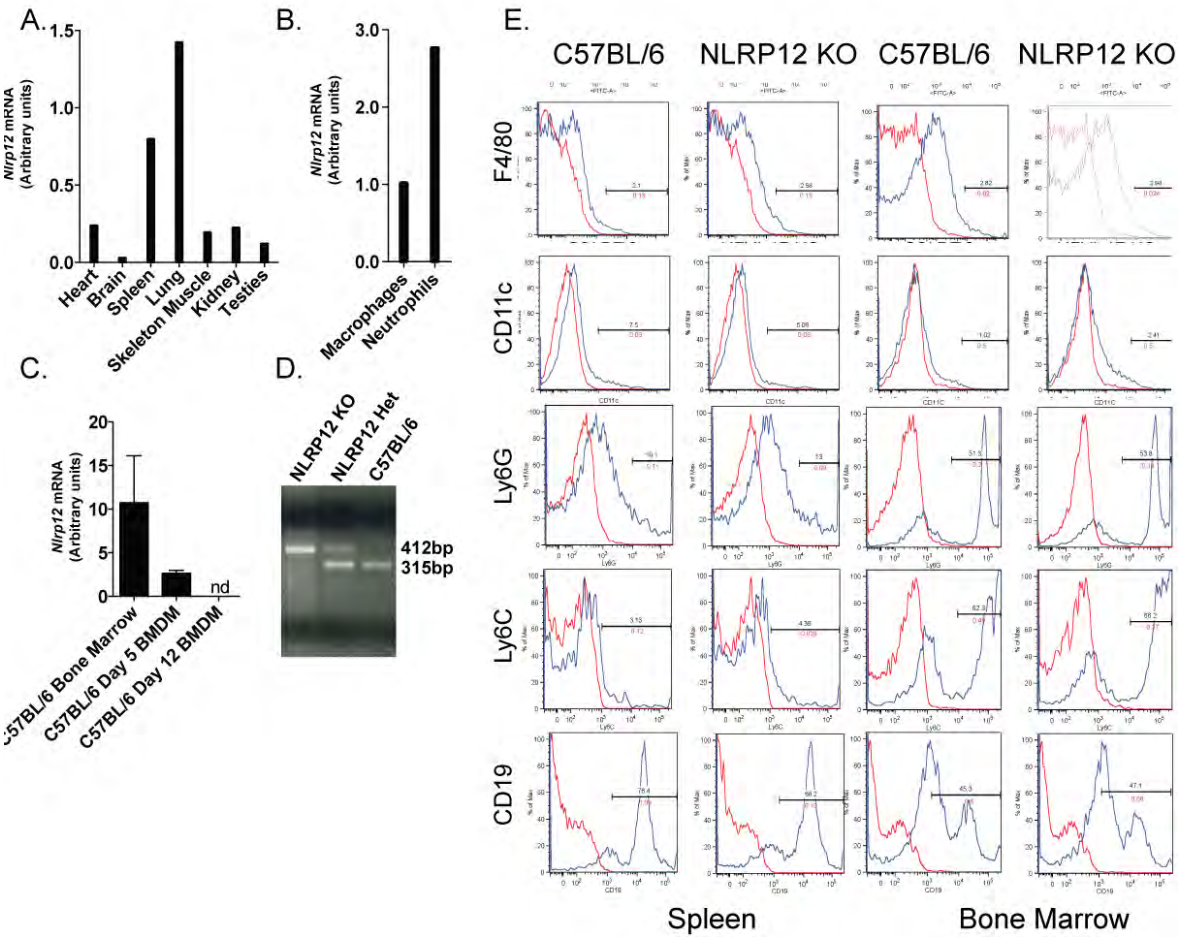


**Figure 3.4: Characterizing the NLRP12 KO mouse**

(A-C) Nlrp12 mRNA measured by qPCR using cDNA (A) from multiple tissues, or (B-C) reverse transcribed cell lysate RNA, results are given as arbitrary units. Samples were normalized towards β-actin. (C) Lysates from bone marrow, freshly harvested or matured with M CSF (in L929 supernatant) for 5 or 12 days, were used for generating mRNA (D) RT-PCR from NLRP12 genotyping of a NLRP12-KO mouse (left lane), a +/- heterozygous animal (het - middle) and a wild-type C57Bl/6 mouse (right). DNA was extracted from tail clippings. (E) Flow cytometric analysis of F4/80 positive macrophages, CD11c positive dendritic cells, Ly6G positive granulocytes, and CD19 positive B cells in spleen cell suspensions or bone marrow of C57BL/6 and NLPR12 KO uninfected mice. Blue lines show specific stain, red lines show isotope controls. Cell populations in WT and NLRP12 KO spleens and bone marrows are comparable. Results are representative out of at least three experiments.

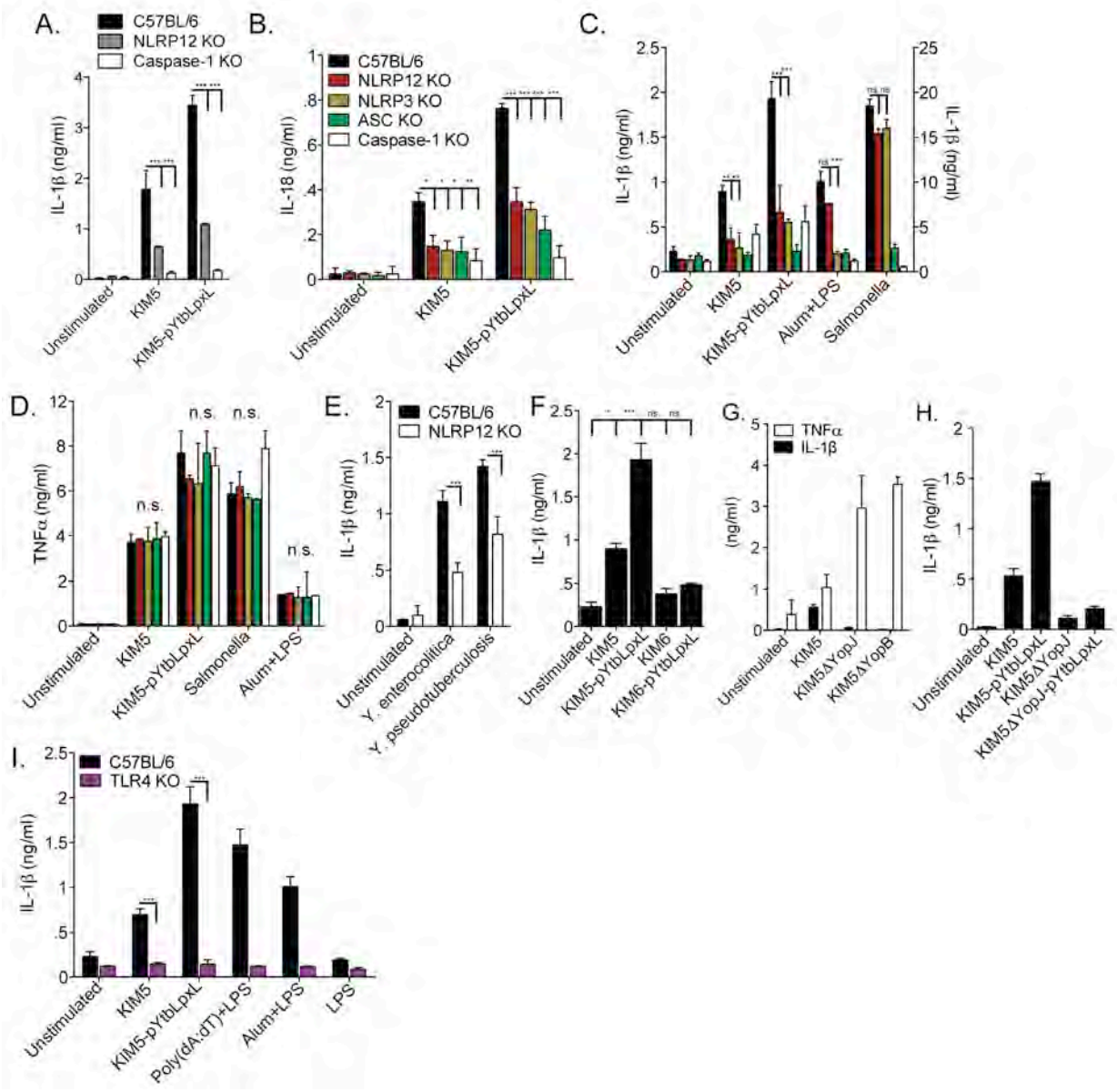


**Figure 3.5: NLRP12 Mediates *Y. pestis*-Induced Release of IL-1β and IL-18**
(A) IL-1β released from neutrophil-enriched peritoneal cells from C57BL/6 (black bars), NLRP12 KO (gray bars), and Casp1$^{-/-}$(Caspase-1 KO, white bars) mice. (B–I) IL-18 (B), IL-1β (C and E–I) and TNF (D and G) released from C57BL/6, NLRP12 KO, NLRP3 KO, ASC KO, and caspase-1 KO BMDM (B–D); C57BL/6 and NLRP12 KO BMDM (E); C57BL/6 BMDM (F and G); or C57BL/6 and TLR4 KO BMDMs (I). Infection with *Yersinia* strains occurred for 6 hr, with an addition of 50 mg/ml gentamicin to limit bacterial growth after 3 hr. *Yersinia* strains were added at 10 m.o.i., S. typhimurium at 1 m.o.i. Alum (130 mg/ml) stimulations (C and I) lasted 6 hr after priming for 3 hr with 10 ng/ml KIM5-YtbLpxL LPS. Shown are mean for triplicate cultures (with SD) in representative experiments out of three to ten performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

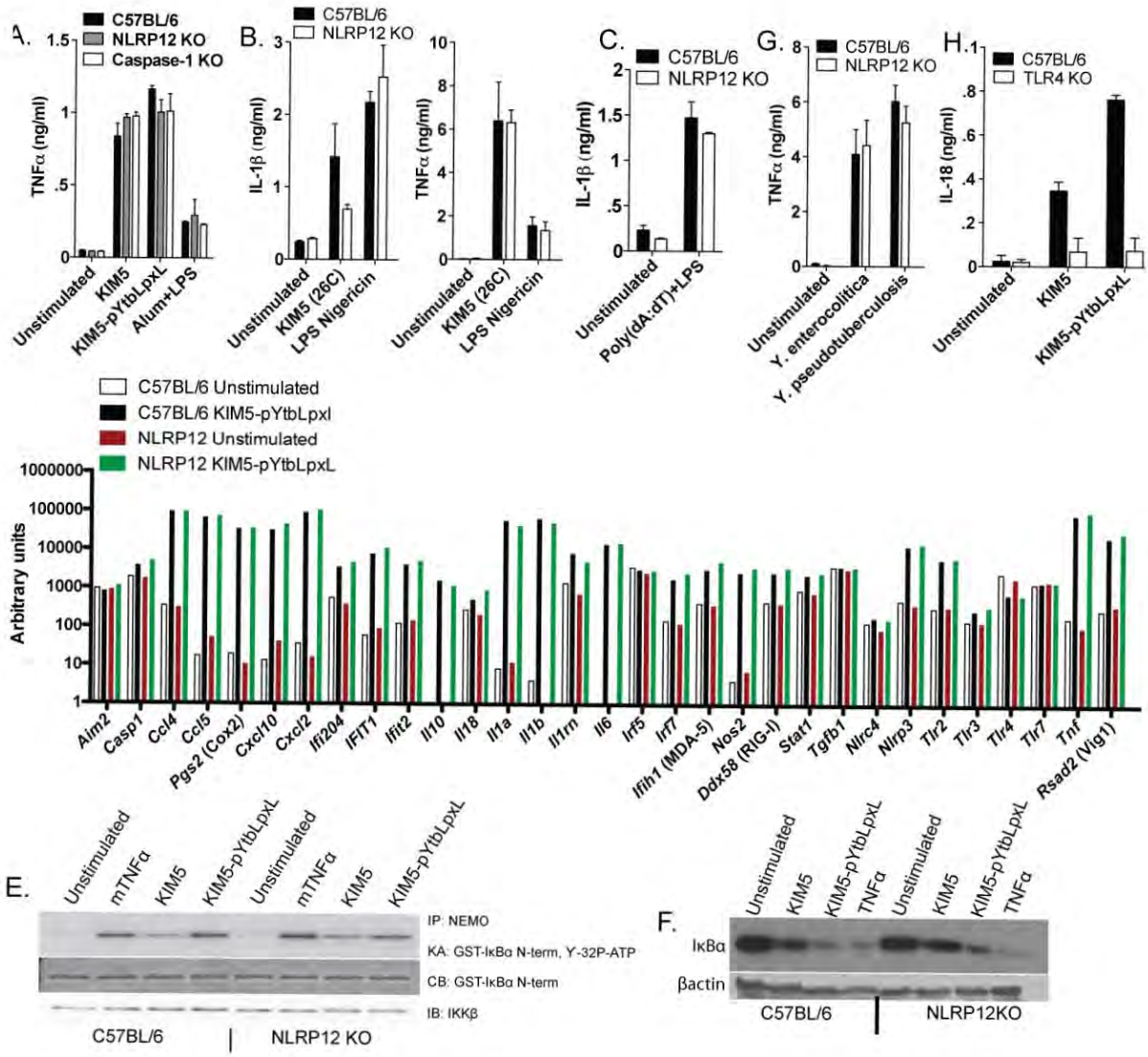
A.
C57BL/6
NLRP12 KO
Caspase-1 KO
TNFα (ng/ml)
Unstimulated
KIM5
KIM5-pYtbLpxL
Alum+LPS
B.
C57BL/6
NLRP12 KO
IL-1β (ng/ml)
TNFα (ng/ml)
Unstimulated
KIM5 (26C)
LPS Nigericin
C.
C57BL/6
NLRP12 KO
IL-1β (ng/ml)
Unstimulated
Poly(dA:dT)+LPS
G.
C57BL/6
NLRP12 KO
TNFα (ng/ml)
Unstimulated
Y. enterocolitica
Y. pseudotuberculosis
H.
C57BL/6
TLR4 KO
IL-18 (ng/ml)
Unstimulated
KIM5
KIM5-pYtbLpxL
C57BL/6 Unstimulated
C57BL/6 KIM5-pYtbLpxl
NLRP12 Unstimulated
NLRP12 KIM5-pYtbLpxL
Arbitrary units
Aim2
Casp1
Ccl4
Ccl5
Pgs2 (Cox2)
Cxcl10
Cxcl2
Ifi204
IFIT1
Ifit2
Il10
Il18
Il1a
Il1b
Il1rn
Il6
Irf5
Irf7
Ifih1 (MDA-5)
Nos2
Ddx58 (RIG-I)
Stat1
Tgfb1
Nlrc4
Nlrp3
Tlr2
Tlr3
Tlr4
Tlr7
Tnf
Rsad2 (Vig1)
E.
Unstimulated
mTNFα
KIM5
KIM5-pYtbLpxL
IP: NEMO
KA: GST-IκBα N-term, Y-32P-ATP
CB: GST-IκBα N-term
IB: IKKβ
C57BL/6
NLRP12 KO
F.
Unstimulated
KIM5
KIM5-pYtbLpxL
TNFα
IκBα
βactin
C57BL/6
NLRP12KO

**Figure 3.6: WT and NLRP12 KO BMDM have comparable non-inflammasome phenotypes**

(A-C) ELISA of (A,B right) TNF or (B left, C) in the supernatants of (A) WT, NLRP12 KO or Caspase-1 KO peritoneal cells enriched for neutrophils or (B) WT or NLRP12 BMDM after 6 hours stimulation (including a gentamicin of infected wells at 3hpi) with (A) KIM5, KIM5-pYtbLpxL (MOI=10) or Alum following a three hour LPS priming, (B) KIM5 pre-grown at 26$^{o}$C, for 6 hours as above; Nigericin was used for 1 hour after LPS priming, or (C) poly(dA:dT) 1 μg/ml (transfected with Genejuice) for 3 hours after LPS prime. (D) Expression level of 31 genes related to innate immunity and inflammatory responses from RNA of C57BL/6 and NLRP12-deficient BMDM. Cells were infected with KIM5-pYtbLpxL (MOI=10) for 4 hours or left unstimulated, and gentamicin was added after 2 hrs to limit bacterial growth. RNA samples were subject to analysis by Nanostring nCounter instrumentation using a custom multiplex codeset. Values are given as arbitrary units, normalized to internal standards: GAPDH and HPRT1 and reflect non-amplified transcript levels. Shown is one representative experiment out of two performed. (E) Kinase Assay (top) detecting IKK kinase activity was performed using lysates from WT or NLRP12 KO BMDM infected with KIM5 or KIM5-pYtbLpxL for 1 hour (MOI=10), or treated with recombinant mouse TNFα for 10 minutes. BMDM lysates were subject to IP with anti-IKKɣ/NEMO and then incubation with purified GST-IκBα and ɣ- p32 labeled ATP. Loading controls (middle) show coomassie blue staining of total loaded protein after the IP, and (bottom) IKKb WB shows that the NEMO IPs co-IP'd similar amounts of the kinase subunit. (F) Western blot of lysates using (top) anti-IκBα from BMDM stimulated as in Figure S3E, loading control (bottom) showing β-actin. (G- H) ELISA for (G) TNFα or (H) IL-18 in supernatants of (G) WT or NLRP12 KO BMDM (H) WT and TLR4 KO BMDM stimulated with (G) Y. enterocolitica and Y. pseudotuberculosis (10 m.o.i.) or (H) KIM5 and KIM5-pYbLpxL for 6 hours, bothwith a gentamicin (50 μg/ml) treatment at 3hpi. Cytokines were measured by ELISA in one representative experiment out of three.

These observations are consistent with the survival data (**Figure 3.3**), which indicated that host recognition of *Y. pestis* involves NLRP12. Cells deficient in ASC and caspase-1 also had decreased IL-18 and IL-1β release (**Figure 3.5 B-C**). Thus, NLRP12 signaling may occur parallel to or in cooperation with additional inflammasome components, as NLRP12 deficiency did not completely block cytokine release. NLRP12 KO macrophages responded normally to alum, *S. typhimurium* (**Figure 3.5 C**), nigericin and poly(dA:dT) (**Figure 3.6 B-C**) suggesting that NLRP12 may not participate in NLRP3, AIM2 or NLRC4 inflammasomes formed in response to those stimuli. None of the inflammasome proteins had an impact on TNF release (**Figure 3.5 D**, **Figure 3.6 A**). Furthermore, NLRP12 deficiency had little impact on the expression of 31 selected macrophage genes, including IL-1β, in the absence or presence of bacteria (**Figure 3.6 C**). Many of those genes are controlled by NF-kB and/or MAPK. In a more detailed study, NF-kB signaling measured by IKK kinase assay and IkB degradation was also largely preserved in NLRP12 deficient cells (**Figure 3.6 D-E**). *Y. pestis* pre-grown at 26ºC naturally expresses a hexa-acylated LPS (Montminy et al., 2006), and release of IL-1β in response to infection by 26ºC-grown bacteria was also influenced by NLRP12 (**Figure 3.6 B**). Upon infection of wild-type and NLRP12 KO BMDM with the human pathogens *Y. pseudotuberculosis* and *Y. enterocolitica*, ancestors of *Y. pestis* (Chain, 2004), we observed a reduction in secreted IL-1β from the cells lacking NLRP12 (**Figure 3.5 E**) while TNF release was normal (**Figure 3.6 F**). By using KIM6, a derivative of KIM5 that lacks the pCD1 virulence plasmid containing genes for the T3SS (Perry and Fetherston, 1997), we found that the secretion system was necessary for stimulating IL-1β release, even in the presence of a highly stimulatory LPS as found in KIM6-pYtbLpxL (**Figure 3.5 F**). YopJ may participate in inflammasome

activation (Zheng et al., 2011) and the deletion of YopJ or the T3SS translocon protein YopB reduced IL-1β release (**Figure 3.5 G**). Experiments performed using a strain with the expression of LpxL on a YopJ mutant background suggested that YopJ is a key player controlling IL-1β release, even in the presence of a stimulatory LPS (**Figure 3.5 H**), although other T3SS-dependent factors may also regulate IL-1β (Brodsky et al., 2010). The data suggest that the ligand(s) responsible for NLRP12 activation are dependent on the *Yersinia* T3SS. TLR4 plays a critical role in the IL-1β and IL-18 production after infection of the mouse macrophages (**Figure 3.5 I**, and **Figure 3.6 G**), although the relative importance of mouse vs. human TLR4/MD-2 in inducing *Y. pestis* responses may differ. Rodent cells have higher ability to recognize hypo-acylated lipid A (Lien et al., 2000; Montminy et al., 2006). This may be influenced by a shallow positioning of the hypoacylated lipid A in mouse MD-2 compared to human MD-2, and the enabling of enhanced ionic interactions between hypoacylated lipid A and mouse TLR4, facilitating receptor cluster dimerization and signaling (Park et al., 2009). Our results indicate a role for both TLR4 and NLRP12 in the pro-inflammatory macrophage response against *Y. pestis* strains.

*NLRP12 is an inflammasome component*

Upregulation of NLRP3 has been suggested to positively affect the activity of the NLRP3 inflammasome (Bauernfeind et al., 2009). We therefore studied expression levels of NLRP12 and NLRP3 (**Figure 3.7 A-B**) after infection of macrophages with KIM5 or KIM5-pYtbLpxL. Expression of NLRP12 in BMDM was markedly increased after infection with *Y. pestis* strains and this may boost host responses to an infection. Treatment with LPS alone upregulated NLRP12 levels (**Figure 3.8 A**).

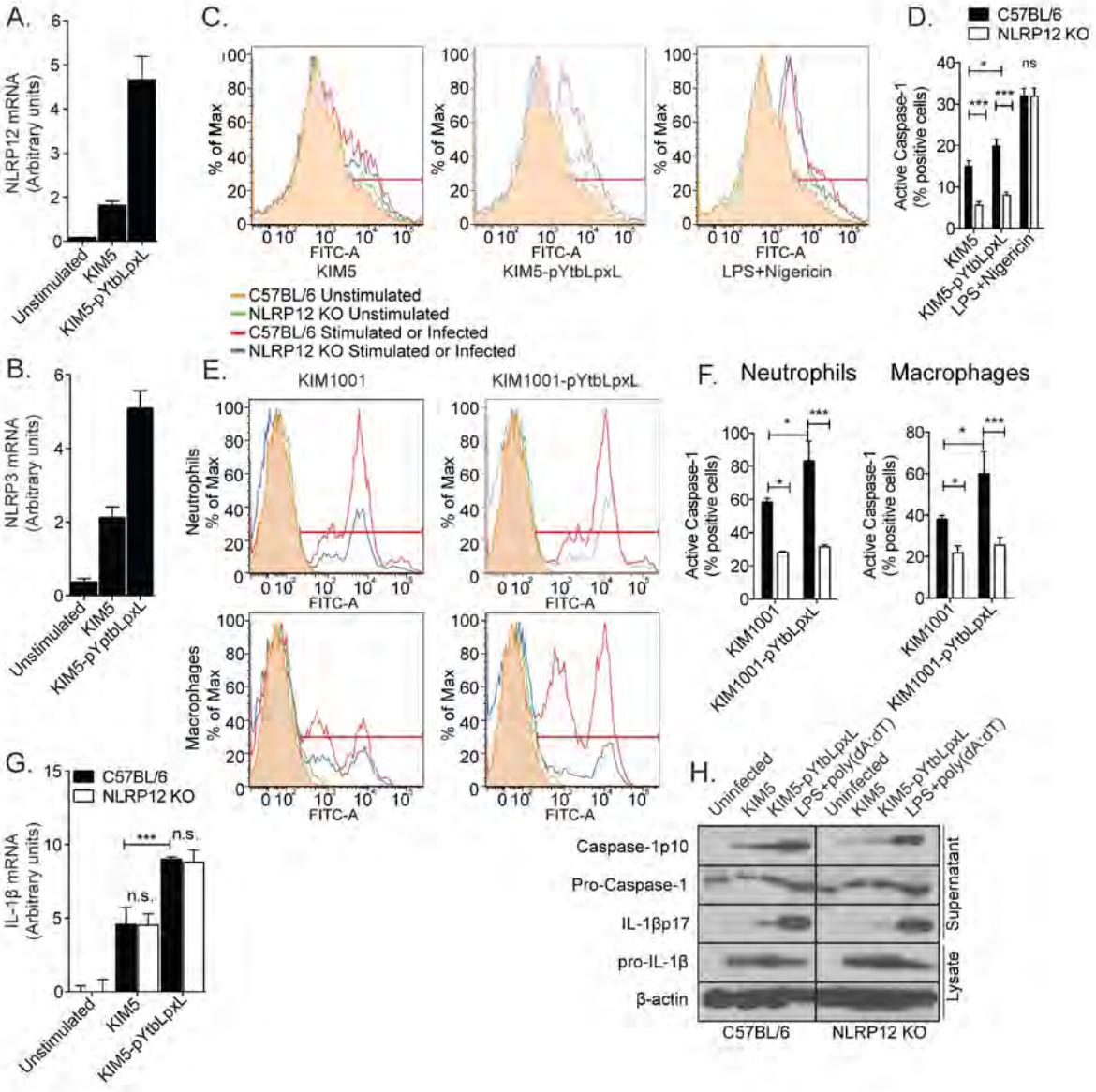
A.
NLRP12 mRNA (Arbitrary units)
Unstimulated
KIM5
KIM5-pYtbLpxL
B.
NLRP3 mRNA (Arbitrary units)
C.
% of Max
FITC-A
KIM5
KIM5-pYtbLpxL
LPS+Nigericin
C57BL/6 Unstimulated
NLRP12 KO Unstimulated
C57BL/6 Stimulated or Infected
NLRP12 KO Stimulated or Infected
D.
C57BL/6
NLRP12 KO
Active Caspase-1 (% positive cells)
ns
E.
KIM1001
KIM1001-pYtbLpxL
Neutrophils
Macrophages
F.
Neutrophils
Macrophages
G.
C57BL/6
NLRP12 KO
IL-1β mRNA (Arbitrary units)
n.s.
H.
Uninfected
KIM5
KIM5-pYtbLpxL
LPS+poly(dA:dT)
Caspase-1p10
Pro-Caspase-1
IL-1βp17
pro-IL-1β
β-actin
Supernatant
Lysate
C57BL/6
NLRP12 KO

**Figure 3.7: NLRP12 Is Necessary for Optimal Maturation of IL-1β and Caspase-1 after Infection with *Y. pestis***

(A, B, and G) Q-PCR of (A) Nlrp12, (B) Nlrp3, or (G) Il1b from BMDMs infected with 10 m.o.i. of Y. pestis KIM5 or KIM5-pYtbLpxL for (G) 4 hr or (A and B) 6 hr, with gentamicin addition after 3 hr. Error bars represent the SD. (C–F) FACS histograms (C and E) showing active caspase-1 after FLICA reagent staining with corresponding (D and F) percent positive cells of (C and D) bone marrow cells after 6 hr of challenge with 10 m.o.i. of *Y. pestis* strains (gentamicin added after 3 hr) or (E and F) Ly6G- or F4/80-positive splenocytes from mice infected with 500 CFUs of KIM1001 or KIM1001-pYtbLpxL for 24 hr. Values from unstimulated cells are subtracted in (D). LPS primed cells treated with nigericin (10 mM) served as a control (C and D). Error bars in (D) and (F) represent the SD.

(H) Immunoblot for caspase-1 p10 or IL-1b p17 in supernatant or cell lysate from BMDMs exposed for 10 hr to poly(dA:dT) (LPS primed as in Figure 3), KIM5, or KIM5-pYtbLpxL. Shown is a representative of two (E–G) or three to five (A–D, H, and I) experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Also see Figure S4.

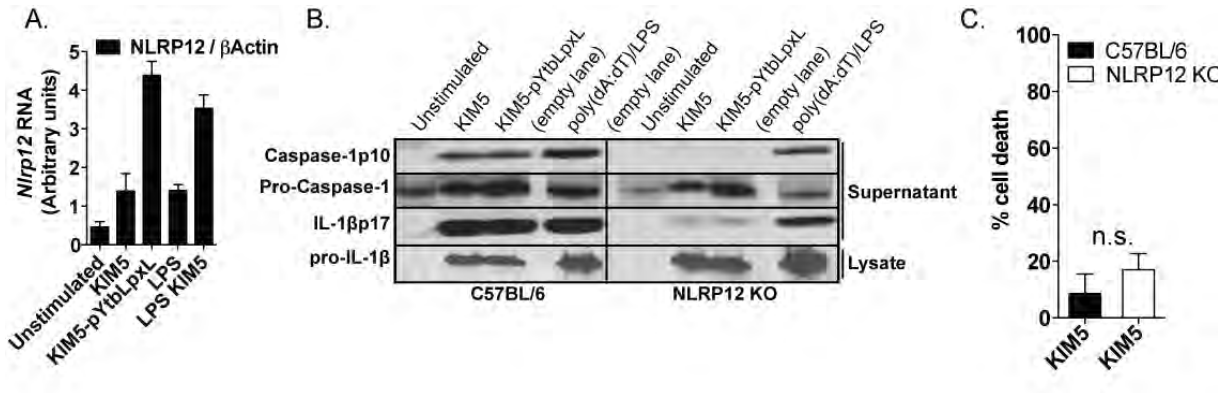


**Figure 3.8: NLRP12 is Upregulated After Stimulation and Plays a Role in Caspase-1 Activation, but not in Cell Death**

Figure S4: (A) Nlpr12 q-PCR of cDNA from RNA purified from WT BMDM stimulated with KIM5, KIM5- pYtbLpxL, LPS (100ng/ml) or KIM5 after 3 hours of LPS priming (100 ng/ml). (B) Caspase-1 and IL-1β maturation following infection of BMDM for 10 hrs with high MOI of the given bacterial strains, 10ng/ml LPS and 2,6 ug/ml of poly dA:dT was used as a control stimulation. (C) Cell death in C57Bl/6 and NLRP12 KO BMDM, 6 hrs after infection of 20 c.f.u. of KIM5 bacteria and centrifugation, measured by calcein stain. Gentamicin was added to a total concentration of (50 μg/ml) 1 hour into the infection. Results are representative out of at least three experiments.

Furthermore, *Y. pestis*-induced formation of cleaved and active caspase-1, as measured by an assay showing binding of active caspase-1 to a fluorescent substrate, was also impaired in NLRP12-deficient cells, providing evidence for NLRP12-dependent inflammasome function (**Figure 3.7 C-D**). Caspase-1 cleavage measured by this assay is also decreased in spleen macrophages or neutrophils from NLRP12 KO mice 24 hours after infection with KIM1001 or KIM1001-pYtbLpxL (**Figure 3.7 E-F**). IL-1β gene expression was similar in infected wild type cells and NLRP12 KO cells infected with *Y. pestis* **(Figure 3.7 G**, and **Figure 3.8 D**). The macrophages infected *in vitro* showed a reduction in caspase-1 and IL-1β processing by western blot (**Figure 3.7 H**), also cells infected at a higher MOI (**Figure 3.8 B**). Thus, several lines of evidence support the hypothesis that NLRP12 is a component of inflammasomes formed after *Y. pestis* infection. Macrophage cell death induced by *Y. pestis* has been reported to be caspase-1 independent (Lilo et al., 2008). We confirmed those data (not shown), and in line with this observation, NLRP12 deficient cells did not show an altered cell death in response to *Y. pestis* infection (**Figure 3.8 C**). Cell death may be induced by other mechanisms than pyroptosis in macrophages infected with *Y. pestis*.

*NLRP12 and IL-18 mediate host resistance to Y. pestis infection*

As shown in **Figures 3.2** and **3.3**, NLRP12 KO and IL-18 KO mice are more susceptible than wild-type mice to infection with *Y. pestis*-pYtbLpxL. To monitor changes in IL-18 and IL-1β in tissues during systemic disease, we subjected WT and NLRP12 mice to i.v. infection with fully virulent or attenuated *Y. pestis* (KIM1001 or KIM1001-pYtbLpxL). At 44 hours post infection with KIM1001, IL-18 cytokine levels were significantly lower in the NLRP12 KO mice, expressed as both cytokine normalized to the spleen bacterial load in each particular animal (**Figure 3.9 A**) or simply as cytokine concentration in homogenate (**Figure 3.10 A**).

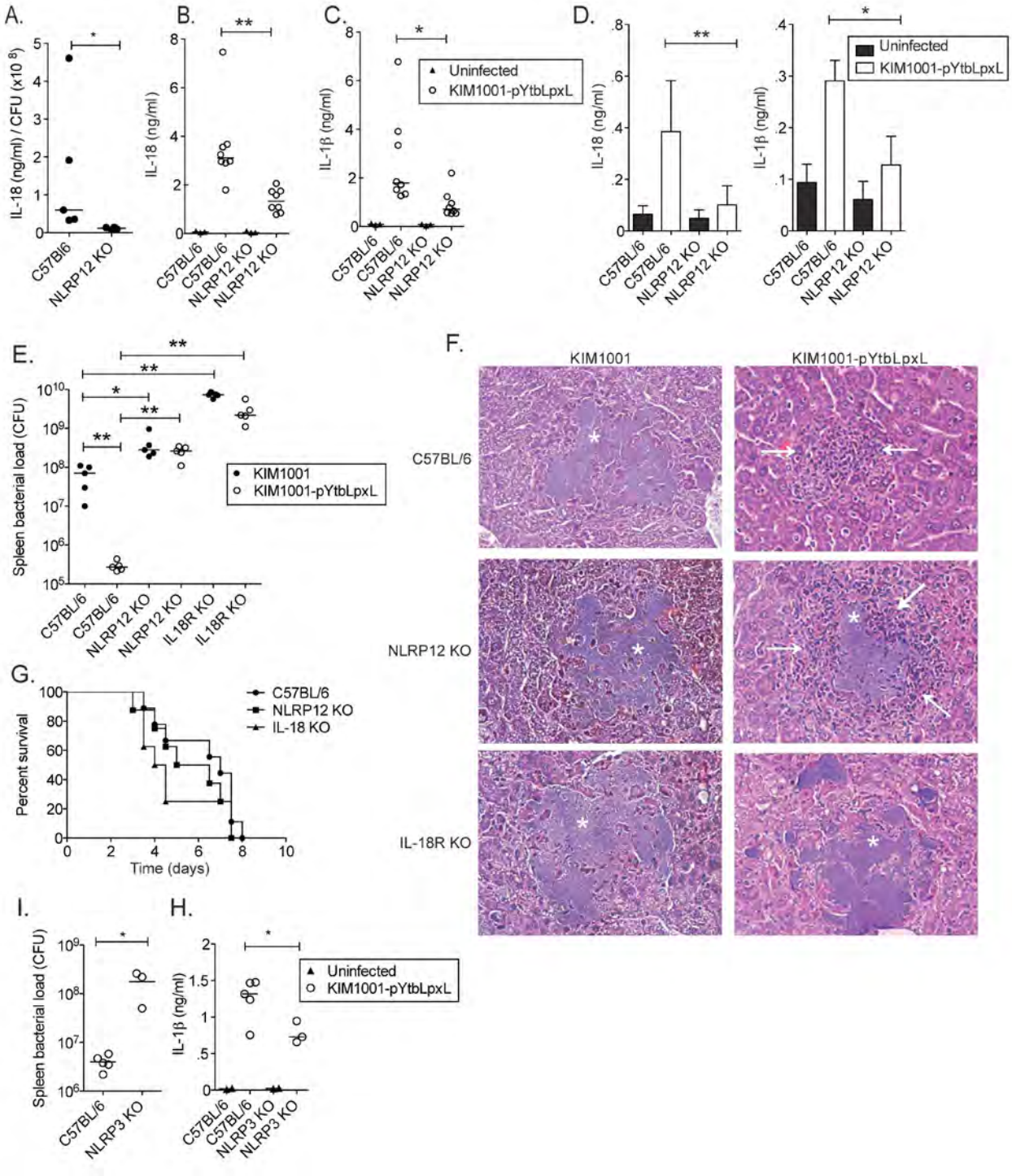
A.
IL-18 (ng/ml) / CFU (x10 8)
C57Bl/6
NLRP12 KO
B.
IL-18 (ng/ml)
C57BL/6
C57BL/6
NLRP12 KO
NLRP12 KO
C.
IL-1β (ng/ml)
Uninfected
KIM1001-pYtbLpxL
D.
IL-18 (ng/ml)
IL-1β (ng/ml)
Uninfected
KIM1001-pYtbLpxL
E.
Spleen bacterial load (CFU)
KIM1001
KIM1001-pYtbLpxL
IL18R KO
IL18R KO
F.
KIM1001
KIM1001-pYtbLpxL
C57BL/6
NLRP12 KO
IL-18R KO
G.
Percent survival
Time (days)
C57BL/6
NLRP12 KO
IL-18 KO
I.
Spleen bacterial load (CFU)
NLRP3 KO
H.
IL-1β (ng/ml)
NLRP3 KO
NLRP3 KO

**Figure 3.9: NLRP12 and IL-18 Control Infection with *Y. pestis In Vivo***

(A) IL-18 in serum (ng/ml) of C57Bl/6 or NLRP12 KO mice infected i.v. with 500 CFU *Y. pestis* KIM1001 for 44 hr, normalized by bacterial loads (for each animal); n = 5. (B–D) IL-18 or IL-1β in (B and C) spleen or (D) serum of WT or NLRP12 KO mice infected i.v. with 500 CFU KIM1001-pYtbLpxL for 44 hr. In (B) and (C), uninfected mice: n = 3; infected animals: n = 8. In (D), uninfected mice: n = 4; infected mice: n = 5. (E) Spleen CFUs of mice infected i.v. with either KIM1001 or KIM1001-pYtbLpxL (n = 5). Horizontal lines indicate mean values. (F) Histology of fixed H&E stained liver sections from (top) WT, (middle) NLRP12 KO, or (bottom) IL-18R KO mice infected i.v. with (left) KIM1001 or (right) KIM1001-pYtbLpxL for 44 hr. Asterisks represent bacterial clusters; arrows represent foci of inflammatory cells, primarily neutrophils. (G) Survival of C57Bl/6 (n = 10), IL-18 KO, and NLRP12 KO (n = 8) mice with 10 CFU s.c. of KIM1001. (H and I) Spleen CFU (H) or spleen IL-1b (I) of C57Bl/6 and NLRP3 KO mice infected for 44 hr with 500 CFU i.v. of KIM1001-pYtbLpxL. Shown is a representative of three performed experiments. *$p < 0.05$; **$p < 0.001$. Also see Figure S5.

A decrease of IL-18 and IL-1β in the spleen (**Figure 3.9 B-C**) and serum (**Figure 3.9 D-E**) was also observed after KIM1001-pYtbLpxL infection in the NLRP12 KO mice as compared to wild-type mice. Experiments with IL-1R KO, IL-1β KO and IL-18R KO suggested that IL-18 signaling had the greatest impact on resistance to *Y. pestis*-pYtbLpxL, as 100% of IL-18 and IL-18R KO animals died after infection (**Figures 3.2 B and 3.2 D**). IL-18R KO mice had reduced levels of IL-18 and IL-1β in the spleens compared to wt mice after infection with KIM1001-pYtbLpxL (**Figure 3.10 B**), suggesting a positive feed-back loop via IL-18R for IL-1β and IL-18 production.

A reduction of several orders of magnitude in spleen bacterial load was seen when mice were infected i.v. with KIM1001-pYtbLpxL compared to wild-type KIM1001 (**Figure 3.9 E**), indicating beneficial host responses induced by the presence of the hexa-acylated LPS. These differences in systemic bacterial load between the two bacterial strains were absent in NLRP12 KO and IL-18R KO mice. NLRP12 KO and IL-18R KO mice also had increased bacterial loads compared to wild-type mice when infected with the virulent *Y. pestis* KIM1001 (**Figure 3.9 E**, $p=0.01$, WT vs. NLRP12 KO; $p<0.001$, wt vs. IL-18R KO). This is important in that it shows that NLRP12 and IL-18 participate in host resistance in vivo towards both virulent and attenuated strains of *Y. pestis*. Thus, it appears that *Y. pestis* has an inherent ability to activate NLRP12-dependent recognition, and that the potent LPS found in strains expressing LpxL increases the formation of pro-forms and subsequently mature forms of inflammasome-controlled cytokines such as IL-1β (**Figures 3.2 G** and **3.5 C**). Livers from animals infected with wild type *Y. pestis* have large extracellular clusters of bacteria (**Figure 3.9 F**, left panels, marked with *) and remarkably few signs of inflammation, likely reflecting active suppression of

immunity combined with stealth via limited initiation of TLR4 signaling. Livers from animals infected with *Y. pestis*-pYtbLpxL display foci consisting of inflammatory cells (**Figure 3.9 F**, upper right, arrows) and absence of visible bacterial masses, suggesting that recruitment of phagocytes limits bacterial growth (Montminy et al., 2006). Livers from NLRP12 KO infected with KIM1001-pYtbLpxL had recruitment of inflammatory cells (**Figure 3.9 F**, arrows). Such masses of inflammatory cells typically contain large number of neutrophils and some mononuclear cells (Montminy et al., 2006), and a calculation of number of recruited cells showed no significant difference between infected wild type versus NLRP12 KO livers (**Figure 3.10 C**). However, this cell recruitment did not correlate with suppression of bacterial growth, as bacterial masses were visible (**Figure 3.9 F**). These results suggest that NLRP12 may not play a major role in the attraction of phagocytes to infected sites in the liver; but is central to the effective anti-bacterial actions they perform. Few, if any inflammatory cells were visible in livers of IL-18R KO mice (**Figure 3.9 F**), indicating failures of both cell recruitment and anti-bacterial defenses.

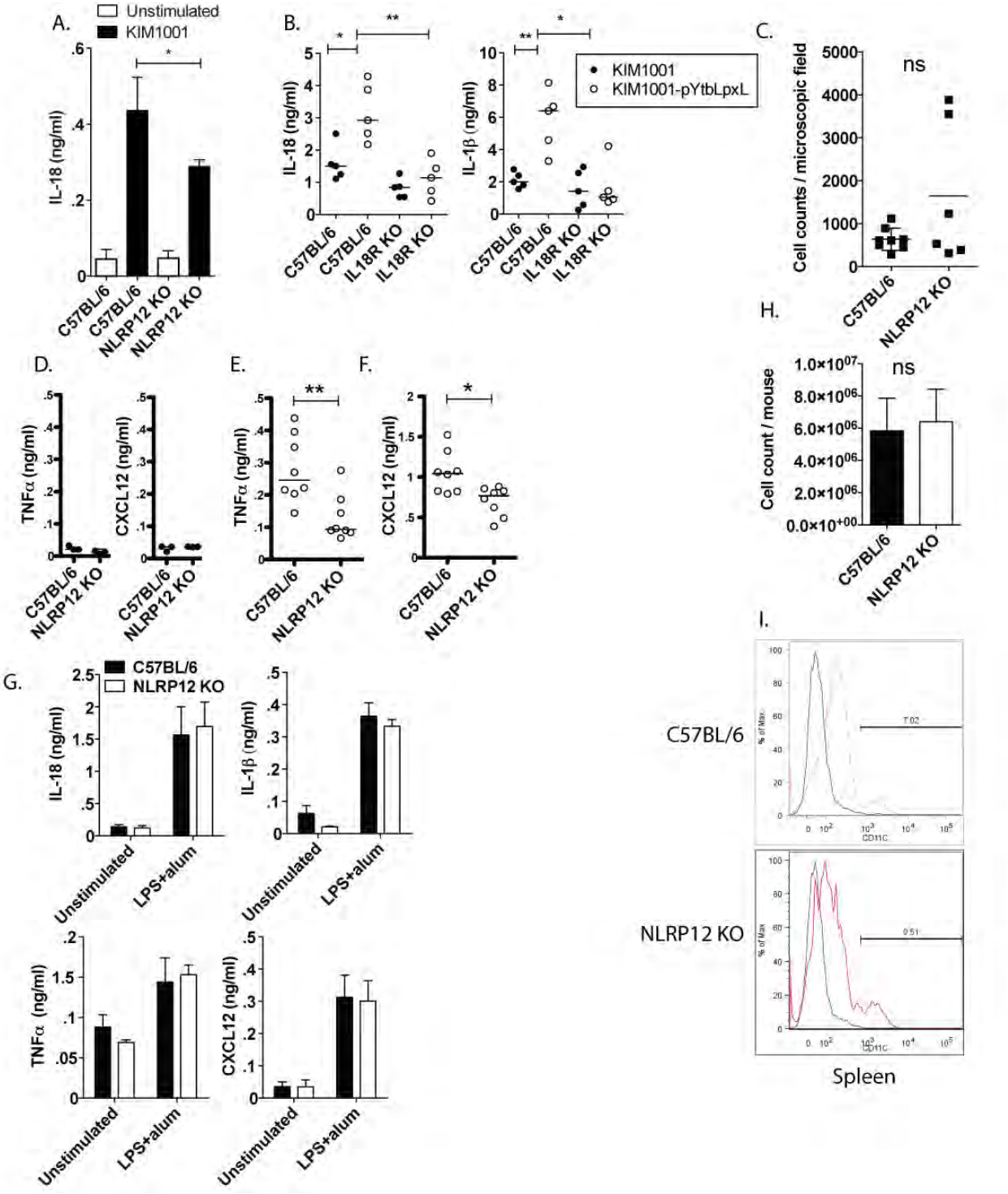
A.
Unstimulated
KIM1001
IL-18 (ng/ml)
C57BL/6
C57BL/6
NLRP12 KO
NLRP12 KO
B.
IL-18 (ng/ml)
IL-1β (ng/ml)
KIM1001
KIM1001-pYtbLpxL
C57BL/6
C57BL/6
IL18R KO
IL18R KO
C.
Cell counts / microscopic field
ns
C57BL/6
NLRP12 KO
D.
TNFα (ng/ml)
CXCL12 (ng/ml)
C57BL/6
NLRP12 KO
C57BL/6
NLRP12 KO
E.
TNFα (ng/ml)
C57BL/6
NLRP12 KO
F.
CXCL12 (ng/ml)
C57BL/6
NLRP12 KO
H.
Cell count / mouse
ns
C57BL/6
NLRP12 KO
G.
C57BL/6
NLRP12 KO
IL-18 (ng/ml)
IL-1β (ng/ml)
Unstimulated
LPS+alum
Unstimulated
LPS+alum
TNFα (ng/ml)
CXCL12 (ng/ml)
Unstimulated
LPS+alum
Unstimulated
LPS+alum
I.
C57BL/6
NLRP12 KO
Spleen

**Figure 3.10: NLRP12 is upstream from IL-18 and IL-1β production after *Y. pestis* infection while recruitment is unchanged.**
(A) IL-18 in spleen homogenates from C57Bl/6 or NLRP12 KO, infected for 44 hrs with KIM1001 (500 c.f.u. i.v.). (B) IL-18 or IL-1β in spleen homogenates of WT or IL-18R KO mice infected i.v. with 500 c.f.u. of KIM1001 or KIM1001-pYtbLpxL and sacrificed after 48 hours post infection. (C) Inflammatory cells, mainly neutrophils, were counted in microscopic fields (100x) from different mice (C57Bl/6 or NLRP12 KO) infected with 500 c.f.u. of KIM1001-pYtbLpxL. (D) Baseline serum levels of (left) TNFα or (right) CXCL12 from uninfected mice. (E-F) Serum levels of (E) TNFα or (F) CXCL12 from mice infected i.v. with 500 c.f.u. of KIM1001-pYtbLxpL, sacrificed at 44 hrs post infection. (G) Levels of IL-18, IL-1β, TNFα, or CXCL12 in the serum of mice either naive or 4h after i.p. injection of 200μg alum pre-mixed with 100 ng KIM5-pYtbLpxL LPS in PBS of WT (black bar) and NLRP12 KO (white bar) mice. (H) Mice were injected with 1 ml of thioglycollate i.p., 4 hrs later peritoneal lavage was performed and neutrophil recruitment assessed. (J) Spleen suspensions from mice infected with 500 c.f.u. of KIM1001-pYtbLpxL were analyzed for CD11c+ DC contents by FACS.

Taken together, the results suggest that NLRP12 and IL-18 contribute to host resistance against *Y. pestis* and *Y. pestis*-pYtbLpxL. We also found that NLRP12 KO mice infected with KIM1001-pYtbLpxL had reduced levels of TNF and the chemokine CXCL12 compared to C57Bl/6 (**Figure 3.10 D-F**), possibly secondary effects of reduced IL-1β and IL-18 levels, as primary NLRP12 KO cells in culture did not display decreased TNF release (**Figure 3.3**). In contrast, NLRP12 KO mice injected with an alum/LPS mixture did not show decreased serum levels of IL-1β, IL-18, TNFα and CXCL12 (**Figure 3.10 G**). Furthermore, we found similar recruitment of neutrophils to the peritoneum of wild type mice or NLRP12 deficient mice injected i.p. with sterile thioglycollate (**Figure 3.10 H**). Movement of neutrophils (Figure 3.9 F, **Figure 3.10 C**) and DC (**Figure 3.10 I**) during infection of NLRP12 KO may be largely preserved. Differences in survival between NLRP12 KO or IL-18 KO mice and wild-type mice after s.c. infection with only 10 c.f.u. of fully virulent KIM1001 were not significant (**Figure 3.9 G**). This result is of uncertain importance because the very low LD50 of Y. pestis (less than 10 c.f.u.) makes it difficult to demonstrate reductions in host resistance impacting survival without the use of very large numbers of animals. Tissue bacterial loads (**Figure 2.9 E**) appear to be more sensitive assays for analyzing host resistance to *Y. pestis*.

NLRP3 has also been proposed as an inflammasome component recognizing *Y. pestis* (Zheng et al., 2011) (**Figure 3.3, Figure 3.5**). NLRP3-deficient animals also were less resistant to infection by KIM1001-pYtbLpxL, in that they displayed increased bacterial loads in the spleen (Figure 3.9 H) that correlated with reduced spleen cytokine levels (**Figure 3.9 I**). To sum up, NLRP12 and NLRP3 both contribute to the host resistance towards *Y. pestis* strains.

*NLRP12, NLRP3 and IL-18 signaling induce IFNγ that limits infection*

IL-18 is a known inducer of IFNγ (Okamura et al., 1995), a key protein in many host responses to pathogens. This suggests that signaling via NLRP12, NLRP3, and the IL-18R, resulting in the release of IFNγ, could mediate resistance to *Y. pestis*-pYtbLpxL. We infected IFNαβR x IFNγR double KO (dKO) animals with *KIM1001*-pYtbLpxL s.c., and we found that all the dKO animals succumbed to the infection (**Figure 3.11 A**). This phenomenon was largely attributed to IFNγR signaling, as only a few IFNαβR single KO died upon infection, whereas almost all IFNγR single KO succumbed (**Figure 3.11 B**). No differences in IFNγ levels were observed between spleens of uninfected wt, NLRP12 KO, NLRP3 KO and IL18R KO mice (**Figure 3.11 C-D**). However, the IFNγ levels in spleens from KIM1001-pYtbLpxL infected NLRP12 KO and NLRP3 KO mice compared to wild-type were drastically reduced (**Figure 3.11 D**), as was also true for the IL-18R KO mice (**Figure 3.11 D**). Thus, we propose a novel cascade of signals from NLRP12 and NLRP3 to IL-18 maturation that in turn mediates IFNγ release following infection with *Y. pestis* strains.

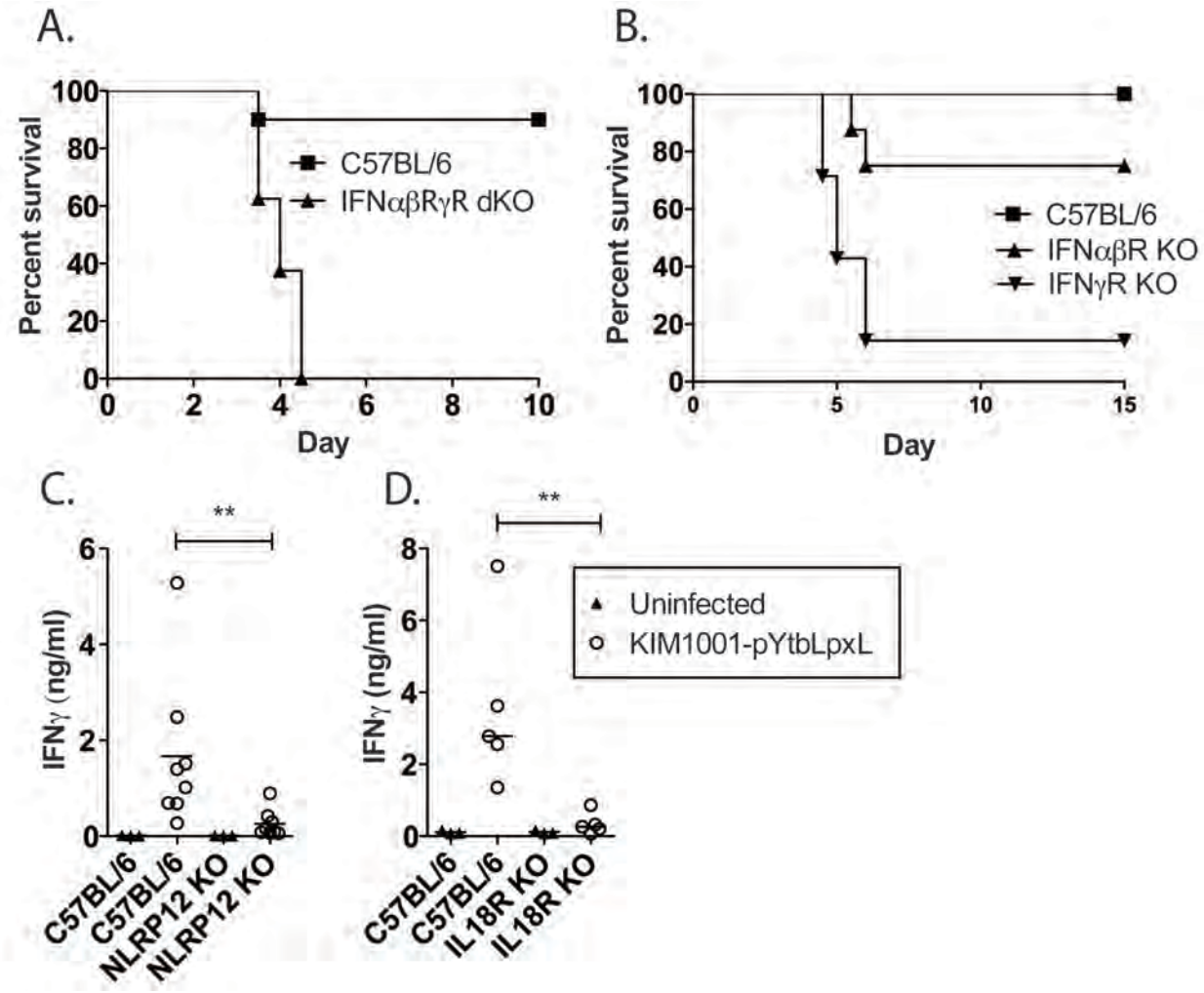


**Figure 3.11: NLRP12 Induces IFN-g via IL-18 Signaling**

(A and B) Survival of mice: in (A), C57Bl/6: n = 10 and Ifnar1$^{-/-}$, 3 Ifngr1$^{-/-}$ (IFNabR 3 IFN-gR DKO; triangles): n = 8, and in (B), C57Bl/6 (squares): n = 10, IFNabR KO: n = 8, and IFN-ɣR KO: n = 7, infected s.c. with 500 CFUs of KIM1001-pYtbLpxL. (C and D) IFN-ɣ in spleen homogenates from C57BL/6 and NLRP12 KO mice infected i.v. with 500 CFU of KIM1001-pYtbLpxL. Uninfected mice: n = 3; in- fected mice: n = 8 (C) and n = 5 (D). Samples were harvested 46 hr after infection. Horizontal lines indicate median values. Experiments shown are representative out of three performed. *$p < 0.05$; **$p < 0.001$.

***Discussion***

We propose that recognition of *Y. pestis* expressing a stimulatory LPS by TLR4 leads to upregulation of NLRP12 and pro-inflammatory cytokines such as IL-18 and IL-1β. NLRP12 then recognizes a ligand produced upon *Y. pestis* infection and assembles into an inflammasome that processes IL-18 and IL-1β. Although the precise nature of the true NLRP12 ligand is unknown, and may be a host or bacterial protein, the generation of the ligand appears to require the virulence-associated T3SS of *Yersinia*. Models for activation may include possibilities that cells sense membrane damage associated with the T3SS, secreted effectors or other molecules channeled by the T3SS, and modified host proteins. NLRP3 also contributes to IL-18/IL-1β release. IL-18 seems to be more critical than IL-1β, and plays a key role in induction of IFNγ.

Most importantly, we show that NLRP12 is an inflammasome component recognizing *Y. pestis*, and contributes to *in vivo* resistance to infection with *Y. pestis* strains, as do NLRP3 inflammasomes. This is the first demonstration of a clear role for NLRP12 in resistance to infection. Our data suggest an inflammasome role for NLRP12 in pathogen recognition, and that the NLRP12 - IL-18 - IFNγ axis is effective in limiting infection with *Y. pestis*-pYtbLpxL. We also show that the expression of *Y. pseudotuberculosis* LpxL in *Y. pestis* increases TLR4-dependent release of IL-18 and IL-1β. This increase correlates with increased resistance to the modified pathogen. In fact, the results indicate that a major consequence of producing LPS with low TLR4-activating potential could be lack of priming necessary for effective synthesis of active IL-1β and IL-18.Therefore, *Y. pestis* is able to utilize inflammasome-activating components like the T3SS to neutralize the immune response without an effective activation of

an inflammatory response. This phenomenon may have played a role in evolution of high virulence in *Y. pestis*.

These findings support the view that inflammasomes, the cellular protein complexes cleaving IL-18 and IL-1β into mature forms, are fundamental components of the host response to many pathogens. Indeed, several viral, bacterial and fungal microbes have strongly increased ability to induce disease in the absence of IL-1β, IL-18 and inflammasome components (Hise et al., 2009; Lamkanfi and Dixit, 2009; Broz et al., 2010; Rathinam et al., 2010; Davis et al., 2011b). In spite of this, only a few mammalian NLRs out of a family of more than 20 members have currently been shown to directly participate in host defenses. Here we are the first to show that NLRP12 participates in host responses to wild type *Y. pestis* and modified *Y. pestis* strains expressing a potent LPS, although the factor(s) in *Y. pestis* responsible for directly activating the NLRP12 inflammasome are still unknown.

NLRP12 may also be involved in resisting infections caused by other human pathogens. It is unclear how NLRP12 may interact with other inflammasome components. NLRP12 deficiency did not cause a complete reduction in ability to release IL-18 and IL-1β following exposure to *Y. pestis* and *Y. pestis*-pYtbLpxL infection (**Figure 3.5 and 3.7**). Also, the increased mortality observed in NLRP12-deficient mice did not appear as great as observed in IL-18 deficient animals (**Figures 3.2**, **3.3**), and NLRP3 also play a role in host defenses (**Figures 3.3**, **3.7**). Redundancy between NLRs may occur, and other NLRs may also participate in optimal responses to infection. This may support the idea that NLRs work together for optimal protection of the host (Broz et al., 2010). The generation of animals with combined deficiencies in NLRP12 and other NLRs may clarify how NLRP12 functions in cooperation with other signaling

components. NF-κB signaling following bacterial challenge appeared normal in NLRP12-deficient cells (**Figure 3.4**).

IL-18, IL-1β and IFNγ are all cytokines active at the interface between innate and adaptive immunity. We have found that *Y. pestis* strains generating a hexa-acylated LPS could function as effective live vaccines (Montminy et al., 2006). It would be of interest to investigate the role of NLRP12 in promoting the development of adaptive immunity and protection following vaccination with both live and subunit/adjuvant vaccines.

The emerging role of inflammasomes as key players in host defenses during many infections makes them desirable targets for therapeutic intervention and drug development. We note that alum, one of the first components known to activate specific inflammasomes, already is in widespread use as one of the few vaccine adjuvants licensed for human use. However, a delicate balance between pathological effects and enhanced host defenses arising from inflammasome-stimulating treatments will be necessary. Mutations in NLRs are linked to inflammatory diseases (Hawkins and Lachmann, 2003; Jéru et al., 2011a), and anti-IL-1 treatment does in fact reduce symptoms in many such patients. More knowledge on the role of NLRs in inflammation and homeostasis is needed in order to fine-tune future NLR-based therapies.

***Material and Methods***

*Bacterial strains and growth conditions*

*Y. pestis* KIM is originally a clinical isolate from a Kurdistan Iran man (Brubaker, 1970; Perry and Fetherston, 1997). *Y. pestis* strains KIM5, KIM5-pEcLpxL (containing *E. coli* LpxL, earlier called pLpxL) and KIM1001 were as reported (Montminy et al., 2006). *Y. pseudotuberculosis*

IP2666 (containing a complementation of PhoP/PhoQ deficiency) and *Y. enterocolitica* 8081 were provided by Joan Mecsas. Strains were grown in tryptose-beef extract (TB) broth with 2.5mM CaCl2 all by shaking at 37°C. lpxL of *Y. pseudotuberculosis* IP2666 including 480 basepairs upstream and 266 basepairs downstream from coding region was cloned using Pfu Ultra (Stratagene) and was ligated into the BamHI and SalI sites of pBR322, creating pSP::YtbLpxL (or 'pYtbLpxL'). The resulting plasmid was electroproated into *Y. pestis* KIM5 (Goguen et al., 1984) or Y. pestis KIM1001 (Sodeinde et al., 1992) and bacteria were selected by growth on TB agar supplemented with 2.5mM CaCl2 in the presence of 100µg/ml of ampicillin. All strains containing plasmids above remained tetracycline sensitive. KIM1001 (pPCP1+, pCD1+, pMT1+) is highly virulent (Perry and Fetherston, 1997) whereas, KIM5 bears the chromosomal deletion 'Δpgm' which substantially attenuates virulence. The pgm locus contains no genes thought to affect LPS biosynthesis. KIM6 is a KIM5 derivative lacking the T3SS-containing pCD1 virulence plasmid . KIM5-ΔYopB was provided by Greg Plano (Torruellas J, 2005). For the generation of KIM5-ΔYopJ, the following method was used. An in-frame deletion removing codons 4-287 was created via allelic exchange. PCR products made with primer sets A (ATAGAGCTCCACTACTGATTCAACTTGGACG), B (TCCGATCATTTATTTATCCTTATTCA) and C (TGAATAAGGATAAATAAATGATCGGATAATGTATTTTGGAAATCTTGCT), D (GGGTCTAGACTGATGTCGTTTATTTCTGGGTAT), respectfully, were used to make a fused product by overlap PCR using primers A and D. This product was cloned in the allelic exchange vector pRE107 in E.coli K12 strain B2155, transferred to *Y. pestis* by conjugation, and recombinants selected on TB medium containing 100µg/ml ampicillin but no diaminopimelic

acid. Following counter selection with 5% sucrose, deletion mutants were identified by PCR. For *in vitro* infections, bacteria were grown overnight at 37ºC in TB broth with or without ampicillin, diluted 1:4 in fresh media and cultured for three more hours at 37ºC, then washed three times with PBS and resuspended in DMEM or RPMI. *S. enterica* serovar typhimurium strain SL1344 was provided by Mary O'Riordan, and strain M525P by Clare Bryant.

*Cell stimulations*

Mouse BMDM were prepared by maturing fresh bone marrow cells for 5-7 days in the presence of M-CSF containing supernatant from L929 cells. Mouse neutrophils were enriched by injecting 1ml of thioglycolate i.p., peritoneal cells (typically >80% Ly6G-positive cells, (Nilsen N, 2004) cells were harvested 4 hrs later after flushing with RPMI. Mouse BMDMs were plated at $2x10^5$ per well in 96-well plates for ELISA or $2x10^6$ per well in 12-well plates for western blot. Stimulation was for 6 hours and supernatants were collected for cytokine analysis. Three hours after bacterial infections, 50μg/ml of gentamycin was added. Alum was from Pierce, nigericin and poly(dA:dT) was from Sigma. IL-1β p17 and Caspase-1 p10 western blots were conducted mainly as described (Hornung et al., 2008) using antibodies from Santa Cruz Biotechnology (caspase-1 p10) and R&D (IL-1β). The antibody against β-actin was from Sigma. Q-PCR for NLRP12 and NLRP3 in resting or infected BMDM or magnetic bead (StemCell Technologies)-isolated neutrophils was performed by RNeasy Mini Kit (Qiagen), and iScript cDNA Synthesis Kit (BioRad). PCR was performed on transcribed cDNA or mouse tissue cDNA (Clontech) with primers for detection of mouse NLPR12 (5'-TGCAAGCTTCGAGTCCTGT-3', 5'-CCTGGTCGGCTTCATTCTG-3'), NLRP3 (5' AACCAATGCGAGATCCTGAC 3', 5' ATGCTGCTTCGACATCTCCT 3'), or pro-IL-1β (5'-GCCCATCCTCTGTGACTCAT-3', 5'-

AGGCCACAGGTATTTTGTCG-3') using SYBR green (BioRad) according to the manufacturer's instructions. ELISA kits for IL-1β, TNFα, IL-8, CXCL12, IFNγ (R&D) and IL-18 (MBL) were used for cytokine detection. Reagents for FACS detection of active and cleaved caspase-1 by FLICA/FITC substrate were from Immunochemistry Technologies.

*Mice*

All experiments involving live animals were approved by the Institutional Animal Care and Use Committee. ASC (Pycard-/-), NLRP6, NLRP3 (Nlrp3-/- ), and NLRP12-deficient (Nlrp12-/-) mice were generated by Millennium Pharmaceuticals and were backcrossed eight to eleven generations to C57BL/6 background. Mice deficient in TLR4 (TLR4-/-) and MyD88 (Myd88-/-) were from S. Akira, and mice lacking caspase-1 (Casp1-/-) were from Michael Starnbach. C57BL/6 and mice deficient in IL-1R1 (Il1r1-/-), IL-18R (Il18r1-/-), IL-18 (Il18-/-), TNFR1 (Tnfr1-/-), IL-12p40 (Il12b-/-), and IFNγR (Ifngr1-/-) were all from Jackson Laboratories. J. Sprent (The Scripps Research Institute) provided the IFNαβR1 (Ifnar1-/-) and IFNγR1 x IFNαβR1 doubly deficient mice. IL-1β (Il1b-/-) deficient mice were provided by Y. Iwakura. Wild-type (from Jackson Laboratories or bred at UMass) or knock-out mice were infected s.c. in the nape of the neck with Y. pestis and their survival monitored twice a day for 30 days. Infection with *S. typhimurium*: mice were infected with 1000 CFU of M525P i.p. and survival was monitored as described above. For cytokine and CFU analysis, mice were infected either s.c. or i.v. and sacrificed at the indicated time points. Serum was generated by centrifugation in microtainer tubes (BD), and spleens were homogenized in 0.5 ml PBS using a closed system Miltenyi gentleMACS dissociator and c-tubes to preserve intact cells, subsequently cells/debris were removed by centrifugation. Samples for cytokine analysis were subjected to protease

inhibitor (Roche) treatment. Cytokine levels corrected by bacterial loads were calculated by dividing IL-18 levels (ng/ml) by the bacterial load (CFU x 108) for each animal. Hematoxylin and eosin (H&E) staining and microscopy was performed as published (Montminy et al., 2006). *In vivo* caspase-1 cleavage analysis: mice were infected with 500 CFU of *Y. pestis* i.p. After 24 hrs, spleens were harvested, homogenized and cell suspensions were stained with caspase-1 FLICA reagent. Wild type and NLRP12 KO mice were injected i.p. with pre- mixed Y. pestis KIM5-pYtbLpxL LPS (100 ng per mouse) and alum (Imject, Thermo Scientific, 200 µg/mouse) as indicated. Blood was harvested 4 hrs post- injection and serum cytokines were analyzed by ELISA.

*Statistical analysis*

*In vitro* cytokine release was analyzed by two-way ANOVA with Bonferroni post-test. Differences in spleen and serum cytokine concentrations were analyzed by the unpaired t-test. Differences in survival were studied using Kaplan-Meyer analysis and the logrank test. Differences in spleen CFU or cytokine/CFU values between genotypes of mice were evaluated with the Mann-Whitney test, or in more complex comparisons involving multiple mouse genotypes, with a generalized linear regression model of cubic transformed log CFU values (95% confidence interval) to meet normality assumptions. P values $<0.05$ were considered significant.

*Lipid A analysis*

Lipid A from *Y. pestis* KIM5, KIM5-pYtbLpxL and *Y. pseudotuberculosis* IP2666 were isolated as described (Montminy et al., 2006) and analyzed by MALDI-TOF mass spectrometry (Paquette et al. unpublished).

*Q-PCR analysis of Nlrp12 expression in mouse tissues*

Multiple tissue cDNA panel was from Clontech Laboratories.

*Genotyping of NLRP12 deficient mice*

RT-PCR genotyping of NLRP12 KO mice was performed using the primers GCAGCGCATCGCCTTCTATC (neo) combined with GAAGCAACCTCCGAATCAGAC and CCCACAAAGTGATGTTGGACTG, using 35 cycles at 94-62-74°C.

*Gene expression analysis by NanoString*

nCounter CodeSets were constructed to detect genes of interest and control genes. WT and NLRP12 KO BMDM were plated at $2x10^6$ cells per well in a 12-well plate and were incubated for 4 hours with or without KIM5-pYtbLpxL at MOI=10. At 2 hours post infection, 50µg/ml gentamicin was added to culture. Total RNA was purified using the Qiagen RNeasy mini kit per manufactures directions. Normalized total RNA was hybridized overnight to the CodeSet and then loaded into the nCounter Prep Station followed by assessment by the nCounter Digital Analyzer, following the manufactures directions. nCounter data was normalized first for processing efficiency by standardizing measurements of all samples to positive controls included by the nCounter during analysis. Second, we normalized expression levels to two control genes (Gapdh and Hprt). These gene transcript levels did not vary between experimental conditions. The weighted average of the mRNA counts of the control genes were used to normalize every sample's values by multiplying each transcript count by the weighted average of the controls.

*Neutrophil recruitment*

1 ml of 3% thioglycollate was injected i.p. into mice. Four hours later, peritoneal lavage cells were harvested, counted, and % Ly6G/Ly6C double positive cells were determined.

*Cell death*

Cell death was evaluated 6 hrs after infection of BMDM with KIM5 (MOI 40, bacteria were centrifuged onto the cells) by using calcein stain (Invitrogen). 100% lysis was obtained by treatment of cells with water.

*IKK kinase assay*

Cells were lysed in whole cell lysis Buffer (10% Glycerol, 20 mM Tris-HCL pH 7.4, 150 mM NaCL, 2 mM EDTA, 1% Triton X-100, 25 mM beta-glycerophosphoate, 1 mM dithiotreitol, 1 mM sodium orthovanadate, and mammalian protease inhibitor cocktail from Sigma-Aldrich (cat # P8340). Lysates were cleared by centrifugation (16000 rpm, 12 min). Protein concentrations were determined by Bradford assay. 200 µg of whole cell lysate was incubated with anti-IKKγ/ NEMO (Santa Cruz # 8330) for 1.5 hours at 4$^{o}$C followed by addition of protein A beads (Pierce # A23330). After overnight incubation at 4$^{o}$C, IPs were washed twice in lysis buffer and twice in kinase buffer (20 mM HEPES pH 7.6, 20 mM beta-glycerophosphate, 10 mM Magnesium Chloride, 50 mM Sodium Chloride, and 1 mM diothreitol). IP beads were split into two aliquots: one aliquot was subjected to SDS-PAGE followed by immuno-blotting for IKKβ (Santa Cruz # 56918) and the other aliquot was incubated with purified GST-IkBα N-term (0.5 µg), ATP (100 µM), and gamma-P32-labeled ATP (5 µCi) for 30 minutes at 30$^{o}$C. Reactions were stopped by the addition of 3X sample buffer containing 20 mM EDTA. Reactions were immediately boiled, subjected to 10% SDS-PAGE, and visualized by autoradiography.

*IkB degradation*

Cell lysates were prepared essentially as for kinase assays described above and subjected to 10% SDS-PAGE. Gels were transferred to PVDF membranes, followed by western blotting using

antibodies against IkBa (Cell Signaling) and secondary antibodies (anti-rabbit IgG HRP) from Bio-Rad.

*Flow Cytometry*

Bone marrow or homogenized spleen cells suspensions (fixed for infected animals) from mice were stained with antibodies against F4/80 (clone BM8, PerCP-Cy5.5), CD11c (N418, eFluor®450) (eBiosciences), Ly6G (1A8, PE), Ly6C (AL-21, FITC), and CD19 (1D3, Pe-Cy7) (BD). Cells were treated with Fc block (clone 93, rat anti CD16/CD32, eBiosciences) 15 minutes prior to stain. Cells were analyzed using a LSRII instrument (BD), with Diva and FlowJo software.

*Calculation of neutrophil recruitment to infected organs*

Inflammatory cell infiltration, largely consisting of neutrophils, in livers from mice infected with 500 c.f.u. of KIM1001-pYtbLpxL was assessed by analyzing HE-stained sections. 10 microscopic fields were analyzed from different mice and inflammatory cell influx was counted.

*Vaccination*

C57Bl/6 mice were vaccinated s.c. with 1000 CFU of Y. pestis KIM1001- pYtbLpxL. Thirty days later, vaccinated or naive mice were challenged s.c. with fully virulent Y. pestis KIM1001 and survival was monitored up to 28 days.

# PREFACE to CHAPTER 4

This section is a version of a manuscript currently being drafted:

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This chapter represents a thesis project of G.I.V., who generated all data except that indicated below. G.I.V. created all of the figures from primary data, and was the principal writer and coordinator of the manuscript text.

Co-authors contributed the following data:
R.M.R. contributed significantly to the experimental planning, data collection, and analysis of many of the experiments in this paper, especially the cellular recruitment and activation data. This work could not have been performed without her assistance. M.M.P. designed and made the Yop-deficient strains of *Y. pestis*, and D.W. assisted as needed during collection of sample material.

# CHAPTER 4:

## Innate immune responses via NLRP12, NLRP3, and IL-18 Control Pneumonic Plague Infection

***Abstract***

Pneumonic plague caused by *Yersinia pestis* can reach 100% mortality if intervention is not started within 24 hours. The initial immune response activated against *Y. pestis* in the lungs has yet to be determined, though we know it is able to suppress production of many host inflammatory factors during primary infection allowing it to become systemic and expand to an extremely high concentration in tissues. Here, we sought to elucidate the role of the inflammasome and the pro-inflammatory cytokine IL-18 during pneumonic plague. We found IL-18 to be necessary in limiting the initial pathogen spread, and IL-18-deficient mice had increased susceptibility to *Y. pestis*. Moreover, the NLRP12 and NLRP3 inflammasome were important regulators controlling IL-18 production after *Y. pestis* infection in the airway, and mice lacking these inflammasomes had decreased IL-18 responses, cellular recruitment to the lungs, and were more susceptible to bacterial challenge. This is the first evidence of NLR-mediated protection against *Y. pestis* lung infection and could be key to understanding the how plague virulence determinants differ during peripheral vs airway infection.

***Introduction***

*Yersinia pestis*, the causative agent of plague, is typically transmitted by a the flea vector from infected rodents to humans causing bubonic disease, or directly between individuals as pneumonic plague (Perry and Fetherston, 1997; Hinnebusch, 2005). Following systemic spread of bacteria during bubonic plague, pulmonary infection can develop resulting in secondary pneumonic plague. Primary pneumonic plague also occurs, which can involve spread from person to person via infectious respiratory droplets. Currently, there is no licensed vaccine effective against pneumonic plague even though it is the expected disease form to result from a *Y. pestis* based biowarfare attack.

Pulmonary *Y.pestis* infection has a rapid onset and high mortality - close to 100% if not treated (Perry and Fetherston, 1997; Perry Ja, 2005; Butler, 2009). Initial pneumonic plague infection may differ from many pulmonary infections as the first 36 hours are characterized by rapid bacterial replication during which infected animals may display a muted pro-inflammatory response (Lathem et al., 2005). This may allow *Y. pestis* to expand exponentially in the lungs and other organs before the immune system is able to contain the infection. In spite of the violent nature of pneumonic plague, several lines of evidence suggest that the bacteria is less able to mount a deadly infection via the airways than via the skin. Pneumonic plague is much less common, as 80-90% of natural cases of *Y. pestis* infections are bubonic plague (Perry and Fetherston, 1997), although some pneumonic plague epidemics have been described (Stenseth et al., 2008). Early studies of human transmission suggested that preventive measures such as simple surgical masks could prevent infection (Kool and Weinstein, 2005). A close contact between subjects resulting in inhalation of large respiratory droplets and likely transmission of

larger amount of bacteria was also believed to be necessary for transmission (Kool and Weinstein, 2005).

Finally, the LD50 for *Y. pestis* infection in mouse models via the pulmonary route is reported to be around 500 CFU. This is indeed 50-100 times higher than for infection via the peripheral route - subcutaneous (s.c.) or intradermal (i.d.) delivery with LD50 of less than 10 bacteria, modeling spread of bubonic plague via flea bites into the skin. This is in contrast to another highly virulent bacteria, *Francisella tularensis* type A, where LD50 via pulmonary and peripheral routes are both very low (estimated to less than 10 CFU). Many studies of *Y. pestis* focus on its ability to evade host immunity (Smiley, 2008) and less attention has been paid to the initial protective innate immune responses limiting pneumonic plague . The observations indicated above may suggest that innate immune responses could contribute to limiting development of pneumonic plague, and that a higher amount of bacteria is needed to overcome these initial responses during lung infection than via the peripheral route, to generate a productive infection. Thus, the immune response may still be significant enough in the airways to mediate some level of protection to limit pneumonic plague. The description of the innate immune responses contributing to limiting airway infection could lead to the development of additional therapeutic options and also guide in finding optimal vaccines against pneumonic plague.

*Y. pestis* virulence depends on multiple factors including a plasmid-encoded T3SS (type III secretion system) that injects effector proteins called Yops into host cells to alter cell functions such as signaling pathways and phagocytosis (Cornelis, 2002a). The T3SS is strongly conserved among all human-pathogenic Yersinia species, although other members of this family

(*Y. pseudotuberculosis* and *Y. entrocolitica*) typically cause self-limiting gastroenteritis compared to the serious infection caused by *Y. pestis* (Perry and Fetherston, 1997). Therefore, differences in the T3SS cannot explain the major increase in virulence during evolution of *Y. pestis*. Acquisition of the surface protease Pla (Sodeinde et al., 1992; Lathem et al., 2007) and loss of ability to potently trigger TLR4 signaling by production of a tetra-acylatedlipid A/LPS in *Y. pestis* may have contributed to dramatically increased ability to cause systemic disease in the evolutionary split from *Y. pseudotuberculosis* (Kawahara K, 2002; Montminy et al., 2006; Vladimer et al., 2012)(Vladimer et al unpublished.)

Previously, we have implicated the pro-inflammatory cytokines IL-1β and, more importantly, IL-18 in host resistance to disease induced by subcutaneous infection of virulent and attenuated strains of *Y. pestis* expressing a modified lipid A/LPS (Vladimer et al., 2012) (and Vladimer et al. unpublished.) We determined that high levels of IL-18 and IL-1β in the serum and the spleen following i.v. infection were dependent on the inflammasome forming nucleotide-binding domain and leucine-rich repeat containing (NLRs) NLRP3, NLRP12. Moreover, mice deficient in these NLRs, as well as NLRC4-deficient animals, had reduced host resistance against *Y. pestis* induced delivered via the peripheral route. We hypothesized that inflammasome signaling and IL-18 production in the lung may be key factors during the primary immune response during pneumonic plague. Here, we propose a mechanism for how inflammasome function and downstream cytokines during pneumonic plague infection mediate protection by controlling anti-bacterial defenses, inflammation,  and cellular recruitment. Thus, the inflammasome-directed production of IL-18 after transmission of bacteria may limit spread of

infection during early stages of pneumonic plague, and therefore also limit the spread of pneumonic plague during epidemics.

***Results***

*Pneumonic plague infection is controlled by IL-18 early in infection*

*Y. pestis* rapidly disseminates into the lymphatic system of mice after a s.c./i.d. infection while yielding immune response activation and pro-inflammatory responses, leading to bubonic plague. The model has an LD50 of less than 10 CFU (Perry and Fetherston, 1997; Vladimer et al., 2012). In contrast, when mice are infected via the pulmonary route (intratracheal, i.t.) an increase in the LD50 to approximately 500 CFU is observed (**Figure 4.1A**). This suggests that plague may be more susceptible to the early innate immune response in the airway compared to the dermal tissues. Normally, *Y. pestis* produces a tetra-acylated lipid A/LPS at 37$^{o}$C with reduced ability to activate TLR4 signaling. Previously, we characterized modified strains of plague which generate a hexa-acylated, potent, TLR4-activating lipid A / LPS after expression of *lpxL*, a “late” acyl-transferase which was deleted during evolution of *Y. pestis* from *Y. pseudotuberculosis*. The *Y. pestis* strain expressing *Y. pseudotuberculosis* LpxL is called *Y. pestis*-pYtbLpxL (Montminy et al., 2006; Vladimer et al., 2012). This strain induces increased immune responses via TLR4, and allowed us to study signaling pathways effective in increasing resistance to *Y. pestis* infections (Vladimer et al., 2012). Indeed, when we infected mice i.t. with 5000 CFU of KIM1001-pYtbLpxL or KIM1001 i.t., we found that the mortality of animals is strongly reduced when bacteria produce a potent LPS (**Figure 4.1B**). This provides evidence that protection from pulmonary infection can be increased by boosting the immune response though activation of TLR4. It also means that an efficient innate immune response could limit *Y. pestis* infection via the airways. Thus, we hypothesized that early cytokine production is necessary for

controlling the lung infection. We have previously proposed the pro-inflammatory cytokine, IL-18, as an important mediator in resistance to bubonic plague (Vladimer et al., 2012). This cytokine is protective for pneumonic plague as mice deficient in IL-18 die rapidly after infection with 500 CFU i.t. of fully virulent *Y. pestis* (strain KIM1001) (**Figure 4.1C**). Furthermore, IL-18 is important in controlling bacterial replication very early during pneumonic plague infection as IL-18 KO animals have significantly increased bacterial loads in lung homogenates at 24 and 48hpi, compared to WT animals (**Figure 4.1D**). Once primary pneumonic plague has colonized the alveoli, it can spread to the bloodstream and to other organs and tissues via the circulatory system (Perry and Fetherston, 1997). At 24hpi, we observe *Y. pestis* in the spleens of IL-18-deficient animals infected *Y. pestis*, in comparison, there are no detectable bacteria with spleens in WT mice (**Figure 4.1E**). Thus IL-18 contributes to limiting early spread of plague from the primary site of infection in the lung to other organs. Degree of pneumonia can be measured by tracking elevated lung weight as fluid retention increases (**Figure 4.2A**); IL-18 also contributes to reduce pneumonia by this measure as lungs from IL-18 KO mice have increased weights compared to C57BL/6 controls (**Figure 4.1F**).

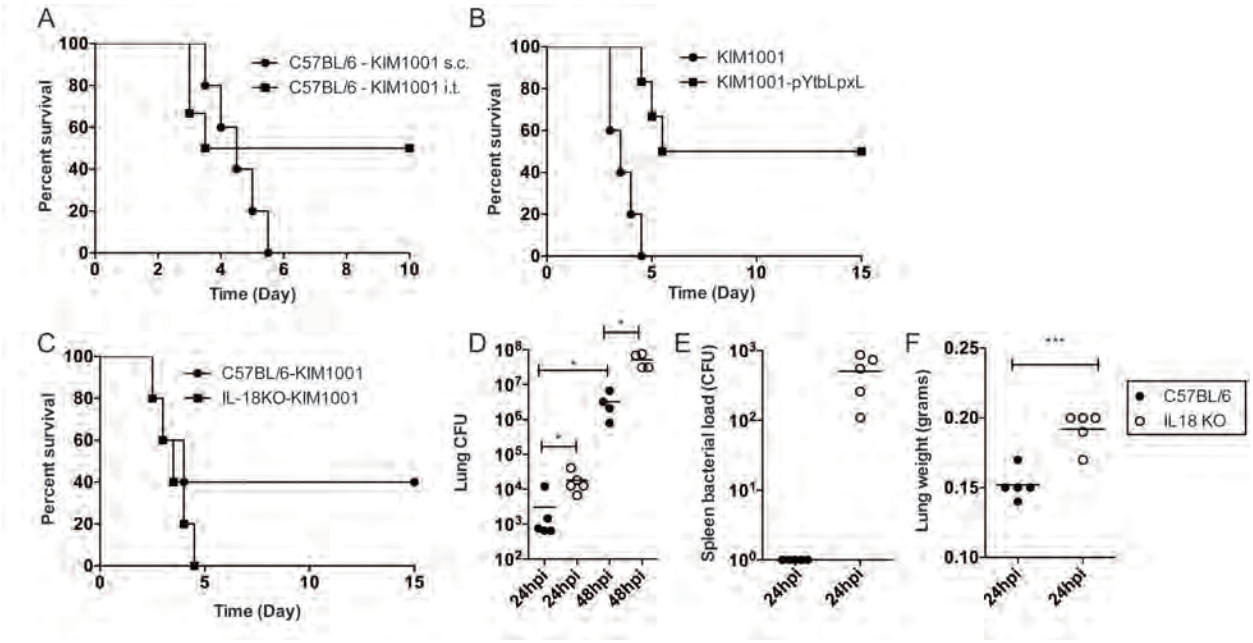



**Figure 4.1: IL-18 contributes to control of pneumonic plague early in infection**
(A-C) Survival of animals infected (A) s.c. or i.t. or (B-C) i.t. with (A,C) 500 CFU KIM1001 or (B) 5000 CFU KIM1001 or KIM1001-pYtbLpxL. (A) groups contained n=10, (B-C) n=5. (D-E) CFU in (D) lung or (E) spleen homogenates from WT or IL-18 KO infected i.t. with 1000 CFU KIM1001 and sacrificed at indicated time. (F) Weights of lungs from WT or IL-18 KO animals infected i.t. with 1000 CFU KIM1001. *$p < 0.05$; **$p < 0.001$.

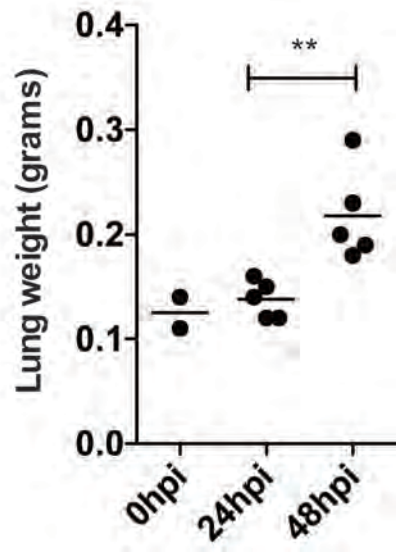
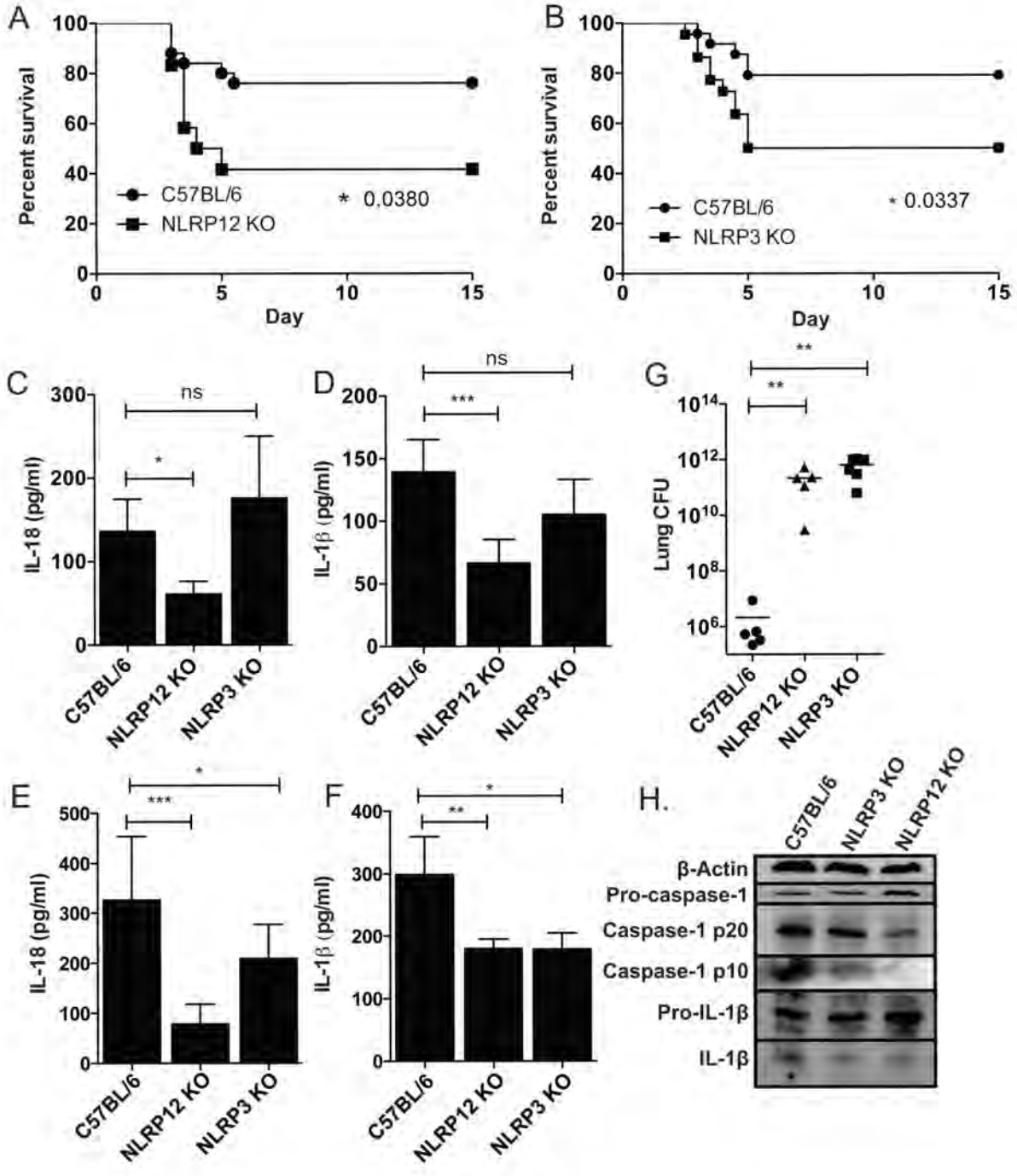



**Figure 4.2: Progression of pneumonia can be tracked by lung weight**
(A) Weights of lungs from C57BL/6 animals infected i.t. with 1000 CFU KIM1001; entire lungs were extracted while minimizing blood contamination on organ. **$p < 0.001$.

*The NLRP12 and NLRP3 participate in controlling IL-18 release in the lung and pneumonic plague*

IL-18 and IL-1β are proteolytically matured by caspase-1 following inflammasome activation (as reviewed in Vladimer *et al., 2013.*) We have proposed an important role for inflammasomes involving NLRP12, NLRP3 (Vladimer et al., 2012) and NLRC4 (Vladimer *et al.* unpublished, see chapter 2) in the protection to *Y. pestis* strains after s.c. and i.v. injection. We hypothesized that NLRP12 and NLRP3 could mediate IL-18 and IL-1β release and subsequent protection during early events in pneumonic plague. We infected NLRP12- and NLRP3-deficient animals with fully virulent *Y. pestis* KIM1001 i.t. and found that those mice are more susceptible to pneumonic plague than WT control mice (**Figure 4.3A,B**). The NLR-deficient animals infected i.t. with 350 CFU of WT plague succumb to the infection to a greater extent than C57BL/6 animals indicating that these NLRs play a role in protection from pneumonic plague within the airways. Further investigation of the BAL (bronchoalveolar lavage) fluid at 48hpi from animals infected with KIM1001 i.t. shows a significant decrease of IL-1β (**Figure 4.3C)** and IL-18 (**D**) in NLRP12-, but not NLRP3-, deficient animals. Furthermore, we compared BAL samples to lung homogenates from WT, NLRP12-, or NLRP3-deficient animals infected with KIM1001 i.t.; IL-18 (**Figure 4.3E**) and IL-1β (**F**) levels were decreased in both NLR-deficient animals indicating possible differences in temporal or spatial roles for these NLRs during protection. In contrast, IL-6, released after activation of inflammasome-independent pathways, is detected at similar levels in lung homogenates from WT or the NLR-deficient animals (**Figure 4.4A**). NLRP12- and NLRP3-deficient animals also have a higher bacterial load in the lungs at 48hpi in comparison to WT animals (**Figure 4.3G**) infected i.t. with 5000 CFU of KIM1001. Inflammasome activation can be directly visualized by detecting the processing of caspase-1

(Martinon et al., 2002). Western blots for caspase-1 and IL-1β in lung lysates from NLRP12- and NLRP3-deficient animals show reduced inflammasome activation and cytokine processing compared to lysates of WT animals infected i.t. with 5000CFU KIM1001 at 48hpi (**Figure 4.3H**). Lastly, to determine the cellular expression of NLRP12 and NLRP3 in uninfected lungs we analyzed the gene expression from sorted resident $Ly6G^+$ neutrophils and $F4/80^+$, $CD11C^+$ alveolar macrophages (**Figure 4.4B**) in C57BL/6 mice. Neutrophils in resting-state lungs express both NLRP3 and NLRP12 along with both pro-IL-1β and pro-IL-18. In comparison the alveolar macrophages only express NLRP3 and pro-IL-18 (**Figure 4.4B**) suggesting a possible cell-type specific innate immune response after lung infection. However, we have previous described an increase of NLRP12 gene expression in BMDM stimulated with *Y. pestis* (Vladimer et al., 2012), therefore, the expression profiles of these cells may change during pneumonic infection.


A
Percent survival
Day
C57BL/6
NLRP12 KO
* 0.0380
B
Percent survival
Day
C57BL/6
NLRP3 KO
* 0.0337
C
IL-18 (pg/ml)
ns
*
C57BL/6
NLRP12 KO
NLRP3 KO
D
IL-1β (pg/ml)
ns
***
C57BL/6
NLRP12 KO
NLRP3 KO
G
Lung CFU
**
**
C57BL/6
NLRP12 KO
NLRP3 KO
E
IL-18 (pg/ml)
*
***
C57BL/6
NLRP12 KO
NLRP3 KO
F
IL-1β (pg/ml)
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C57BL/6
NLRP12 KO
NLRP3 KO
H.
C57BL/6
NLRP3 KO
NLRP12 KO
β-Actin
Pro-caspase-1
Caspase-1 p20
Caspase-1 p10
Pro-IL-1β
IL-1β

**Figure 4.3: NLRP12 and NLRP3 control IL-18 and IL-1β in the lungs**
(A-B) Survival of C57Bl/6 and (A) NLRP12 KO or (B) NLRP3 KO animals infected i.t. with 500 CFU KIM1001. Data shown are pooled from two to four experiments. (C-F) Concentrations of (C,E) IL-18 or (D,F) IL-1β in (C-D) BAL or (E-F) lung homogenates of mice infected i.t. with (C-D) 5000 CFU or (E-F) 500 CFU KIM1001, measured by ELISA. (G) CFU in lung homogenates after i.t. infection with 5000 CFU KIM1001. (H) Western blot of lung lysates for pro- and mature-caspase-1 and IL-1β at 48hpi after 5000CFU infection of KIM1001 i.t. Animals in C-H were sacrificed at 48hpi Groups contain (A) C57BL/6 n=25, NLRP12 n=12 (B) NLRP3 n=19, (C-D) n=5, (E-G) n=7, (H) n=1. BAL was collected by washing the aveolar space 1x with 1ml PBS via the mouse trachea.

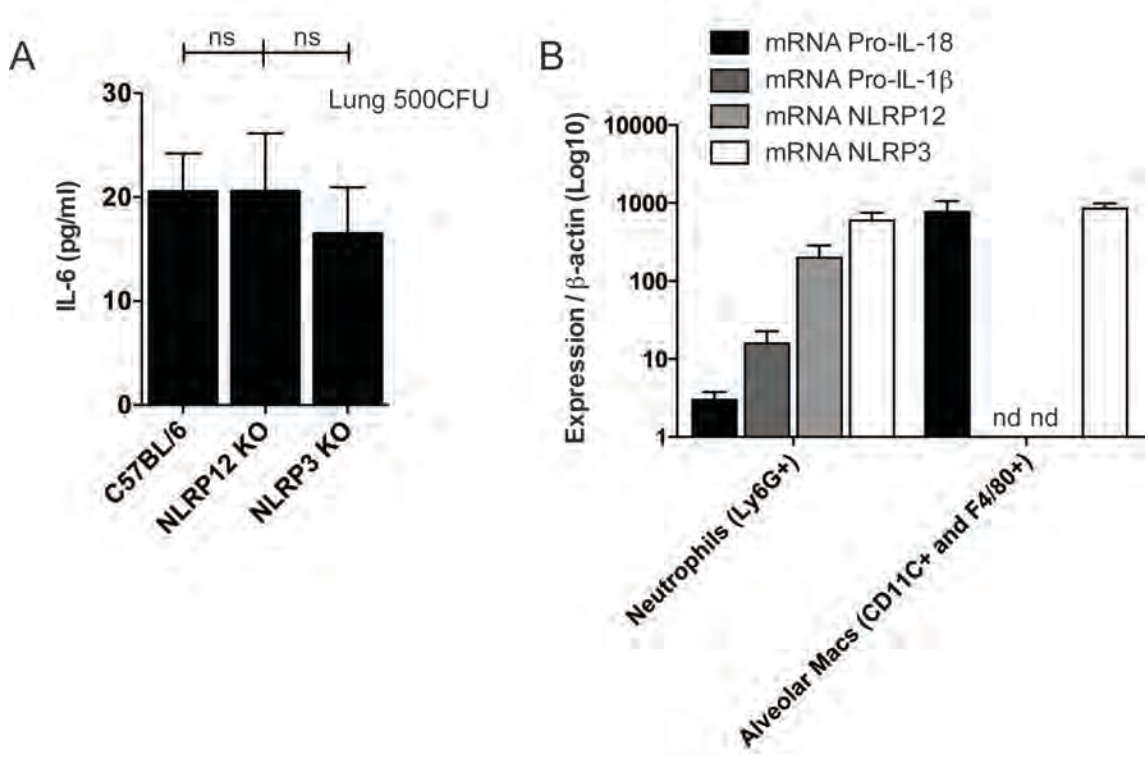


**Figure 4.4: NLRP12 and NLRP3 in lungs of C57BL/6 mice**
(A) Concentration of IL-6 in lung homogenates of mice infected i.t. with 500 CFU KIM1001 for 48 hours, n=7 for each genotype. (B) Q-PCR for mRNA detection of pro-cytokines or inflammasome components in FACS sorted neutrophils (Ly6G+) or alveolar macrophages (CD11C+, F4/80+) cells pooled lung homogenates of three uninfected C57BL/6 mice.

Tissue destruction can be visualized by H&E staining of sections from infected lung harvested at 48hpi (**Figure 4.5A**). The analysis of lungs from WT mice compared to those from the NLRP3KO and NLRP12KO animals infected with 500 CFU KIM1001 i.t. (second row) shows decreased alveolar hemorrhage (arrows, and **Figure 4.6A**) and decreased fibrosis (marked by **x**). Staining of lung tissue samples infected with 5000 CFU i.t. at 4x and 20x show (third and forth rows) shows an increase destruction of alveoli and an increased loss of normal lung architecture in the NLR-deficient animals as compared to uninfected controls (first row). This can be measured as a loss of airway space (**Figure 4.6B**). Examination of the loci of infection at 40x (**Figure 4.5A**) shows increased cell-recruitment and inflammation and the absence of visible bacteria in WT lungs (forth row, left panel, marked by >) and mild fibrosis (marked by a **x**), whereas, NLRP12 KO and NLRP3 KO lungs (forth row, middle and right panel) shows areas with visible bacterial clusters (marked with a *****), diminished cellular recruitment, greater fibrosis, and edema (marked by a #). This data indicates that NLRP12 and NLRP3 play an important role in the early detection and amelioration of pneumonic plague by production of potent pro-inflammatory cytokines IL-18 and IL-1β in a possible cell-recruitment dependent manner.

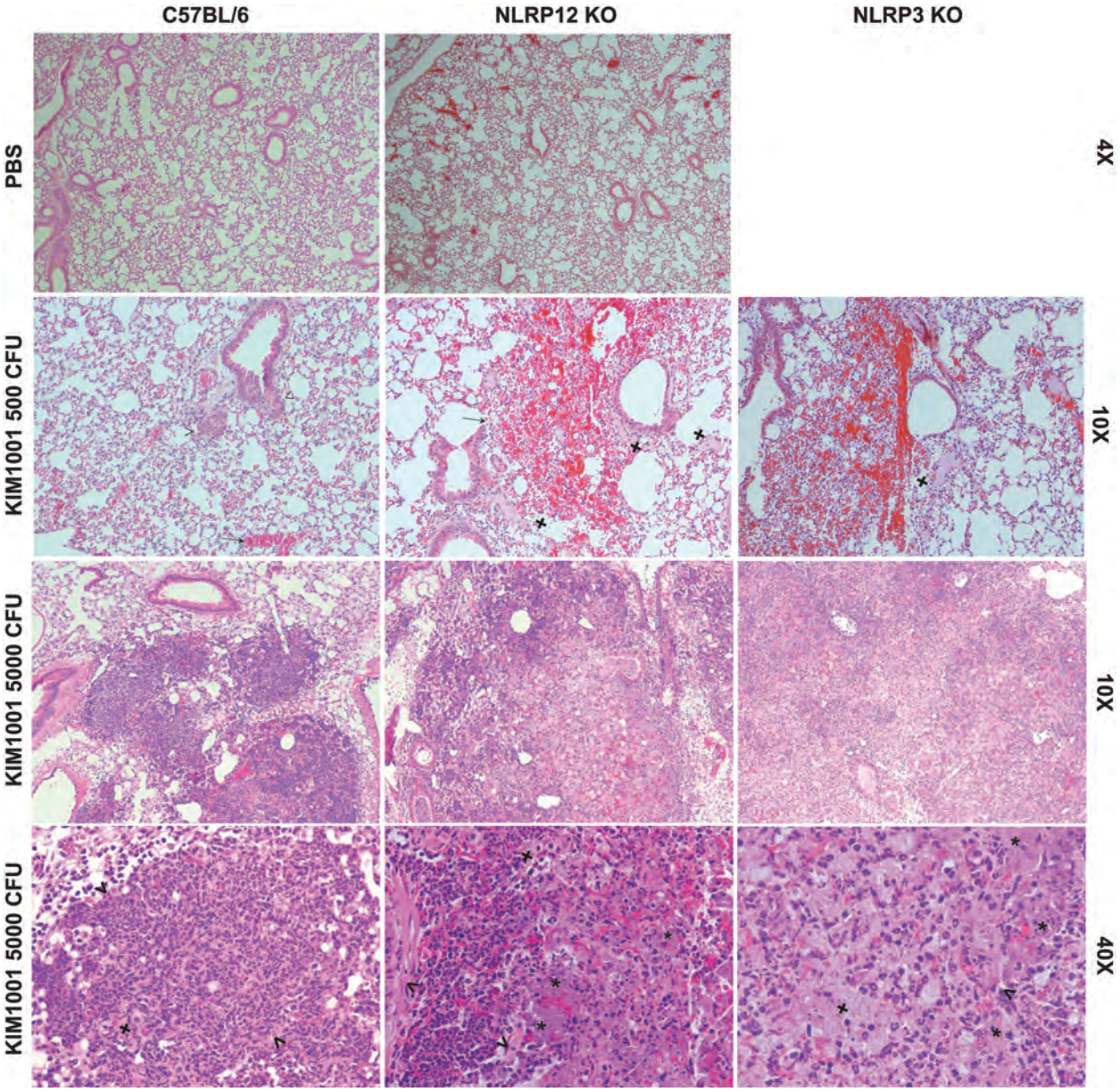


**Figure 4.5: Lung histology suggests increased lung disease in NLR-deficient animals**

(A) Histology analysis of fixed H&E stained lung sections from (left) WT, (middle) NLRP12 KO, or (right) NLRP3 KO mice (top row) uninfected or infected i.t. with (second row) 500 CFU or (third and fourth row) 5000 CFU of KIM1001 for 48 hr. Arrows (second row only) represent alveolar hemorrhage, X marks fibrosis, open arrow heads show loci of inflammatory cells, primarily neutrophils, asterisks show bacterial clusters (bottom row only).

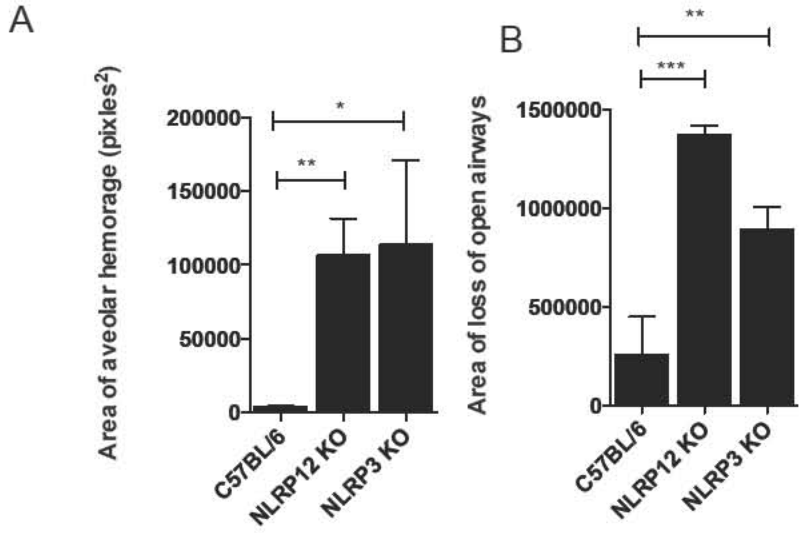


**Figure 4.6: Loss of normal alveolar architecture during pneumonic infection in NLR-deficient animals**

(A-B) Measurement of area (in pixles$^2$) of (A) aveolar hemorrhage and (B) loss of open airways 48hpi after 500 CFU infection i.t. with 500 CFU KIM1001. (A) Area was measured by specifically selecting red targets in H&E stained fixed lung sections and (B) by measuring around loci of cellular recruitment at 5x magnification. n=5 for each group and images used were representative of multiple experiments. *$p < 0.05$; **$p < 0.001$.

*NLRP12 and NLRP3 control cellular recruitment and immune activation in infected lungs*

The strong IL-1β and IL-18 production resulting from inflammasome activation has been shown to drive production of other cytokine (such as IFNɣ) and chemokine, and recruitment of other pro-inflammatory cells. Inflammasome deficiencies have resulted in impaired chemokine production and neutrophil recruitment during bacterial lung infection and murine gout (Tiemi Shio et al., 2009; Amaral et al., 2012) and have been linked to decreased dendritic and myeloid cell recruitment (Arthur et al., 2010). Here we show limited neutrophil recruitment to alveoli of NLRP12- and NLRP3-deficient animals compared to WT mice after infection with 5000cfu of KIM1001 i.t. (**Figure 4.7A**), possibly resulting from the decrease in IL-1β and IL-18. In contrast, the $CD11c^+$, $F4/80^+$ alveolar macrophage population, as measured by % of total cells, is unchanged (**Figure 4.7B**.) However, the induction of IFNɣ provides an activation feed-back to macrophages; macrophage activation can be tracked by decreased surface expression of F4/80 (Ezekowitz and Gordon, 1982). After pneumonic infection with 500cfu of KIM1001, the Geometric Mean of the Mean Fluorescence Intensity (MFI) level of F4/80 on macrophages ($F4/80^+$, $CD11C^-$) is higher compared to WT animals (**Figure 4.7C**) suggesting that there is less cellular activation. The NLRP12-deficient animals have comparable baseline composition of $Ly6G^+$ neutrophils and $F4/80^+$, $CD11C^+$ alveolar macrophages in lung tissue to C57BL/6 (**Figure 4.8A-B**). Therefore, the lack of pro-inflammatory activation by NLRP12 and NLRP3 not only limits cytokine production locally, but also cellular recruitment and activation at the site of infection, likely contributing to reduced resistance against pneumonic plague.

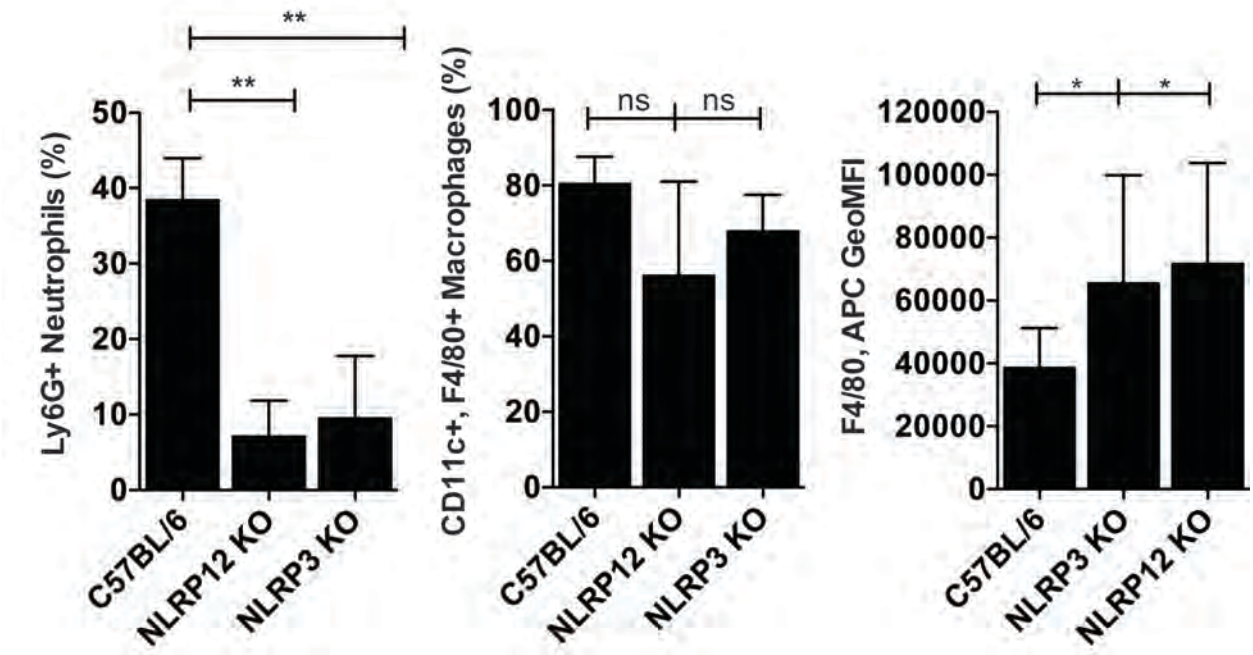


**Figure 4.7: NLRP12 and NLRP3 KO animals have deficiencies in cellular recruitment and activation during pneumonic plague.**

FACS of BAL fluid from animals infected i.t. with (A-B) 5000 CFU or (C) 500 CFU of KIM1001 after 24 hours. Population percentages for (A) Ly6G+ neutrophils, (B) CD11C+, or F4/80+ resident macrophages were determined. (C) Geometric mean of F4/80 staining on F4/80+, CD11C- cells was determined by averaging each event and averaging the samples together. n=5 (C57BL/6), 4 (NLRP12 and NLRP3). *$p < 0.05$; **$p < 0.001$.

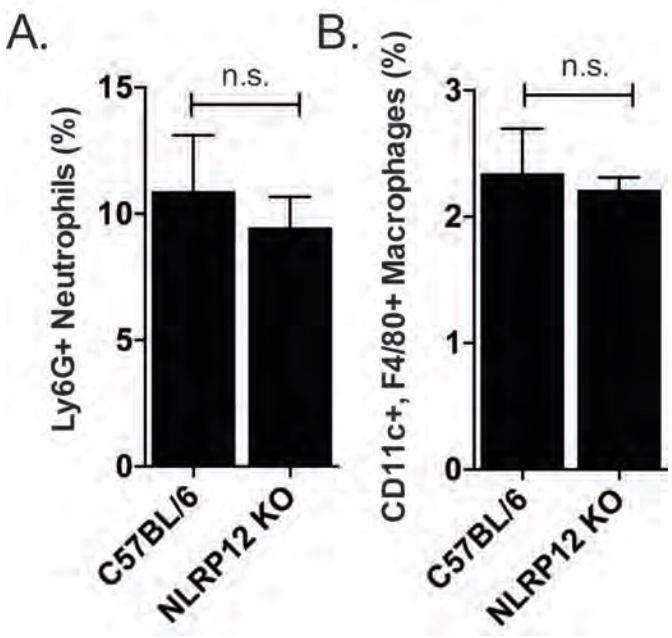


**Figure 4.8: C57BL/6 and NLRP12 KO mice have comparable baseline cellular levels in lung homogenates**

(A-B) Percent of (A) Ly6G+ neutrophils and (B) CD11C+, F4/80+ alveolar macrophages present in lung homoginates of uninfected C57BL/6 or NLRP12 KO mice. n=3 C57Bl/6, 2 NLRP12 KO.

***Discussion***

Pneumonic plague is the most likely form of the disease to result from a bioterrorist attack, and also the most deadly form with rapid onset pneumonia that reaches 100% fatality if not treated within 24 hours (Perry and Fetherston, 1997; Butler, 2009). Little was known about the initial immune response early during pneumonic plague infection, in fact it was was previously thought that the immune response in the lung was limited during the first stages of pneumonic infection, suppressed by the pathogen virulence factors (Bubeck et al., 2007; Smiley, 2008). However, we have elucidated roles of the inflammasome forming NLRs NLRP12 and NLRP3, as well as pro-inflammatory cytokines IL-18 and IL-1β within the lung that assist in controlling early pneumonic *Y. pestis* infection. These pathways had yet to be described in the lung during pneumonic plague infection and are important for the survival of infected mice.

We previously described NLRP12 and NLRP3 in protection of mice against both WT and an LPS-modified strain of *Y. pestis*; here, we use WT *Y. pestis* to begin to understand the significant differences of LD50 exist between the peripheral and pneumonic routes. The LD50 for i.t. plague infection with KIM1001 in mice is approximately 500CFU compared to less than approximately 10CFU s.c. The subversion of NLR activation after bubonic plague infection is possibly due to the fast decimation of plague or a lack of resident expression in comparison to the lung, which could be responsible for the changes in protection and the much lower LD50. Moreover, the differences in NLRP12 and NLRP3 dependence in lung homogenate vs BAL fluid could be due to resident cellular population and expression differences at baseline before infection. We have published the upregulation of NLRs after macrophage stimulation with *Y.*

*pestis* (Vladimer et al., 2012) and believe the same is occurring in the resident macrophages in the BAL.

Previous studies have shown that NLRP3 is responsible for complete cellular recruitment to the lung after influenza infection (Allen et al., 2009) as well, NLRP12-deficiencies resulted in decreased cell recruitment due to contact hypersensitivity (Arthur et al., 2010). We have not only shown that NLRP3 and 12 are important for cellular recruitment to the lung during pneumonic plague infection, but this is the first study that links NLRs to cellular immune activation in the airway.

Together, this study has shown that NLR driven IL-18 production in the lung is necessary and to and can protect against pneumonic plague infection in mice; and inflammasome activation may result in the higher LD50 necessary to cause infection. This is also the second study to show an important role for the novel inflammasome NLRP12 in the protection against a gram-negative bacterial infection. The NLRs are emerging as important mediators of innate immunology and more work must be done to elucidate their exact role for possible therapy and vaccine targets.

## ***Materials and Methods***

### *Bacterial Strains and Growth Conditions*

*Y. pestis* KIM is originally a clinical isolate from a Kurdistan Iran man (Perry and Fetherston, 1997). *Y. pestis* strains KIM1001 and KIM1001-pYtbLpxL were as reported (Vladimer et al., 2012). Strains were grown in tryptose-beef extract (TB) broth with 2.5 mM CaCl2 all by shaking at 37C. All strains containing plasmids above remained tetracycline sensitive. KIM1001 (pPCP1+, pCD1+, and pMT1+) is highly virulent *Y. pestis* (Perry and Fetherston, 1997).

*Mice*

All experiments involving animals were approved by the Institutional Animal Care and Use Committee. All work involving virulent pathogens was performed in a secure ABL3 facility approved by the Center for Disease Control, protocols were approve by the Institutional Biosafety Committee. NLRP3- ($Nlrp3^{-/-}$) and NLRP12-deficient ($Nlrp12^{-/-}$) mice were generated by Millennium Pharmaceuticals and were backcrossed 8–11 generations to C57BL/6 background. C57BL/6 mice were bread in-house, and mice deficient in IL-1R1 ($Il1r1^{-/-}$), and IL-18 ($Il18^{-/-}$) were all from Jackson Laboratories. IL-1b ($Il1b^{-/-}$)-deficient mice were provided by Y. Iwakura. Wild-type and knockout mice were infected i.t. by pipetting 50µl of PBS containing *Y. pestis* into the mouth after sub-lethal isoflurane administration, and performing a "tongue-pull" to prevent swallowing of the pathogen until breathing was sufficient for full infection to the respiratory tract. Survival was monitored twice a day for 30 days. For cytokine and CFU analysis, mice were infected i.t. and sacrificed at the indicated time points. Lungs and spleens were homogenized in 0.5 ml PBS with a closed system Miltenyi gentleMACS dissociator; subsequently cells/debris were removed by centrifugation. Lungs were washed with 1ml of PBS (including void-volume) though the trachea of sacrificed animals to collect BAL samples. Samples for cytokine analysis were subjected to protease inhibitor (Roche) treatment. Hematoxylin and eosin (H&E) staining and microscopy were performed as published (Montminy et al., 2006). Microscopy was analyzed with ImageJ software. Analysis of alveolar hemorrhage was measured by color selecting the RBCs in 5 10x fields per genotype and then measuring the area of selected color. Loss of airway space was measured by selecting the loci of infection and measuring the area from 5 20x fields per genotype, then subtracting the area of the whole field.

*ELISA, Flow Cytometry, and Q-PCR*

Q-PCR for Nlrp12, Nlrp3, and Il1b performed on cell-suspensions of naive C57BL/6 lungs after cell-sorting by BD biosciences FACSAria II. Cells were stained with antibodies against F4/80 (clone BM8, PerCP-Cy5.5), CD11c (N418, eFluor®450) (eBiosciences) and Ly6G (1A8, PE) (BD). Cells were treated with Fc block (clone 93, rat anti CD16/32, eBiosciences) 15 minutes prior to staining, on ice. Alveolar macrophages are $CD11C^{+}$, $F4/80^{+}$. Primers for detection of mouse Nlpr12, mouse Nlrp3, and mouse Il1b were as described (Vladimer et al., 2012) with SYBR green (BioRad) in accordance with the manufacturer's instructions. ELISA kits for IL-1b, IL-6, (R&D), and IL-18 (MBL) were used for cytokine detection. BAL cells were stained for Flow Cytometry with antibodies above and with CD4, CD8, CD19, CD69, MHCII... BAL populations were analyzed using a LSRII (BD) with Diva and Flowjo Software. MFI was calculated by taking the sum of florescent intensity of each event and the geometric mean of each samples sum.

# Chapter 5: Discussion

This thesis research has focused on the evolution of *Y. pestis* virulence, as well as furthered the understanding of innate immune pathways that limit plague early in infection. Beginning with elucidating the pathogenesis of *Y. pestis* after the loss of LpxL, a key factor in the increased virulence from *Y. pseudotuberculosis*, this work implicates the cytosolic, multi-protein complex, the inflammasome, as key in controlling plague infections. Together, this information can aid in creating a useful vaccine against a pathogen that has caused worldwide pandemics, and persists as a constant biowarfare threat.

*Y. pestis* uses multiple factors to efficiently, and concurrently, suppress many innate immune pathways to quell local inflammatory responses and cause systemic infection. *Y. pestis* also has the unique ability to evade immune recognition by TLR4 thereby limiting initial response. We previously found that the expression of a poorly stimulatory lipid A/LPS was necessary to cause bubonic plague (Montminy et al., 2006). Here, we continued to clarify the importance of the evolutionary loss of LpxL on *Y. pestis* virulence by noting that all closely related *Yersiniae* bacteria with known sequences of the relevant chromosome segments contain the *lpxL* gene (**Figure 2.3**). Moreover, adding back *lpxL* genes from several evolutionary ancestors results in significant loss of virulence *in vivo* (**Figure 2.5 A-B**) while cellular stimulations result in a notable increase in cytokine production.

The study and creation of these modified strains of plague gives us the ability to evaluate various components that assist in controlling bubonic plague infection that would have otherwise been unknown. Bubonic plague has an estimated LD50 of less than 10 CFU in mouse models, therefore, infection is difficult to control, and increased susceptibility in mice is also challenging to monitor. In comparison, the LD50 of a strain of *Y. pestis* expressing a stimulatory LPS is

greater than $1x10^7$ (Montminy et al., 2006). Using this strain, we determined an important role for the pro-inflammatory cytokines IL-1β and IL-18 in the protection of mice against bubonic plague as mice deficient in these cytokines and their receptors succumb to infection in much higher rates (**Figure 2.3 B-D**). Furthermore, mice deficient in the adapter protein MyD88 are also very susceptible to the modified plague strain (Montminy et al., 2006). MyD88 KO mice are the only immune deficient animals who die faster than C57BL/6 mice after bubonic plague infection with fully virulent strain of *Y. pestis* (**Figure 3.1C**). This provides evidence that MyD88 could be an important adapter in multiple pathways of innate immune responses to WT plague: TLR4 signaling as well as IL-1β/IL-18 signaling.

We investigated mechanisms upstream of the cytokine production and determined the NLRP3, NLRP12, and NLRC4 inflammasomes to be protecting mice from infection (**Figure 2.7A, 3.3A-B**). We also provided evidence that these NLRs are responsible for circulating IL-1β/ IL-18 cytokines during septicemic plague infection with both WT and modified hexa-acylated LPS-producing *Y. pestis*. Most importantly, we show that NLRP12 is an inflammasome component recognizing *Y. pestis*, and this is the first clear role for NLRP12 in resistance to infection. Our data suggests the NLRP12/IL-18/IFNɣ axis is effective at limiting infection with a modified *Y. pestis* strain.

Though bubonic plague is the most prevalent form of the natural disease worldwide, pneumonic plague has higher mortality, and is the most likely form that would result from the use of *Y. pestis* by bioterrorism. Moreover, the role of innate immunity, inflammasomes, and inflammasome-dependent cytokine production in the lung during *Y. pestis* infection is not fully understood. We determined that NLRP12 and, to a lesser extent, NLRP3, are responsible for

IL-18 and IL-1β during the initial disease progression. Moreover, we have shown that these NLRs affect both cellular recruitment and activation, which is in agreement with published data on NLRP12 and NLRP3 (Allen et al., 2009; Arthur et al., 2010). The differences in NLR-dependence may be due to expression of each protein in different cells, present at different times during the infection. This evidence defines the role for the inflammasome complex in both local inflammasome as well as elicit activation of the innate immune system.

Inflammasome formation culminates in caspase-1 activation to mature the inactive pro-IL-18 and IL-1β. This study has shown that survival following infection of mice is dependent on multiple inflammasome forming NLRs as well as caspase-1 dependent pro-inflammatory cytokines. However, we have observed that caspase-1-deficient animals (caspase-1/caspase-11 functional dKO) do not succumb to infection at all (s.c. infection of 500 CFU of KIM1001-pYtbLpxL), or have not significantly difference in survival compared to C57BL/6 mice (500 cfu KIM1001 i.t.), respectively (**Figure 5.1 A-B**.) This is curious as several other mouse strains deficient in genes in this pathway cannot control *Y. pestis* infection. It is possible that, in vivo, there are redundant inflammatory caspases, such as caspase-8 (Kang et al., 2012) maturing pro-IL-1β and pro-IL-18 that are also NLR activation dependent. Evidence of non-caspase pro-IL-1β maturation also exists, for example in neutrophils (Netea et al., 2010), perhaps mediated by enzymes such as cathepsins, elastase, and PR3. Our observed caspase-1 independence could be a partially neutrophil driven phenotype via action of these enzymes. Moreover, the differences in caspase-1 mediated survival could be greatly be dependent on route of primary infection and may explain some of the LD50 differences. More work must be done to elucidate the dependence

of caspase-1,caspase-11 and other IL-1β/IL-18 processing enzymes on cell death and inflammation during plague infection.

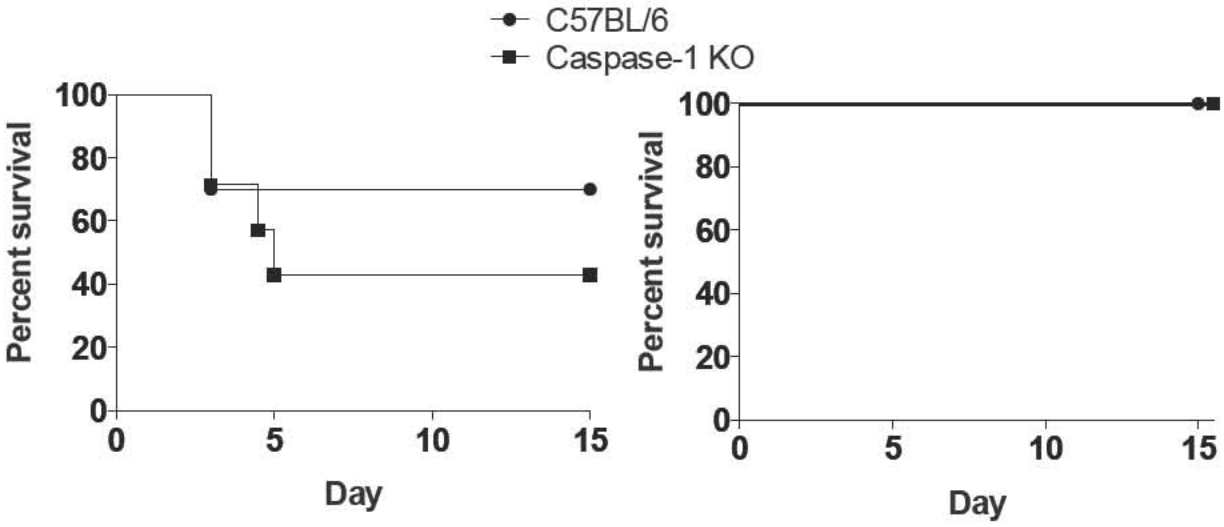


**Figure 5.1: Caspase-1 KO Animals Have Varying Phenotypes Depending on Route of Infection**

(A-B) Survival of mice infected with 500 CFU of (A) KIM1001-pYtbLpxL s.c. or (B) KIM1001 i.t. n=(A)C57BL/6, 10, Caspase-1, 7 and (B) C57BL/6, 15, Caspase-1 KO, 7

We and others have proposed a role for the conserved *Yersiniae* effector protein YopJ (YopP in *Y. enterocolitica*) in promoting inflammasome activation (**Figure 3.5H, Figure 5.1**), data not shown, and (Brodsky et al., 2010; Zheng et al., 2012). This indicates that all the pathogenic *Yersiniae* can promote YopJ-mediated inflammasome function and caspase-1 activity, in spite of the detrimental effects this could have on the infection. YopJ was conserved during the evolution of *Y. pestis* from *Y. pseudotuberculosis*, thereby conveying the important role it plays to promote infection, other than inflammasome activation.

Though the mechanism is unknown, it is speculated that YopJ may directly acetylate an NLR, or an NLR detects inappropriately-acylated host proteins (vis-a-vie plant R-proteins), leading to inflammasome activation (**Figure 5.2**). As well, studies in the intestinal lumen after oral *Y. pseudotuberculosis* infection show YopJ's role in barrier disruption (Jung et al., 2012; Meinzer et al., 2012). Interestingly, the balance of YopJ cytokine-blocking and cell death-induction is necessary for optimal pathogenesis as *Y. pseudotuberculosis* strains lacking YopJ fail to induce cell-death, cytokine down-regulation, and dissemination from a oral infection model. However, *Y. pseudotuberculosis* expressing hypercytotoxic YopP (from *Y. entrocolitica*), which enables stronger inhibition of cytokine production and more cell death, are also attenuated *in vivo*.

Other *Yersiniae* Yops were found to play roles during inflammasome activation: YopM was recently described to perhaps bind caspase-1 and sequester it into the nucleus, thereby halting pro-IL-1β/pro-IL-18 maturation (LaRock and Cookson, 2012). Interestingly, caspase-1 KO animals succumb quickly to infection with the YopM deficient *Y. pseudotuberculosis*, demonstrating the importance of caspase-1 to mediate infection. We however have found that

caspase-1 animals (caspase-1/caspase-11 dKO mutants) do not succumb to *Y. pestis* KIM1001-pYtbLpxL s.c. infection, and only trending (non-significant) towards more susceptibility to KIM1001 i.t. infection, therefore, the route of infection or bacterial strains used may determine caspase-1 importance. YopM may also have other host targets other than caspase-1 and also *Yersinia* YopM may have variations like YopJ has in *Y. pestis* strains.

YopK mutants strongly activated the inflammasome complex, thereby hinting at a role of YopK in regulating NLR function (Brodsky et al., 2010) (and Vladimer *et al.,* data not shown, **Figure 5.1**.) The direct pathway of YopK mediated inflammasome control is not clear, though there is evidence that YopK regulates Yop delivery and therefore the overabundance of Yops could be driving inflammasome formation (**Figure 5.1**) (Dewoody et al., 2012).

No connection has yet to be made of YopE and YopT to inflammasome activation, but we can hypothesize that their activity on cell-structure could lead to an increase in cellular danger signals, which would drive the NLRP3 inflammasome. Studies of the roles of *Yersiniae* Yops in cell-death, pro- or anti-inflammatory activity, and phagocytosis is important in discovering novel microbial-therapies. However, it seems likely that it is the balance of Yop expression and activity that regulates their overall function during pathogenesis and findings from single Yop-mutant bacteria probably do not represent the overall function. Yops must be studied in the context of the others, and more studies reviewing the disregulation of Yop delivery must be done to understand their role during infection.

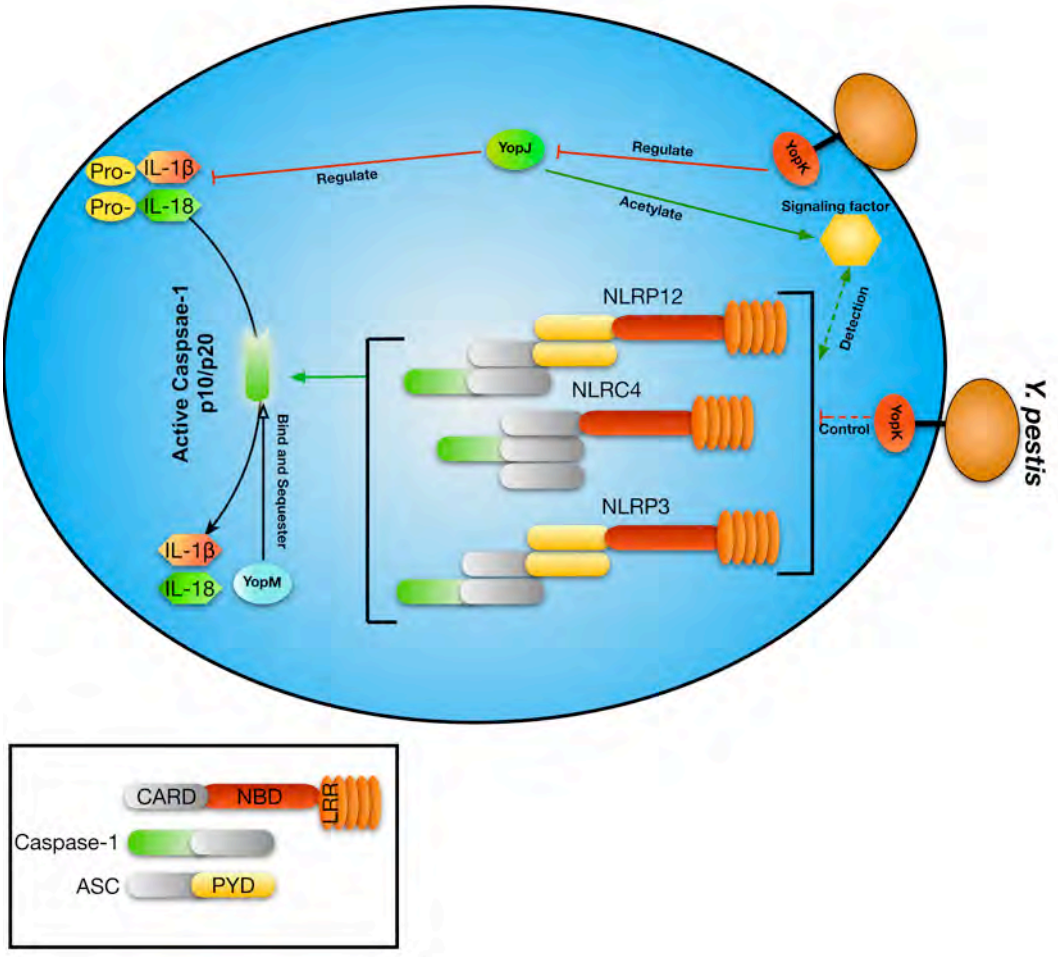


**Figure 5.2: Multiple Inflammasome-Forming NLRs are Activated by *Y. pestis*.** NLRP12, NLRP3 and NLRC4 are all activated by *Y. pestis* and are necessary in controlling plague infection. Multiple *Yersiniae* virulence factors regulate inflammasome function such as YopJ (modification of host proteins and down-regulation of Nf-kB indused pro-cytokines), YopK (inflammasome control, possibly directly or by regulation of other Yop delivery to the cytoplasm), and YopM (binding and sequestering pro- and active-caspase-1.) Dashed lines are hypothetical pathways, solid lines are summarized pathways.

We believe the loss of *lpxL* was necessary to limit pro-IL-1β and pro-IL-18 production downstream from NF-κB. By limiting the caspase-1 dependent cytokine substrates, activation of many inflammasomes (NLRP12, NLRP3, and NLRC4) has no anti-microbial effects on the *Y. pestis* infection.

*Y. pestis,* which we have shown to activate three known inflammasomes, is a convenient model system in determining the relationship of NLRP12, NLRP3, and NLRC4 during anti-microbial responses. Published evidence shows only one inflammasome per cell, therefore, we would assume that the multiple activated NLRs would hetero-, rather then homooligomerize upon activation.

All known inflammasomes, other then AIM2, contain a NACHT domain where oligomerization may occur. FRET, or IP studies could be done to further analyze the relationship of the separate NLRs. The model for single-inflammasomes per cell is based off of visualizing ASC speckles in activated cells under a fluorescence microscope. It is possible that the sensitivity of microscopes is not great enough to detect multiple individual inflammasomes - which could be evidence of single/homooligomerized inflammasome complexes. Moreover, this work indicates that knocking out single NLRs does not completely abrogate cytokine production, therefore, overlapping and redundant pathways of the multiple activated NLRs may exist.

NLRP12 has been described previously in anti-inflammatory roles regulating colitis and NF-κB driven responses (Lich et al., 2007; Zaki et al., 2011b; Allen et al., 2012), however, in this pestis model, we see no effects on NF-κB in the NLRP12-deficient animal or cells (**Figure 3.5A, 3.6 A-B,G, 3.10 E-F, Figure 4.4A**). This could be due to the regulatory effects of YopJ on NF-κB and MAPK, thereby, regulating the loss of NLRP12 controlled activation of these

transcription factors (Sweet et al., 2007; Paquette et al., 2012). Though YopJ plays many roles of immune-suppression and inflammasome activation *in vitro,* the role of YopJ *in vivo* remains somewhat controversial (described above in Chapter 1.) The variations in published work could be due to route of infection or pathogen preparation prior to infection (bubonic vs pneumonic plague, and growth temperature and characteristics.) We see C57BL/6 animals survive challenge with a YopJ-deficient strain of KIM1001 after i.t. injection, but not s.c. (data not shown). We believe YopJ may act in a tissue-specific manner.

In contrast to *Y. pestis,* which has an LD50 of less than 10 CFU for s.c. infection but an LD50 of approximately 500 CFU for i.t. infection, *F. tularensis,* has an LD50 of less than 10 CFU for both routes. This drastic difference in virulence could be due to limited inflammasome activation in the lungs during tularemia as *F. tularensis* primarily signals though AIM2 (Fernandes-Alnemri et al., 2010), which pestis does not (data not shown.) This could be due to AIM2-differential expression in the lung vs other tissues, and the lack of peripheral spread of *F. tularensis* in comparison to *Y. pestis*. The drastic difference of NLR success in controlling plague infection after intratracheal (pneumonic) infection compared to subcutaneous (bubonic) infection may be due to resident cellular expression of localized cells in tissues. Moreover, the *Yersinia* Pla protease promotes rapid decimation of the pathogen though the tissue, limiting the time the pathogen spends in the dermis.

These studies reveal a role for multiple inflammasomes in host resistance against pathogens, and minimizing inflammasome activation may have been a central factor in evolution of the high virulence of *Y. pestis*. This is also the first study to elucidate a pro-inflammatory role for NLRP12 during bacterial infection. Our work has identified signaling pathways, that once

activated, could assist in limiting and controlling plague infection. Intervention must typically be started within 24 hours of the first onset of plague symptoms to limit the pathogen spread and increase chances of survival. With the increased challenges of treating antibiotic resistant bacteria, targeting NLRs to produce greater amounts of IL-18 could be an effective treatment for plague and other bacterial infections within an expanded post-symptom window. Lastly, by studying the immune mediating effect of *Yersiniae* Yops, more knowledge about their ability to activate and inhibit certain signaling pathways could lead to the development of treatments for modulating local inflammation.

Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K.A., and Hornung, V. (2009). RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III|[ndash]|transcribed RNA intermediate. Nat Immunol *10*, 1065–1072.

Achtman, M., Morelli, G., Zhu, P., Wirth, T., Diehl, I., Kusecek, B., Vogler, A.J., Wagner, D.M., Allender, C.J., Easterday, W.R., et al. (2004). Microevolution and history of the plague bacillus, Yersinia pestis. Proc Natl Acad Sci USA *101*, 17837–17842.

Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A., and Carniel, E. (1999). Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis. Proc Natl Acad Sci USA *96*, 14043–14048.

Agostini, L., Martinon, F., Burns, K., McDermott, M.F., Hawkins, P.N., and Tschopp, J. (2004). NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity *20*, 319–325.

Akira, S., Yamamoto, M., and Takeda, K. (2003). Role of adapters in Toll-like receptor signalling. Biochem Soc Trans *31*, 637–642.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature *413*, 732–738.

Allen, I.C., Scull, M.A., Moore, C.B., Holl, E.K., McElvania-Tekippe, E., Taxman, D.J., Guthrie, E.H., Pickles, R.J., and Ting, J.P.-Y. (2009). The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity *30*, 556–565.

Allen, I.C., Wilson, J.E., Schneider, M., Lich, J.D., Roberts, R.A., Arthur, J.C., Woodford, R.-M.T., Davis, B.K., Uronis, J.M., Herfarth, H.H., et al. (2012). NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-κB signaling. Immunity *36*, 742–754.

Amaral, F.A., Costa, V.V., Tavares, L.D., Sachs, D., Coelho, F.M., Fagundes, C.T., Soriani, F.M., Silveira, T.N., Cunha, L.D., Zamboni, D.S., et al. (2012). NLRP3 inflammasome-mediated neutrophil recruitment and hypernociception depend on leukotriene B4 in a murine model of gout. Arthritis Rheum *64*, 474–484.

Anand, P.K., Malireddi, R.K.S., Lukens, J.R., Vogel, P., Bertin, J., Lamkanfi, M., and Kanneganti, T.-D. (2012). NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. Nature *488*, 389–393.

Anderson, G.W., Leary, S.E., Williamson, E.D., Titball, R.W., Welkos, S.L., Worsham, P.L., and Friedlander, A.M. (1996). Recombinant V antigen protects mice against pneumonic and bubonic

plague caused by F1-capsule-positive and -negative strains of Yersinia pestis. Infect Immun *64*, 4580–4585.

Andersson, K., Magnusson, K.E., Majeed, M., Stendahl, O., and Fällman, M. (1999). Yersinia pseudotuberculosis-induced calcium signaling in neutrophils is blocked by the virulence effector YopH. Infect Immun *67*, 2567–2574.

Andrade, W.A., Silva, A.M., Alves, V.S., Salgado, A.P.C., Melo, M.B., Andrade, H.M., Dall'orto, F.V., Garcia, S.A., Silveira, T.N., and Gazzinelli, R.T. (2010). Early endosome localization and activity of RasGEF1b, a toll-like receptor-inducible Ras guanine-nucleotide exchange factor. Genes and Immunity.

Arlehamn, C.S.L., Pétrilli, V., Gross, O., Tschopp, J., and Evans, T.J. (2010). The role of potassium in inflammasome activation by bacteria. Journal of Biological Chemistry *285*, 10508–10518.

Arthur, J.C., Lich, J.D., Aziz, R.K., Kotb, M., and Ting, J.P.-Y. (2007). Heat shock protein 90 associates with monarch-1 and regulates its ability to promote degradation of NF-kappaB-inducing kinase. The Journal of … *179*, 6291–6296.

Arthur, J.C., Lich, J.D., Ye, Z., Allen, I.C., Gris, D., Wilson, J.E., Schneider, M., Roney, K.E., O'Connor, B.P., Moore, C.B., et al. (2010). Cutting edge: NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity. The Journal of Immunology *185*, 4515–4519.

Averette, K.M., Pratt, M.R., Yang, Y., Bassilian, S., Whitelegge, J.P., Loo, J.A., Muir, T.W., and Bradley, K.A. (2009). Anthrax lethal toxin induced lysosomal membrane permeabilization and cytosolic cathepsin release is Nlrp1b/Nalp1b-dependent. PLoS ONE *4*, e7913.

Bae, J.Y., and Park, H.H. (2011). Crystal structure of NALP3 protein pyrin domain (PYD) and its implications in inflammasome assembly. Journal of Biological Chemistry *286*, 39528–39536.

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

Barchet, W., Krug, A., Cella, M., Newby, C., Fischer, J., Dzionek, A., Pekosz, A., and Colonna, M. (2005). Dendritic cells respond to influenza virus through TLR7- and PKR-independent pathways. Eur J Immunol *35*, 236–242.

Battafaraono, R., Dahlberg, P., Ratz, C., Johnston, J., Gray, B., Haseman, J., Mayo, K., and Dunn, D. (1995). Peptide derivatives of three distinct lipopolysaccharide binding proteins inhibit lipopolysaccharide-induced tumor necrosis factor- alpha secretion in vitro. Surgery *118*, 318–324.

Bauer, C., Duewell, P., Mayer, C., Lehr, H.A., Fitzgerald, K.A., Dauer, M., Tschopp, J., Endres, S., Latz, E., and Schnurr, M. (2010). Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. Gut *59*, 1192–1199.

Bauer, S., Kirschning, C.J., Häcker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., and Lipford, G.B. (2001). Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci USA *98*, 9237–42.

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., et al. (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol *183*, 787–791.

Beal, M.F. (2003). Mitochondria, oxidative damage, and inflammation in Parkinson's disease. Ann N Y Acad Sci *991*, 120–131.

Becker, M., Diamond, G., Verghese, M., and Randell, S. (2000). CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. J Biol Chem *275*, 29731–6.

Bergsbaken, T., and Cookson, B. (2009). Innate immune response during Yersinia infection: critical modulation of cell death mechanisms through phagocyte activation. J Leukoc Biol.

Black, D.S., and Bliska, J.B. (1997). Identification of p130Cas as a substrate of Yersinia YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. Embo J *16*, 2730–2744.

Black, D.S., and Bliska, J.B. (2000). The RhoGAP activity of the Yersinia pseudotuberculosis cytotoxin YopE is required for antiphagocytic function and virulence. Mol Microbiol *37*, 515–527.

Black, D.S., Marie-Cardine, A., Schraven, B., and Bliska, J.B. (2000). The Yersinia tyrosine phosphatase YopH targets a novel adhesion-regulated signalling complex in macrophages. Cell Microbiol *2*, 401–414.

Boland, A., and Cornelis, G.R. (1998). Role of YopP in Suppression of Tumor Necrosis Factor Alpha Release by Macrophages during YersiniaInfection.

Bos, K.I., Schuenemann, V.J., Golding, G.B., Burbano, H.A., Waglechner, N., Coombes, B.K., McPhee, J.B., DeWitte, S.N., Meyer, M., Schmedes, S., et al. (2011). A draft genome of Yersinia pestis from victims of the Black Death. Nature.

Brodsky, I.E. (2012). Cell death programs in Yersinia immunity and pathogenesis. 1–7.

Brodsky, I.E., Palm, N.W., Sadanand, S., Ryndak, M.B., Sutterwala, F.S., Flavell, R.A., Bliska, J.B., and Medzhitov, R. (2010). A Yersinia effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. Cell Host Microbe *7*, 376–387.

Broz, P., Newton, K., Lamkanfi, M., Mariathasan, S., Dixit, V.M., and Monack, D.M. (2010). Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. Journal of Experimental Medicine *207*, 1745–1755.

Broz, P., Ruby, T., Belhocine, K., Bouley, D.M., Kayagaki, N., Dixit, V.M., and Monack, D.M. (2012). Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. Nature.

Brozek, K., and Raetz, C. (1990). Biosynthesis of lipid A in Escherichia coli. Acyl carrier protein- dependent incorporation of laurate and myristate. J Biol Chem *265*, 15410–15417.

Brubaker, R.R. (1970). Interconversion of Purine Mononucleotides in Pasteurella pestis.

Bruey, J.-M., Bruey-Sedano, N., Luciano, F., Zhai, D., Balpai, R., Xu, C., Kress, C.L., Bailly-Maitre, B., Li, X., Osterman, A., et al. (2007). Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. Cell *129*, 45–56.

Brydges, S.D., Mueller, J.L., McGeough, M.D., Pena, C.A., Misaghi, A., Gandhi, C., Putnam, C.D., Boyle, D.L., Firestein, G.S., Horner, A.A., et al. (2009). Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. Immunity *30*, 875–887.

Bubeck, S.S., Cantwell, A.M., and Dube, P.H. (2007). Delayed inflammatory response to primary pneumonic plague occurs in both outbred and inbred mice. Infect Immun *75*, 697–705.

Butler, T. (2009). Plague into the 21st century. Clin Infect Dis *49*, 736–742.

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D.V. (1996). TRAF6 is a signal transducer for interleukin-1. Nature *383*, 443–446.

Cassel, S.L., Eisenbarth, S.C., Iyer, S.S., Sadler, J.J., Colegio, O.R., Tephly, L.A., Carter, A.B., Rothman, P.B., Flavell, R.A., and Sutterwala, F.S. (2008). The Nalp3 inflammasome is essential for the development of silicosis. Proceedings of the National Academy of Sciences *105*, 9035–9040.

Cavanaugh, D.C., Elisberg, B.L., Llewellyn, C.H., Marshall, J.D., Rust, J.H., Williams, J.E., and Meyer, K.F. (1974). Plague immunization. V. Indirect evidence for the efficacy of plague vaccine. J Infect Dis *129*, Suppl:S37–Suppl:S40.

Celhar, T., Magalhães, R., and Fairhurst, A.-M. (2012). TLR7 and TLR9 in SLE: when sensing self goes wrong. Immunol Res *53*, 58–77.

Centers for Disease Control and Prevention (CDC) (2011). Fatal laboratory-acquired infection with an attenuated Yersinia pestis Strain--Chicago, Illinois, 2009. MMWR Morb. Mortal. Wkly. Rep. *60*, 201–205.

Chain, P.S.G. (2004). Insights into the evolution of Yersinia pestis through whole-genome comparison with Yersinia pseudotuberculosis. Proceedings of the National Academy of Sciences *101*, 13826–13831.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., et al. (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol *4*, 702–707.

Chen, G.Y., Liu, M., Wang, F., Bertin, J., and Nuñez, G. (2011). A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. The Journal of Immunology *186*, 7187–7194.

Choe, J., Kelker, M.S., and Wilson, I.A. (2005). Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science *309*, 581–585.

Clementz, T., Bednarski, J., and Raetz, C. (1996). Function of the htrB high temperature requirement gene of Escherchia coli in the acylation of lipid A: HtrB catalyzed incorporation of laurate. J Biol Chem *271*, 12095–12102.

Coope, H.J. (2002). CD40 regulates the processing of NF-kappaB2 p100 to p52. The EMBO Journal *21*, 5375–5385.

Cornelis, G. (2002a). The Yersinia Ysc-Yop “type III” weaponry. Nat Rev Mol Cell Biol *3*, 742–752.

Cornelis, G. (2002b). Yersinia type III secretion: send in the effectors. J Cell Biol *158*, 401–408.

Cornelis, G.R. (2000). Molecular and cell biology aspects of plague. Proc Natl Acad Sci USA *97*, 8778–8783.

Costa, A., Gupta, R., Signorino, G., Malara, A., Cardile, F., Biondo, C., Midiri, A., Galbo, R., Trieu-Cuot, P., Papasergi, S., et al. (2012). Activation of the NLRP3 inflammasome by group B streptococci. The Journal of Immunology *188*, 1953–1960.

Davis, B.K., Roberts, R.A., Huang, M.T., Willingham, S.B., Conti, B.J., Brickey, W.J., Barker, B.R., Kwan, M., Taxman, D.J., Accavitti-Loper, M.-A., et al. (2011a). Cutting edge: NLRC5-dependent activation of the inflammasome. The Journal of Immunology *186*, 1333–1337.

Davis, B.K., Wen, H., and Ting, J.P.-Y. (2011b). The Inflammasome NLRs in Immunity, Inflammation, and Associated Diseases. Annu Rev Immunol *29*, 707–735.

Deng, W., Burland, V., Plunkett, G., Boutin, A., Mayhew, G., Liss, P., Perna, N., Rose, D., Mau, B., Zhou, S., et al. (2002). Genome sequence of Yersinia pestis KIM. J Bacteriol *184*, 4601–4611.

DEVIGNAT, R. (1951). [Varieties of Pasteurella pestis; new hypothesis]. Bull. World Health Organ. *4*, 247–263.

Dewoody, R., Merritt, P.M., and Marketon, M.M. (2012). YopK controls both rate and fidelity of Yop translocation. Mol Microbiol.

Dewoody, R., Merritt, P.M., Houppert, A.S., and Marketon, M.M. (2011). YopK Regulates the Yersinia pestis Type III Secretion System from Within Host Cells. Mol Microbiol.

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

Du, Y., Rosqvist, R., and Forsberg, A. (2002). Role of fraction 1 antigen of Yersinia pestis in inhibition of phagocytosis. Infect Immun *70*, 1453–1460.

Duewell, P., Kono, H., Rayner, K.J., Sirois, C.M., Vladimer, G., Bauernfeind, F.G., Abela, G.S., Franchi, L., Nuñez, G., Schnurr, M., et al. (2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature *464*, 1357–1361.

Duncan, J.A., Bergstralh, D.T., Wang, Y., Willingham, S.B., Ye, Z., Zimmermann, A.G., and Ting, J.P.-Y. (2007). Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. Proceedings of the ….

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., et al. (2011). NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. Cell *145*, 745–757.

Evdokimov, A.G., Anderson, D.E., Routzahn, K.M., and Waugh, D.S. (2001). Unusual molecular architecture of the Yersinia pestis cytotoxin YopM: a leucine-rich repeat protein with the shortest repeating unit. J Mol Biol *312*, 807–821.

Ezekowitz, R.A., and Gordon, S. (1982). Down-regulation of mannosyl receptor-mediated endocytosis and antigen F4/80 in bacillus Calmette-Guérin-activated mouse macrophages. Role of T lymphocytes and lymphokines. J Exp Med *155*, 1623–1637.

Fan, Y., Yu, Y., Shi, Y., Sun, W., Xie, M., Ge, N., Mao, R., Chang, A., Xu, G., Schneider, M.D., et al. (2010). Lysine 63-linked polyubiquitination of TAK1 at lysine 158 is required for tumor necrosis factor alpha- and interleukin-1beta-induced IKK/NF-kappaB and JNK/AP-1 activation. Journal of Biological Chemistry *285*, 5347–5360.

Faustin, B., Lartigue, L., Bruey, J.-M., Luciano, F., Sergienko, E., Bailly-Maitre, B., Volkmann, N., Hanein, D., Rouiller, I., and Reed, J.C. (2007). Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. Mol Cell *25*, 713–724.

Feodorova, V.A., and Corbel, M.J. (2009). Prospects for new plague vaccines. Expert Rev Vaccines *8*, 1721–1738.

Feodorova, V.A., Pan'kina, L.N., Savostina, E.P., Kuznetsov, O.S., Konnov, N.P., Sayapina, L.V., Dentovskaya, S.V., Shaikhutdinova, R.Z., Ageev, S.A., Lindner, B., et al. (2009). Pleiotropic effects of the lpxM mutation in Yersinia pestis resulting in modification of the biosynthesis of major immunoreactive antigens. Vaccine *27*, 2240–2250.

Fernandes-Alnemri, T., Yu, J.-W., Juliana, C., Solorzano, L., Kang, S., Wu, J., Datta, P., McCormick, M., Huang, L., McDermott, E., et al. (2010). The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. Nat Immunol *11*, 385–393.

Fitzgerald Ka, R. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med *198*, 1043.

Fitzgerald, K., Rowe, D., Barnes, B., Caffrey, D., Visintin, A., Latz, E., Monks, B., Pitha, P., and Golenbock, D. (2003). LPS-TLR4 Signaling to IRF-3/7 and NF-{kappa}B Involves the Toll Adapters TRAM and TRIF. J Exp Med *198*, 1043–1055.

Fitzgerald, K.A., Palsson-McDermott, E.M., Bowie, A.G., Jefferies, C.A., Mansell, A.S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M.T., et al. (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature *413*, 78–83.

Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., and Nuñez, G. (2009). The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol *10*, 241–247.

Franchi, L., Kamada, N., Nakamura, Y., Burberry, A., Kuffa, P., Suzuki, S., Shaw, M.H., Kim, Y.-G., and Nuñez, G. (2012a). NLRC4-driven production of IL-1β discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. Nat Immunol *13*, 449–456.

Franchi, L., Kanneganti, T.-D., Dubyak, G.R., and Núñez, G. (2007). Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. J Biol Chem *282*, 18810–18818.

Franchi, L., Muñoz-Planillo, R., and Nuñez, G. (2012b). Sensing and reacting to microbes through the inflammasomes. Nat Immunol *13*, 325–332.

Gack, M.U., Shin, Y.C., Joo, C.-H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S., et al. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature *446*, 916–920.

Gazzinelli, R., Ropert, C., and Campos, M. (2004). Role of the Toll/interleukin-1 receptor signaling pathway in host resistance and pathogenesis during infection with protozoan parasites. Immunol Rev *201*, 9–25.

Girardin, S.E., Boneca, I.G., Carneiro, L.A.M., Antignac, A., Jéhanno, M., Viala, J., Tedin, K., Taha, M.-K., Labigne, A., Zähringer, U., et al. (2003a). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science *300*, 1584–1587.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J., and Sansonetti, P.J. (2003b). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem *278*, 8869–8872.

Goguen, J.D., Yother, J., and Straley, S.C. (1984). Genetic analysis of the low calcium response in Yersinia pestis mu d1(Ap lac) insertion mutants. *160*, 842–848.

Guan, Y., Ranoa, D.R.E., Jiang, S., Mutha, S.K., Li, X., Baudry, J., and Tapping, R.I. (2010). Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. The Journal of Immunology *184*, 5094–5103.

Guinet, F., Avé, P., Jones, L., Huerre, M., and Carniel, E. (2008). Defective innate cell response and lymph node infiltration specify Yersinia pestis infection. PLoS ONE *3*, e1688.

Haas, T., Metzger, J., Schmitz, F., Heit, A., Müller, T., Latz, E., and Wagner, H. (2008). The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. Immunity *28*, 315–323.

Halle, A., Hornung, V., Petzold, G.C., Stewart, C.R., Monks, B.G., Reinheckel, T., Fitzgerald, K.A., Latz, E., Moore, K.J., and Golenbock, D.T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol *9*, 857–865.

Hawkins, P., and Lachmann, H. (2003). Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. New Engl J Med.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature *410*, 1099–1103.

Håkansson, S., Galyov, E.E., Rosqvist, R., and Wolf-Watz, H. (1996). The Yersinia YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. Mol Microbiol *20*, 593–603.

Heath, D.G., Anderson, G.W., Mauro, J.M., Welkos, S.L., Andrews, G.P., Adamovicz, J., and Friedlander, A.M. (1998). Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. Vaccine *16*, 1131–1137.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science *303*, 1526–1529.

Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W.Z., Strowig, T., Thaiss, C.A., Kau, A.L., Eisenbarth, S.C., Jurczak, M.J., et al. (2012). Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature *482*, 179–185.

Higuchi, K., Kupferberg, L.L., and Smith, J.L. (1959). … ON THE NUTRITION AND PHYSIOLOGY OF PASTEURELLA PESTIS III. Effects of Calcium Ions on the Growth of Virulent and Avirulent Strains ofPasteurella pestis. Journal of Bacteriology.

Hinnebusch, B.J. (2005). The evolution of flea-borne transmission in Yersinia pestis. Curr Issues Mol Biol *7*, 197–212.

Hinnebusch, B.J., Rudolph, A.E., Cherepanov, P., Dixon, J.E., Schwan, T.G., and Forsberg, A. (2002). Role of Yersinia murine toxin in survival of Yersinia pestis in the midgut of the flea vector. Science *296*, 733–735.

Hise, A.G., Tomalka, J., Ganesan, S., Patel, K., Hall, B.A., Brown, G.D., and Fitzgerald, K.A. (2009). An Essential Role for the NLRP3 Inflammasome in Host Defense against the Human Fungal Pathogen Candida albicans. Cell Host Microbe *5*, 487–497.

Hoffman, H.M., and Brydges, S.D. (2011). Genetic and Molecular Basis of Inflammasome-mediated Disease. Journal of Biological Chemistry *286*, 10889–10896.

Holmstrom, A., Rosqvist, R., Wolf-Watz, H., and Forsberg, A. (1995). Virulence plasmid-encoded YopK is essential for Yersinia pseudotuberculosis to cause systemic infection in mice. Infect Immun *63*, 2269–2276.

Holmström, A., Petterson, J., Rosqvist, R., Håkansson, S., Tafazoli, F., Fällman, M., Magnusson, K.E., Wolf-Watz, H., and Forsberg, A. (1997). YopK of Yersinia pseudotuberculosis controls translocation of Yop effectors across the eukaryotic cell membrane. Mol Microbiol *24*, 73–91.

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., and Fitzgerald, K.A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature *458*, 514–518.

Hornung, V., and Latz, E. (2010). Intracellular DNA recognition. Nature Reviews Immunology *10*, 123–130.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., Rock, K.L., Fitzgerald, K.A., and Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol *9*, 847–856.

Hornung, V., Ellegast, J., Kim, S., Brzózka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.-K., Schlee, M., et al. (2006). 5'-Triphosphate RNA is the ligand for RIG-I. Science *314*, 994–997.

Horvath, G., and Schrum, J. (2011). Intracellular sensing of microbes and danger signals by the inflammasomes - Horvath - 2011 - Immunological Reviews - Wiley Online Library. Immunological ….

Husebye, H., Halaas, Ø., Stenmark, H., Tunheim, G., Sandanger, Ø., Bogen, B., Brech, A., Latz, E., and Espevik, T. (2006). Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity. Embo J *25*, 683–692.

Iriarte, M., and Cornelis, G.R. (1998). YopT, a new Yersinia Yop effector protein, affects the cytoskeleton of host cells. Mol Microbiol *29*, 915–929.

Ishii, K.J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., Uematsu, S., Takeuchi, O., Takeshita, F., Coban, C., et al. (2008). TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature *451*, 725–729.

Ishikawa, H., and Barber, G.N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature *455*, 674–678.

Ishikawa, H., Ma, Z., and Barber, G.N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature *461*, 788–792.

Janeway, C.A., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol *20*, 197–216.

Jéru, I., Duquesnoy, P., Fernandes-Alnemri, T., Cochet, E., Yu, J.W., Lackmy-Port-Lis, M., Grimprel, E., Landman-Parker, J., Hentgen, V., Marlin, S., et al. (2008). Mutations in NALP12 cause hereditary periodic fever syndromes. Proc Natl Acad Sci USA *105*, 1614–1619.

Jéru, I., Hentgen, V., Normand, S., Duquesnoy, P., Cochet, E., Delwail, A., Grateau, G., Marlin, S., Amselem, S., and Lecron, J.-C. (2011a). Role of interleukin-1β in NLRP12-associated autoinflammatory disorders and resistance to anti-interleukin-1 therapy. Arthritis Rheum *63*, 2142–2148.

Jéru, I., Le Borgne, G., Cochet, E., Hayrapetyan, H., Duquesnoy, P., Grateau, G., Morali, A., Sarkisian, T., and Amselem, S. (2011b). Identification and functional consequences of a recurrent NLRP12 missense mutation in periodic fever syndromes. Arthritis Rheum *63*, 1459–1464.

Jéru, I., Marlin, S., Le Borgne, G., Cochet, E., Normand, S., Duquesnoy, P., Dastot-Le Moal, F., Cuisset, L., Hentgen, V., Fernandes-Alnemri, T., et al. (2010). Functional consequences of a germline mutation in the leucine-rich repeat domain of NLRP3 identified in an atypical autoinflammatory disorder. Arthritis Rheum *62*, 1176–1185.

Jin, M.S., Kim, S.E., Heo, J.Y., Lee, M.E., Kim, H.M., Paik, S.-G., Lee, H., and Lee, J.-O. (2007). Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell *130*, 1071–1082.

Jung, C., Meinzer, U., Montcuquet, N., Thachil, E., Château, D., Thiébaut, R., Roy, M., Alnabhani, Z., Berrebi, D., Dussaillant, M., et al. (2012). Yersinia pseudotuberculosis disrupts

intestinal barrier integrity through hematopoietic TLR-2 signaling. J. CLin. Invest. *122*, 2239–2251.

Juris, S.J., Rudolph, A.E., Huddler, D., Orth, K., and Dixon, J.E. (2000). A distinctive role for the Yersinia protein kinase: actin binding, kinase activation, and cytoskeleton disruption. Proc Natl Acad Sci USA *97*, 9431–9436.

Jurk M, H. (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol *3*, 499.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A.M., Wagner, H., Lipford, G., and Bauer, S. (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol *3*, 499.

Kagan, J.C., and Medzhitov, R. (2006). Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell *125*, 943–955.

Kalali, B.N., Köllisch, G., Mages, J., Müller, T., Bauer, S., Wagner, H., Ring, J., Lang, R., Mempel, M., and Ollert, M. (2008). Double-stranded RNA induces an antiviral defense status in epidermal keratinocytes through TLR3-, PKR-, and MDA5/RIG-I-mediated differential signaling. The Journal of Immunology *181*, 2694–2704.

Kalis, C., Kanzler, B., Lembo, A., Poltorak, A., Galanos, C., and Freudenberg, M.A. (2003). Toll-like receptor 4 expression levels determine the degree of LPS-susceptibility in mice. Eur J Immunol *33*, 798–805.

Kang, J.Y., Nan, X., Jin, M.S., Youn, S.-J., Ryu, Y.H., Mah, S., Han, S.H., Lee, H., Paik, S.-G., and Lee, J.-O. (2009). Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity *31*, 873–884.

Kang, T.-B., Yang, S.-H., Toth, B., Kovalenko, A., and Wallach, D. (2012). Caspase-8 Blocks Kinase RIPK3-Mediated Activation of the NLRP3 Inflammasome. Immunity.

Kanneganti, T.-D. (2010). Central roles of NLRs and inflammasomes in viral infection. Nature Reviews Immunology *10*, 688–698.

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T.S., Fujita, T., and Akira, S. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. Journal of Experimental Medicine *205*, 1601–1610.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., et al. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature *441*, 101–105.

Kawahara K, T. (2002). Modification of the structure and activity of lipid A in Yersinia pestis lipopolysaccharide by growth temperature. Infect Immun *70*, 4092–4098.

Kawahara, K., Tsukano, H., Watanabe, H., Lindner, B., and Matsuura, M. (2002). Modification of the structure and activity of lipid A in Yersinia pestis lipopolysaccharide by growth temperature. Infect Immun *70*, 4092–4098.

Kawai, T., and Akira, S. (2007). Signaling to NF-kappaB by Toll-like receptors. Trends in Molecular Medicine *13*, 460–469.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol *11*, 373–384.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., and Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol *6*, 981–988.

Kawasaki, T., Kawai, T., and Akira, S. (2011). Recognition of nucleic acids by pattern-recognition receptors and its relevance in autoimmunity. Immunol Rev *243*, 61–73.

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., et al. (2011). Non-canonical inflammasome activation targets caspase-11. Nature *479*, 117–121.

Keating, S.E., Baran, M., and Bowie, A.G. (2011). Cytosolic DNA sensors regulating type I interferon induction. Trends Immunol *32*, 574–581.

Khare, S., Dorfleutner, A., Bryan, N.B., Yun, C., Radian, A.D., de Almeida, L., Rojanasakul, Y., and Stehlik, C. (2012). An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages. Immunity *36*, 464–476.

Kim, S., Bauernfeind, F., Ablasser, A., Hartmann, G., Fitzgerald, K.A., Latz, E., and Hornung, V. (2010). Listeria monocytogenes is sensed by the NLRP3 and AIM2 inflammasome. Eur J Immunol *40*, 1545–1551.

Kim, Y.-M., Brinkmann, M.M., Paquet, M.-E., and Ploegh, H.L. (2008). UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature *452*, 234–238.

Knirel, Y.A., Lindner, B., Vinogradov, E., Shaikhutdinova, R.Z., Senchenkova, S.N., Kocharova, N.A., Holst, O., Pier, G.B., and Anisimov, A.P. (2005). Cold temperature-induced modifications to the composition and structure of the lipopolysaccharide of Yersinia pestis. Carbohydrate Research *340*, 1625–1630.

Kofoed, E.M., and Vance, R.E. (2011). Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature *477*, 592–595.

Kool, J.L., and Weinstein, R.A. (2005). Risk of Person-to-Person Transmission of Pneumonic Plague. Clinical Infectious Diseases.

Kraushaar, B., Dieckmann, R., and Wittwer, M. (2011). Characterization of a Yersinia enterocolitica biotype 1A strain harbouring an ail gene - Kraushaar - 2011 - Journal of Applied Microbiology - Wiley Online Library. Journal of Applied ….

Kumar, H., Kawai, T., and Akira, S. (2011). Pathogen recognition by the innate immune system. Int. Rev. Immunol. *30*, 16–34.

Lachmann, H.J., Lowe, P., Felix, S.D., Rordorf, C., Leslie, K., Madhoo, S., Wittkowski, H., Bek, S., Hartmann, N., Bosset, S., et al. (2009). In vivo regulation of interleukin 1beta in patients with cryopyrin-associated periodic syndromes. Journal of Experimental Medicine *206*, 1029–1036.

Lamkanfi, M., and Dixit, V.M. (2009). Inflammasomes: guardians of cytosolic sanctity. Immunol Rev *227*, 95–105.

Lamkanfi, M., and Kanneganti, T.-D. (2012). Regulation of immune pathways by the NOD-like receptor NLRC5. Immunobiology *217*, 13–16.

LaRock, C.N., and Cookson, B.T. (2012). The Yersinia Virulence Effector YopM Binds Caspase-1 to Arrest Inflammasome Assembly and Processing. Cell Host Microbe *12*, 799–805.

Lathem, W.W., Crosby, S.D., Miller, V.L., and Goldman, W.E. (2005). Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. Proc Natl Acad Sci USA *102*, 17786–17791.

Lathem, W.W., Price, P.A., Miller, V.L., and Goldman, W.E. (2007). A plasminogen-activating protease specifically controls the development of primary pneumonic plague. Science *315*, 509–513.

Latz E, V. (2002). Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. J Biol Chem *277*, 47834.

Latz, E. (2010). The inflammasomes: mechanisms of activation and function. Curr Opin Immunol *22*, 28–33.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K., Monks, B., Knetter, C., Lien, E., Nilsen, N., Espevik, T., and Golenbock, D. (2004). TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol *5*, 190–198.

Latz, E., Verma, A., Visintin, A., Gong, M., Sirois, C.M., Klein, D.C.G., Monks, B.G., McKnight, C.J., Lamphier, M.S., Duprex, W.P., et al. (2007). Ligand-induced conformational changes allosterically activate Toll-like receptor 9. Nat Immunol *8*, 772–779.

Latz, E., Visintin, A., Lien, E., Fitzgerald, K.A., Monks, B.G., Kurt-Jones, E.A., Golenbock, D.T., and Espevik, T. (2002). Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. J Biol Chem *277*, 47834–47843.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973–983.

Li, Q., and Verma, I.M. (2002). NF-kappaB regulation in the immune system. Nature Reviews Immunology *2*, 725–734.

Lich, J.D., and Ting, J.P.-Y. (2007). Monarch-1/PYPAF7 and other CATERPILLER (CLR, NOD, NLR) proteins with negative regulatory functions. Microbes and Infection *9*, 672–676.

Lich, J.D., Williams, K.L., Moore, C.B., Arthur, J.C., Davis, B.K., Taxman, D.J., and Ting, J.P.-Y. (2007). Monarch-1 suppresses non-canonical NF-kappaB activation and p52-dependent chemokine expression in monocytes. J Immunol *178*, 1256–1260.

Lien, E., Means, T., Heine, H., Yoshimura, A., Kusumoto, S., Fukase, K., Fenton, M., Oikawa, M., Qureshi, N., Monks, B., et al. (2000). Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J. CLin. Invest. *105*, 497–504.

Lightfield, K.L., Persson, J., Brubaker, S.W., Witte, C.E., Moltke, von, J., Dunipace, E.A., Henry, T., Sun, Y.-H., Cado, D., Dietrich, W.F., et al. (2008). Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. Nat Immunol *9*, 1171–1178.

Lilo, S., Zheng, Y., and Bliska, J.B. (2008). Caspase-1 activation in macrophages infected with Yersinia pestis KIM requires the type III secretion system effector YopJ. Infect Immun *76*, 3911–3923.

Lu, B., Nakamura, T., Inouye, K., Li, J., Tang, Y., Lundbäck, P., Valdes-Ferrer, S.I., Olofsson, P.S., Kalb, T., Roth, J., et al. (2012). Novel role of PKR in inflammasome activation and HMGB1 release. Nature *488*, 670–674.

Lund J, S. (2003). Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med *198*, 513.

Manji, G.A., Wang, L., Geddes, B.J., Brown, M., Merriam, S., Al-Garawi, A., Mak, S., Lora, J.M., Briskin, M., Jurman, M., et al. (2002). PYPAF1, a PYRIN-containing Apaf1-like protein

that assembles with ASC and regulates activation of NF-kappa B. J Biol Chem *277*, 11570–11575.

Manuse, M.J., Briggs, C.M., and Parks, G.D. (2010). Replication-independent activation of human plasmacytoid dendritic cells by the paramyxovirus SV5 Requires TLR7 and autophagy pathways. Virology *405*, 383–389.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell *10*, 417–426.

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., and Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature *440*, 237–241.

Mayor, A., Martinon, F., de Smedt, T., Pétrilli, V., and Tschopp, J. (2007). A crucial function of SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune responses. Nat Immunol *8*, 497–503.

Meinzer, U., Barreau, F., Esmiol-Welterlin, S., Jung, C., Villard, C., Léger, T., Ben-Mkaddem, S., Berrebi, D., Dussaillant, M., Alnabhani, Z., et al. (2012). Yersinia pseudotuberculosis Effector YopJ Subverts the Nod2/RICK/TAK1 Pathway and Activates Caspase-1 to Induce Intestinal Barrier Dysfunction. Cell Host Microbe *11*, 337–351.

Meinzer, U., Esmiol-Welterlin, S., Barreau, F., Berrebi, D., Dussaillant, M., Bonacorsi, S., Chareyre, F., Niwa-Kawakita, M., Alberti, C., Sterkers, G., et al. (2007). Nod2 mediates susceptibility to Yersinia pseudotuberculosis in mice. PLoS ONE *3*, e2769.

Meissner, T.B., Li, A., Biswas, A., Lee, K.-H., Liu, Y.-J., Bayir, E., Iliopoulos, D., van den Elsen, P.J., and Kobayashi, K.S. (2010). NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. Proceedings of the National Academy of Sciences *107*, 13794–13799.

Meng, J., Lien, E., and Golenbock, D.T. (2010). MD-2-mediated ionic interactions between lipid A and TLR4 are essential for receptor activation. Journal of Biological Chemistry *285*, 8695–8702.

Miao, E.A., Ernst, R.K., Dors, M., Mao, D.P., and Aderem, A. (2008). Pseudomonas aeruginosa activates caspase 1 through Ipaf. Proceedings of the National Academy of Sciences *105*, 2562–2567.

Miao, E.A., Mao, D.P., Yudkovsky, N., Bonneau, R., Lorang, C.G., Warren, S.E., Leaf, I.A., and Aderem, A. (2010). Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. Proceedings of the National Academy of Sciences *107*, 3076–3080.

Miao, E.A., Rajan, J.V., and Aderem, A. (2011). Caspase-1-induced pyroptotic cell death. Immunol Rev *243*, 206–214.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J.M., and Cornelis, G. (1990). Secretion of Yop proteins by Yersiniae. Infect Immun *58*, 2840–2849.

Mittal, R., Peak-Chew, S.-Y., and McMahon, H.T. (2006). Acetylation of MEK2 and I kappa B kinase (IKK) activation loop residues by YopJ inhibits signaling. Proc Natl Acad Sci USA *103*, 18574–18579.

Moltke, von, J., Trinidad, N.J., Moayeri, M., Kintzer, A.F., Wang, S.B., van Rooijen, N., Brown, C.R., Krantz, B.A., Leppla, S.H., Gronert, K., et al. (2012). Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. Nature.

Monack, D.M., Mecsas, J., Ghori, N., and Falkow, S. (1997). Yersinia signals macrophages to undergo apoptosis and YopJ is necessary for this cell death. Proc Natl Acad Sci USA *94*, 10385–10390.

Montie, T.C. (1981). Properties and pharmacological action of plague murine toxin. Pharmacol. Ther. *12,* 491–499.

Montminy Sw, K. (2006). Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response. Nat Immunol *7*, 1066.

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

Morelli, G., Song, Y., Mazzoni, C.J., Eppinger, M., Roumagnac, P., Wagner, D.M., Feldkamp, M., Kusecek, B., Vogler, A.J., Li, Y., et al. (2010). Yersinia pestis genome sequencing identifies patterns of global phylogenetic diversity. Nat Genet *42*, 1140–1143.

Motshwene, P.G., Moncrieffe, M.C., Grossmann, J.G., Kao, C., Ayaluru, M., Sandercock, A.M., Robinson, C.V., Latz, E., and Gay, N.J. (2009). An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. J Biol Chem *284*, 25404–25411.

Mukherjee, S., Keitany, G., Li, Y., Wang, Y., Ball, H.L., Goldsmith, E.J., and Orth, K. (2006). Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. Science *312*, 1211–1214.

Munford, R. (2008). Sensing gram-negative bacterial lipopolysaccharides: a human disease determinant? Infect Immun *76*, 454–465.

Munford, R., Hall, C., Lipton, J., and Dietschy, J. (1982). Biological activity, lipoprotein-binding behavior, and in vivo disposition of extracted and native forms of Salmonella typhimurium lipopolysaccharides. J. CLin. Invest. *70*, 877–888.

Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002). Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat Immunol *3*, 667–672.

Nakhaei, P., Hiscott, J., and Lin, R. (2009). STING-ing the Antiviral Pathway. Journal of Molecular Cell Biology.

Netea, M.G., Nold-Petry, C.A., Nold, M.F., Joosten, L.A.B., Opitz, B., van der Meer, J.H.M., van de Veerdonk, F.L., Ferwerda, G., Heinhuis, B., Devesa, I., et al. (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood *113*, 2324–2335.

Netea, M.G., Simon, A., van de Veerdonk, F., Kullberg, B.J., van der Meer, J.W.M., and Joosten, L.A.B. (2010). IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog *6*, e1000661.

Neyt, C., and Cornelis, G.R. (1999). Insertion of a Yop translocation pore into the macrophage plasma membrane by Yersinia enterocolitica: requirement for translocators YopB and YopD, but not LcrG. Mol Microbiol *33*, 971–981.

Nilsen N, N. (2004). Lipopolysaccharide and Double-stranded RNA Up-regulate Toll-like Receptor 2 Independently of Myeloid Differentiation Factor 88. Journal of Biological Chemistry *279*, 39727–39735.

Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., and Hattori, K. (1995). Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature *378*, 88–91.

Orth K, X. (2000). Disruption of signaling by Yersinia effector YopJ, a ubiquitin-like protein protease. Science (New York, N.Y.) *290*, 1594.

Ozkurede, V.U., and Franchi, L. (2012). Immunology in clinic review series; focus on autoinflammatory diseases: role of inflammasomes in autoinflammatory syndromes. Clin Exp Immunol *167*, 382–390.

Paquette, N., Conlon, J., Sweet, C., Rus, F., Wilson, L., Pereira, A., Rosadini, C.V., Goutagny, N., Weber, A.N.R., Lane, W.S., et al. (2012). Serine/threonine acetylation of TGFβ-activated kinase (TAK1) by Yersinia pestis YopJ inhibits innate immune signaling. Proceedings of the National Academy of Sciences *109*, 12710–12715.

Park, B., Brinkmann, M.M., Spooner, E., Lee, C.C., Kim, Y.-M., and Ploegh, H.L. (2008). Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nat Immunol *9*, 1407–1414.

Park, B.S., Song, D.H., Kim, H.M., Choi, B.-S., Lee, H., and Lee, J.-O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature *458*, 1191–1195.

Perry Ja, O. (2005). Cutting edge: the acquisition of TLR tolerance during malaria infection impacts T cell activation. J Immunol *174*, 5921–5925.

Perry Rd, S. (1998). DNA sequencing and analysis of the low-Ca2+-response plasmid pCD1 of Yersinia pestis KIM5. Infect Immun *66*, 4611–4623.

Perry, R., and Fetherston, J. (1997). Yersinia pestis--etiologic agent of plague. Clin Microbiol Rev *10*, 35–66.

Pilsl, H., Killmann, H., Hantke, K., and Braun, V. (1996). Periplasmic location of the pesticin immunity protein suggests inactivation of pesticin in the periplasm. Journal of Bacteriology.

Pinheiro, A.S., Proell, M., Eibl, C., Page, R., Schwarzenbacher, R., and Peti, W. (2010). Three-dimensional structure of the NLRP7 pyrin domain: insight into pyrin-pyrin-mediated effector domain signaling in innate immunity. Journal of Biological Chemistry *285*, 27402–27410.

Poeck, H., Bscheider, M., Gross, O., Finger, K., Roth, S., Rebsamen, M., Hannesschläger, N., Schlee, M., Rothenfusser, S., Barchet, W., et al. (2009). Recognition of RNA virus by RIG-I

results in activation of CARD9 and inflammasome signaling for interleukin 1beta production. Nat Immunol.

Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., and Beutler, B. (2000). Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. Proc Natl Acad Sci USA *97*, 2163–2167.

Posch, C., Kaulfersch, W., and Rappersberger, K. (2012). Cryopyrin-Associated Periodic Syndrome. Pediatr Dermatol.

Pouliot, K., Pan, N., Wang, S., Lu, S., Lien, E., and Goguen, J.D. (2007). Evaluation of the Role of LcrV-Toll-Like Receptor 2-Mediated Immunomodulation in the Virulence of Yersinia pestis. Infect Immun *75*, 3571–3580.

Price Sb, C. (1991). The Yersinia pestis V antigen is a regulatory protein necessary for Ca2(+)-dependent growth and maximal expression of low-Ca2+ response virulence genes. J Bacteriol *173*, 2649–2657.

Py, B.F., Kim, M.-S., Vakifahmetoglu-Norberg, H., and Yuan, J. (2012). Deubiquitination of NLRP3 by BRCC3 Critically Regulates Inflammasome Activity. Molecular Cell.

Qu, Y., Misaghi, S., Izrael-Tomasevic, A., Newton, K., Gilmour, L.L., Lamkanfi, M., Louie, S., Kayagaki, N., Liu, J., Kömüves, L., et al. (2012). Phosphorylation of NLRC4 is critical for inflammasome activation. Nature.

Raetz Cr, R. (2007). Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem *76*, 295–329.

Rathinam, V.A.K., Jiang, Z., Waggoner, S.N., Sharma, S., Cole, L.E., Waggoner, L., Vanaja, S.K., Monks, B.G., Ganesan, S., Latz, E., et al. (2010). The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol *11*, 395–402.

Rathinam, V.A.K., Vanaja, S.K., and Fitzgerald, K.A. (2012a). Regulation of inflammasome signaling. Nat Immunol *13*, 333–332.

Rathinam, V.A.K., Vanaja, S.K., Waggoner, L., Sokolovska, A., Becker, C., Stuart, L.M., Leong, J.M., and Fitzgerald, K.A. (2012b). TRIF Licenses Caspase-11-Dependent NLRP3 Inflammasome Activation by Gram-Negative Bacteria. Cell *150*, 606–619.

Rebeil, R., Ernst, R., Gowen, B., Miller, S., and Hinnebusch, B. (2004). Variation in lipid A structure in the pathogenic yersiniae. Mol Microbiol *52*, 1363–1373.

Rebeil, R., Ernst, R.K., Jarrett, C.O., Adams, K.N., Miller, S.I., and Hinnebusch, B.J. (2006). Characterization of late acyltransferase genes of Yersinia pestis and their role in temperature-dependent lipid A variation. Journal of Bacteriology *188*, 1381–1388.

Reuter, B.K., and Pizarro, T.T. (2004). Commentary: the role of the IL-18 system and other members of the IL-1R/TLR superfamily in innate mucosal immunity and the pathogenesis of inflammatory bowel disease: friend or foe? Eur J Immunol *34*, 2347–2355.

Roggenkamp, A., Geiger, A.M., Leitritz, L., Kessler, A., and Heesemann, J. (1997). Passive immunity to infection with Yersinia spp. mediated by anti-recombinant V antigen is dependent on polymorphism of V antigen. Infect Immun *65*, 446–451.

Ronald, P.C., and Beutler, B. (2010). Plant and animal sensors of conserved microbial signatures. Science *330*, 1061–1064.

Rowe, D.C., McGettrick, A.F., Latz, E., Monks, B.G., Gay, N.J., Yamamoto, M., Akira, S., O'Neill, L.A., Fitzgerald, K.A., and Golenbock, D.T. (2006). The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc Natl Acad Sci USA *103*, 6299–6304.

Saitoh S, A. (2004). Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. International Immunology *16*, 961–969.

Sander, L.E., Davis, M.J., Boekschoten, M.V., Amsen, D., Dascher, C.C., Ryffel, B., Swanson, J.A., Müller, M., and Blander, J.M. (2011). Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature *474*, 385–389.

Sato S, S. (2003). Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like re. J Immunol *171*, 4304.

Schilling B, M. (2007). Characterization of lipid A acylation patterns in Francisella tularensis, Francisella novicida, and Francisella philomiragia using multiple-stage mass spectrometry and matrix-assisted laser desorption/ionization on an intermediate vacuum source linear ion. Analytical Chemistry *79*, 1034.

Shahzad G Raja, G.D.D. (2007). Eritoran: the evidence of its therapeutic potential in sepsis. Core Evidence *2*, 199.

Sharma, S., Sharma, S., Deoliveira, R.B., Deoliveira, R.B., Kalantari, P., Kalantari, P., Parroche, P., Parroche, P., Goutagny, N., Goutagny, N., et al. (2011). Innate Immune Recognition of an AT-Rich Stem-Loop DNA Motif in the Plasmodium falciparum Genome. Immunity *35*, 194–207.

Shaw, M.H., Reimer, T., Kim, Y.-G., and Nuñez, G. (2008). NOD-like receptors (NLRs): bona fide intracellular microbial sensors. Curr Opin Immunol *20*, 377–382.

Shenoy, A.R., Wellington, D.A., Kumar, P., Kassa, H., Booth, C.J., Cresswell, P., and MacMicking, J.D. (2012). GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. Science *336*, 481–485.

Shi, C.-S., Shenderov, K., Huang, N.-N., Kabat, J., Abu-Asab, M., Fitzgerald, K.A., Sher, A., and Kehrl, J.H. (2012). Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. Nat Immunol 1–11.

Shimada, K., Crother, T.R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., Ramanujan, V.K., Wolf, A.J., Vergnes, L., Ojcius, D.M., et al. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. Immunity *36*, 401–414.

Shimamoto, A., Chong, A.J., Yada, M., Shomura, S., Takayama, H., Fleisig, A.J., Agnew, M.L., Hampton, C.R., Rothnie, C.L., Spring, D.J., et al. (2006). Inhibition of Toll-like receptor 4 with eritoran attenuates myocardial ischemia-reperfusion injury. Circulation *114*, I270–I274.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med *189*, 1777–1782.

Skrzypek, E., Cowan, C., and Straley, S.C. (1998). Targeting of the Yersinia pestis YopM protein into HeLa cells and intracellular trafficking to the nucleus. Mol Microbiol *30*, 1051–1065.

Smiley, S.T. (2008). Immune defense against pneumonic plague. Immunol Rev *225*, 256–271.

Sodeinde, O., Subrahmanyam, Y., Stark, K., Quan, T., Bao, Y., and Goguen, J. (1992). A surface protease and the invasive character of plague. Science *258*, 1004–1007.

Stenseth, N.C., Atshabar, B.B., Begon, M., Belmain, S.R., Bertherat, E., Carniel, E., Gage, K.L., Leirs, H., and Rahalison, L. (2008). Plague: past, present, and future. *5*, e3.

Stetson, D.B., and Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity *24*, 93–103.

Stutz, A., Golenbock, D.T., and Latz, E. (2009). Inflammasomes: too big to miss. J. CLin. Invest. *119*, 3502–3511.

Sweet, C.R., Conlon, J., Golenbock, D.T., Goguen, J., and Silverman, N. (2007). YopJ targets TRAF proteins to inhibit TLR-mediated NF-kappaB, MAPK and IRF3 signal transduction. Cell Microbiol *9*, 2700–2715.

Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., Ninomiya-Tsuji, J., and Matsumoto, K. (2000). TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol Cell *5*, 649–58.

Takagi, H., Kanai, T., Okazawa, A., Kishi, Y., Sato, T., Takaishi, H., Inoue, N., Ogata, H., Iwao, Y., Hoshino, K., et al. (2003). Contrasting action of IL-12 and IL-18 in the development of dextran sodium sulphate colitis in mice. Scand. J. Gastroenterol. *38*, 837–844.

Takaoka, A., Wang, Z., Choi, M.K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., et al. (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature *448*, 501–505.

Takeda, K., and Akira, S. (2004). TLR signaling pathways. Semin Immunol *16*, 3–9.

Tanimura, N., Saitoh, S., Matsumoto, F., Akashi-Takamura, S., and Miyake, K. (2008). Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. Biochem Biophys Res Commun *368*, 94–99.

Tatematsu, M., Ishii, A., Oshiumi, H., Horiuchi, M., Inagaki, F., Seya, T., and Matsumoto, M. (2010). A molecular mechanism for Toll-IL-1 receptor domain-containing adaptor molecule-1-mediated IRF-3 activation. J Biol Chem.

Terra, J.K., Cote, C.K., France, B., Jenkins, A.L., Bozue, J.A., Welkos, S.L., LeVine, S.M., and Bradley, K.A. (2009). Cutting Edge: Resistance to Bacillus anthracis Infection Mediated by a Lethal Toxin Sensitive Allele of Nalp1b/Nlrp1b. The Journal of Immunology *184*, 17–20.

Therisod, H., Karibian, D., Perry, M., and Caroff, M. (2002). Structural analysis of Yersinia pseudotuberculosis ATCC 29833 lipid A. International Journal of Mass Spectrometry *219*, 549–557.

Tiemi Shio, M., Tiemi Shio, M., Eisenbarth, S.C., Savaria, M., Vinet, A.F., Bellemare, M.-J., Harder, K.W., Sutterwala, F.S., Bohle, D.S., Descoteaux, A., et al. (2009). Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases. PLoS Pathog *5*, e1000559.

Tobias, P., Soldau, K., Iovine, N., Elsbach, P., and Weiss, J. (1997). Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. J Biol Chem *272*, 18682–18685.

Torruellas J, J. (2005). The Yersinia pestis type III secretion needle plays a role in the regulation of Yop secretion. Mol Microbiol *57*, 1719.

Vallabhapurapu, S., and Karin, M. (2009). Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol *27*, 693–733.

Vladimer, G., Weng, D., Paquette, S.W.M., Vanaja, S.K., Rathinam, V.A.K., Aune, M.H., Conlon, J.E., Burbage, J.J., Proulx, M.K., Liu, Q., et al. (2012). The NLRP12 Inflammasome Recognizes Yersinia pestis. Immunity *37*, 96–107.

Vyleta, M.L., Wong, J., and Magun, B.E. (2012). Suppression of ribosomal function triggers innate immune signaling through activation of the NLRP3 inflammasome. PLoS ONE *7*, e36044.

Wang, L., Manji, G.A., Grenier, J.M., Al-Garawi, A., Merriam, S., Lora, J.M., Geddes, B.J., Briskin, M., DiStefano, P.S., and Bertin, J. (2002). PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. J Biol Chem *277*, 29874–29880.

Wang, S., Miura, M., Jung, Y.K., Zhu, H., Li, E., and Yuan, J. (1998). Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell *92*, 501–509.

Warren, S.E., Mao, D.P., Rodriguez, A.E., Miao, E.A., and Aderem, A. (2008). Multiple Nod-like receptors activate caspase 1 during Listeria monocytogenes infection. J Immunol *180*, 7558–7564.

Watanabe, H., Gaide, O., Pétrilli, V., Martinon, F., Contassot, E., Roques, S., Kummer, J.A., Tschopp, J., and French, L.E. (2007). Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. J Invest Dermatol *127*, 1956–1963.

Watari, M., Watari, H., Nachamkin, I., and Strauss, J. (2000). Lipopolysaccharide induces expression of genes encoding pro-inflammatory cytokines and the elastin-degrading enzyme, cathepsin S, in human cervical smooth-muscle cells. J Soc Gynecol Investig *7*, 190–198.

Wesche, H., Henzel, W., Shillinglaw, W., Li, S., and Cao, Z. (1997). MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity *7*, 837–847.

Williams, K.L., Lich, J.D., Duncan, J.A., Reed, W., Rallabhandi, P., Moore, C., Kurtz, S., Coffield, V.M., Accavitti-Loper, M.A., Su, L., et al. (2005). The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor alpha-, and Mycobacterium tuberculosis-induced pro-inflammatory signals. J Biol Chem *280*, 39914–39924.

Williams, K.L., Taxman, D.J., Linhoff, M.W., Reed, W., and Ting, J.P.-Y. (2003). Cutting edge: Monarch-1: a pyrin/nucleotide-binding domain/leucine-rich repeat protein that controls classical and nonclassical MHC class I genes. J Immunol *170*, 5354–5358.

Williamson, E.D., Eley, S.M., Griffin, K.F., Green, M., Russell, P., Leary, S.E., Oyston, P.C., Easterbrook, T., Reddin, K.M., and Robinson, A. (1995). A new improved sub-unit vaccine for plague: the basis of protection. FEMS Immunol Med Microbiol *12*, 223–230.

Worsham, P.L., Stein, M.P., and Welkos, S.L. (1995). Construction of defined F1 negative mutants of virulent Yersinia pestis. Contrib. Microbiol. Immunol. *13*, 325–328.

Wright, S., Ramos, R., Tobias, P., Ulevitch, R., and Mathison, J. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science *249*, 1431–1433.

Xia, Z.-P., Sun, L., Chen, X., Pineda, G., Jiang, X., Adhikari, A., Zeng, W., and Chen, Z.J. (2009). Direct activation of protein kinases by unanchored polyubiquitin chains. Nature *461*, 114–119.

Yamamoto M, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science (New York, N.Y.) *301*, 640.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., et al. (2003a). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640–643.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003b). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol *4*, 1144–1150.

Yarovinsky, F., Kanzler, H., Hieny, S., Coffman, R.L., and Sher, A. (2006). Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4+ T cell response. Immunity *25*, 655–664.

Ye, Z., and Ting, J.P.-Y. (2008). NLR, the nucleotide-binding domain leucine-rich repeat containing gene family. Curr Opin Immunol *20*, 3–9.

Ye, Z., Kerschen, E.J., Cohen, D.A., Kaplan, A.M., van Rooijen, N., and Straley, S.C. (2009). Gr1+ cells control growth of YopM-negative yersinia pestis during systemic plague. Infect Immun *77*, 3791–3806.

Ye, Z., Lich, J.D., Moore, C.B., Duncan, J.A., Williams, K.L., and Ting, J.P.-Y. (2008). ATP binding by monarch-1/NLRP12 is critical for its inhibitory function. Mol Cell Biol *28*, 1841–1850.

Zaki, M.H., Boyd, K.L., Vogel, P., Kastan, M.B., Lamkanfi, M., and Kanneganti, T.-D. (2010). The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. Immunity *32*, 379–391.

Zaki, M.H., Lamkanfi, M., and Kanneganti, T.-D. (2011a). The Nlrp3 inflammasome: contributions to intestinal homeostasis. Trends Immunol *32*, 171–179.

Zaki, M.H., Vogel, P., Malireddi, R.K.S., Body-Malapel, M., Anand, P.K., Bertin, J., Green, D.R., Lamkanfi, M., and Kanneganti, T.-D. (2011b). The NOD-Like Receptor NLRP12 Attenuates Colon Inflammation and Tumorigenesis. Cancer Cell *20*, 649–660.

Zamboni, D.S., Kobayashi, K.S., Kohlsdorf, T., Ogura, Y., Long, E.M., Vance, R.E., Kuida, K., Mariathasan, S., Dixit, V.M., Flavell, R.A., et al. (2006). The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of Legionella pneumophila infection. Nat Immunol *7*, 318–325.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., and Kagan, J.C. (2011). CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell *147*, 868–880.

Zhao, Y., Yang, J., Shi, J., Gong, Y.-N., Lu, Q., Xu, H., Liu, L., and Shao, F. (2011). The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature *477*, 596–600.

Zheng, Y., Lilo, S., Brodsky, I.E., Zhang, Y., Medzhitov, R., Marcu, K.B., and Bliska, J.B. (2011). A Yersinia effector with enhanced inhibitory activity on the NF-κB pathway activates the NLRP3/ASC/caspase-1 inflammasome in macrophages. PLoS Pathog *7*, e1002026.

Zheng, Y., Lilo, S., Mena, P., and Bliska, J.B. (2012). YopJ-induced caspase-1 activation in Yersinia-infected macrophages: independent of apoptosis, linked to necrosis, dispensable for innate host defense. PLoS ONE *7*, e36019.

Zhou Z, L. (1999). Lipid A modifications characteristic of Salmonella typhimurium are induced by NH4VO3 in Escherichia coli K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. J Biol Chem *274*, 18503.

Zhou, D., and Yang, R. (2009). Molecular Darwinian evolution of virulence in Yersinia pestis. Infect Immun *77*, 2242–2250.

Zhou, H., Monack, D., Kayagaki, N., Wertz, I., Yin, J., Wolf, B., and Dixit, V. (2005). Yersinia virulence factor YopJ acts as a deubiquitinase to inhibit NF-kappa B activation. J Exp Med *202*, 1327–1332.

Zhou, R., Yazdi, A.S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature *469*, 221–225.

Zumbihl, R., Aepfelbacher, M., Andor, A., Jacobi, C.A., Ruckdeschel, K., Rouot, B., and Heesemann, J. (1999). The cytotoxin YopT of Yersinia enterocolitica induces modification and cellular redistribution of the small GTP-binding protein RhoA. J Biol Chem *274*, 29289–29293.