2016-05-11

Activation and Inhibition of Multiple Inflammasome Pathways by the Yersinia Pestis Type Three Secretion System: A Dissertation

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ACTIVATION AND INHIBITION OF MULTIPLE INFLAMMASOME PATHWAYS BY THE *YERSINIA PESTIS* TYPE THREE SECRETION SYSTEM

A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester,

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 11, 2016

PROGRAM IN IMMUNOLOGY AND VIROLOGY

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This work was undertaken at the Graduate School of Biomedical Sciences
Program in Immunology and Virology

Under the mentorship of

Dr. Egil Lien, Thesis Advisor

The signatures of the Dissertation Defense Committee signify
completion and approval as to style and content of the Dissertation

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Dr. Lee Wetzler, External Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets
the requirements of the Dissertation Committee

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Dr. Kate Fitzgerald, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies
that the student has met all graduation requirements of the School.

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Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

May 11 2016

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Acknowledgements

I wish to express my deepest thanks and appreciation to my advisor, Dr. Egil Lien, for his phenomenal support and guidance through this journey. He was always ready to discuss and consider new ideas, which at times resulted in very creative experiments and approaches in this study. Importantly, having independence to try new things and to work according to my own rhythm has sustained my motivation to keep pursuing this project in earnest. Flexibility was especially crucial in the months after I became a father, and I cannot thank Egil enough for unwavering support and understanding when I would work at odd hours or take off unexpectedly to help out at home. Through his respectful, kind, and flexible approach to mentoring, Egil inspired trust, loyalty and gratitude from this student.

I also want to express my appreciation for the ways in which Egil's style differs from my own. Egil is meticulously attentive to quality control in experiments, which balances my tendency to favor speed, multitasking, and large experimental setups at the possible expense of quality. In some mentoring relationships such differences can turn into a source of friction, but our stylistic differences were complementary and resulted in high quality, comprehensive experiments at a fast rate. I also admire Egil's tempered approach to data – he remains remarkably composed when presented with exciting data, but also when assays do not work or when results may otherwise be disappointing. My relationship with Egil has been the most positive, productive, and transformative factor in my research training, and I thank him with all my heart.

I would like to thank my thesis committee, Dr. Kate Fitzgerald, Dr. Jon Goguen, Dr. John Harris, and Dr. Neal Silverman for their guidance and input during the course of this research. Dr. Jon Goguen has been a great ally and teacher through the toughest parts of this research, and I want to especially thank him for the insightful discussions and advice on topics ranging from how to apply inductive reasoning in this project, to how to best organize my data for presentation. I also thank Dr. Lee Wetzler for taking the time to read and evaluate this dissertation as my external committee member.

I thank all the members of the Lien lab who have supported me through these years and contributed to the completion of this work either by training me, through help with experiments, or through invaluable moral support. I especially want to thank Pontus Ørning for his friendship and support; it's probably not a coincidence that his arrival in the lab coincided with the turning point when my research progress accelerated dramatically. I also thank Dr. Kristian Starheim and Dr. Kimberly Pouliot for their advice, discussion, and moral support, and to Dr. Robyn Marty-Roix for the time she took to train and assist me in BSL3 work. I thank Gail Germain for the indispensable and difficult work of maintaining our animal resources and managing competing pleas for animal availability and budget-consciousness.

This work would not have been possible without the bacterial strains generated by Megan Proulx in the Goguen lab. Heartfelt thanks to her, to Samantha Palace, and once more to Dr. Jon Goguen for providing me with this support. Furthermore, I greatly thank Dr. Chris Sassetti for his help with help with a particularly technically challenging mouse experiment; Dr. Donghai Wang for discussions and insight about Pyrin; Dr. Sanjay Ram for

helping to draw blood from human donors; Drs. Mikhail Gavrilin, Dr. Mark Wewers, Dr. Joan Mecsas, Dr. Emad Alnemri, Dr. Peter F Johnson, and Dr. Ray Welsch for their kind reagent gifts.

Thanks also to all the wonderful, kind, and generous people in the Department of Medicine who readily share their reagents, equipment, insight and advice. Special thanks to Roland Elling, Sandhya Ganesan, Shruti Sharma, Zhaozhao Jiang, Srinjoy Chakraborti, and Dr. Vijay Rathinam for their help and advice. My gratitude and appreciation also goes to Kim West for maintaining the BSL3 lab and helping to train me in using that facility.

I also want to express how important the support of MD/PhD students is for one another, and particularly thank my classmates Abhishek Satischandran, Evelyn Santos, Victor Liu, Barry Kriegsman, Jacob Schrum, Gregory Orlowski, and Brian Quattrochi for support, discussion, and camaraderie.

Finally, appreciation that would be shameful to express merely as a 'thanks' goes to my family, who have vicariously experienced the ups and downs of my student experience and stuck it out with me. My wonderful and supportive partner, Sveta, and my mother have worried and jumped for joy with and for me during these years. They, along with my son Savi, my grandmother, and my parents in law, have been as much a part of this experience as I have, and I could not have done this without their presence in my life. Our whole family looks forward to the days where the awful word "Yop" regains its rightful place as a curseword in our Russian-speaking home.

Abstract

Host survival during plague, caused by the Gram-negative bacterium *Yersinia pestis*, is favored by a robust early innate immune response initiated by IL-1β and IL-18. Precursors of these cytokines are expressed downstream of TLR signaling and are then enzymatically processed into mature bioactive forms, typically by caspase-1 which is activated through a process dependent on multi-molecular structures called inflammasomes. *Y. pestis* evades immune detection in part by using a Type three secretion system (T3SS) to inject effector proteins (Yops) into host cells and suppress IL-1β and IL-18 production. We investigated the cooperation between two effectors, YopM and YopJ, in regulating inflammasome activation, and found that *Y. pestis* lacking both YopM and YopJ triggers robust caspase-1 activation and IL-1B/IL-18 production *in vitro*. Furthermore, this strain is attenuated in a manner dependent upon caspase-1, IL-1β and IL-18 *in vivo*, yet neither effector appears essential for full virulence. We then demonstrate that YopM fails to inhibit NLRP3/NLRC4 mediated caspase-1 activation and is not a general caspase-1 inhibitor. Instead, YopM specifically prevents the activation of a Pyrin-dependent inflammasome by the Rho-GTPase inhibiting effector YopE. Mutations rendering Pyrin hyperactive are implicated in the autoinflammatory disease Familial Mediterranean Fever (FMF) in humans, and we discuss the potential significance of this disease in relation to plague. Altogether, the *Y. pestis* T3SS activates and inhibits several inflammasome pathways, and the fact that so many T3SS components are involved in manipulating IL-1β/IL-18 underscores the importance of these mechanisms in plague.

List of Publications

Ratner D, Orning MP, Starheim KK, Marty-Roix R, Proulx MK, Goguen JD, Lien E. **Manipulation of IL-1β and IL-18 production by *Yersinia pestis* effectors YopJ and YopM and redundant impact on virulence**. J Biol Chem. 2016 Feb 16. pii: jbc.M115.697698. [Epub ahead of print]

Mueller C, **Ratner D**, Zhong L, Esteves-Sena M, Gao G. **Production and Discovery of Novel Recombinant Adeno-Associated Viral Vectors**. Curr Protoc Microbiol. 2012 Aug;Chapter 14:Unit14D.1

Ratner D, Mueller C. **Immune Responses in Cystic Fibrosis; Are they Intrinsically Defective?** Am J Respir Cell Mol Biol. 2012 Jun;46(6):215-22.

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CHAPTER I

Introduction

Innate Immunity

Cells of innate immunity comprise the first line of defense against microbes that breach integumentary barriers. Macrophages, dendritic cells, and neutrophils respond to direct or indirect pathogen contact, tissue damage, and other signals by e ngaging in specialized cell-specific roles to ensure that infection does not progress. These responses include phagocytosis and 'sampling,' production of cytokines to communicate with other cells, recruitment of other immune cells, inflammation, killing of invading microbes, by tissue repair. Lastly, cross-presentation of antigens serves to engage adaptive immunity to develop molecular memory preventing future infection.

Innate immune cells sense conserved and recognizable pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) through pattern recognition receptors (PRRs)[1, 2]. Activation of these receptors turns on specific intracellular pathways to relay the signal to the DNA level and activate transcription of cytokines, chemokines, interferons, and other genes important for priming an immune response. PRR families include NOD-like receptors (NLRs), Toll-like receptors (TLRs), AIM2-like receptors, and others.

TLRs represent an important class of PRRs, capable of recognizing a r ange of bacterial and viral molecules to arrest infection in the early stages. These receptors are transmembrane proteins found in cell membranes and endosomes. Ligands are bound by horseshoe-shaped leucine-rich regions (LRR), followed by a conformational change in the Toll-interleukin receptor (TIR) domain on the cytoplasmic side of the membrane and the recruitment of adaptor molecules to trigger downstream signaling pathways[3]. TLRs 2, 4, 5, and 6 a re located at the cell surface and recognize PAMPs including Pam2Cys4 from

Gram-positive bacteria (TLRs 2 and 6), LPS from Gram-negative bacteria (TLR4), and bacterial flagellin (TLR5) among others[1]. TLRs 3, 7, 8, 9, 11 , and 13 a re found on endosomal membranes and primarily recognize nucleic acids.

TLR4, together with MD-2 and CD14, recognize LPS - the major constituent of the cell wall of Gram-negative bacteria. Specifically, TLR4/MD-2 homodimerize around the hexa-acyl chains of the lipid A component of the LPS molecule, five out of six acyl chains buried within a hydrophobic pocket in MD-2, to trigger downstream adaptors MyD88, Mal, TRIF, and TRAM[4, 5]. The integrity of signaling downstream of TLR4 is protected by molecular oversight mechanisms to ensure proper signal propagation. Checkpoint mechanisms include RIP kinases, TAK1, cIAPs, caspase-8, and others [6-12]. Pharmacologic or pathogen-mediated inhibition of some of these pathways may lead to cell death, often accompanied by the release of additional cytokines to signal danger to other immune cells.

TLR4 signaling feeds into a sophisticated cross-signaling network which leads to activation of different transcription pathways depending on inputs through other receptors. Classically, TLR4 signaling is described to activate the MyD88-dependent and independent signaling cascades, followed by activation of MAP Kinases and/or NEMO, and finally initiation of gene expression by t he NFkB, AP-1, and C/EBPbeta transcription factors; however, it is increasingly clear that linear representations of this process are gross oversimplifications[13-16]. Systems biology approaches show that complex cross-talk following the activation of multiple PRRs is precisely what confers specificity and effectiveness to immune responses.

Activation of NFkB downstream of TLR4 results in production of chemotactic and pro-inflammatory cytokines including TNFa, IL-6, IL-8, IL-1β, and IL-18. However, IL-1β and IL-18 are translated as pro-forms which cannot be secreted until they are enzymatically cleaved into their mature forms. This is classically accomplished by molecular complexes called inflammasomes. These complexes form upon activation of additional PRR sensors, thus ensuring the specificity of IL-1β/IL-18-dependent immune responses.

Inflammasomes and their Role in Disease

Inflammasomes are increasingly recognized as critical orchestrators of immunity[17, 18]. These large protein complexes are at the center of a variety of pathways in innate immune cells, including cytokine production[19], cytoskeletal remodeling[20], and cell death[21]. The primary function of an inflammasome may be to produce biologically active IL-1β and IL-18 cytokines in response to an activating event. Typically, inflammasome formation is initiated when a sensor, such as Nucleotide-binding domain Leucine-rich Repeat containing protein (NLR) proteins NLRP3 or NLRC4, recognize pathogen or danger-associated molecular patterns (PAMP or DAMP). This allows the adaptor protein Asc to nucleate and oligomerize at the site of the NLR, and recruit pro-caspase-1 to its CARD domain[22, 23]. Dimers of pro-caspase-1 are then cleaved to active caspase-1 through autoproteolysis, which then catalyzes the final processing of pro-IL-1β and pro-IL-18 into their mature secreted forms. Activation of caspase-1 is also accompanied by an inflammatory form of apoptosis, termed pyroptosis. Non-canonical caspase-11

inflammasomes, as well as pathways dependent on caspase-8 or neutrophil proteases may also lead to IL-1β and IL-18 processing [24-27].

Inflammasome-dependent secretion of IL-1β and IL-18 is critical for immune control of many microbes[17, 28-32], and some have suggested they may play an important role in signaling induced by vaccine adjuvants[33-35]. However, dysregulation or inappropriate activation of inflammasomes can also produce severe autoinflammation[36-39] and contribute to autoimmune disorders[40-42], Alzheimer's disease[43], Parkinson's Disease[44], and many other pathologic processes.

To some extent, the roles of IL-1β and IL-18 overlap. Both are considered pro-inflammatory cytokines, both are processed by inflammasome-dependent caspase-1 activation, both are controlled by NF-kB-mediated transcription, and both have the capacity to induce NF-kB-mediated transcription[45-47]. Nevertheless, there are also important differences. Unlike IL-1β, IL-18 does not induce fever or inflammatory prostaglandin production[48]; moreover, in human epithelial cells IL-18 appears to activate the MAP kinase pathway but not NF-kB-mediated gene transcription, suggesting that its specific effect varies by cell type. Another important role of IL-18 is to induce NK cells and T-cells to produce IFN-g, which activates macrophages[49].

Prominent effects of IL-1β include recruitment of neutrophils to sites of infection, promoting endothelial cell adhesion, and stimulating adaptive Th17 as well as allergic Th2 responses[50, 51]. Activation of the IL-1β receptor leads to NF-kB-mediated expression of TNFa, RANTES and chemokines IL8 and KC which lead to rapid recruitment of neutrophils[52-61]. Thus, IL-1β in particular has the capacity to cause host tissue damage,

although this robust induction of inflammation is necessary in the context of certain infections, such as Staphylococcus aureus[60, 61]. By contrast, IL-18 tends to have a less toxic effect while still playing an important role in controling infection. The difference in the physiology of IL-1β and IL-18 may be particularly critical in the lung, where IL-18 tends to play a more protective role while IL-1β is generally associated with increased tissue damage[54, 62-65]. Yet under different circumstances, both cytokines can be be important for the clearance of intracellular pathogens, and for efficient activation of adaptive immune responses. Consequently, inflammasome-activated caspase-1 and subsequent levels of IL-1β and IL-18 secretion are key events in many infectious and non-infectious diseases.

Heterogeneity of Inflammasome structure, activation, and regulation mechanisms

Consistent with the delicate balance needed between a sufficiently robust immune response and minimal tissue damage, sophisticated mechanisms exist to tightly regulate the specificity and sensitivity of inflammasome pathways. While the general model of Ligand-NLR-Asc-Caspase-1 from early inflammasome studies is useful for a basic conceptualization of this system, the extent of its heterogeneity is being increasingly recognized and appreciated.

NLR sensor molecules such as NLRP1, NLRP3, NLRP6, and NLRP12 contain LRRs which are believed to be involved in activation, an ATPase NACHT domain (except NLRP1), and a pyrin domain through which they interact with Asc. However NLRC4 (sometimes referred to as IPAF) contains a CARD domain, which recruits Asc but can also directly recruit caspase-1. Although NLRC4 activation is more robust in the presence of

Asc, it is not required. The sensors NLRP1b[66] and NOD1[67] can also activate caspase-1 independently of Asc.

Non-NLR sensors such as Pyrin, IFI16, and AIM2 also exist; these sensors contain Asc-interacting pyrin domains, but lack the LRR domains present on many other sensors. Instead, AIM2 and IFI16 contain DNA-sensing HIN domains[68], and have been reported to respond to viral as well as bacterial DNA in the cytosol[69-71]. In the case of the Pyrin inflammasome, a directly activating pathogen ligand has not been established but it has been proposed that this sensor responds to pathologic Rho-GTPase activity induced by multiple Gram-negative pathogens[36].

The case of Pyrin also demonstrates that inflammasome activation is not necessarily the result of a direct interaction of a sensor with a PAMP or DAMP ligand. Pyrin was initially proposed to be an inhibitor of caspase-1 activity, based on evidence that Pyrin knockdown leads to increased NLRP3 activity *in vitro*[72]. Yet more recent studies point to a different mechanism, whereby the Pyrin inflammasome is spontaneously activated by mutations in the SPRY domain[73]. In humans, such mutations result in Familial Mediterranean Fever (FMF)[74]. Activity of the Pyrin inflammasome has been reported to be influenced by PSTPIP1[75, 76], Siva[77], certain 14-3-3 isoforms[78, 79], the leading edge of polymerizing actin[80, 81], and a diverse variety of microbial molecules[82]. Many aspects of the activation mechanism remain unknown, but the emerging picture is one where Pyrin is triggered by changes in intracellular homeostasis, which are sensed by the endogenous binding partners of Pyrin rather than by direct binding of a pathogenic ligand.

Other inflammasomes require cofactors for activation as well. The NLRC4 inflammasome is a well-established sensor of flagellin, yet it does not bind flagellin directly; instead, the presence of flagellin is relayed to NLRC4 by NAIP proteins[83, 84]. NLRP3, often regarded as the quintessential classical inflammasome, also has a complex mechanism of activation which senses DAMPs and PAMPs indirectly. For example, NLRP3 sensing of double stranded DNA (dsDNA) occurs downstream of TRIM33-dependent ubiquitination of DXH33, which allows it to interact with NLRP3 and activate it[85, 86]. In general, NLRP3 can be activated by a variety of triggers including excessive influxes of calcium and/or efflux of potassium, oxidative damage, elevated ATP levels, and bacterial pore-forming toxins, crystallized molecules such as silica or uric acid, oxidized mitochondrial DNA, and many others[87]. Activation by mitochondrial DNA appears attractive as a unifying mechanism, since the other activating events may trigger the upstream damage which causes the release and oxidation of mitochondrial DNA. Whether NLRP3 interacts with mitochondrial DNA directly or via intermediate sensors is still incompletely understood. The mitochondrial DNA hypothesis may also explain observations that autophagy is associated with reduced inflammasome activation[88], as turnover of damaged mitochondria as well as ubiquitinated inflammasome components increases[89-91]. If so, a recent study by Orlowski et al showing that multiple endogenous cathepsins potentiate NLRP3 activity may reveals another important mechanism of inflammasome regulation[92], considering that cathepsin activity is known to inhibit autophagy[93-95].

Another important NLRP3-activating mechanism occurs through upstream recognition of intracellular LPS by caspase-11. In this pathway, termed the non-canonical

inflammasome, LPS-activated caspase-11 cleaves gasdermin, which then activates both pyroptosis and NLRP3-dependent caspase-1 activation[96, 97]. Caspase-11 activity depends on interferon pathways, as TLR4, TRIF, and IFNAR1 deficient cells show heavily impaired caspase-11 processing[98]. Recently it was shown that type-I interferons activate guanylate binding proteins (GBPs) which are involved in trafficking proteins to the plasma membrane or membranes of intracellular organelles, and are required for activation of the inflammasomes pathways as well as other antimicrobial actions in response to vacuolar Gram negative bacteria[99-103]. However, while the non-canonical caspase-11 inflammasome plays a critical role in host defense against intracellular Gram negatives[98, 104-106], mice lacking gasdermin or caspase-11 are also protected from LPS-mediated septic shock[96, 107].

It is also important to note that some NLR sensors, such as NLRP6 and NLRP12, also have major anti-inflammatory functions[108, 109]. NLRP6 was shown to negatively regulate NF-kB driven innate immune responses and actually impede clearance of bacterial pathogens[110]. Interestingly, in gut epithelial cells and neurons NLRP6 has a protective effect independent of inflammasome activity[111, 112]. NLRP6 was recently shown to recognize dsRNA together with Dhx15 (another cofactor?), and play an important role in defense against norovirus in the gut independently of caspase-1[113]. The unusual functions of NLRP6 raise the question whether it forms an inflammasome at all; currently, the evidence for this is lacking.

Like NLRP6, NLRP12 negatively regulates NF-kB[114] and limits autoinflammatory immune responses both in the gut and in neurons[115, 116]. Yet in

certain disease contexts NLRP12 may also forms an Asc-dependent inflammasome and promote caspase-1 activation. NLRP12 contributes to caspase-1 activity and IL-1β production in response to malaria parasites [117], *Y. pestis*[118], and *K. pneumonia*[119]. To date, however, no specific trigger of an NLRP12 inflammasome has been identified.

A number of pathways are also capable of processing IL-1β and IL-18 independently of inflammasomes. Indeed, the IL-1β response to certain stimuli can be nearly unchanged in mice lacking caspase-1 or Asc[120-122]. Several neutrophil proteases including serine proteinase-3, cathepsin G, and neutrophil elastase are known to directly process IL-1β and IL-18[123]. In addition, caspase-8 can activate caspase-1 through an incompletely understood mechanism which may or may not involve inflammasomes[11, 124]; however, caspase-8 has also been reported to process IL-1β independently of caspase-1[27, 125, 126].

Finally, it should be noted that inflammasome expression varies by cell type, stage of maturation, and type of activation. Expression of NLRP3, for example, is generally too low under resting conditions and needs to be induced by priming before it may be activated. By contrast, expression of NLRC4 relative to NLRP3 may inverse within hours of stimulation in some cells[127], which may have important implications for studies involving long periods of priming or infection. Priming is also sometimes necessary to restore expression of Pyrin[75], which Gavrilin and colleagues showed to be lost in macrophages upon differentiation; however, monocytes and PBMCs differentiated in the presence of additional growth factors restores Pyrin expression[128]. Similarly, NLRP12 is not expressed in fully differentiated macrophages but is present in neutrophils[129]. For these

reasons, inflammasome studies in any cell line should be carefully scrutinized for appropriate expression of relevant components. However, some inflammasome components may not even be expressed in certain mouse strains, a good example being NLRP1b[130]. Expression of cofactors involved in regulation of various inflammasomes may also differ significantly. Therefore, caution is warranted when drawing conclusions about negative results in inflammasome studies, both *in vitro* and *in vivo*.

In summary, the remarkable variety in these pathways raises questions about what defines an inflammasome, and should caution against generalizations about their mechanisms.

Secretion Systems of Bacterial Pathogens

The ability to export molecules to manipulate the host environment is an essential ability of many bacterial pathogens. Some of the most virulent bacteria have evolved type 3, 4 and 6 secretion systems capable of penetrating host cells and injecting effector proteins to alter normal cellular processes in ways that benefit the pathogen. Examples of such bacteria - *Salmonella, Shigella, Francisella, Legionella, Burkholderia, Pseudomonas, Yersinia*, and others - cause worldwide infections in the hundreds of millions, and deaths in the millions annually. In addition to the health & economic burden owed to these pathogens, some are candidates for bioterror and biowarfare.

There has been significant progress in understanding the structural and mechanistic aspects of bacterial secretion systems[131]. Yet the complex roles they play in the host-pathogen interaction, particularly as they pertain to immune responses, are

only beginning to be recognized and appreciated. Considering the breadth of pathways involved in inflammasome regulation described earlier, it should be reasonably expected that molecules delivered by bacterial secretion systems with the design to manipulate host cell homeostasis would in one way or another influence inflammasome pathways. The functions of many of these molecules remain unknown or incompletely characterized, keeping this field rich with questions and opportunity for inquiry.

Of the six secretion systems known in bacteria, the type 3 secretion system, (T3SS), type IV secretion system (T4SS), and type VI secretion system (T6SS) are associated with the most virulent human pathogens. Examples of pathogens with a T4SS are *Legionella* and *Burkholderia* pathogens, as well as *Helicobacter pylori* (not discussed in this review). The T6SS was discovered relatively recently, and is present in Vibrio, *Pseudomonas*, *Burkholderia*, and *Francisella* species.

Of these secretion systems, the T3SS is the best studied and common to some of the most important and deadly bacterial pathogens (*Yersinia*, *Salmonella*, *Shigella*, *Burkholderia*, *Pseudomonas*, and others). The delivery apparatus of the T3SS has remained well conserved across species, and consists of the basal body, the needle, and a pore-forming complex at the tip. The needle/translocon, also called the needle/translocon, spans both the inner and out bacterial membranes and protrudes from the cell. The basal body is partially in the bacterial cytoplasm, anchored to the membrane. Its function is to recruit specific effector proteins and secrete them through the needle, assisted by an ATPase complex. Effectors pass from the base to the needle via an inner rod, and rod components can become secreted through the needle itself. The needle is a multimeric

structure composed of a single protein, approximately 60-80nm in length, with a 3nm diameter hole. The tip of the needle is composed of a pore-forming complex and translocon, which inserts into the host cell membrane and allows passage of effector proteins through the needle into the host cytoplasm[131].

The T3SS structure is critically important for pathogen virulence; however, some key components cannot be easily altered without significantly compromising the ability to deliver effectors[132-140]. Perhaps for this reason the secretion systems of several pathogenic species have become recognizable immune targets, or pathogen-associated molecular patterns (PAMPs) - molecules which are pathognomonic with bacterial infection for host immunity. Cytokine responses to the T3SS tend to be quite robust and involve the activation of toll-like receptors and inflammasomes[83, 141-143]. Likewise, T3SS needle/translocon proteins, particularly those involved in attachment and penetration of the host cell, have a disproportionate number of immune epitopes compared to other bacterial proteins (IEDB.org); several of these are established protective antigens that confer adaptive immunity against the pathogen[144]. Consequently, there is constant evolutionary pressure on T3SS pathogens to limit or manipulate the host response to its T3SS, and likewise there is pressure on the host to develop sophisticated methods of immune recognition with minimal immunotoxic harm to self.

Interactions of specific bacterial secretion systems with inflammasomes

Salmonella

Species of the Gram-negative *Salmonella* genus are the leading source of acute gastroenteritis worldwide, resulting from foodborne poisoning through consumption of contaminated poultry, pork, eggs, and milk. In total, *Salmonella* causes 1.3 billion cases of human disease each year and approximately 800,000 deaths [145-147]. *Salmonella typhi*, spread through contaminated water, causes up to 20 million additional cases and 220,000 deaths per year globally[148]. The combined 15.2 million disability-adjusted life years (DALYs) lost per year due to typhoidal and non-typhoidal *Salmonella*[149, 150] make it the second greatest bacterial contributor to global disease burden after tuberculosis.

Salmonella enterica serovar *typhimurium*, one of the most common serovars causing nontyphoidal salmonellosis, is a facultative intracellular bacterium able to survive and reproduce both inside and outside of host cells. This adaptability requires a large number of genes which are distributed throughout the *Salmonella* genome, distinguishing it from many other pathogens whose virulence genes are typically more compartmentalized[151]. Horizontal transfer of pathogenicity islands (so called because of their absence in nonpathogenic serovars) gives some *Salmonella* enterica serovars the ability to survive inside host cells and effectively evade the immune system.

The two major virulence determinants of *Salmonella* are the pathogenicity islands SPI-1 and SPI-2. These gene clusters encode two type III secretion systems (T3SS) capable of forming needle-like structures on the surface of the bacteria through which

more than thirty specialized effector proteins can be injected directly into host cells[152, 153].

SPI-1 is a 40-kb region which encodes two distinct regulatory proteins, InvF and HilA, in addition to a T3SS termed Inv/Spa and a cluster of effector proteins. This secretion system was shown to be necessary for initial bacterial contact with host cells, and effectors secreted through this system trigger host cell pathways to internalize the bacteria[154].

The second pathogenicity island, SPI-2, encodes a t wo-component regulatory system as well as another distinct T3SS (Spi/Ssa) which is a major virulence factor found in all subspecies of *Salmonella* enterica[155, 156]. The Spi/Ssa T3SS of the SPI-2 pathogenicity island differs in structure and function from the Inv/Spa T3SS of the SPI-1, and while Inv/Spa mediates uptake of the bacterium, Spi/Ssa enables the survival and replication inside the host cell[155, 156].

During enteric infection *Salmonella* invades the intestinal mucosa, followed by phagocytic uptake or entry into non-phagocytic enterocytes[146]. The bacteria restricts the expression of SPI-1 and SPI-2 until it encounters the appropriate host environment, at which point it expresses the T3SS genes required for further survival and propagation[157, 158]. Culturing *Salmonella typhimurium* at different conditions can mimic different host environments. For example, bacteria is grown to log-phase will express SPI-1[159], but at stationary phase expression of SPI-1 decreases while expression of SPI-2 will increase [160]. Thus, the bacteria seem to sense whether it is in

an extracellular or intracellular environment, and alters the expression of its virulence factors for optimal adaptation.

The host immune system is able to sense and react to these bacterial factors. *Salmonella typhimurium* expressing SPI-1 and the Inv/Spa T3SS induces rapid macrophage cell death and IL-1β production, which is dependent on NLRC4 as well as the NLR apoptosis inhibitory proteins (NAIPs)[83, 161]. As mentioned previously, NAIPs interact with NLRC4 upon sensing PAMPs and DAMPs, and trigger activation of the NLRC4 inflammasome. Mice express four NAIP paralogs (NAIP 1, 2, 5 and 6) of which NAIP5 and NAIP6 detect bacterial flagellin, NAIP2 detects the Inv/Spa T3SS inner rod protein PrgJ[83, 161], and NAIP1 and its human homolog NAIP detect the Inv/Spa T3SS needle protein PrgI[143, 162]. Interestingly, there is only one known human NAIP protein, which raises the question whether it may singularly be responsible for detecting both PrgI and flagellin. A study in human cells suggests this may indeed be the case[163], which warrants further investigation of the mechanism.

As mentioned above, *S. typhimurium* grown to stationary phase (mimicking an intracellular niche) will upregulate SPI-2 while downregulating SPI-1. Macrophages infected with these bacteria will undergo a much slower cell death (12-17 hours compared to 1-2 hours for log-phase bacteria), which is not dependent on the Inv/Spa T3SS. Instead, this cell death is triggered by the Spi/Ssa T3SS and occurs predominantly through NLRP and the noncanonical caspase-11 inflammasome, and to a lesser extent through NLRC4[164]. The Spi/Ssa T3SS is used by the bacteria to inject effector proteins into the cell cytoplasm, but it also allows translocation of flagellin protein which triggers

the NLRC4 inflammasome. Using a Δflag mutant which does not produce flagellin, Broz et al showed that *S. typhimurium* initiates two host inflammasome pathways, with flagellin and SPI-2 respectively triggering NLRC4 and caspase-11[164].

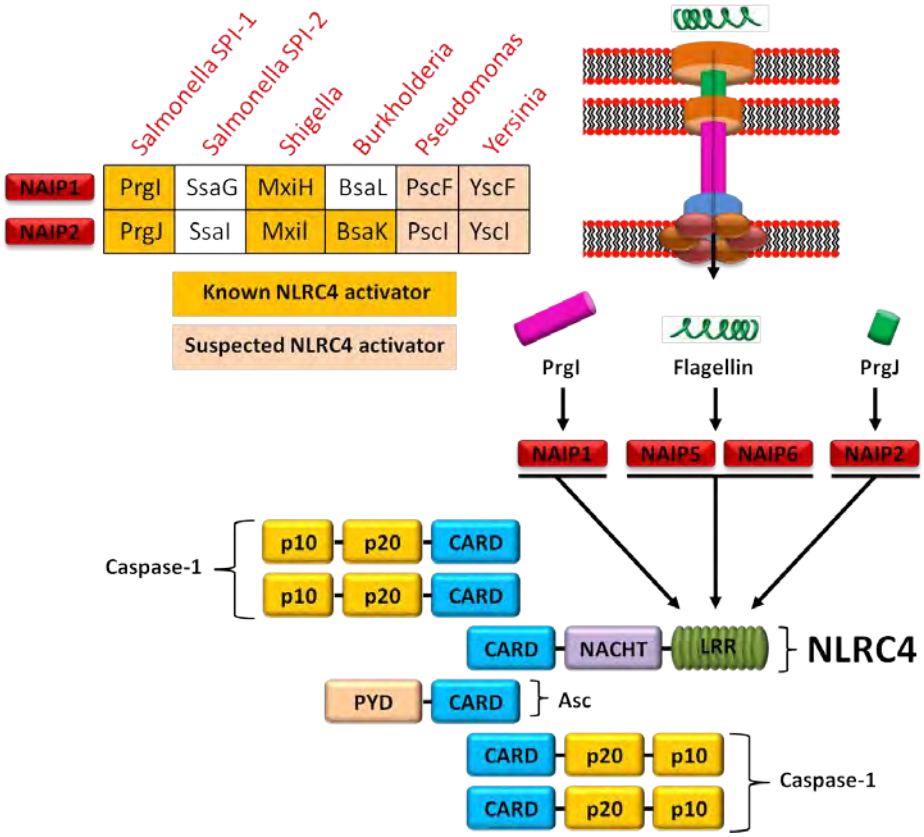

Figure 1.1. NLRC4 inflammasome structure and common recognition patterns of flagellin and T3SS molecules. Known activators include *Salmonella*, *Shigella*, *Burkholderia*, *Pseudomonas*, and *Yersinia*. In the upper left are shown homologs of PrgI and PrgJ which are known or predicted to activate NLRC4 via NAIP1 or NAIP2, respectively.

Caspase-11 is capable of detecting intracellular LPS through an TRIF/interferon assisted pathway[98] and activate what has been termed the noncanonical inflammasome, leading to release of IL-1β and IL18 and initiation of pyroptosis[24, 107, 165]. It was proposed that caspase-11 binds directly to LPS[166] leading to cleavage of gasdermin D

and initiation of cell death[96, 167]. The Spi/Ssa T3SS encoded by SPI-2 likely introduces bacterial LPS into the host cytoplasm, activating caspase-11. The fact that some caspase-11 dependent IL-1β production still occurs in the absence of SPI-2 suggests an alternative mechanism for LPS to enter the cytoplasm.

S. typhimurium has several mechanisms to avoid immune detection and maintain an intracellular growth niche. The bacteria shifts from SPI-1 to SPI-2 expression, and also down-regulates flagellin expression in order to minimize activation of NLRC4. SPI-2 drives the expression of the Spi/Ssa T3SS, which the bacteria uses to secrete effector proteins that help it persist in its vacuolar niche. One secreted protein, SifA, induces stabilization of the vacuole by microtubules, and is critically important for virulence[105, 168]. Yet the Spi/Ssa T3SS, which is required by the bacteria for virulence, also activates inflammasome pathways by the mechanisms described above and results in the eventual clearance of the pathogen.

Burkholderia

Burkholderia species are closely related to *Pseudomonas*, and include several opportunistic pathogens which can cause serious disease in humans. *B. pseudomallei* causes the highly lethal disease melioidosis, and has even been considered as a candidate for biowarfare. Many species of *Burkholderia* are considered harmless; however, CF patients are uniquely susceptible to chronic lung infection with *Burkholderia* species, including ones which normally do not cause disease in humans.

Burkholderia pathogens are able to survive inside macrophages, and infection is typically eventually resolved by adaptive immunity. Nevertheless, in the early stages of infection, the *Burkholderia* secretion systems interact with several inflammasomes with important consequences for the course of disease. Some polymorphisms of NLRC4, for example, significantly impact survival in melioidosis in humans[169].

In general, it is difficult to distinguish whether effectors or secretion systems themselves are responsible for activating an inflammasome, but it is even more challenging when multiple interacting secretion systems are present. *B. cenocepacia* has a T2SS, T3SS, T4SS, and T6SS, each of which may contribute to activation NLRP3 and possibly to a lesser extent NLRC4[170]. The T6SS and T2SS cooperate in the delivery of metalloproteinases zmpA and zmpB, which are essential for intracellular survival and also partially contribute to NLRP3 activation. Yet there seem to be other NLRP3 activators which have yet to be identified, and may include structural components of the secretion systems or other translocated proteins.

By contrast, *B. pseudomallei* does not seem to trigger NLRP3 activation in macrophages, but instead the early inflammasome response appears entirely dependent on NLRC4[171]. This inflammasome activity arrests replication of intracellular bacteria. The NLRC4 activation appears to be primarily driven by the flagellin protein FliC and the basal body protein BsaK - a homolog of the NLRC4-activating PrgJ protein in *Salmonella*. Later in infection, IL-1β secretion is driven by an NLRC4-independent pathway, and is curiously accompanied by caspase-1 independent cell death. Both NLRC4 and TLR5 are required for host survival and resolution of *B. pseudomallei* lung

infection *in vivo*, but indeed there appears to be another unidentified inflammasome activated later during infection[169]. Although some inflammasome activity is essential for the host response, production of IL-1β specifically leads to excessive neutrophil recruitment and elastase-mediated lung damage[172]. Rather than resolution of infection, this results in increased host mortality and systemic invasion by the pathogen. Instead, it is IL-18 production by inflammasome activity which appears to assist survival and bacterial clearance.

B. cenocepacia has also been shown to activate the Pyrin inflammasome in human monocytic cells by G avrilin and colleagues[173], and this was recently also demonstrated in mice[82]. The *B. cenocepacia* T6SS, but not the T3SS activates Pyrin and induces its recruitment to phagosomes. The *B. cenocepacia* T6SS is known to disrupt Rho-GTPases and cytoskeletal regulation[174-176], which are events that have been hypothesized to activate Pyrin[36, 80-82, 177]. Loss of Pyrin is associated with increased intracellular bacterial survival, but also reduced inflammation in the lungs of infected mice[82]. This may be an important insight for CF lung infection with *Burkholderia* species, as evidence suggests higher mortality and ineffective clearance of the related *P. aeruginosa* pathogen associated with increased inflammasome responses.

Pseudomonas

Certain *Pseudomonas* species, particularly *Pseudomonas aeruginosa*, are important opportunistic and nosocomial pathogens. They are particularly dangerous for immunocompromised and severely ill patients, as well as individuals with cystic fibrosis

(CF). *P. aeruginosa* is able to establish chronic lung infection in CF patients due to the uniquely permissive environment of the CF lung; the ensuing inflammation results in progressive lung damage and is currently the leading cause of death among CF patients. Given the high hazard that *P. aeruginosa* poses to the sizable population of individuals with CF worldwide, inflammasome responses to this pathogen in the context of the CF lung deserve special attention.

The relationship between *Pseudomonas* infection and inflammasomes is complex and controversial. Some studies indicate reduced bacterial clearance when inflammasome activation is defective[178]. Yet a majority of studies suggest that inflammasome activation is counterproductive to bacterial clearance, particularly in the lungs, and may even exacerbate tissue damage and mortality[127, 179-183]. In the study by Faure et al, it appears that inflammasome-driven IL-18 dampens IL-17 activity, which is critical for clearing *Pseudomonas* lung infection. It is possible that these discrepancies highlight a difference between corneal infection, where an IL-1β/IL-18 response is beneficial to the host, and lung infection, where the same type of response is inappropriate. A curious observation is that *P. aeruginosa* appears to exploit the regulation of host autophagy by IL-1β and caspase-1, with the net result that inflammasome activation promotes the survival of the pathogen[127, 184, 185].

Pseudomonas is primarily known to activate NLRC4 and NLRP3, although a caspase-1 independent pathway will also be discussed. The AIM2 inflammasome does not appear to be activated[186]. The *Pseudomonas* RhsT protein has been suggested to be an inflammasome activator, however the authors do not elaborate on pos sible

mechanisms[182]. RhsT contains conserved sequence homology with the C. difficile toxin B (TcdB), a known activator of the Pyrin inflammasome. Whether this protein indeed activates Pyrin or another inflammasome warrants investigation, as the RhsT family proteins are widespread and conserved among many pathogens, including several that have been shown to activate Pyrin[82]. *P. aeruginosa* also contains two potent GAP enzymes, ExoS and ExoT, which inhibit RhoA[187-189]. Based on the hypothesis of Rho GTPase inhibition but forth by Xu and colleagues[82], these toxins could potentially be Pyrin activators.

The NLRC4 inflammasome is activated by multiple *Pseudomonas* molecules, likely including the T3SS needle/translocon itself, both *in vivo* and *in vitro*[179, 190]. Potentially recognized needle/translocon components include PscI (a homologue of the NLRC4-activating *Salmonella* basal body protein PrgJ), and PscF (a homologue of *Yersinia* needle protein YscF). NLRC4 is also known to be activated by flagellin proteins of many bacterial species, and *Pseudomonas* flagellin follows this pattern as well[183]. It is worth noting that NLRC4 activation correlates with bacterial motility, and some investigators have suggested that it may be flagellar motility, rather than the flagellin protein per se, which leads to phagocytosis and inflammasome activation[191, 192]. Yet this view is challenged by experiments showing that surfactant protein A directly binds recombinant flagellin as well as live *Pseudomonas in vitro* and *in vivo*, enhancing the phagocytosis and capacity of both to activate NLRC4[193]. Findings by Anantharajah and colleagues also suggest that IL-1β release and pyroptosis are not correlated to flagellar motility[194]. Thus, it is possible that decreased inflammasome activation by

non-motile *Pseudomonas* is due to reduced contact with host cells; this is especially worth considering given that clinical isolates of *Pseudomonas* from chronically infected lungs are typically mucoid strains, which are resistant to contact with immune cells and phagocytosis.

The *P. aeruginosa* T3SS has been shown to activate both NLRP3 and, surprisingly, NLRC4 by inducing mitochondrial damage and DNA release[127, 184]. In the latter study, Jabir et al demonstrated mitochondrial DNA binding to NLRC4 downstream of *Pseudomonas* infection, uncovering aspects of the NLRC4 mechanism which may have been previously overlooked[185, 195]. *Pseudomonas*-triggered inflammasome activation induces autophagy, which seems to be associated with defective killing of the bacteria. Moreover, in an acidic microenvironment, as is typically the case in bacterial infection foci, *Pseudomonas* T3SS triggers enhances inflammasome activation immune cells[196]. This may be significant because acidic conditions are known to favor autophagy[197-199], further assisting bacterial survival. Cumulatively this adds to a growing body of evidence that in most cases, inflammasome activation infection with *P. aeruginosa* is ineffective and histotoxic - particularly in the lung where excessive inflammatory damage is associated with worse clinical outcome.

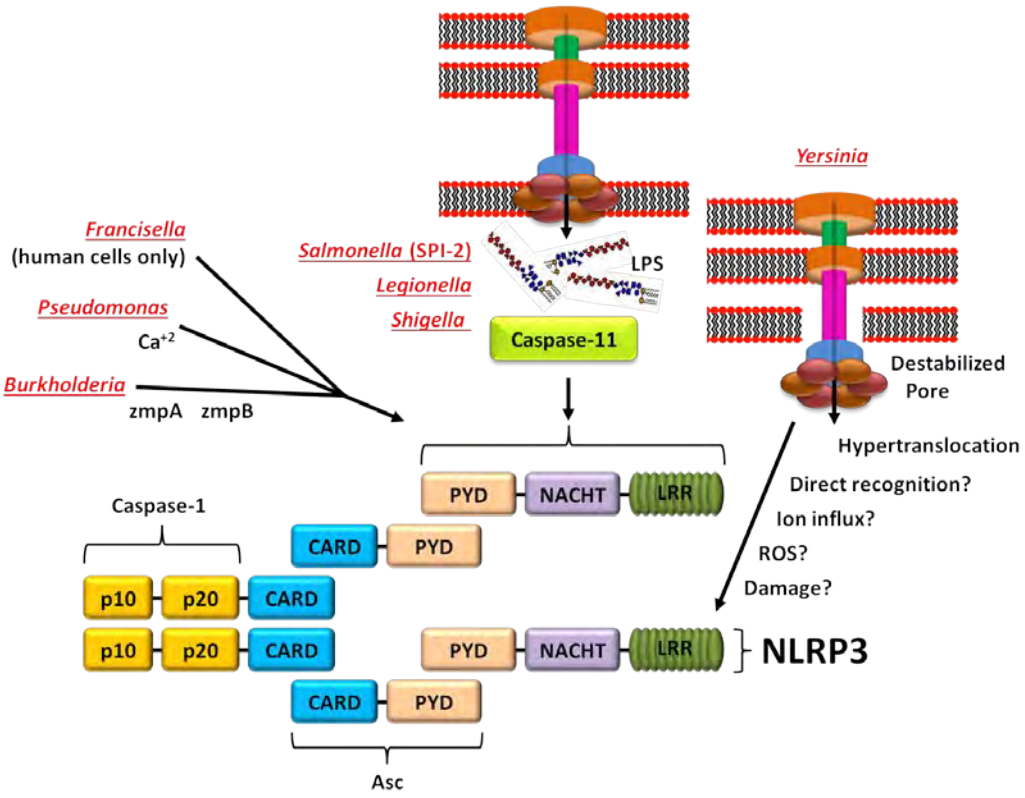

Figure 1.2. NLRP3 inflammasome structure and function, including the non-canonical caspase-11 pathway. Known activators are in red, and triggering molecules and processes are detailed.

These findings may help explain why CF patients are more vulnerable to *P. aeruginosa* lung infection. First, the pH of the CF lung is more acidic than in healthy individuals[200, 201], which was shown to negatively impact bacterial killing by Pezzulo and colleagues[202]. According to the studies cited earlier, this lower pH would be associated with even greater inflammasome activation and decreased bacterial clearance. Second, a recent elegant study by Rimessi and colleagues demonstrated that *Pseudomonas* activates NLRP3 and NLRC4 more strongly in CF cells due to intrinsically impaired calcium homeostasis[203]. CFTR is a chloride ion channel, and its deficiency leads to abnormally high intracellular and mitochondrial calcium levels. *P. aeruginosa*

infection triggers additional calcium entry via the mitochondrial calcium uniporter, resulting in greater mitochondrial damage, oxidative stress and subsequent NLRP3 activation in CF cells. Thus, the intrinsically aberrant calcium homeostasis and increased acidity exacerbate the inappropriate inflammasome activation in response to *P. aeruginosa*, and lead to exaggerated neutrophil influx with subsequent lung damage by neutrophil elastase despite perpetual failure to clear the bacteria. Indeed, inhibiting the Pannexin-1 (P2X7) channel with probenecid prior to *Pseudomonas* infection successfully prevents calcium influx-driven caspase-1 activation, and reduces the severity of infection *in vivo*[204, 205].

Yet if caspase-1 inhibition in the lung is beneficial for the host and detrimental for *Pseudomonas*, then what is the significance of ExoU - a *Pseudomonas* toxin which is reported to be a potent caspase-1 inhibitor? According to Anantharajah and colleagues, IL-1β secretion is abrogated in the presence of ExoU, and pyroptosis is replaced with rapid cell death[194], which is not caspase-8 dependent[11] and is likely necrotic[206]. Neither the mechanism of cell death nor caspase-1 inhibition are fully understood. ExoU is a phospholipase, which is unique among T3SS effectors[206], and it is apparently able to effectively suppress both NLRC4-dependent and independent caspase-1 processing. However, *Pseudomonas* strains which lack ExoU appear to have a competitive advantage over ExoU(+) strains[207]. Over time, the clinical isolates recovered from chronic *Pseudomonas* lung infections tend to becomes ExoU(-), non-motile, and often completely lacking a T3SS.

In addition to NLRP3 and NLRC4, *Pseudomonas* also appears to trigger a non-canonical inflammasome pathway. This pathway appears to be activated by pilin and requires a functional T3SS, but is not dependent on NLRP3, NLRC4, or Asc[208]. Karmakar et al also identified a pathway of IL-1β production by neutrophils in response to *Pseudomonas* corneal infection which is independent of Asc and caspase-1, but dependent on the activity of neutrophil elastase and serine proteases[178]. Here, IL-1β was found to be necessary for bacterial clearance from the cornea, in contrast to the detrimental effects of IL-1β in *Pseudomonas* lung infection. Others have also reported a neutrophil-driven pathway in response to *Pseudomonas* with similar non-canonical characteristics[180, 209], with potential regulation by Pstpip2[209]. If these studies indeed describe a single pathway, then the fact that it is independent of caspase-1 narrows the possibilities of enzymes known to directly cleave IL-1β to caspase-8, neutrophil elastase, proteinase 3, and cathepsin G. Although evidence to confidently exclude a role for caspases-8 and 11 is incomplete, currently it appears *Pseudomonas* does not activate these pathways[11, 24, 98]. Synthesizing all of these results suggests a scenario where pilin is secreted by the *Pseudomonas* T3SS, and activates direct processing of IL-1β and IL-18 by neutrophil serine proteases independently of inflammasomes or caspase-1.

Francisella

The facultative intracellular bacterium *Francisella* tularensis is the causative agent of tularemia, an acute systemic disease with high mortality. It is a highly virulent pathogen which, like *Yersinia pestis*, is classified as a category A select agent with the

potential to be used for bioterror and biowarfare. For bench research, the model of choice is *F. novicida* because it is virulent in mice but attenuated in humans[210-212].

F. novicida avoids degradation by phagocytes by escaping from the phagosome into the cytosol, where it is then free to replicate. However, upon e scape from the phagolysosome, *F. novicida* triggers caspase-1 cleavage and IL-1β secretion[213]. Unlike other pathogens discussed in this review, *Francisella* does not seem to activate neither NLRC4 nor NLRP3[214] in mice, but in human cells both NLRP3 and AIM2 are triggered[215]. Activation of NLRP3 was recently corroborated by another study where human monocytes produced IL-1β in response to *Francisella* bacteria and ATP[216]; this IL-1β secretion also required K+ influx, strongly suggesting an NLRP3-dependent mechanism. Perhaps a *Francisella* effector is able to inhibit NLRP3 activation in mice but not in humans[217], or perhaps mice and humans have other significant differences in NLRP3 regulation. Another mouse-human difference concerns *Francisella* activation of the Pyrin inflammasome. Gavrilin and colleagues showed that in human monocyte-derived macrophages and THP-1 cells, *Francisella* triggers the Pyrin inflammasome[128]. This contrasts with findings by F ernandes-Alnemri et al, who showed that mice lacking Pyrin still produce IL-1β in response to *Francisella*, while mice lacking AIM2 produce little to none. Significant differences in the sequence and function of mouse Pyrin versus human Pyrin may be at the core of this discrepancy[218].

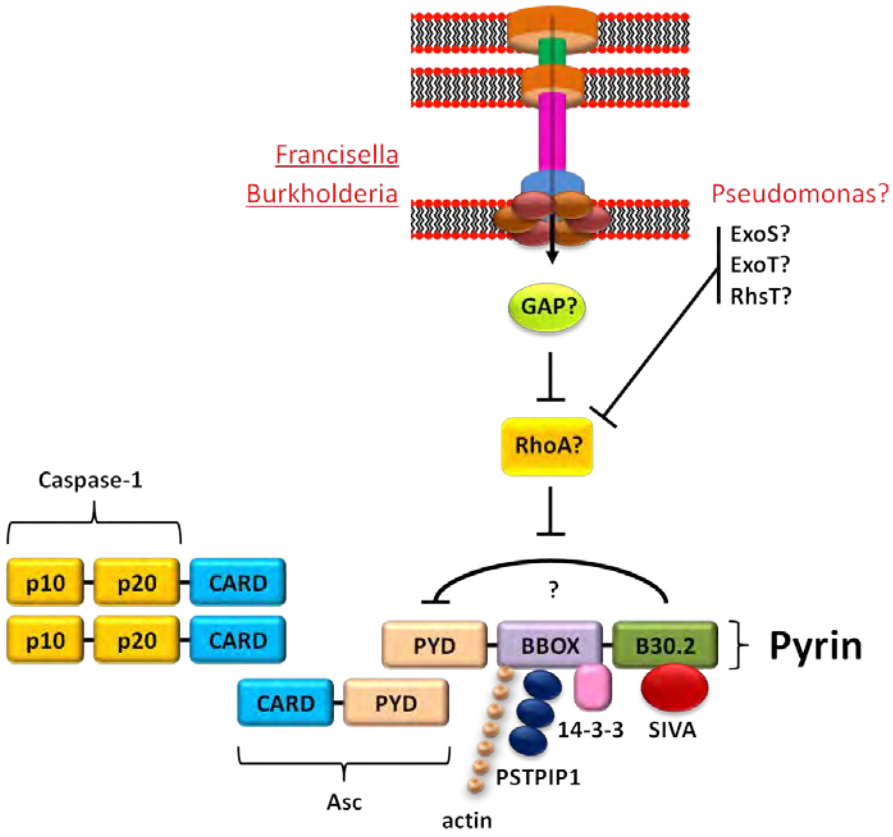

Figure 1.3. Pyrin inflammasome structure and function. *Francisella* and *Burkholderia* are known activators of Pyrin. *Pseudomonas* is a suspected activator based on the GAP activity of ExoS and ExoT, as well as the homology of RhsT to known Pyrin activator TcdB.

The mechanism by which *F. novicida* activates AIM2 is also unusual, occuring through an IRF-1 dependent pathway[219]. Activation of the cytosolic DNA sensor cGAS and STING in response to cytosolic *F. novicida* leads to IRF-1 mediated transcription of GBPs; specifically, GBP 2 and 5 were found to induce AIM2, but not NLRP3, in a dsDNA dependent manner in mouse macrophages. As mentioned earlier, the AIM2 inflammasome assembles upon directly binding dsDNA via the HIN domain[68, 220-222]. Yet the study by Man and colleagues shows that although AIM2 and cGAS can

both bind dsDNA, cGAS activation is upstream of AIM2 and is necessary for inflammasome formation in response to *F. novicida*. Both AIM2 and IRF1 were required for restriction of *F. novicida* replication *in vitro*, and survival *in vivo*[219]. Potentially, interferon signaling could be necessary to increase AIM2 expression, however other studies show that even small amounts of transfected dsDNA are enough to rapidly trigger activation of the AIM2 inflammasome[223]. A specific trigger of this pathway is not known, although one possibility is that the *F. novicida* activator of STING is a secreted cyclic nucleotide, similar to *L. monocytogenes*[224].

Cytosolic LPS from the intracellular *F. novicida* would be expected to also trigger caspase-11 activation. However, *Francisella* produces tetra-acylated rather than hexa-acylated LPS, which loses its ability to bind and activate caspase-11[107]. This is a similar strategy to that of *Y. pestis*, limiting activation of TLR4[225], and downstream expression of inflammasome factors such as NLRP3, pro-caspase-1, pro-IL-1β, and pro-IL-18.

The *Francisella* pathogenicity island (FPI) encodes 16-19 genes which express a Type VI secretion system (T6SS). The secreted IglC protein induces phagosome rupture and allows *Francisella* to escape into the cytosol[211]. This is in contrast to *S. typhimurium*, which secretes factors in order to stabilize the phagosome and avoid cytosolic entry. *Francisella* lacking functional IglC fail to escape the phagosome, and also fail to trigger the AIM2 inflammasome[219]. This may suggest that activation of AIM2 requires the presence of the bacteria in the cytosol, and bacterial secretion of

effectors and other factors into the cytosol from inside the phagosome is not sufficient to trigger inflammasome activity.

The function of IglC is still under investigation, and it may potentially be part of the T6SS apparatus itself[226]; indeed, it appears to be a homolog of Hcp, which is thought to form the tube-like structure of the T6SS for delivery of effectors[227, 228]. In this case, an IglC mutant may fail to activate AIM2 simply because the activating molecule is not translocated into the cytosol. This also raises a question: if IglC is part of the T6SS apparatus, how is it secreted? Notably, its Hcp homolog is secreted as well[227], and some components of the T3SS such as PrgJ are also secreted. From this a common pattern suggests itself, where conserved homologues of PrgJ activate NLRC4, homologues of Hcp may potentially activate AIM2, and each of these proteins is necessary for the proper function of the its respective secretion system.

Legionella

Legionella pneumophila is a Gram-negative intracellular pathogen responsible for the respiratory infection known as Legionnaire's disease. The ability of *Legionella* to survive inside macrophages and the way it interacts with inflammasomes is in many ways comparable to *Salmonella*. After phagocytosis, survival and replication by *L. pneumophila* requires inhibition of phagosome-lysosome fusion, so that the bacteria may persist in a protected vacuole. The requirement to stabilize this intracellular niche is evident from the fact that bacteria that are incapable of growing inside host cells are also incapable of causing disease in animals[229]. However, upon sufficient replication the

bacteria induces rupture of the vacuole followed by lysis of the infected cell[230]. This releases the bacteria into the host environment, allowing it infect more cells and for the infection to continue.

Legionella has a type IV secretion system (T4SS) encoded by a region of the genome called *icm* (intracellular multiplication). The T4SS translocates hundreds of effector proteins into the cytoplasm in order to stabilize the bacterial vacuole and establish a replicative niche[229-232]; this high number of effectors distinguishes *Legionella* among pathogens with secretion systems. Most of the effectors are involved in manipulating host pathways to prevent fusion of the bacterial vacuole with lysosomes[233]. Some effectors such as S idF and SdhA prevent the host cells from undergoing apoptosis in order to limit inflammatory responses and immune detection[234, 235].

Despite having a T4SS rather than a T3SS, *Legionella* also triggers activation of the non-canonical caspase-11 dependent NLRP3 inflammasome [236, 237]. Pyroptosis requires caspase-11, but not NLRP3 in cells infected with *Legionella*, which is consistent with the gasdermin-dependent mechanism proposed by K ayagaki and colleagues[96]. Interestingly, it has also been reported that activated caspase-11 induces fusion of the *L. pneumophila*-containing phagosome to the lysosome through actin remodeling[106].

Legionella flagellin translocated into the cytoplasm through its secretion system is detected by the NLRC4 inflammasome through the adaptor molecule Naip5[32, 237-240]. In other bacteria, the T3SS needle protein PrgJ and its homologs may also trigger NLRC4, however *Legionella* lacks a T3SS and flagellin appears to be the only NLRC4

activator in this pathogen. This NLRC4 pathway seems to be sufficient for controlling bacterial replication, based on evidence that deficiency of caspase-1 but not caspase-11 impairs bacterial clearance both *in vitro* and *in vivo*[241]. However, lack of caspase-1 is functionally similar to a lack of caspase-11 and NLRC4, so it is not possible to evaluate the relative importance of the caspase-11 pathway using a capase-1 deficient model. Given the robust caspase-11 dependent activation of caspase-1 by *L. pneumophila* lacking flagellin[237], as well as its role in fusing the bacterial vacuole with lysosomes[106], it is conceivable that caspase-11 may be redundant with NLRC4 and sufficient for bacterial control on an NLRC4 -/- background. *Legionella* lacking the T4SS (dotA -/-) does not show any inflammasome activation or cell death[237], most likely because these mutant bacteria fail to secrete LPS and flagellin along with essential effector proteins to stabilize the vacuole; these mutants traffic to the lysosome where they are efficiently neutralized[238].

Shigella

The Gram-negative *Shigella* is the causative agent of shigellosis, a foodborne illness prevalent in developing countries. *Shigella* results in severe gastrointestinal disease in humans, but does not seem to cause significant disease in other animals. Its invades the colonic and rectal mucosa leading to leukocyte recruitment, severe inflammation, and often bloody diarrhea (dysentery) which leads to further spread of infection in poorly sanitized regions. Like several other pathogens discussed in this review, the ability of *Shigella* to survive intracellularly is central to its infection strategy.

Shigella is closely related to *Salmonella*, but one major distinction is that *Shigella* lacks flagellin. Like *S. typhimurium*, Pathogenic *Shigella* species are capable of entering gut epithelial cells as well as macrophages, and trigger rapid cell death. *Shigella* also uses a T3SS to secrete effectors which induce vacuole rupture and release the bacteria into the cytosol. Although *Shigella* lacks flagellin it still readily triggers the NLRC4 inflammasome through the same T3SS it uses to escape this vacuole. NLRC4 is activated by Naip2, which detects the inner rod protein MxiI[242], and Naip1, which recognizes the needle component MxiH[143].

Release of *Shigella* into the cytoplasm of the host cell also triggers IFN dependent caspase-11 activation[98], with downstream activation of caspase-1 through the non-canonical inflammasome, secretion of IL-1β and IL-18, and pyroptosis via gasdermin D[96]. It was also reported that caspase-4, the human homolog of caspase-11, is involved in host resistance to *Shigella*[243]; however, *Shigella* secretes the effector protein OspC3 which inhibits caspase-4 activation. It is interesting that OspC3 is highly specific to caspase-4 and does not inhibit caspase-11, suggesting the preference *Shigella* has for infecting humans

The *Shigella* T3SS also appears to induce autophagy[244], which is known suppress inflammasome activation; inhibition of autophagy promoted cell death in infected macrophages, which is again suggestive of pyroptosis. In the absence of caspase-1 or NLRC4 autophagy was dramatically enhanced, which is consistent with reports that caspase-1 negatively regulates autophagy[185].

Similar to *Yersinia*, *Shigella* modifies its LPS in order to evade immune detection[245]. Paciello and colleagues show that during intracellular replication, *Shigella* predominantly expresses tri- and tetra-acylated LPS with fewer acyl chains in lipid A than when it is cultured in growth media. This hypoacylated LPS is much less potent in activating TRL4, resulting in limited expression of pro-caspase-1, pro-IL-1β, and pro-IL-18. However, the authors also suggest that in late infection, when *Shigella* is obligated to proliferate extracellularly due to decreasing access to live local cells to infect, the bacteria reverts to production of immunopotent hexa-acylated LPS. This allows leukocytes to respond to the pathogen more effectively and eventually clear it from the body.

Yersinia

Yersinia pestis is the etiologic agent of plague, causing the deadliest pandemics in human history, with deaths in the hundreds of millions. *Y. pestis* continues to cause disease worldwide, particularly impacting the African sub-continent[246]. However, *Y. pestis* is endemic in rodents in the Western North America, and sporadic cases of infection and death in the United States.

Pathogenic *Yersiniae* share a conserved T3SS with essentially identical needle structure and injected effector proteins (Yops). The structure and function of the needle/translocon is analogous to that of related pathogens. The *Y. pestis* needle/translocon is assembled from 25 Ysc proteins, forming a structure which spans both the inner and outer membranes, and protrudes from the bacterial surface. At the tip

of the structure are the translocon proteins, YopB, YopD and LcrV, whose role is to penetrate the host cell membrane and form a pore through which other Yop effectors can be delivered into the cytoplasm[247, 248].

The Yops play an important role in suppressing host immune functions and promoting bacterial survival. Avoiding immune surveillance is particularly key to the biological strategy of the *Y. pestis*, the etiologic agent of the bubonic plague; immune evasion enables this pathogen to cause systemic disease with extremely high mortality. Lack of a functional T3SS renders *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* essentially avirulent[249-254].

In the case of *Yersinia*, a robust early immune response orchestrated by Interleukin-1β (IL-1β) and IL-18 favors host survival[118, 225]. The expression of these cytokines is effectively suppressed by injected Yops, despite evidence that *Yersinia* can activate the NLRP3, NLRC4, and NLRP12 inflammasomes[118, 255], as well as a non-canonical caspase-8 pathway[11, 124]. How specific *Yersinia* molecules activate and inhibit these pathways is not fully understood.

In the case of NLRP3 and NLRC4, activation depends on the presence of the functional T3SS apparatus[255, 256]. The extent to which these inflammasomes are triggered by the needle/translocon itself or by secreted components remains to be determined. Notably, Brodsky and colleagues suggested that the rate at which effectors are injected into cells may be an important contributor to inflammasome activation, and demonstrated that the effector YopK controls the rate of delivery[255].

The *Yersinia* effector YopM also limits caspase-1 mediated IL-1β/IL-18 production through another incompletely understood mechanism. YopM was originally proposed to directly bind and inhibit caspase-1[257], yet on followup this mechanism has been challenged; instead, the indirect ability of YopM to inhibit caspase-1 seems to depend on the presence of the cytoskeletal scaffolding protein Iqgap1[258]. Furthermore, the confirmed bindings partners of YopM are kinases Prk1/2 and Rsk1/2 (also known as p90 ribosomal S6 kinase), neither of which has previously been associated with inflammasome regulation[259, 260]. There is also disagreement as to whether YopM inhibits NF-kB dependent cytokine production, and whether its main role may be in promoting IL-10 production to silence innate immunity. Also, YopM is currently classified as a E3-ubiquitin ligase based on its homology to IpaH (*Shigella*) and SspH1 (*Salmonella*); however YopM is not known to ubiquitinate any targets, and neither SspH1 nor IpaH are known to regulate caspase-1. Thus, both the mechanism of caspase-1 inhibition by YopM and its biological role remain elusive and warrant continued investigation.

Another effector, YopJ, robustly inhibits transcription of pro-inflammatory cytokines by NF-kB, and induces caspase-8/RIP1-mediated apoptosis, caspase-1 cleavage, and IL-1β activation - effects which are dependent on its enzymatic activity[11, 124, 261]. Caspase-8 is important in host defense against *Y. pestis*[11, 225], but it is not clear whether this is due to its role in processing IL-1β/IL-18, its pro-apoptotic activity, or its role in regulating other NF-kB dependent cytokines. YopJ was originally reported to behave as a deubiquitinase[262], however subsequent *in vitro* studies indicated that it is an acetyltransferase targeting IKKβ[263], MAP Kinase Kinases[264, 265], and

Tak1[266, 267]. Yet despite its powerful effects *in vitro*, YopJ appears perplexingly dispensable during *Y. pestis* infection *in vivo*[268, 269].

Host survival of infection by *Y. pestis* is associated with increased recruitment and activation of monocytes, macrophages, dendritic cells, and T-cells[270-276]. Consistent with this, IL-18 is indispensible for defense against plague, considering that it differs from IL-1β in its enhanced ability to activate these cell types[118]. Notably, the same study shows that IL-1β is also critical for defense against plague, while another study suggests that early neutrophil recruitment to the lung plays a protective role[277]. This is interesting since excessive IL-1β production and associated neutrophil recruitment to the lung can be detrimental to the host in the context of other lung infections; yet in plague this does not appear to be the case. This could be explained by the fact that neutrophils are resistant to the cytotoxic action of YopJ, and may be more effective at intracellular killing of the bacteria[278, 279].

Yersinia* pestis vs other *Yersinia

The enteric pathogens *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are closely related to *Y. pestis* genetically but are adapted to a different biological niche, and are not nearly as dangerous to humans. All three species contain a plasmid encoding nearly identical T3SS and Yop proteins; pCD1 in *Y. pestis*, pIB1 in *Y. pseudotuberculosis*, pYV in *Y. enterocolitica*[249]. In *Y. pseudotuberculosis* and *Y. enterocolitica*, the T3SS confers the ability to invade the intestinal epithelium and proliferate in mesenteric lymph nodes despite the presence of immune cells[280, 281].

Y. pestis is the most recently evolved of the three pathogens, marked by acquisition of the pPCP1 and pMT1 plasmids. The genes encoded by these plasmids adapted *Y. pestis* for transmission between flea and host, enabled infection via a peripheral route, and allowed it to gain access to systemic circulation through the lymphatic system, rather than through an oro-fecal route[282]. This niche transition was accompanied by pressure to achieve maximum immune silence, since systemic immune surveillance is much less tolerant to the presence of bacteria and components such as LPS compared to the intestine. Distinguishing examples of *Y. pestis* adaptation to this niche include: fraction 1 (F1) capsule protein, which hinders contact with phagocytes[283]; plasminogen activator (Pla), critical for fibrinolysis and tissue invasion[284-288]; outer membrane protein Ail, which reduces complement-mediated lysis[289]; loss of flagellin, which is a major PAMP recognized by TLR5 and NLRC4[290]; and the shift to tetra-acylated LPS production, which is an extremely weak agonist of TLR4 compared to hexa-acylated LPS[225]. Many of these and other *Y. pestis* virulence factors are under temperature control, and are rapidly expressed when the bacteria switches from flea (26°C) to mammalian host temperature (37°C)[291, 292].

Despite the drastic differences in the way *Y. pestis* infection presents compared to other *Yersinia*, these microbes share nearly identical T3SSs with similar *in vitro* phenotypes. For this reason, studies in one *Yersinia* species are often expected to be analogous in other *Yersinia* species. While such parallels can provide important insight into pathways across *Yersinia* species, it is important to keep in mind that *in vivo* studies

of enteric *Yersinia* are not reflective or predictive of the dynamics at play during *Y. pestis* infection.

Y. pestis as a model for T3SS interaction with Inflammasomes

A particularly fascinating feature of *Y. pestis* is the fact that it activates many inflammasome pathways, but its T3SS effectively suppresses almost all of them using just 7 translocated Yops. Furthermore, loss of these 7 effectors brings *Y. pestis* from an LD50 of ~10 CFU to being essentially avirulent[282]. Compared to *Y. pestis*, other T3SS relatives have a much higher diversity of effectors (e.g. *Salmonella* has >60)[293]. The low number of Yops and their powerful phenotypes make a genetic approach particularly feasible for their study. For these reasons, we believe *Y. pestis* is an excellent candidate for describing the first complete model of interaction between inflammasomes and a T3SS.

Objectives for Thesis

1) Determine the independent and combined effects of *Y. pestis* effectors YopM and YopJ on pathways regulating inflammasome activation *in vitro* and *in vivo*.

2) Characterize the mechanism through which YopM achieves caspase-1 inhibition.

3) Investigate the dynamics between inflammasome activation and cell death pathways by the *Y. pestis* needle/translocon/translocon and the seven injected Yops.

Preface to CHAPTER II

This section is a version of a manuscript published in the Journal of Biological Chemistry.

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Manipulation of IL-1β and IL-18 production by Yersinia pestis effectors YopJ and YopM and redundant impact on virulence. J Biol Chem. 2016 Feb 16. pii: jbc.M115.697698. [Epub ahead of print]

This chapter represents a thesis project of Dmitry Ratner, who generated all the data except that indicated below. Dmitry Ratner and Egil Lien prepared the manuscript.
Data contributed by co-authors: Pontus Orning performed the experiments shown in Figure 2.2A.

Reagents, cells and mice: Megan K. Proulx and Jon D. Goguen created the *Y. pestis* strains used both *in vitro* and *in vivo*. Dr. Ray Welsh provided the TCRβ/δ-/- mice.

CHAPTER II

Y. pestis YopM and YopJ

Inhibit IL-1β/IL-18 Production

Abstract

Innate immunity plays a central role in resolving infections by pathogens. Host survival during plague, caused by the Gram-negative bacterium *Yersinia pestis*, is favored by a robust early innate immune response initiated by Interleukin-1β (IL-1β) and IL-18. These cytokines are produced by a two-step mechanism involving NF-kB mediated pro-cytokine production and inflammasome-driven maturation into bioactive inflammatory mediators. Because of the anti-microbial effects induced by IL-1β/IL-18, it may be desirable for pathogens to manipulate their production. *Y. pestis* type III secretion system effectors YopJ and YopM can interfere with different parts of this process. Both effectors have been reported to influence inflammasome caspase-1 activity; YopJ promotes caspase-8 dependent cell death and caspase-1 cleavage, while YopM inhibits caspase-1 activity via an incompletely understood mechanism. Yet neither effector appears essential for full virulence *in vivo*. Here we report that the sum of influences by YopJ and YopM on IL-1β/IL-18 release is suppressive. In the absence of YopM, YopJ minimally affects caspase-1 cleavage, but suppresses IL-1β, IL-18 and other cytokines and chemokines. Importantly, we find that *Y. pestis* containing combined deletions of YopJ and YopM induces elevated levels of IL-1β/IL-18 *in vitro* and *in vivo*, and is significantly attenuated in a mouse model of bubonic plague. The reduced virulence of the YopJ-YopM mutant is dependent on the presence of IL-1β, IL-18 and caspase-1. Thus, we conclude that *Y. pestis* YopJ and YopM can both exert a tight control of host IL-1β/IL-18 production to benefit the bacteria, resulting in a redundant effect on virulence.

Introduction

Many pathogens rely upon a strong suppression or evasion of host immune responses in order to cause disease. *Yersinia pestis*, the etiologic agent of plague, achieves high virulence in part by actively suppressing the host immune system. A key component of this strategy is the Type III secretion system (T3SS), by which the bacterium delivers seven *Yersinia* outer protein (Yop) effectors into host immune cells. These Yops manipulate intracellular pathways to inhibit phagocytosis, motility, cytokine expression, and other vital immune processes[282]. Two effectors, YopJ and YopM, have been extensively studied in isolation for over two decades, but key questions about their roles in disease remain unanswered.

In vitro studies have revealed YopJ to be an acetyl transferase targeting IKKβ[263], MAP Kinase Kinases[264, 294], and TAK1[266, 267]. YopJ has also been reported to behave like a deubiquitinase[262]. YopJ robustly inhibits NF-kB-mediated transcription of pro-inflammatory cytokines, and induces caspase-8/RIP1-mediated apoptosis, caspase-1 cleavage, and IL-1β activation - effects which are dependent on its enzymatic activity[11, 124, 261]. Yet despite its powerful effects *in vitro*, YopJ appears perplexingly dispensable during *Y. pestis* infection *in vivo*[268, 269].

YopM has also been reported to limit the recruitment of monocytes, neutrophils, and NK cells to infected organs[295, 296]. It was recently demonstrated that YopM inhibits caspase-1[257, 258]. Caspase-1 mediates maturation of IL-1β and IL-18, cytokines which promote a robust early immune response that enhances host resistance against *Y. pestis* infection [118, 225]. Thus, YopM may promote virulence by inhibiting

processing of these cytokines. Yet the impact of YopM on *Y. pestis* virulence appears minor[296, 297], and it is unclear if IL-1β/IL-18 levels would increase *in vivo* during *Y. pestis* infection without YopM.

IL-1β and IL-18 are produced in a two-step process requiring an initial signal to trigger the transcription/translation of their pro-forms, and a second signal to trigger their processing by caspase-1 into their biologically active secreted forms[87]. The first signal is NF-kB-dependent, typically downstream of stimulation via Toll-like receptors by microbial associated molecules such as L PS. The second signal is catalyzed by inflammasomes. A canonical inflammasome is formed when a pathogen or danger-associated molecular patterns (PAMP or DAMP) are sensed by a NOD-like receptor (NLR), such as NLRP3, NLRC4, or AIM2. This leads to recruitment and oligomerization of the adaptor protein ASC, which in turn recruits pro-caspase-1 dimers to be autoproteolysed into the catalytically active p20 form. This process is normally accompanied by an inflammatory form of apoptosis called pyroptosis. Importantly, non-canonical inflammasomes involving caspases-8[298, 299] or 11[24, 25] have also been described.

Because of the potential importance of IL-1β/IL-18 during infection, the interaction of *Y. pestis* with host inflammasomes warrants close attention. Since YopJ and YopM appear to target the IL-1β/18 maturation pathway in distinct ways, we hypothesized that these two effectors may cooperate for optimal immune suppression. We set out to investigate whether such an interaction exists, and what role this may play during infection. Surprisingly, we found that *Y. pestis* lacking both YopJ and YopM induced increased IL-1β/IL-18 compared to parental bacteria or strains lacking one of the

effectors. We also found a strain lacking both effectors to be significantly attenuated in a bubonic plague model in an IL-18/IL-1β/caspase-1 dependent fashion, suggesting that tuning down inflammasome activity and IL-1β/IL-18 release are key features of *Y. pestis* pathogenesis.

Results

YopM and YopJ differentially influence inflammasomes and inflammatory cytokines and chemokines. Deletion of YopM results in increased IL-1β/IL-18 secretion and caspase-1 cleavage (Fig 2.1A, 2.1B) in BMDMs compared to the parental strain. By contrast, loss of YopJ alone results in reduced IL-1β/IL-18 secretion (Fig 2.1B) and caspase-1 activation (Fig 2.1A), despite an increase in pro-IL-1β and pro-caspase-1. Loss of both YopM and YopJ leads to a further increase in ASC/caspase-1 dependent IL-1β and IL-18 secretion but an insignificant increase in caspase-1 activation compared to loss of YopM alone (Fig 2.1A, 2.1B, 2.1D). We confirmed Yop expression in the ΔYopJ and ΔYopM strains (Fig 2.1E).

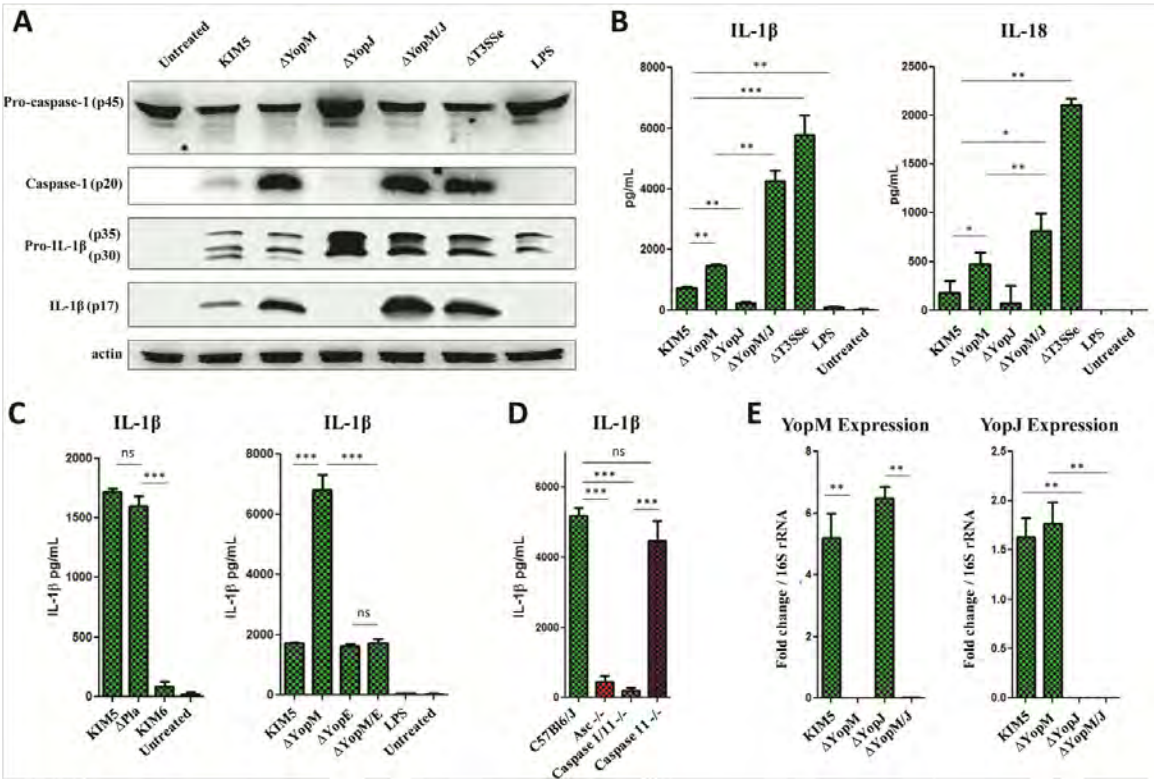
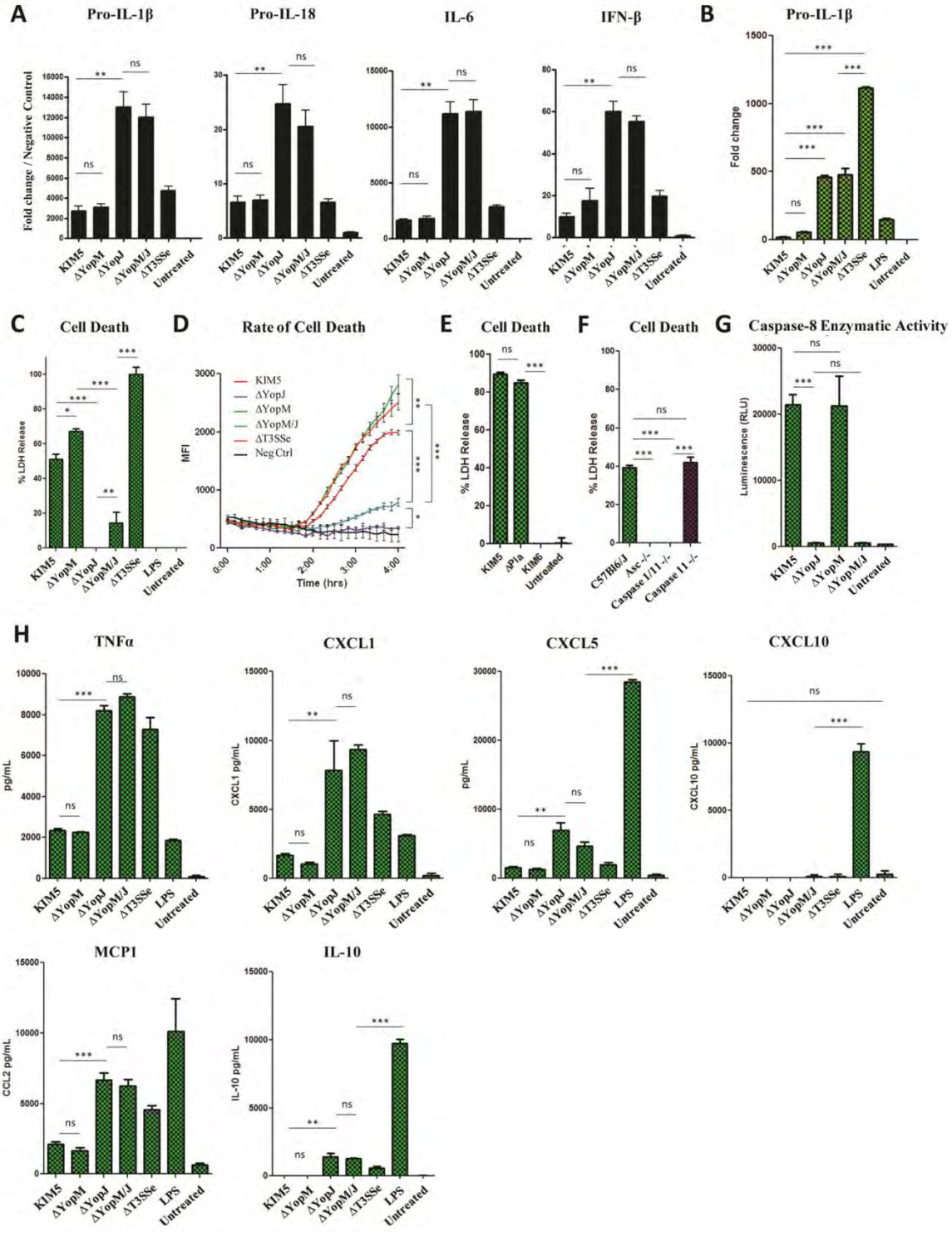


FIGURE 2.1. YopM and YopJ have opposing effects on caspase-1 cleavage, but in sum reduce IL-1β and IL-18 levels during infection. A) Total protein from total BMDM samples (cell lysate and supernatant) at 6 hours post-infection (p.i.) was separated by SDS-PAGE and analyzed by Western Blot for IL-1β and caspase-1. B,C) BMDMs were infected with *Y. pestis* strains at MOI 10 and supernatants were harvested at 6 hours p.i. for analysis of IL-1β or IL-18 for ELISA. D) Supernatants from BMDMs from the indicated mouse genotypes were harvested 6 hours p.i. with KIM5 ΔYopM/J (MOI 10) and assayed for IL-1β by ELISA. E) RNA was extracted from *Y. pestis* (grown at 37°C to up-regulate T3SS expression), and amplified by RT-PCR using primers specific for YopM or YopJ. C(t) values for each primer pair were normalized to *Y. pestis* 16S rRNA internal control C(t) values. Figures are representative of three or more experiments. Shown is mean plus s.d. for triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.


A
Pro-IL-1β
Pro-IL-18
IL-6
IFN-β
Fold change / Negative Control
KIM5
ΔYopM
ΔYopJ
ΔYopM/J
ΔT3SSe
Untreated
B
Pro-IL-1β
Fold change
LPS
C
Cell Death
% LDH Release
D
Rate of Cell Death
MFI
KIM5
ΔYopJ
ΔYopM
ΔYopM/J
ΔT3SSe
Neg Ctrl
Time (hrs)
E
Cell Death
% LDH Release
ΔP1a
KIM6
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Cell Death
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C57Bl6/J
Asc-/-
Caspase 1/11-/-
Caspase 11-/-
G
Caspase-8 Enzymatic Activity
Luminescence (RLU)
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TNFα
pg/mL
CXCL1
CXCL1 pg/mL
CXCL5
pg/mL
CXCL10
CXCL10 pg/mL
MCP1
CCL2 pg/mL
IL-10
IL-10 pg/mL

FIGURE 2.2. YopJ, but not YopM, inhibits expression of Pro-IL-1β and other cytokines, and is a major driver of macrophage cell death. A) RNA from peritoneal macrophages or B) BMDMs were harvested at 2 hours p.i. and amplified by RT-PCR using primers specific for Pro-IL-1β, Pro-IL-18, IL-6, or IFN-β. (C, E) Cell death in BMDMs was assayed either at 5 hours p.i. by assaying LDH release, or D) continuously by measuring intracellular EtHD-1 entry. F) Cell death was assayed at 5 hours p.i. with KIM5 ΔYopM/J at MOI 10 by assaying LDH release. G) Caspase-8 enzymatic activity in BMDMs was assayed at 3 hours p.i. H) BMDMs were infected with *Y. pestis* at MOI 10 and supernatants were harvested at 6 hours p.i. for quantification of cytokines by ELISA. Figures are representative of two or more independent experiments. Shown is mean plus s.d. for triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

RT-PCR analysis demonstrated that YopJ, and not YopM, is primarily responsible for inhibiting the transcription of IL-1β/IL-18 precursors, as well as IL-6 and IFN-β (Fig 2.2A, 2.2B). We found that YopM confers protection from caspase-1 dependent cell death in the absence of YopJ, but has only a weak cytoprotective effect when YopJ is present (Fig 2.2C, 2.2D, 2F). We also confirmed that YopJ, but not YopM, drives the enzymatic activity of caspase-8 (Fig 2.2G). Expression of TNFα, CXCL1, MCP1, CXCL5, and IL-10, which require no inflammasome processing, is also inhibited by YopJ but not YopM (Fig 2.2H). Notably, the pCD1 plasmid containing the T3SS is essential for IL-1β induction, as the KIM6 strain lacking pCD1 failed to induce any IL-1β. (Fig 2.1A-C, Fig 2.2C-E). The lack of another known virulence factor, the plasminogen activator protein (Pla)[288], had no effect on cell death or IL-1β release (Fig 2.1C, Fig 2.2E).

YopM and YopJ have redundant effects on virulence in vivo. We found that in the mouse bubonic plague model, *Y. pestis* KIM1001 lacking both YopM and YopJ was significantly attenuated. In contrast, *Y. pestis* lacking either YopM or YopJ alone induced lethality comparable to the parent strain, with no significant differences from the fully

virulent KIM1001 (Fig 2.3A). In mice lacking caspase-1, IL-1β, or IL-18, the attenuation of the KIM1001ΔM/J strain was significantly reduced (Fig 2.3B), suggesting that these components play central roles in the host defenses towards *Y. pestis* lacking both YopM and YopJ. We note that both WT and IL-18 KO mice die of low doses of the parental KIM1001 strain[118]. On day 4 of subcutaneous infection, bacterial loads were significantly lower in mice infected with KIM1001ΔM/J both in the spleen and lymph nodes, but KIM1001ΔM and KIM1001ΔJ did not result in statistically different bacterial loads from the parental KIM1001 (Fig 2.3C).

The increased activation of innate immunity as observed with the KIM1001ΔM/J may also impact adaptive immune responses. We found that wild-type mice that survive initial challenge with KIM1001ΔM/J were effectively protected from subsequent infection with fully virulent *Y. pestis* (Fig 2.3D), indicating that strains containing deletions of YopM and YopJ, perhaps in combination with expression of LpxL[225] could be promising vaccine candidates. Adaptive immunity may also contribute to resistance of attenuated *Y. pestis* strains[225, 271], and T cells appear to play a key role[270, 300]. To explore this question further, we subjected mice lacking T-cell receptors (TCR β/δ -/- mice lack both αβ and γδ TCRs)[301] to the same s.c. challenge with KIM1001ΔM/J as in Figure 2.3B. We found that the T-cell receptor is very important for mice to survive the initial infection with KIM1001ΔM/J (Fig 2.3E). Furthermore, the three TCR β/δ -/-mice which survived the initial challenge were not protected from KIM1001; when infected with KIM1001 on day 21, all three died by day 30 whereas 100% (total 21 out of 21) of the re-challenged wild-type controls survived

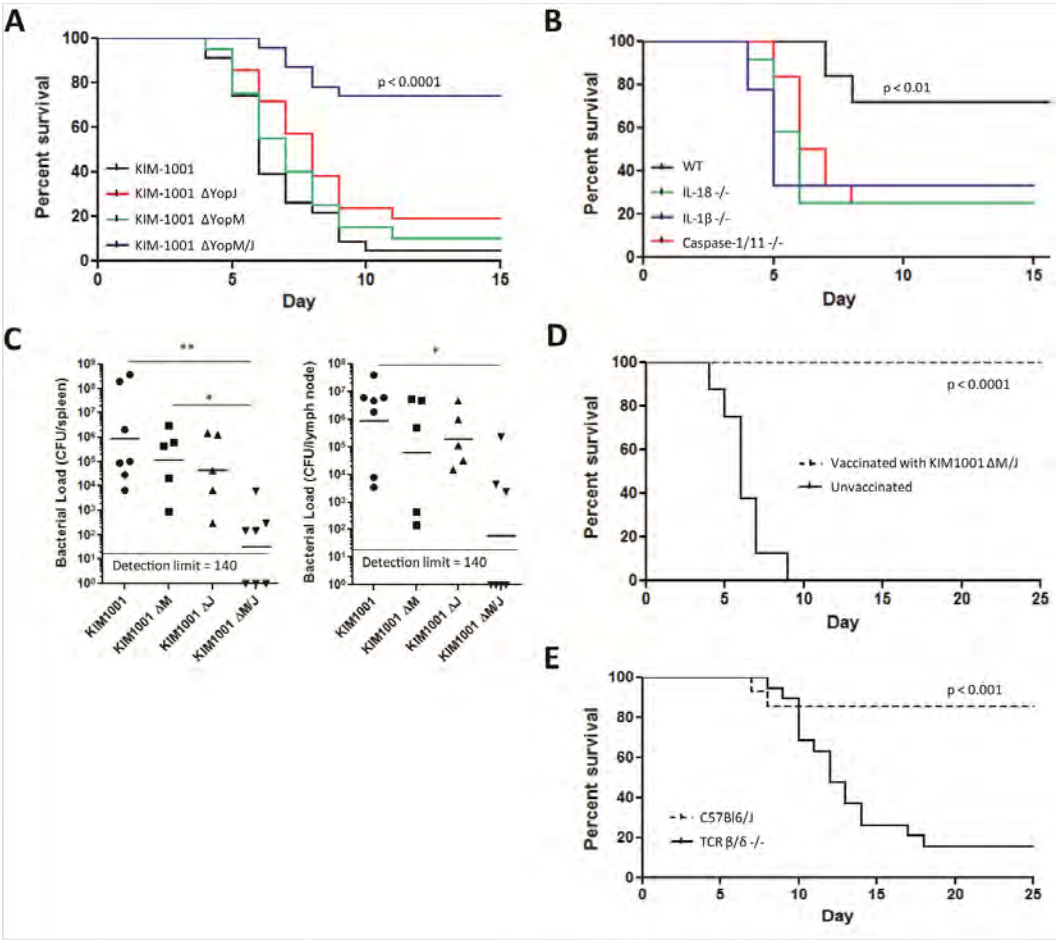

FIGURE 2.3. Deletion of both YopM and YopJ attenuates *Y. pestis* in a manner dependent on Caspase-1, IL-1β, and IL-18. A) C57Bl6/J mice were injected s.c. with 50-140 CFU of KIM1001, KIM1001ΔM, KIM1001ΔJ, or KIM1001ΔM/J and monitored for survival. Results represent pooled data from two separate experiments. B) C57Bl6/J (n=14), IL-18 KO (n=12), IL-1β KO (n=9), or caspase-1/11 KO (n=12) mice were injected with 50-140 CFU of KIM1001ΔM/J s.c. and monitored for survival. P values reflect comparisons between KIM1001ΔM/J and other bacterial strains (A), or between wild-type mice and other mouse strains (B). C) C57Bl6/J mice were injected s.c. with 50-140 CFU of KIM1001 (n=7), KIM1001ΔM (n=5), KIM1001ΔJ (n=5), or KIM1001ΔM/J (n=7); spleens and lymph nodes were harvested 90 hrs p.i. and bacterial load was determined. Geometric mean values are shown. D) C57Bl6/J mice which survived infection with KIM1001ΔM/J (n=7) or naïve controls (n=8) were injected s.c. with 400 CFU of KIM1001 and monitored for survival for 25 days. The p value reflects the comparison of vaccinated vs naive animals. E) C57Bl6/J mice (n=14) or TCR β/δ -/- mice (n=20) were injected s.c. with 120-180 CFU of KIM1001ΔM/J and monitored for survival for 25 days. Results represent pooled data from two separate experiments. P value reflects comparison between wild-type and TCR β/δ -/- mice.

beyond day 40. One limitation of these studies is that we cannot rule out a role of B-cells or antibodies; nevertheless, T cells are crucial for the resistance against bacterial challenge, suggesting participation of both innate and adaptive immune responses in optimal host responses to these bacterial strains.

Inhibition of IL-1β and IL-18 production are overlapping functions of YopM and YopJ in mice, but other effects differ. During the subcutaneous infection described above (Fig 2.3) it is likely that systemic IL-1β and IL-18 levels may correlate to bacterial loads, and thus this experimental setup may not be optimal to study direct effects of YopM/J deletion on cytokine expression. For this reason, we injected the *Y. pestis* strains into mice intravenously and harvested tissue at 42 hours to compare systemic IL-1β and IL-18 with smaller differences in host bacterial loads between the different strains (Fig 2.4). We confirmed that bacterial loads in the spleen at this time were equivalent in all groups with no statistical differences, validating the comparison of cytokines between the groups (Fig 2.4A). We found significantly elevated IL-1β and IL-18 in the serum of mice infected with KIM1001ΔM/J (Fig 2.4B, 2.4C). In contrast, however, we also detected a small but significant reduction in IL-18 when YopJ alone was deleted. Furthermore, while IL-1β and IL-18 expression in the KIM1001ΔM/J group was expressed in a caspase-1 dependent manner, KIM1001 induced low but significant levels of these cytokines independently of caspase-1, perhaps indicating a role of caspase-8[11]. The NF-kB-dependent chemokines CXCL1, CXCL10, CXCL5 and MCP1 were elevated in the absence of YopJ but not YopM (Fig 2.4D-F, 2.4H), similar trends as observed with macrophages *in vitro* (Fig 2.2). Although CXCL5 levels were not significantly different

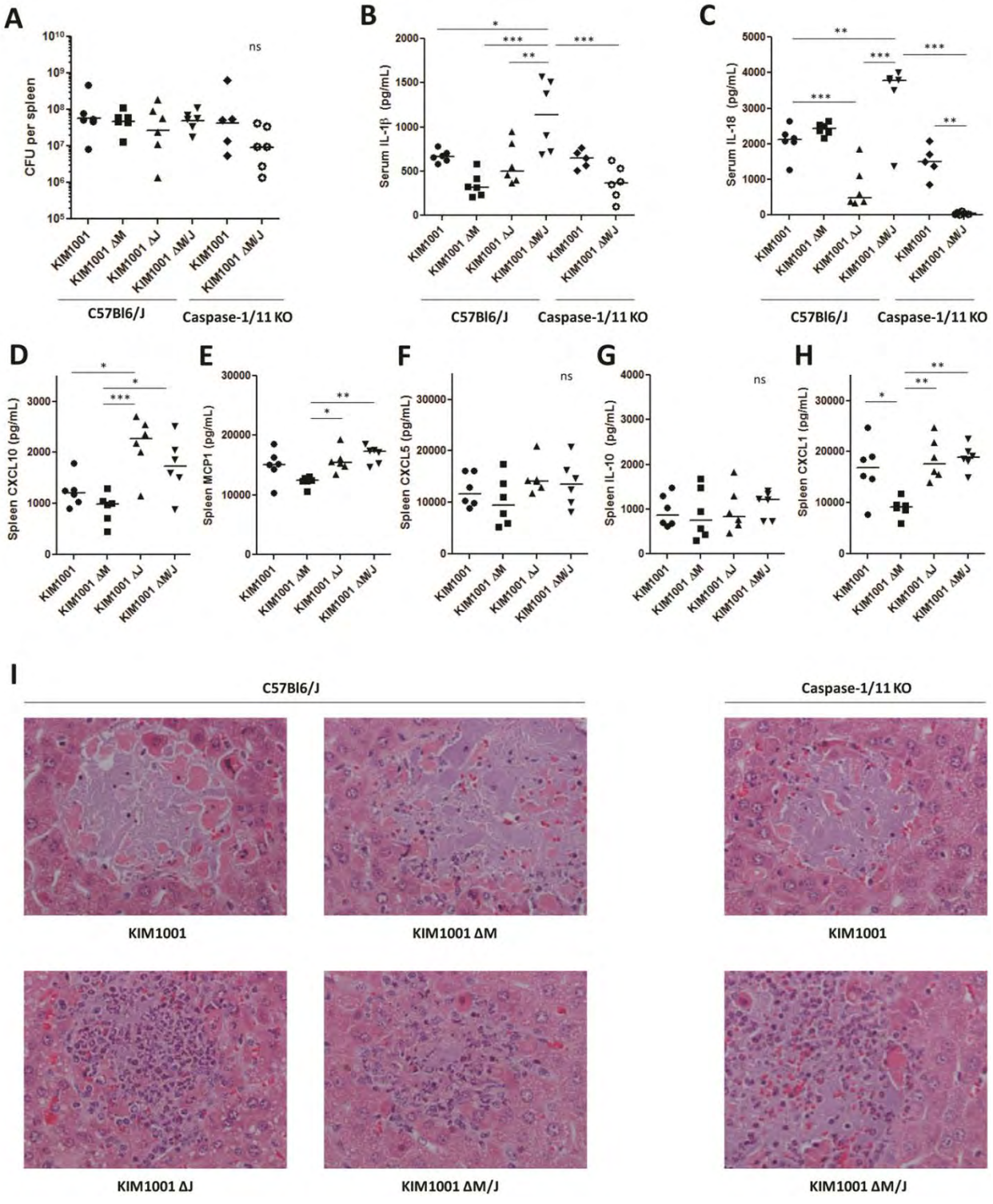
A
CFU per spleen
ns
KIM1001
KIM1001 ΔM
KIM1001 ΔJ
KIM1001 ΔM/J
KIM1001
KIM1001 ΔM/J
C57Bl6/J
Caspase-1/11 KO
B
Serum IL-1β (pg/mL)
C
Serum IL-18 (pg/mL)
D
Spleen CXCL10 (pg/mL)
E
Spleen MCP1 (pg/mL)
F
Spleen CXCL5 (pg/mL)
G
Spleen IL-10 (pg/mL)
H
Spleen CXCL1 (pg/mL)
I
C57Bl6/J
Caspase-1/11 KO
KIM1001
KIM1001 ΔM
KIM1001
KIM1001 ΔJ
KIM1001 ΔM/J
KIM1001 ΔM/J

FIGURE 2.4. YopM predominantly suppresses IL-1β and IL-18, while YopJ limits cell recruitment in part by reducing chemokine expression during infection *in vivo*. C57Bl6J or caspase-1/11 KO mice were injected i.v. with 40-50 CFU of KIM1001, KIM1001ΔM, KIM1001ΔJ, or KIM1001ΔM/J in 200μL PBS (n=5 caspase-1/11 KO mice infected with KIM1001ΔM/J, all other groups n=6). Tissues were harvested at 42 hours p.i. A) Bacterial load was determined from spleen homogenates. (B) Serum was used to quantify IL-1β and (C) IL-18, and D-H) spleen homogenates were used to quantify CXCL10, MCP1, CXCL5, IL-10, and CXCL1 by ELISA. Shown are median values. (I) Livers were stained with H&E and representative foci of infection were selected. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

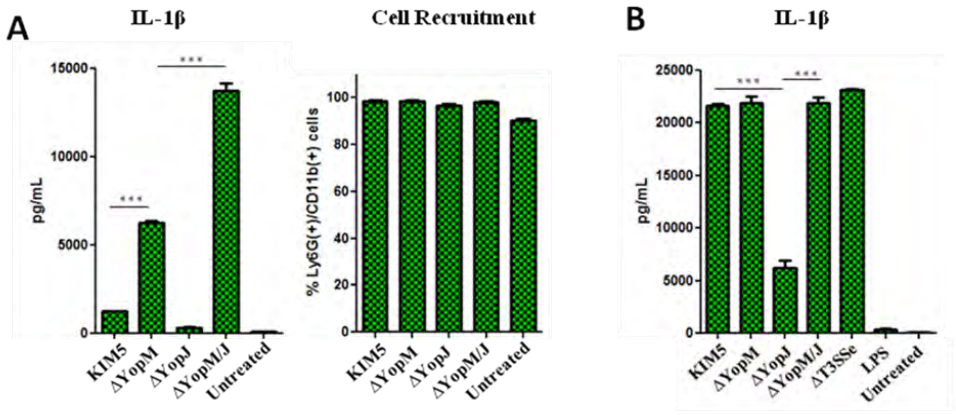

FIGURE 2.5. YopJ and YopM may have different effects on IL-1β in dendritic cells compared to neutrophils and macrophages. A) Mice were injected intraperitoneally with 0.7mL 4% thioglycolate for 3 hou rs to trigger heavy neutrophil influx, followed by injection with $1x10^8$ CFU of the indicated bacterial strains in 0.3mL PBS for an additional 6 hours. Peritoneal lavage fluid was assayed for IL-1β ELISA (left panel); Cells from peritoneal lavage were analyzed for cell surface markers by FACS to confirm comparable and robust influx of Ly6G/CD11b positive cells (right panel: %Ly6G+/CD11b+ of all conditions). B) BMDCs were infected with *Y. pestis* at MOI 10 and supernatants were harvested at 6 hours p.i. and assayed for IL-1β by ELISA. Shown are means of triplicates representative of two separate experiments.

in any strain, they trended higher in the absence of YopJ (Fig 2.4F). IL-10 levels were not affected by either YopM or YopJ *in vivo* (Fig 2.4G). On evaluation of liver histology we noted that deletion of YopJ, but not YopM, appeared associated with increased presence of both polymorphonuclear and mononuclear cells at foci of infection (Fig 2.4C).

YopM and YopJ may have different functions in other cell types. In view of some of the overlapping effects of YopM and YopJ, we investigated how they affect IL-1β production in other innate immune cells[302]. We found that the relative roles of YopM and YopJ in IL-1β production *in vivo* in a neutrophil-enriched (90-98% Ly6G/CD11b+) peritoneal cell population (Fig 2.5A) follow a pattern similar to macrophages *in vitro* (Fig 2.1B). In BMDCs, however, IL-1β secretion is triggered by KIM5 and significantly reduced in the absence of YopJ. By contrast, IL-1β secretion is minimally inhibited by the absence of YopM unless YopJ is absent (Fig 2.5B). These results suggest that the bacterial regulation of IL-1β release in dendritic cells (DCs) is markedly different from macrophages and neutrophils.

Interpretation of the relative roles of YopM and YopJ in vitro and in vivo. We summarize some of our major findings about the respective roles of YopM and YopJ in the regulation of IL-1β/IL-18 production in Figure 2.6. The *Y. pestis* effectors YopJ and YopM have apparent opposite effects on caspase-1 processing; however, in the absence of both effectors, it becomes apparent that the sum of the actions of YopJ and YopM suppresses IL-1β and IL-18 release more effectively than either YopM or YopJ alone. In general, the dampening of caspase-1 processing mediated by YopM is dominant, and when YopM is deleted, YopJ appears to mainly suppress pro-IL-1β and not contribute to caspase-1 cleavage in a major way. Neither of these effectors appears to enhance disease progression when the other is present, and we propose that their actions promote virulence *in vivo* by a redundant net effect on IL-1β and IL-18. Nevertheless, their effects may vary by cell type and reflect the heterogeneity of the pathways regulating IL-1β/IL-

18 production. While their net effects on IL-1β/IL-18 overlap, their roles in regulating other aspects of the immune response are distinct and in some ways appear to oppose one another. Thus our data suggest that YopJ and YopM, rather than being dispensable for plague virulence, are effective promoters of virulence capable of replacing each other's net effect *in vivo* by targeting different arms of the same immune defense mechanism.

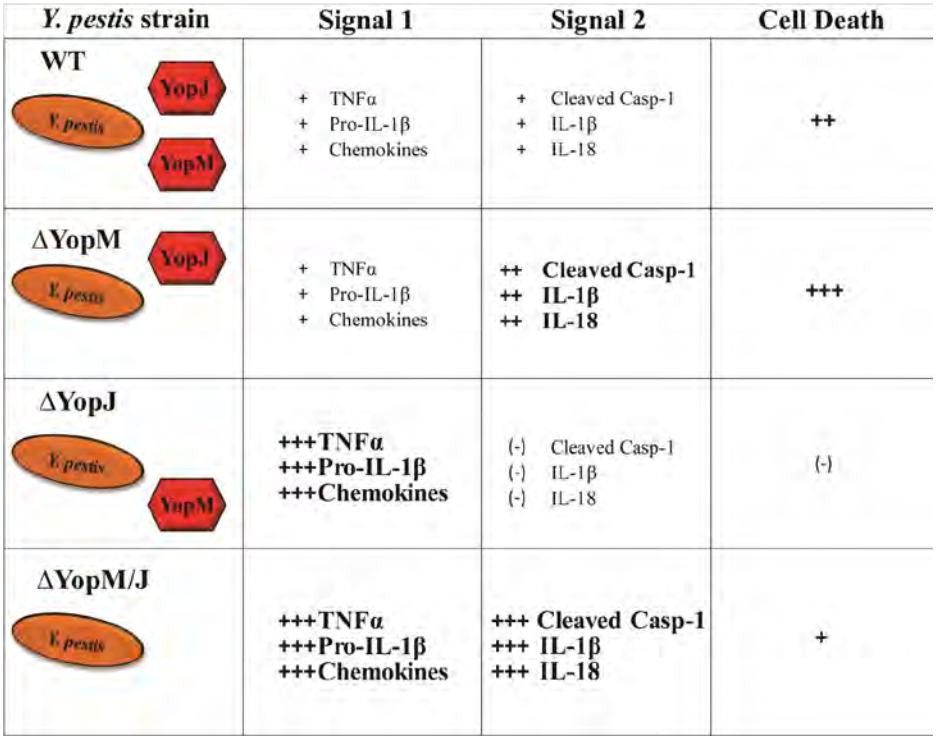

FIGURE 2.6. Summary of proposed YopM and YopJ effects on IL-1β/IL-18 production and cell death in host cells. In the absence of YopJ and YopM, cells respond to *Y. pestis* by expressing high levels of pro-IL-1β and pro-IL-18, and activating robust caspase-1 cleavage accompanied by pyroptotic cell death. YopM can inhibit IL-1β/IL-18 release and cell death by inhibiting inflammasome-mediated caspase-1 cleavage, but it has little effect on expression of pro-IL-1β/IL-18 or other cytokines not requiring inflammasome processing. In contrast, YopJ limits IL-1β/IL-18 production by suppressing expression of precursors, and possibly by inducing rapid cell death which may overshadow the cytoprotective effect of YopM. Additionally, YopJ alone triggers modest caspase-1 cleavage with some degree of IL-1β/IL-18 production, limited by the reduced levels of precursors, including pro-caspase-1 itself.

The parental KIM5 strain normally triggers only low levels of IL-1β/IL-18 release in BMDMs compared to the ΔT3SSe strain which lacks the seven translocated Yops; this reflects the efficient suppression of IL-1β/IL-18 by the cumulative action of these Yops. We and other groups have previously observed that YopJ triggers caspase-8 activation, which triggers some caspase-1 activation and IL-1β/IL-18 release[11, 124, 303]; here, this is likely reflected in Figure 2.1A/2.1B and Figure 2.4B/C. Low levels of IL-1β/IL-18 in response to *Y. pestis in vivo* appear independent of caspase-1 and could be due to direct processing of the precursor forms by caspase-8[304]. Nevertheless, these YopJ-triggered IL-1β/IL-18 levels do not appear to alter the outcome of infection (Fig 2.3, 2.4). It is possible that this YopJ-induced activation is offset by the significant inhibition of pro-IL-1β and pro-IL-18, or other NF-kB-dependent factors (Fig 2.2, 4). This effect on signal 1 may explain why IL-1β/IL-18 levels remain relatively low in the absence of YopM *in vivo* (Fig 2.4B, 2.4C).

By contrast, YopM appears not to have any effect on signal 1 (Fig 2.2), but rather achieves IL-1β/IL-18 suppression by inhibiting caspase-1 activation (Fig 2.1A, 2.1D). Consistent with this role, YopM inhibits caspase-1 dependent cell death although in the presence of YopJ the cytoprotective role of YopM appears minor (Fig 2.2C, 2.2D, 2.2F). It is unclear what the implications of this small effect would be *in vivo*. YopJ appears to be a major driver of cell death, with some inhibitory effects by YopM.

Considering the seeming redundancy of YopM and YopJ which we report here, it is notable that YopM and YopJ sequences are well-conserved in the *Y. pestis* genome and present in all virulent strains of *Y. pestis*, *Y. enterocolitica,* and *Y. pseudotuberculosis*;

however, it is also worth considering that the CO92 strain, which contains a less catalytically active version of YopJ, is more recently evolved than the KIM1001 strain used in this study. Furthermore, *Y. pestis* infects a wide range of animals, with significant differences in immune systems and sensitivities to infection. Since a major strategy of *Y. pestis* is immune evasion to assist propagation, rather than to kill the organism, it is likely that YopM and YopJ evolved to suppress the IL-1β/IL-18 producing pathway in alternative ways without a significant difference in host survival.

Our results underscore some important differences between YopM and YopJ which suggest that while their effects on IL-1β/IL-18 and survival in mice overlap, their functions should not be viewed as completely redundant. There may be other factors important for the fitness of *Y. pestis* in its unique life cycle which make both effectors indispensible, independent of host survival. YopJ inhibits the expression of multiple cytokines and chemokines both *in vitro* (Fig 2.2H) and *in vivo* (Fig 2.4D-H) in addition to IL-1β/IL-18, while YopM appears to primarily inhibit IL-1β/IL-18 maturation by caspase-1. One study shows that YopM barely affects the expression of immunity-related genes *in vivo[305]*, supporting the idea of a more targeted role compared to YopJ. Furthermore, although YopM and YopJ may have similar effects on IL-1β secretion in neutrophils and macrophages (Fig 2.1, Fig 2.5A), their respective roles are less clear in DCs (Fig 2.5B). It appears that the absence of YopM has little bearing on IL-1β secretion in DCs, although YopM may have an effect when YopJ is absent (Fig 2.5B). In any case, we propose that regulation of IL-1β release by YopJ and YopM in DCs is markedly different than in macrophages and neutrophils. Evidence for a direct role of YopM in

DCs is lacking, although YopM appears to limit recruitment of DCs as well as other cell types[295, 306]; however, we believe these observations can be largely explained by the role YopM plays in suppressing IL-1β and IL-18 secretion. YopJ, in contrast, has been shown to inhibit signaling and cytokine production in DCs directly [307, 308]. Importantly, YopJ prevents DCs from activating Natural Killer cells, T-cell proliferation, and IFN-γ induction. Our findings support a critical role of T-cells during *Y. pestis* infection (Fig 2.3E), and studies in *Y. enterocolitica* also argue for the importance of a DC-induced T-cell response[309]. The ability of YopJ to inhibit TNFα and IFN pathways likely contributes to a defective CD8+ T-cell response in pneumonic plague[270], potentially promoting bacterial propagation independently of YopM in some circumstances. Taken together, we think this suggests that YopJ, but not YopM, may be particularly important for regulating immune responses coordinated by DCs and T-cells.

While it is unclear which mechanisms YopM uses for inhibition of inflammasome activation, direct interaction with caspase-1 and the scaffold protein Iqgap1 have been proposed [257, 258]. Interactions betweenYopM and kinases such as RSK1/2 and PKN1/2[259, 260, 310], known for binding to the effector, may also play a role in caspase-1 regulation. Regardless of the method for YopM-mediated inhibition, it is possible that the pathway blocked by YopM could be a dominant pathway in *Yersinia*-induced inflammasome activation. Multiple candidate pathways could potentially be blocked by YopM, as NLRP3, NLRC4, and NLRP12 have all been proposed as mediators of *Yersinia*-induced IL-1β/IL-18 production. We also cannot exclude the possible involvement of additional pathways that sense type III secretion system

molecules. We also cannot disregard the idea that bacterial molecules other than those belonging to the T3SS contribute to modulation of caspase-1 cleavage and IL-1β production *in vivo*[311]. We add to a large body of evidence (Fig 2.1) suggesting minimal macrophage IL-1β release in the absence of the pCD1 T3SS-containing plasmid or the YopB/YopD components of the pore-forming translocon[17].

Conclusion

In summary, our findings illustrate parallel strategies of a microbial pathogen to suppress generation of pro-inflammatory cytokines as a means of resisting host defense. As IL-1β and IL-18 are central mediators of anti-microbial host defenses, it may be particularly desirable to block their production. These cytokines are produced in a multi-step process, potentially as a fail-safe mechanism as excess production may also contribute to shock[164]. However, this complex regulation also opens opportunities for diverse microbial approaches to dampen their production and release. The picture that emerges is complex and this suggests that many T3SS-related molecules together regulate IL-1β/IL-18 release. Our experiments indicate that their relative roles may be dependent upon the presence or absence of other specific molecules.

Preface to CHAPTER III

This section is a version of an unpublished manuscript.

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Specific inhibition of the Pyrin inflammasome by Yersinia pestis type III secretion effector YopM. (unpublished)

This chapter represents a thesis project of Dmitry Ratner, who generated all the data except that indicated below. Dmitry Ratner and Egil Lien prepared the manuscript.
Data contributed by co-authors: Pontus Orning performed the experiments in Figure 3.11.

Reagents, cells and mice: Megan K. Proulx and Jon D. Goguen created the Y. *pestis* strains used both *in vitro* and *in vivo*; *Y. pseudotuberculosis* IP2666ΔYopM and IP2666ΔYopM+recM mutant strains were a kind gift from Dr. Joan Mecsas; HEK293T-Asc-YFP cells were a kind gift from Dr. Kate Fitzgerald; THP-1 YFP-Pyrin and siPyrin cell lines, as well as anti-Pyrin antibody serum were generous gifts from Drs. Mark Wewers and Mikhail Gavrilin; pCDNA3-Pyrin and pCDNA3-14-3-3ε were kind gifts from Dr. Emad Alnemri and Dr. Michaela Gack, respectively.

CHAPTER III

Specific inhibition of the Pyrin inflammasome

by *Yersinia pestis* type III secretion effector YopM

Abstract

Type III secretion systems (T3SS) are central virulence factors for many pathogenic Gram-negative bacteria used to control host innate immune responses. The *Yersinia pestis* T3SS is particularly effective and sophisticated in manipulating the production of pro-inflammatory cytokines IL-1β and IL-18, which are typically processed into their mature forms by active caspase-1 or in some cases caspase-8. The *Y. pestis* T3SS initiates caspase-1 activation by at least three different pathways. In the weakest pathway, the effector YopJ triggers caspase-8- dependent caspase-1 activation; additionally, the T3SS needle/translocon activates NLRP3 and NLRC4-dependent caspase-1 maturation, which is blocked by YopK but not YopM. Importantly, we show here that YopM specifically prevents activation of the Pyrin inflammasome by the RhoA-inhibiting effector YopE, blocking caspase-1 dependent IL-1β/IL-18 production and cell death. We propose a model in which YopM and its binding partners PKN1 and RSK1 kinases interact with Pyrin, with potential roles in its regulation that were not previously considered. Thus, we introduce a novel regulatory pathway for the Pyrin inflammasome which is exploited by a plague effector protein for maximal immune evasion.

Introduction

We and others have previously demonstrated that a key strategy of *Yersinia pestis*, the causative agent of plague, is to actively suppress production of IL-1β and IL-18 by multiple combined mechanisms in order to promote virulence and favor bacterial survival[118, 225, 255, 257, 312]. *Yersinia* can activate the NLRP3, NLRC4, and NLRP12

inflammasomes[118, 255], as well as a non-canonical caspase-8 pathway[11, 124]. However, the expression of IL-1β and IL-18 is effectively suppressed by the injected Yops. The discrepancy between the large number of inflammasome pathways being activated and the small number of Yops sufficient to shut down production of IL-1β and IL-18 substantiates the impressive efficiency of this system (Fig 3.1).

Aside from the high virulence conferred by this small effector toolkit, we believe that the small number of Yops makes *Yersinia* an excellent model for characterizing T3SS functions. Since the survival strategy of *Y. pestis* appears heavily dedicated towards immune evasion and suppression, we posited that mapping the mechanisms by which the *Y. pestis* T3SS activates and inhibits inflammasomes is an auspicious long-term aim.

The T3SS is required for *Y. pestis* virulence, but it also triggers inflammasome activation which is critical for controlling *Y. pestis* infection[17, 118, 225]. One of the strongest drivers of inflammasome activation may be the needle/translocon itself (Fig 2.1B/C, 2.5B). Studies in *Y. pseudotuberculosis* show that NLRP3 and NLRC4 may be activated by the hypertranslocation of the needle/translocon, and possibly by increased delivery rate of Yop effectors[255, 256]; this activation can be prevented by the effector YopK. Consistent with this, we observed that the needle/translocon encoded by the pCD1 plasmid strongly triggers IL-1β and IL-18 in response to *Y. pestis* lacking the seven translocated Yops (we refer to this strain as ΔT3SSe).

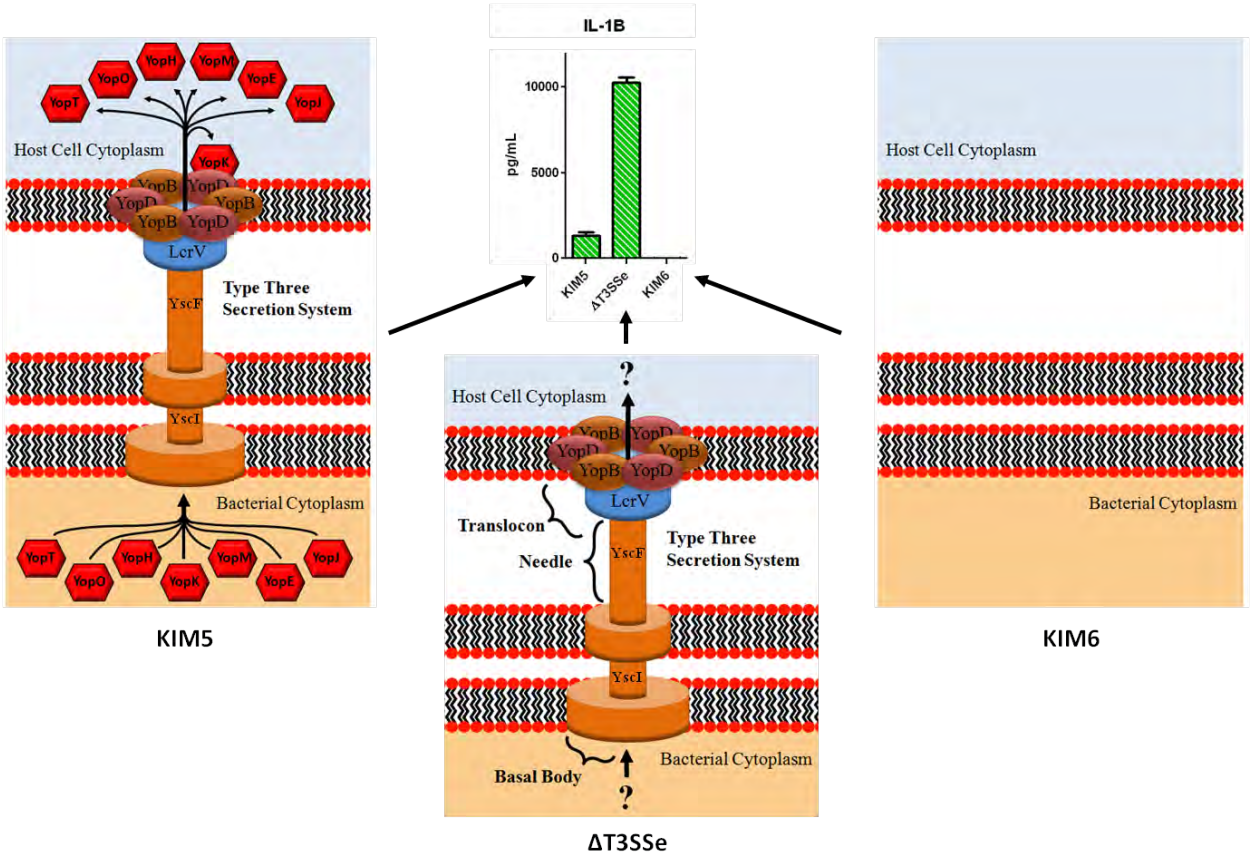


Figure 3.1. The seven tranlocated Yops of *Y. pestis* effectively suppress the IL-1β in response to the needle/translocon to near-zero levels, and confer a high degree of virulence to the pathogen (see Experimental Procedures for strain construction details).

Previously we showed that loss of YopM and YopJ results in high levels of active caspase-1 and IL-1β comparable to ΔT3SSe. Thus, we were interested to investigate whether YopM and/or YopJ would also be sufficient to suppress IL-1β and inflammasome activity if added back to the ΔT3SSe background. YopJ triggers apoptosis mediated by caspase-8 and RIP1, and also caspase-8-dependent caspase-1 processing which leads to a small amount of IL-1β secretion (Fig2.1)[11]. However, YopJ can limit overall production of IL-1β by inhibiting expression of pro-IL-1β precursor (Fig 2.2). YopM was originally proposed to be a direct caspase-1 inhibitor[257], although Chung et

al recently advanced an alternative mechanism for YopM inhibition of caspase-1 which relies on the cytoskeletal scaffolding protein Iqgap1.

We expected that if YopM is a general caspase-1 inhibitor as proposed by LaRock and Cookson[257], then YopM would suppress much of the IL-1β produced in response to the ΔT3SSe strain, save for any non-canonical pathways that do not rely on caspase-1. With regards to YopJ, we expected it to partially inhibit IL-1β production through suppression of transcription, and to partially contribute to IL-1β release through caspase-8-activation. In conjunction, we expected YopM and YopJ to complement one another and bring IL-1β levels down potentially as low as typically seen with the parental *Y. pestis* strain (KIM5).

Instead, we were surprised to discover that YopM is unable to inhibit caspase-1 activation triggered through NLRP3, NLRC4, or caspase-8, but instead it specifically inhibits another signal occurring through a Pyrin-dependent pathway. We identified the effector YopE as the trigger for Pyrin in the absence of YopM, and characterized some key mechanistic features of how this pathway is regulated during *Y. pestis* infection.

Results

YopK, but not YopM, keeps NLRP3 and NLRC4 activation by the Y. pestis needle/translocon in check. We began this investigation first by confirming the dependence of needle/translocon-triggered IL-1β secretion on NLRP3 and NLRC4, based on studies in *Y. pseudotuberculosis* indicating this to be the case[255, 256]. As we discussed in chapter 1, many T3SS bacteria activate NLRC4 via conserved homologues

of the inner rod protein PrgJ and via flagellin, if expressed. Unlike many of its relatives, *Y. pestis* does not express flagellin; however its inner rod protein YscI is a PrgJ homolog, and is thus a potential NAIP-2 dependent activator of NLRC4.

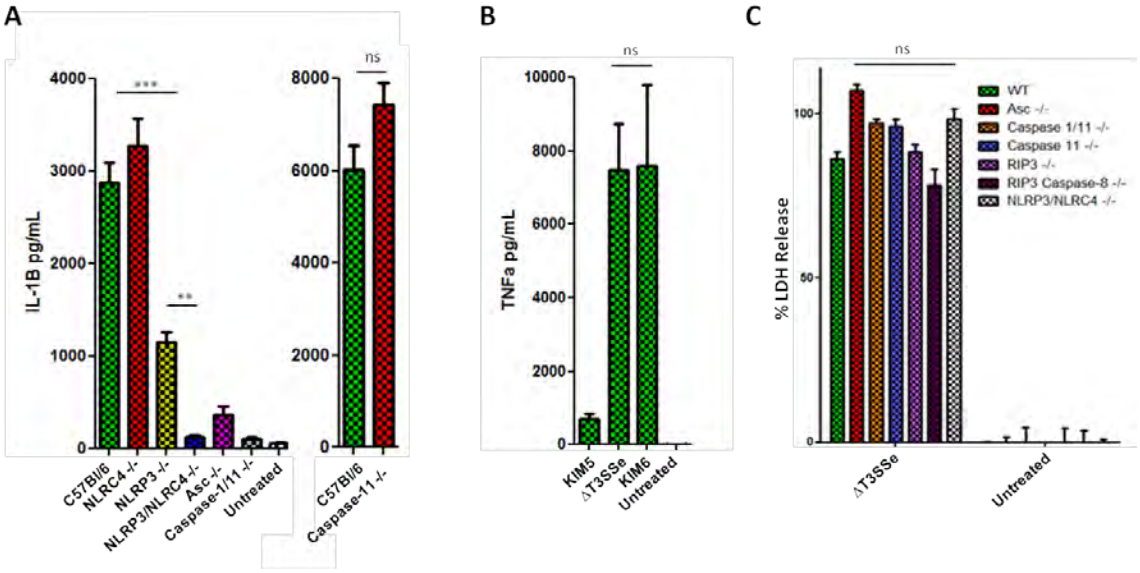


Figure 3.2. The *Y. pestis* needle/translocon triggers robust IL-1β production via NLRP3 and NLRC4, but cell death occurs independently of inflammasomes or the known pro-inflammatory caspases, through an undetermined pathway. A) Unprimed BMDMs of indicated genotypes were infected with *Y. pestis* strain ΔT3SSe (containing a functional secretion structure but lacking secreted effectors) at MOI 10, and supernatants were harvested at 6 hours p.i. for analysis of IL-1β. B) Unprimed wild-type BMDMs were infected with KIM5 (complete T3SS), ΔT3SSe, or KIM6 (lacking the whole T3SS) at MOI 10 and supernatants were analyzed for TNFa by ELISA. C) Unprimed BMDMs of indicated genotypes were infected with ΔT3SSe for measurement of LDH release to assay cell death. Figures are representative of three or more experiments. Shown is mean plus s.d. for triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

We observed a strong IL-1β signal in response to ΔT3SSe, to which NLRP3 and NLRC4 both partially contributed(Fig 3.2A). This signal vanished entirely in BMDMs lacking both NLRP3 and NLRC4. Caspase-1 and Asc, but not caspase-11 were required for the IL-1β response to ΔT3SSe; however, a small amount of IL-1β was still produced

in the absence of Asc, consistent with the fact that NLRC4 is able to directly recruit caspase-1 via its CARD domain. We also measured TNFa production as an additional control to confirm that the presence of the needle/translocon in ΔT3SSe did not significantly impact baseline cytokines compared to the KIM6 strain, lacking all T3SS components (Fig 3.2B).

We expected this apparent robust inflammasome activity to be accompanied by caspase-1 dependent cell death (pyroptosis); indeed, infection with ΔT3SSe unequivocally results in rapid cytotoxicity, but to our surprise this death pathway was not dependent on caspase-1, NLRP3, NLRC4, Asc, caspase-11, or RIP3/Caspase-8 (Fig3.1C). We are currently investigating which alternative cell death pathway that would be accompanied by such dramatic inflammasome-driven IL-1β release. Notwithstanding the fascinating implications of this finding, we concluded that the NLRP3 and NLRC4 inflammasomes are responsible for the entire IL-1β release in response to the *Y. pestis* needle/translocon.

We next investigated whether reconstituting endogenous levels of YopM or YopJ alone on a ΔT3SSe background (inserted back into the original pCD1 plasmid) would inhibit inflammasome activation and IL-1β release. While ΔT3SSe + YopJ demonstrated strong but incomplete inhibition of IL-1β, perhaps reflecting both inhibition of transcription and some activation of the caspase-8-dependent IL-1β pathway, ΔT3SSe + YopM surprisingly had no effect (Fig 3.3A). This is inconsistent with the hypothesis that YopM is a general an inhibitor of caspase-1[257], and it prompted us to explore the role of YopM more closely in subsequent experiments.

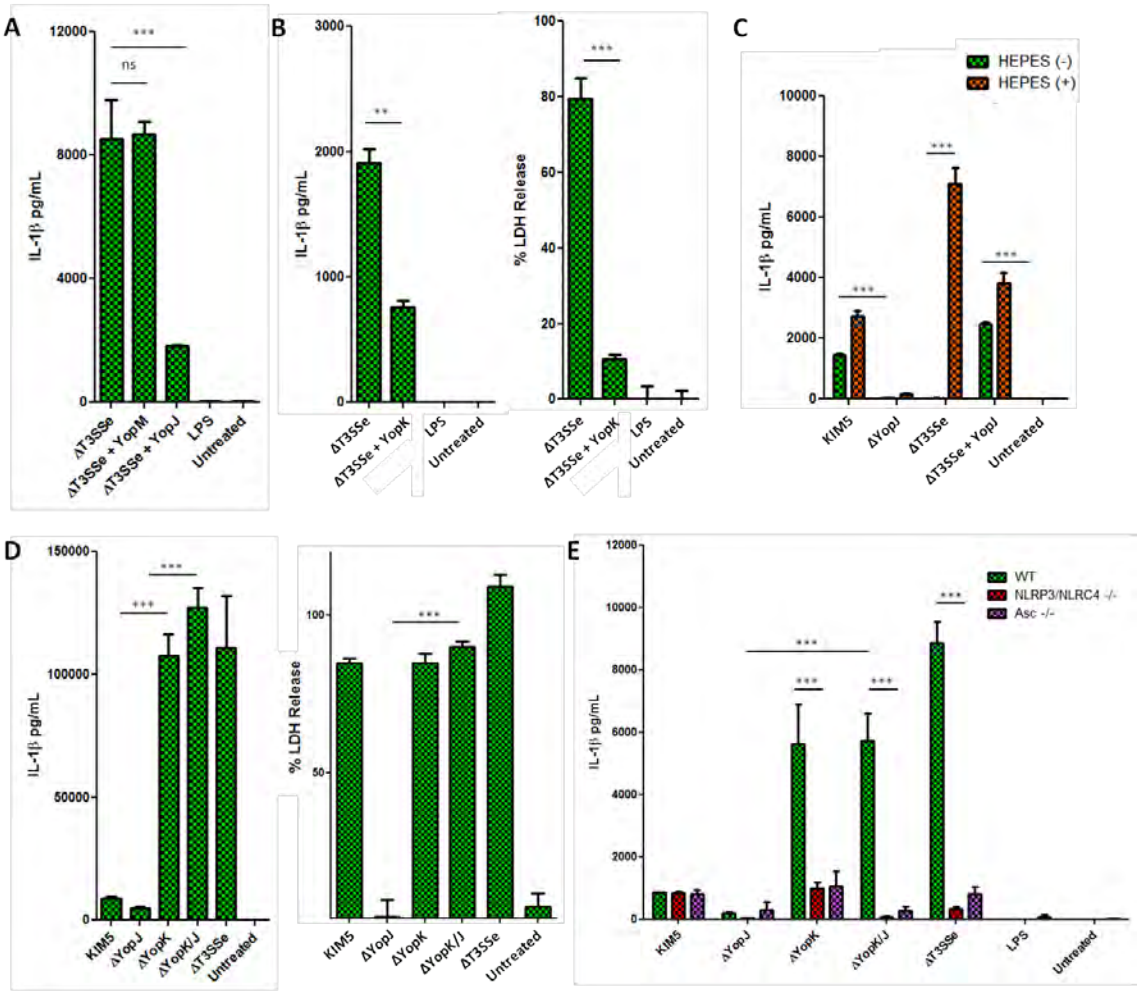


Figure 3-3. The NLRP3/NLRC4-driven IL-1β secretion in response to the *Y. pestis* secretion system is partially suppressed by YopJ, unaffected by YopM, and effectively inhibited by YopK. A-C) BMDMs or D) BMDCs were infected with indicated *Y. pestis* strains at MOI 10 and supernatants were assayed at 6 hours p.i. for IL-1β by ELISA, or for LDH to measure cell death. C) Infections were carried out in media with or without HEPES buffering to demonstrate the pH dependence of the T3SS-activated pathway. E) Shown are IL-1β levels secreted by unprimed BMDMs infected with *Y. pestis* strains lacking YopJ, YopK, or both. Figures are representative of three or more experiments. Shown is mean plus s.d. for triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

As shown in chapter 2, KIM5 lacking YopJ triggers no detectable IL-1β in macrophages. Therefore, if YopJ is not required to completely inhibit the IL-1β response to the needle/translocon (even though it is independently capable of considerably

reducing it), and YopM is not able to, we reasoned that a different Yop must play the dominant role in this inhibition. An earlier report suggests YopK may limit NLRP3 activation, either by preventing hypertranslocation of the pore-forming complex or the hyperinjection of Yops[255, 256], thus we tested responses to the add-back strain ΔT3SSe + YopK. Indeed, YopK reduces ΔT3SSe-triggered IL-1β at least as effectively as YopJ, and also prevents the inflammasome-independent cell death pathway(Fig 3-2B). This is also evident when YopK is deleted from a KIM5 or KIM5 ΔYopJ background(Fig 3-2C), with a sharp rise in YopJ-independent cytotoxicity and IL-1β production. This IL-1β production in response to infection with KIM5 ΔYopK was eliminated in NLRP3/NLRC4 deficient cells (Fig 3-2D). We therefore concluded that YopK is both necessary and sufficient to block inflammasome activation by the *Y. pestis* needle/translocon.

Incidentally, we found that both the inflammasome activation and cell death induced by the needle/translocon are dependent on pH, as lack of buffering by HEPES completely prevented IL-1β secretion(Fig 3.3E) and cytotoxicity(not shown). However, HEPES buffering was not required for IL-1β secretion in response to YopJ (Fig 3.3E), and did not have any remarkable effect on other cytokines such as TNFa (not shown). This is an interesting phenomenon with plausible explanations[196, 199, 313, 314] and important implications in disease[201-203], and deserves a separate inquiry.

YopM specifically inhibits the Pyrin inflammasome, which is activated by YopE

Both we and others have shown that YopM is an inhibitor of caspase-1 activation [257, 258, 312, 315], however our data suggests it is not able to inhibit all types of

caspase-1 activating pathways. KIM5 lacking YopM triggers increased levels of active caspase-1 and IL-1β, indicating that YopM suppresses a bacteria-triggered inflammasome pathway. To identify the pathway inhibited by YopM, we tested whether BMDMs lacking specific inflammasome components would have reduced IL-1β production when YopM is absent (Fig 3.4A). Although YopM inhibits a pathway dependent on Asc and caspase-1, we did not observe any reduction of IL-1β in cells lacking NLRP3, NLRC4, NLRP12, or caspase-11. We also tested cells lacking AIM2, NALP6, RIP3, or caspase-8 (not shown), and found none of them to be required for the IL-1β producing pathway which YopM suppresses. The same pattern was observed for cell death (Fig 3.4B); YopM inhibits caspase-1 dependent cell death (pyroptosis) as we described in chapter 2, but this cell death still occurs in the absence of the inflammasomes tested.

We took a comprehensive approach to cross-analyze how the relative interactions between YopM, YopJ, the needle/translocon, and different inflammasome pathways impact caspase-1 and IL-1β levels. We performed experiments directly comparing all of these factors (Fig 3.5). In general, mature IL-1β and caspase-1 closely reflected ELISA data in Fig 3.2 and Fig 3.4, and agreed with the findings presented in chapter 2 (Fig 2.1). YopJ suppresses pro-IL-1β and pro-caspase-1 production, and triggers a small amount of IL-1β processing in a caspase-8-dependent manner. Notably, caspase-1 and Asc are not

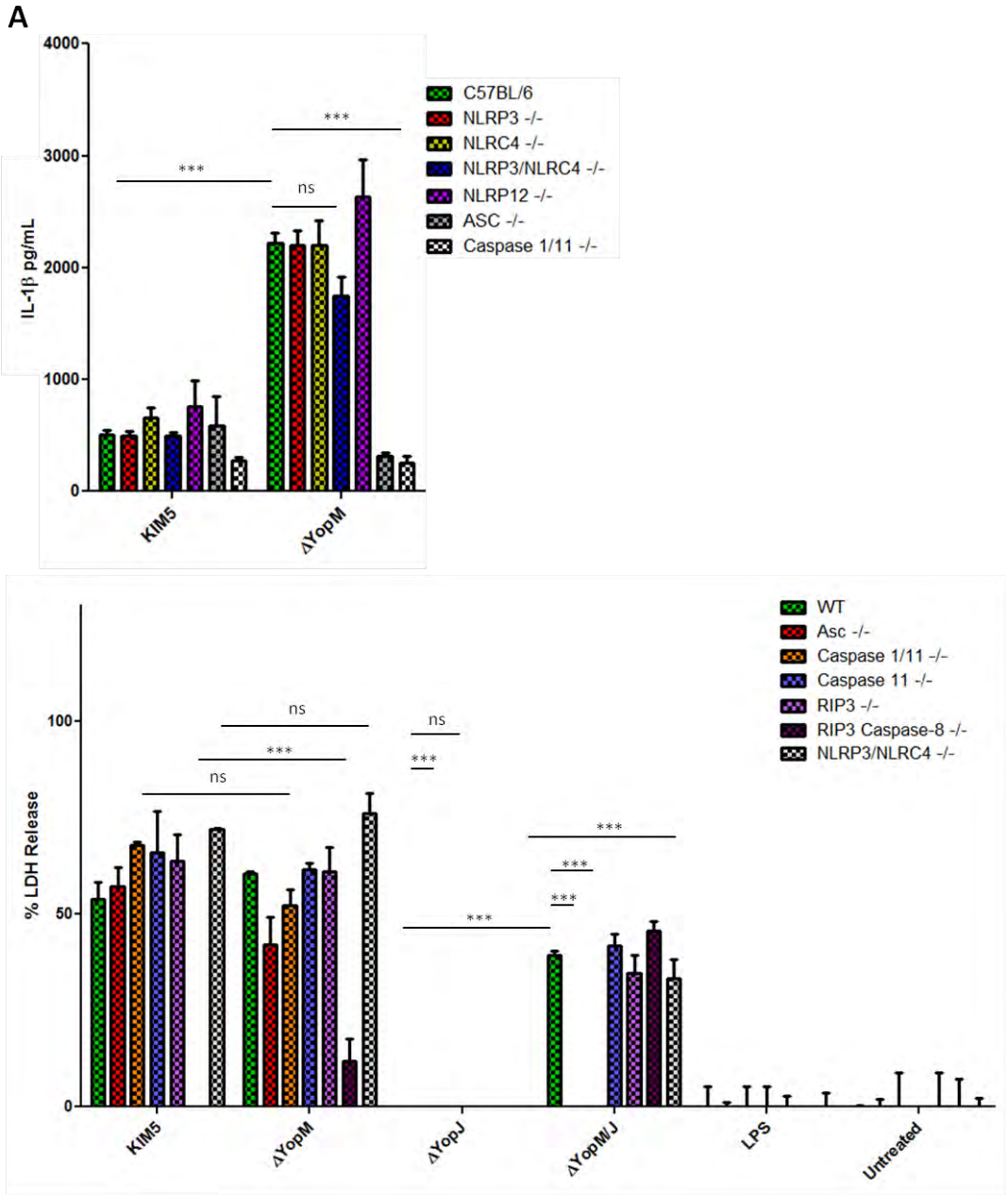


Figure 3-4. YopM is not a general caspase-1 inhibitor, but instead suppresses an inflammasome pathway which is dependent on caspase-1 and Asc, but not on NLRP3, NLRC4, caspase-11, or caspase-8. Primed BMDMs were infected with indicated *Y. pestis* strains at MOI 10 and supernatants were assayed at 6 hours p.i. for A) IL-1β by ELISA, or for B) LDH to measure cell death. Figures are representative of three or more experiments. Shown is mean plus s.d. for triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

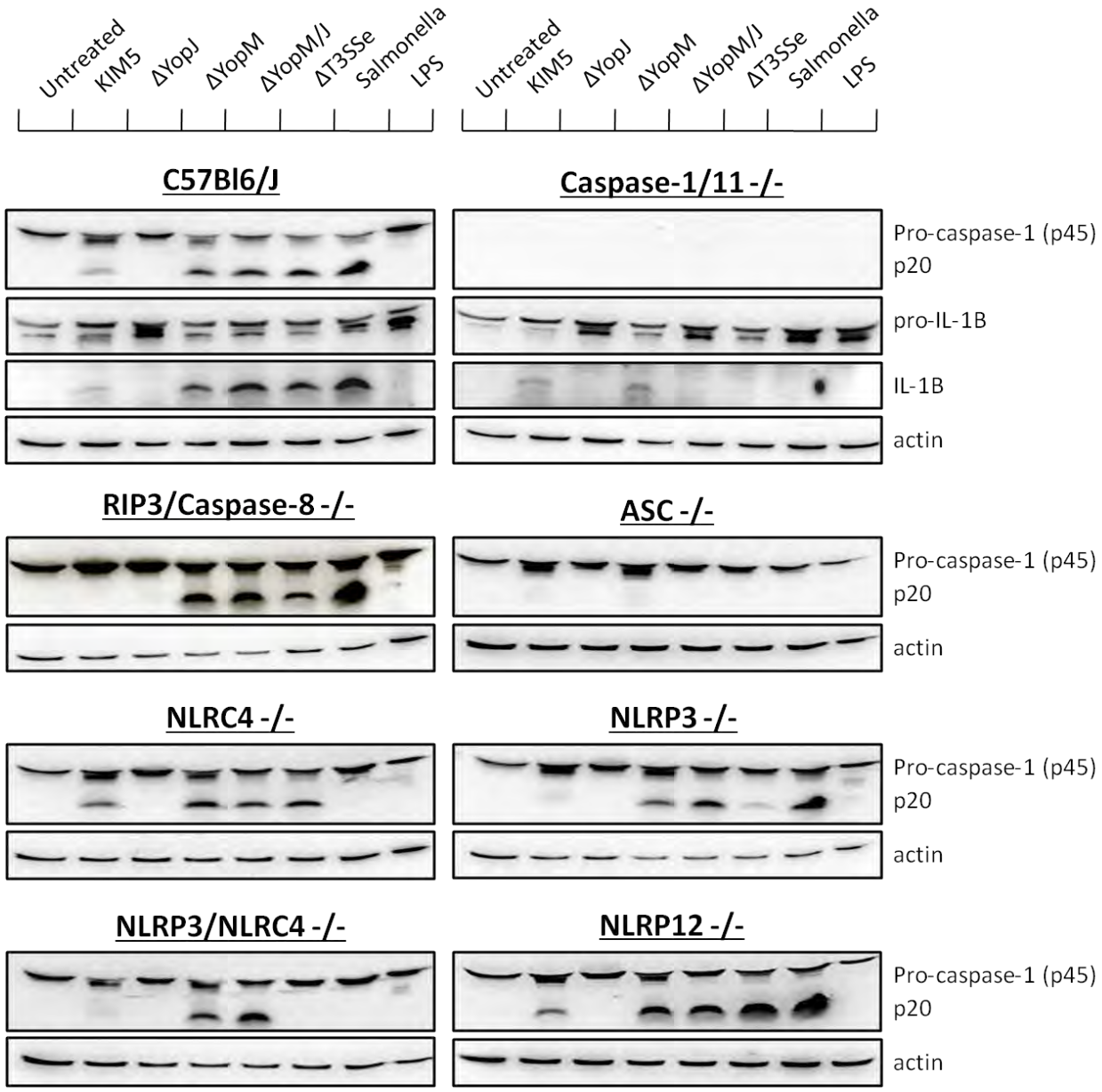


Figure 3-5. Primed BMDMs of indicated genotypes were infected with *Y. pestis* lacking YopM, YopJ, YopM and YopJ, or all seven translocated Yops (ΔT3SSe) at MOI 10 for 6 hours. Total protein from samples (combined cell lysate and supernatant) was separated by SDS-PAGE and analyzed by Western Blot for IL-1β and caspase-1. The banding pattern indicates that YopM inhibits caspase-1 activation triggered by a nother Yop effector through an Asc-dependent pathway which is not mediated by Y opJ-induced caspase-8, nor the by the ΔT3SSe-induced NLRP3 and NLRC4 inflammasomes, nor NLRP12.

required for caspase-8 dependent IL-1β processing in response to YopJ. Caspase-1 activation by the needle/translocon (ΔT3SSe) is fully dependent on NLRP3/NLRC4, while YopM inhibits caspase-1 processing independently of NLRP3, NLRC4, NLRP12, RIP3, or caspase-8. It is however a noteworthy point that in NLRP3/NLRC4 deficient cells, KIM5 ΔYopM/J triggers a substantial amount of caspase-1 and IL-1β processing while ΔT3SSe (which lacks the other 5 translocated Yops) does not; this indicates that YopM inhibits an Asc-dependent inflammasome triggered by another Yop effector.

To identify the effector triggering the unknown inflammasome blocked by YopM, candidate Yops were deleted from the KIM5 ΔYopM background. When we tested this strategy in wild-type BMDMs and BMDMs lacking Pyrin - one of the few remaining Asc-dependent inflammasomes which we had not yet evaluated - we found that the IL-1β signal inhibited by YopM is fully dependent on Pyrin, and appears to be triggered by YopE (Fig 3.6A). By contrast, TNFa secretion was not appreciably impacted (Fig 3.6B). Pyroptosis (Fig 3.6C) and caspase-1 activation (Fig 3.6D) associated with ΔYopM were also abolished in the absence of Pyrin.

We observed a significant Pyrin-dependent reduction in secreted IL-1β in response to ΔT3SSe + YopE, but not to ΔT3SSe alone (Fig 3.6E). However, it is also possible that YopE may inhibit NLRP3/NLRC4-dependent IL-1β production, consistent with a report that YopE may suppress caspase-1 activation in some settings[20]. Thus, to clarify the inflammasome-activating role of YopE, we minimized the contribution of the needle/translocon to the IL-1β signal by conducting infections in NLRP3/NLRC4 deficient cells, or in the presence of YopK (Fig 3.6F). Under these conditions, we

observed unambiguous YopE-dependent IL-1β secretion and cell death, which was suppressed in the presence of YopM. The Pyrin-activating compound, TcdB, was used as a control to demonstrate that NLRP3/NLRC4 deficient cells are not impaired in the ability to activate Pyrin (Fig 3.6G).

We and others have found it is necessary to prime macrophages with LPS in order to see increased IL-1β secretion in the absence of YopM[257, 258, 312, 315]. This is somewhat counter-intuitive, since *Y. pestis* switches to production of non-stimulatory tetra-acylated LPS during infection, and we wondered what parallel such priming may have *in vivo*. We therefore experimented with the timing, dose, and agent used in priming to gain better insight into these mechanisms. In contrast to NLRP3-dependent responses where 3 hours of priming is typically used, we found that the Pyrin-dependent pathway in our system required at least 5 hours of priming to achieve a phenotype(Fig 3.7A). Importantly, use of heat-killed KIM5 instead of LPS also resulted in effective priming, even when the bacteria were grown at 37°C to express tetra-acylated LPS (Fig 3.7B). In this context, it is reasonable to imagine a situation where innate immune cells become primed upon encountering dead bacteria or debris either in the circulation or at the site of an infected lymph node.

Priming also up-regulates pro-IL-1β, and Pyrin itself (Fig 3.7C). This is significant because the baseline level of Pyrin in macrophages is very low[128, 218]. Furthermore, macrophages lacking the transcription factor C/EBPbeta were unable to produce IL-1β specifically in response to KIM5ΔYopM or KIM5ΔYopM/J (data not shown), consistent with the fact that transcription of Pyrin is controlled by

C/EBPbeta[316]. Interestingly, many C/EBPbeta-dependent genes begin to be expressed only 4-8 hours after stimulation with LPS[317]. It is also worth noting that without priming, KIM5ΔYopM produces IL-1β indistinguishable from KIM5, whereas KIM5ΔYopM/J triggers significantly elevated levels of IL-1β (data not shown). This indicates that YopJ suppresses priming that occurs during the course of the 6-hour infection, either by inhibiting NFkB-mediated gene expression, or by inducing apoptosis before sufficient priming can occur.

Pyrin interacts with YopM, Pkn1, Rsk1, Iqgap1, and is controlled by 14-3-3ε

Next we aimed to characterize the mechanism by which YopM inhibits the Pyrin inflammasome. Using a *Yersinia pseudotuberculosis* model reconstituted with YopM mutants on a ΔYopM background[310], we determined that the C-terminus of YopM is needed to inhibit Pyrin-mediated IL-1β release and caspase-1 processing (Fig 3.8). This is also the region of YopM necessary for interaction with Rsk1, one of its known binding partners[260].

Because human pyrin and mouse pyrin exhibit some important differences in structure and function, we wanted to confirm whether YopM is capable of inhibiting Pyrin in human cells. To do this, we isolated human donor peripheral blood mononuclear cells (PBMCs) from whole blood and infected them with *Y. pestis* using our standard *in vitro* infection protocol (Fig 3.9A). For this experiment, cells were not primed as PBMCs are known to produce IL-1β in response to LPS alone in an ERK-dependent manner[318]. Consistent with this, we observed powerful inhibition of IL-1β secretion by YopJ - the

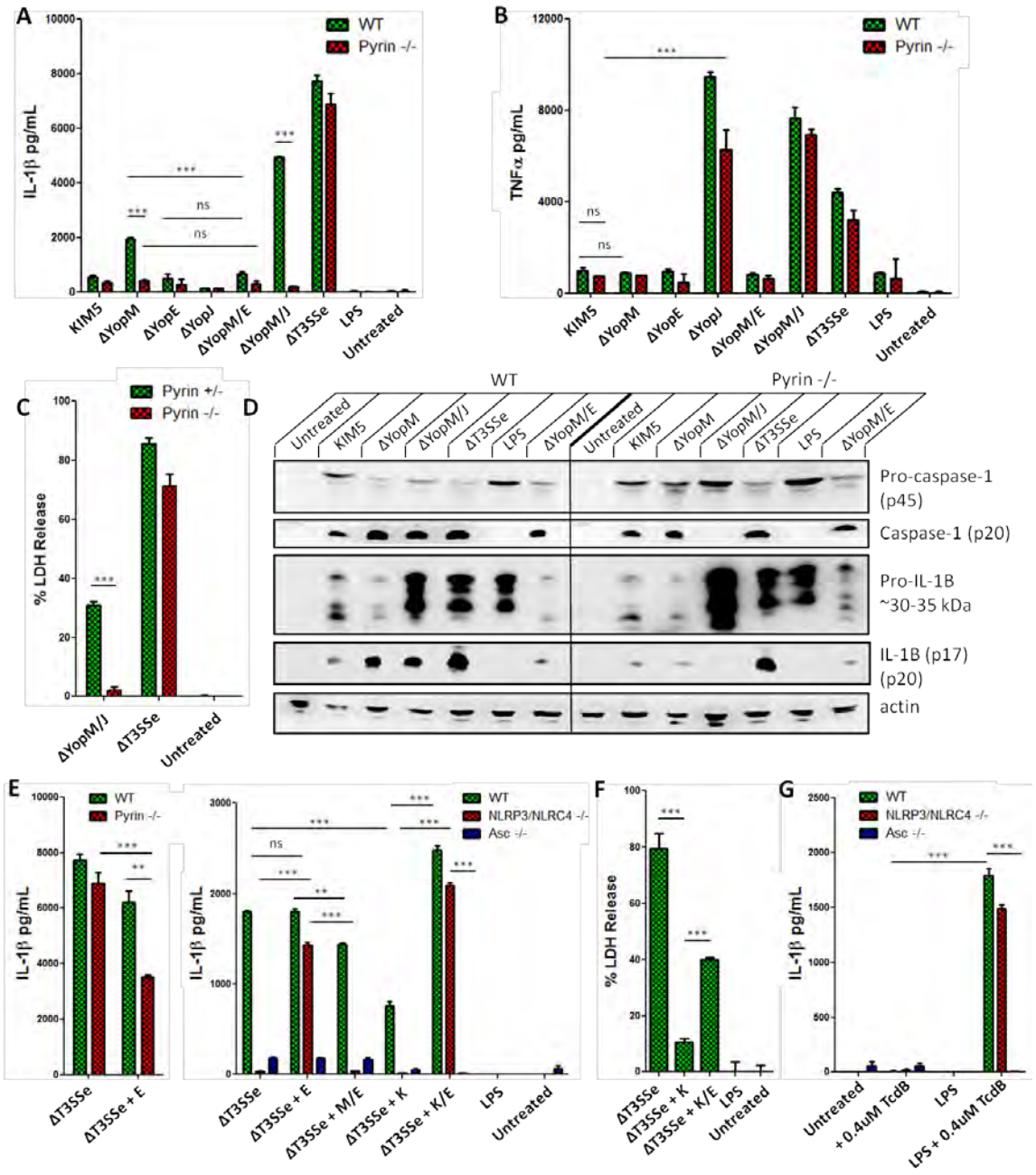

A
IL-1β pg/mL
WT
Pyrin -/-
KIM5
ΔYopM
ΔYopE
ΔYopJ
ΔYopM/E
ΔYopM/J
ΔT3SSe
LPS
Untreated
B
TNF-α pg/mL
C
% LDH Release
Pyrin +/-
Pyrin -/-
D
WT
Pyrin -/-
Pro-caspase-1 (p45)
Caspase-1 (p20)
Pro-IL-1B ~30-35 kDa
IL-1B (p17) (p20)
actin
E
NLRP3/NLRC4 -/-
Asc -/-
ΔT3SSe + E
ΔT3SSe + M/E
ΔT3SSe + K
ΔT3SSe + K/E
F
ΔT3SSe + K
G
+ 0.4uM TcdB
LPS + 0.4uM TcdB

Figure 3-6. YopM specifically silences the Pyrin inflammasome, which is activated in the presence of the effector YopE. Shown are supernatant cytokines (A, B, E), cytotoxicity as measured by LDH release (C, F), and maturation of caspase-1 and IL-1β by Western blot (D) in primed BMDMs after 6 hours of infection with indicated strains of *Y. pestis*. The elevation in A) secreted IL-1β, C) cell death, and D) mature caspase-1 in the absence of YopM disappears entirely when either Pyrin or YopE are also absent, whereas the relative levels of B) TNFa as well as D) caspase-1 and IL-1β proforms are not significantly impacted. E) *Y. pestis* strains expressing YopE on a ΔT3SSe background in combination with YopK or YopM were added to wild-type or NLRP3/NLRC4 deficient cells respectively, in order to reduce confounding needle/translocon-driven activation and isolate YopE-driven E) IL-1β and F) cell death pathways. G) Primed cells treated with 0.4uM TcdB, a Rho-GTPase inhibitor known to activate Pyrin, to mimic YopE. Figures are representative of multiple experiments with similar results. Shown is mean plus s.d. for triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

reverse of what we observed in macrophages, but in line with the known role of YopJ in suppressing the MAPK2 upstream of ERK. Nevertheless, we confirmed that YopM also contributes to inhibition of IL-1β secretion in human PBMCs.

We next tested the effect of YopM in a monocytic human THP-1 cell line overexpressing YFP-Pyrin, and found an IL-1β secretion pattern generally comparable to human PBMCs, where YopM is also capable of inhibiting the Pyrin-mediated IL-1β production (Fig 3.9B). Therefore, we used human THP-1 cells overexpressing YFP-Pyrin as a tool for biochemical analysis of how YopM or its known bindings partners (Rsk1 and Pkn1) interacts with the Pyrin pathway[259, 260, 310]. Rsk1, and recently the cytoskeletal scaffolding protein Iqgap1, have been suggested to be important for caspase-1 inhibition by YopM[258]. Co-IP in these cells using anti-GFP antibody revealed Pyrin interaction with Pkn1 as late as 6 hours p.i., but not in the siPyrin knockdown control (Fig 3.9C). In a separate immunoprecipitation using either anti-Pkn1 antibody and anti-GFP antibody at 3 hours p.i., we confirmed interaction between Pkn1 and Pyrin in the

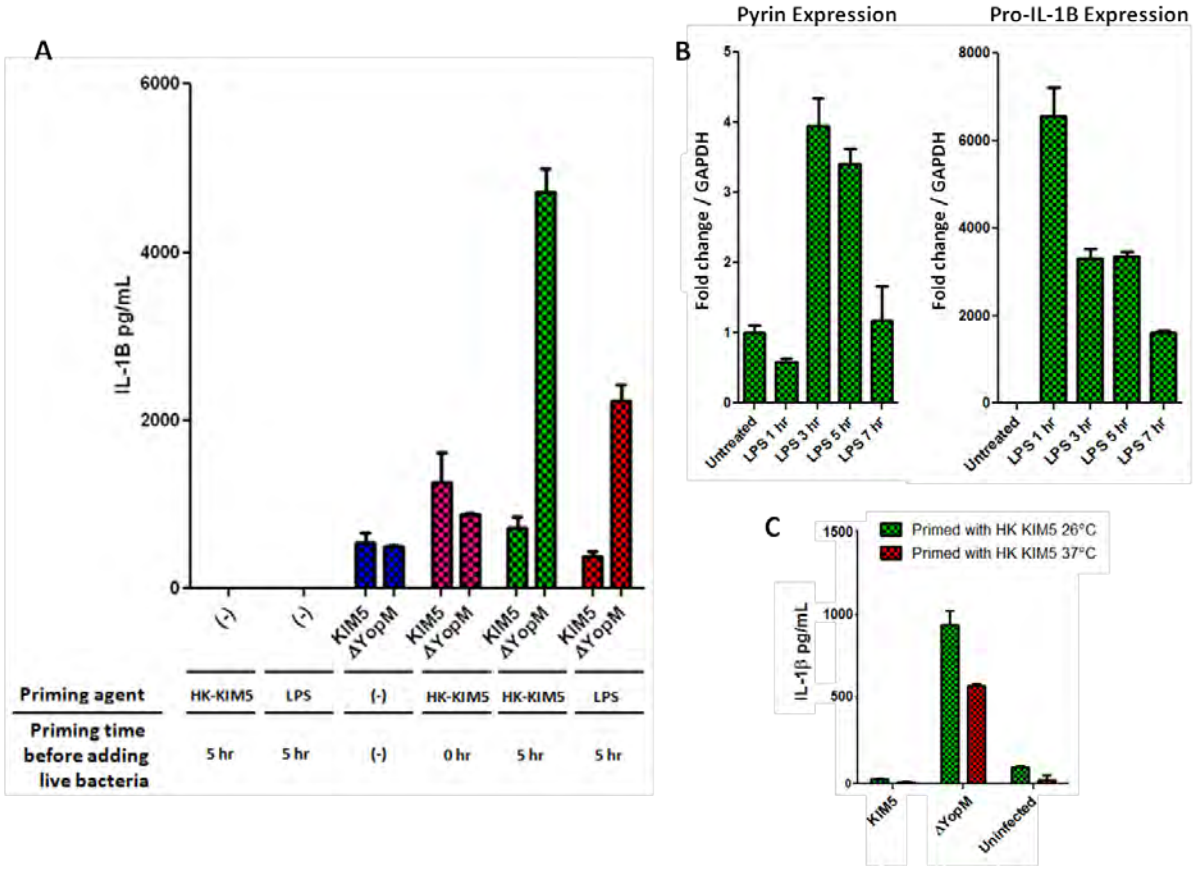


Figure 3-7. Robust activation of the Pyrin pathway requires priming. Priming is necessary to upregulate weakly expressed components, including Pyrin itself. A) 100ng/mL LPS or $1x10^8$ CFU equivalents of heat-killed KIM5 was added to BMDMs either 5 hours before, or simultaneously with live KIM5 or ΔYopM at MOI 10. Supernatant from 6 hours p.i. was assayed for IL-1β by ELISA. B) RNA from BMDMs was harvested at the indicated hours after addition of 100ng/mL LPS, and amplified by RT-PCR using primers specific for Pro-IL-1β or Pyrin. C(t) values for each primer pair were normalized to GAPDH internal control C(t) values, and untreated controls. C) Priming can be achieved with heat-killed *Y. pestis* regardless of whether it is grown at 26°C or 37°C, despite expression of non-stimulatory tetra-acylated LPS.

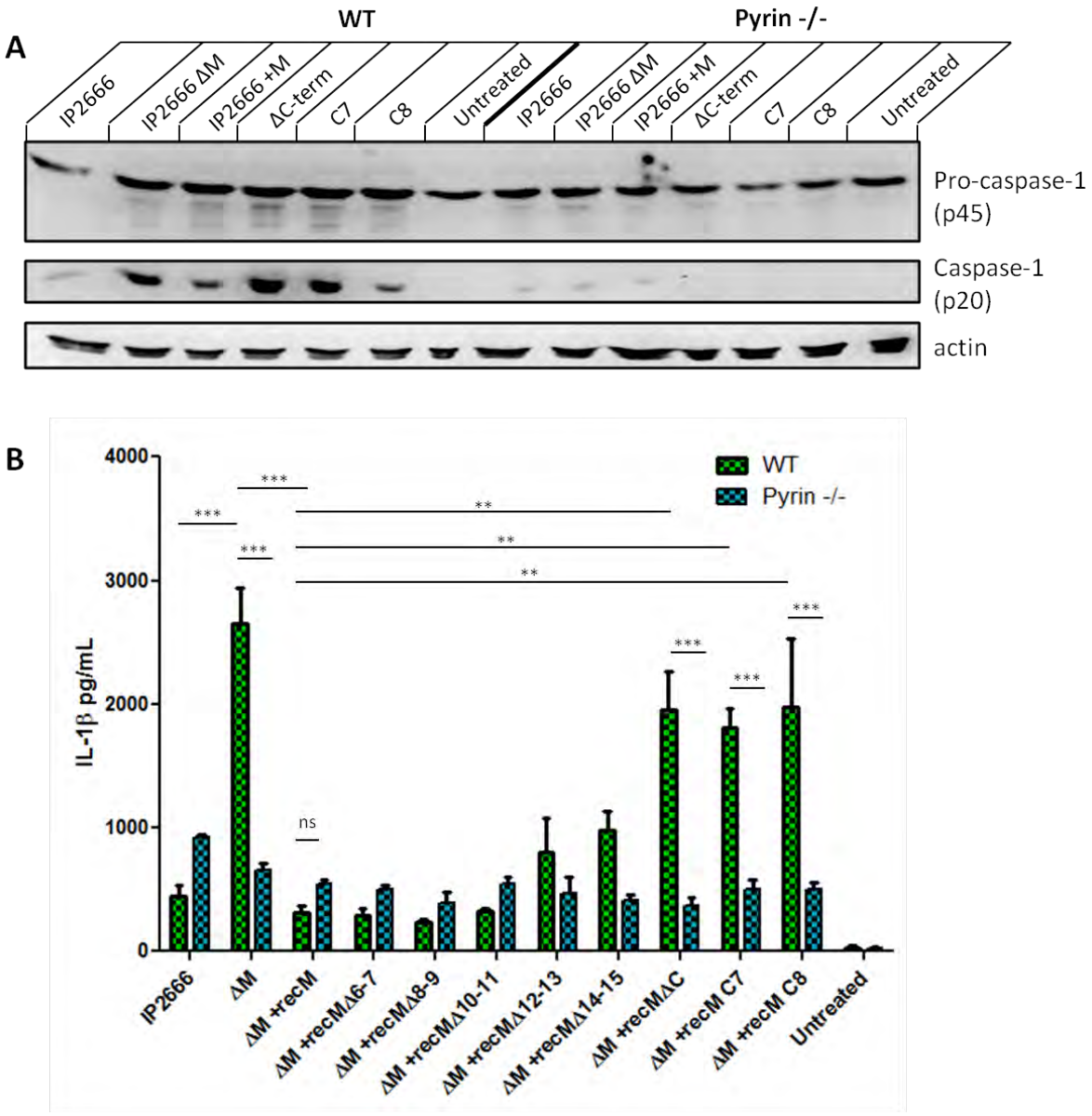


Figure 3-8. Deletions of sequences mapping to the C-terminus of YopM impair its ability to inhibit Pyrin. A-B) Wild-type and Pyrin -/- BMDMs were primed and infected with *Yersinia pseudotuberculosis* ΔYopM strains reconstituted with YopM containing sequential deletions of its LRRs, at MOI 10. A) At 6 hours p.i. protein from samples (combined cell lysate and supernatant) was separated by SDS-PAGE and probed for caspase-1 by Western Blot. B) A fraction of the supernatant was kept and assayed for IL-1β by ELISA.

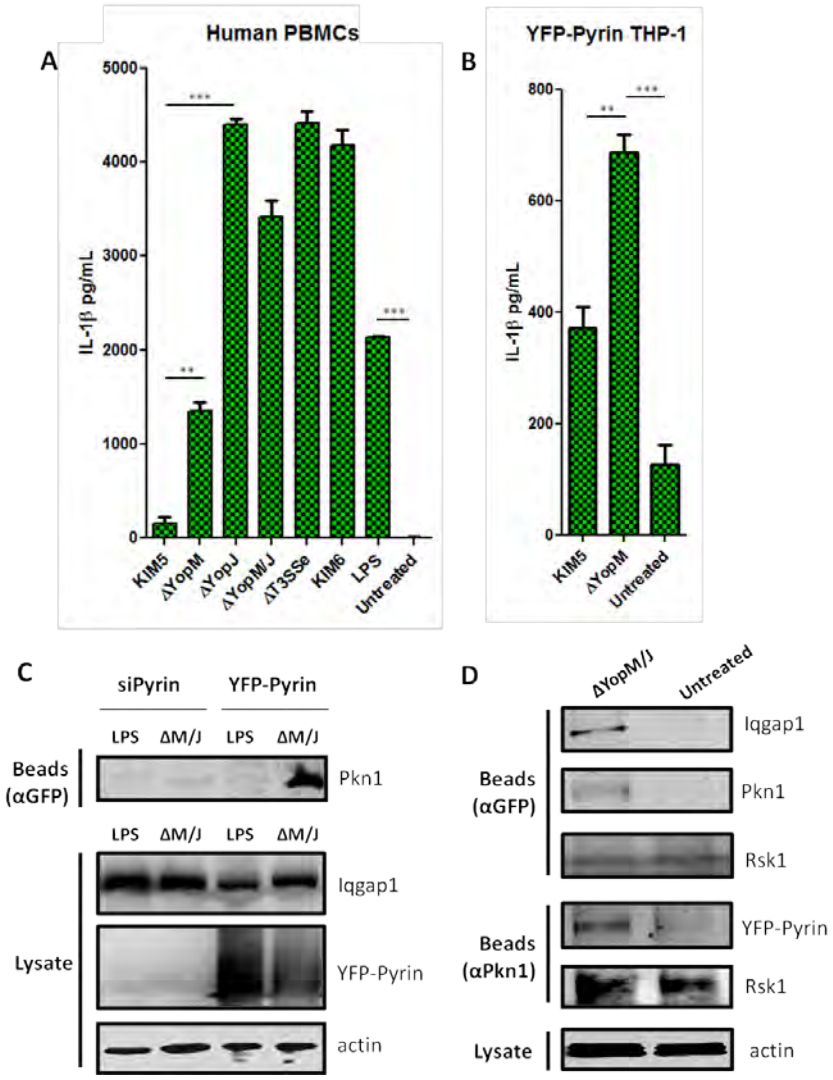


Figure 3-9. YopM maintains an inhibitory phenotype in human PBMCs, and in a human THP-1 cell line overexpressing YFP-Pyrin. Co-IP pulldowns in these cells indicate Pyrin interaction with Pkn1, Rsk1, and Iqgap1. A) PBMCs were isolated from healthy human donor blood and infected at MOI 10 with indicated *Y. pestis* strains without priming. At 6 hours p.i. supernatant was collected for IL-1β detection by ELISA. B-D) Cultured YFP-Pyrin and siPyrin THP-1 cells were differentiated with 100nM Vitamin D3 for 48-72 hours, and either B-C) primed with 100ng/mL LPS for 5 hours before infection or D) infected without priming with indicated *Y. pestis* strains at MOI 10. Shown in B) is IL-1β assayed from supernatants by ELISA. C) cells were harvested at 6 or D) 3 hours p.i., lysed, and either anti-GFP or anti-Pkn1 were used for IP pull-down with protein G beads. Following the IP protocol, bead-bound protein and lysates were separated by SDS-PAGE and analyzed by Western Blot for the proteins indicated.

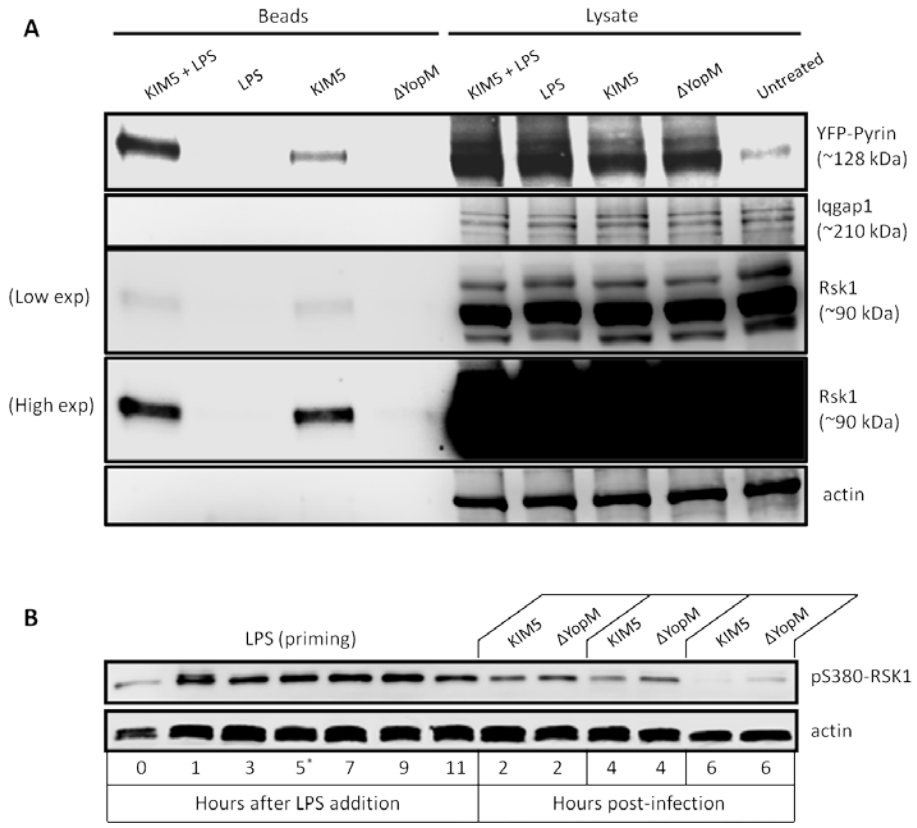


Figure 3-10. YopM interacts with Pyrin and Rsk1 during *Y. pestis* infection in YFP-Pyrin THP-1 cells, and may influence Rsk1 S380-dependent activity. A) Shown are Western blot results of co-IP with anti-YopM using $1x10^7$ Vitamin D3-differentiated, unprimed YFP-Pyrin cells after infection with the indicated strains at MOI 10 for 3 hours. B) BMDMs were primed for 5 hours and infected with either KIM5 or ΔYopM at MOI 10 for 6 hours (hence 11 hours total in LPS). Time course samples were harvested at 2 hour intervals, including the priming stage. Shown is a Western blot detailing the impact of priming and infection on the phosphorylation status of Rsk1-S380, in the presence of absence of YopM.

bound fraction of both immunoprecipitations. We found Iqgap1 interacting with Pyrin in the presence of bacteria, while Rsk1 was detected to interact with both Pyrin and Pkn1 even in the absence of infection (Fig 3.9D).

Pull-down assays in YFP-Pyrin cells using anti-YopM antibody showed that YopM interacts with Rsk1 as expected, although we were unable to detect interaction

with Pkn1 or Iqgap1 (Fig 3.10A). However, we did detect Pyrin in the bound fraction, suggesting that it may directly or indirectly interact with YopM. YopM is known to interact with Rsk1 and Pkn1, and has been shown to prevent dephoshophorylation of Rsk1-S380 in HEK293 cells, keeping it in an activated state[259]. In our model system, however, we did not witness a major effect of YopM on phosphorylation status of Rsk1-S380 in BMDMs (Fig 3.10B). In fact, we observed progressive dephosphorylation and a banding pattern suggestive of Rsk1 degradation over the course of the 6-hour infection, with no e ffect of YopM. It may be that a phoshophorylation site other than S380 is influenced by YopM and important for Pyrin inhibition, such as perhaps S154[319].

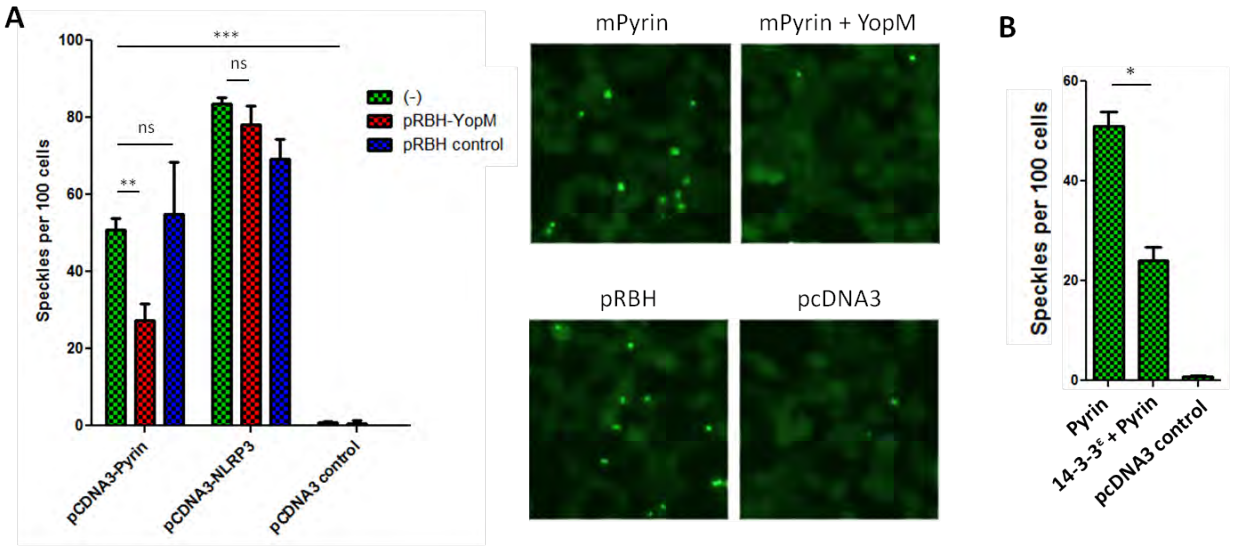


Figure 3-11. YopM prevents the formation of Pyrin-dependent Asc complexes, possibly by interfering with steady-state inhibition of Pyrin by 14-3-3 binding proteins. A) HEK293T cells stably expressing Asc-YFP were transfected with pCDNA3-Pyrin, pRBH-YopM, or both constructs together. pCDNA3-NLRP3 and respective empty vectors were used as positive and negative controls. Asc speckles were visualized, quantified, and normalized to cell number. In a separate experiment, B) pCDNA3-Pyrin was co-transfected with pCDNA3-14-3-3ε and Asc speckles per 100 cells were quantified. Figures are representative of three or more experiments. Shown is mean plus s.d. for triplicate fields quantified. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Finally, we tested the ability of YopM alone to inhibit Pyrin activation. HEK293T cells stably expressing Asc-YFP were transfected with pCDNA3-Pyrin, pRBH-YopM, or both constructs together. We observed significantly increased Asc speckle formation upon transfection of pCDNA3-Pyrin, indicating Pyrin inflammasome assembly (Fig 3.11A). This Asc speckling was significantly reduced upon co-transfection of pRBH-YopM. The pCDNA3-NLRP3 control also triggered the formation of Asc-speckles, but was not affected by the addition of YopM .

Based on reports that 14-3-3 binding proteins τ and ε are downstream of RhoA and interact with Pyrin[78, 79], we posited that Pyrin is kept inhibited by 14-3-3 and becomes activated when YopE inhibits RhoA, resulting in subsequent dephosphorylation and disassociation of 14-3-3 from Pyrin. We probed this possibility by co-transfecting pCDNA3-Pyrin with pCDNA3-14-3-3ε, and we found that, indeed, 14-3-3ε significantly reduced Pyrin-dependent Asc speckling (Fig 3.11B). We believe the inhibition of Pyrin by 14-3-3 proteins is central to the mechanism of Pyrin activation by YopE and inhibition by YopM. Notably, Pkn1 and Rsk1 are known to interact with and influence the function of 14-3-3 isoforms [319-321], with input from RhoA[322, 323]. Thus, it is possible that YopM rescues Rsk1 and Pkn1 activity to keep 14-3-3 phosphorylated and bound to Pyrin despite RhoA inhibition by YopE, although more experiments are needed to describe a detailed mechanism.

Conclusion

In this chapter we confirmed that IL-1β secretion in response to the *Y. pestis* needle/translocon is dependent on NLRP3/NLRC4, and that it is partially suppressed by YopJ, unaffected by YopM, and effectively inhibited by YopK. We also revealed that although YopK can also avert the cytotoxicity induced by the needle/translocon, it proceeds independently of NLRP3, NLRC4, and inflammasome-associated caspases, but instead relies on an undetermined pH-dependent mechanism. We showed that rather than being a general caspase-1 inhibitor, YopM specifically prevents activation of a primed Pyrin inflammasome by YopE. We also showed that the same protein regions of YopM required for it to bind its interaction partner Rsk1 are required for its ability to inhibit Pyrin. Finally, YopM specifically inhibits the Pyrin inflammasome in human cells even outside the context of infection, and we suggest that this occurs in a scenario where Pkn1, Rsk1, Iqgap1, and 14-3-3ε interact with and regulate Pyrin through a novel, as yet unreported pathway.

With the newest addition of Pyrin, the number of distinct caspase-1 processing pathways triggered by *Y. pestis* now numbers five. Specifically, NLRP3 and NLRC4 are activated by the needle/translocon, Pyrin is activated by YopE, caspase-8 is activated by YopJ, and NLRP12 is activated by an as-yet undetermined mechanism[118]. This is the highest diversity of inflammasome activation of any other T3SS pathogen discussed in Chapter 1, and even more impressively, the *Y. pestis* T3SS overcomes these pathways with just seven translocated Yop to achieve nearly absolute suppression of IL-1β production in net.

CHAPTER IV

Discussion

Immune evasion by T3SS pathogens related to *Y. pestis*

Pathogens use secretion systems to export molecules which can manipulate the host environment and promote bacterial fitness. The survival strategy of *Y. pestis* is to maintain maximum immune silence, and consequently its T3SS has evolved to suppress immune responses. Yet for enteric pathogens, such as *Salmonella*, *Shigella*, *Y. pseudotuberculosis* and *Y. enterocolitica*, immune silence is complicated by t he the intrinsic nature of the gut - a niche that is immunologically primed by t he constant presence of LPS and other molecules from a great number of diverse of bacteria. Instead, the T3SS of these pathogens is adapted for penetration and destruction of epithelial barriers and both intracellular and extracellular survival in the presence of phagocytes. This is not necessarily achieved by immunologically quiet mechanisms, in fact all four of these enteric pathogens activate robust NLRC4-dependent IL-1β (see chapter 1) and produce flagellin, which confer motility and epithelial invasiveness. These traits are particularly important in the gut environment, where lack of motility makes it easier to expel the bacteri from the body. At the same time, the non-silent but invasive nature of these enteric pathogens allows for rapid and sufficient replication of enteric bacteria, in order to be shed in the stool and continue its lifecycle. *Salmonella*, *Shigella*, *Y. pseudotuberculosis*, and *Y. enterocolitica* likely cause self-limiting infections precisely because their strategy does not entirely rely on total immune evasion, but rather retention in the gut for just long enough to reproduce in sufficient quantities.

Opportunistic pathogens such as *Pseudomonas*, *Legionella*, and *Burkholderia* typically establish temporary residence in the lung, and benefit from the tight control of

IL-1β production by the host. *Burkholderia* and *Pseudomonas* both apparently benefit when the host produces IL-1β and IL-18, which seems to hinder bacterial clearance while causing greater lung inflammation[127, 172, 179-183]. Why these cytokines fail to assist bacterial clearance is unclear, but one potentialy hypothesis concerns the fact that *Pseudomonas* exploits host regulation of autophagy to prevent immune recognition despite the activation of inflammasoms. This seems particularly true when *Pseudomonas* establishes chronic lung infection in CF patients - in these cases, the IL-1β response is disproportionately augmented and is associated with even less effective bacterial clearance[203]. While the details for this are not completely understood, this concept of IL-1β/IL-18 being beneficial to the bacteria runs counter to the strategy of *Y. pestis*, which is to avoid IL-1β and IL-18 production. Clearly, immune evasion carries different meaning and different requirements in different niches, and the secretion system is not a one-size-fit-all tool.

Inflammasome Activation and Inhibition by the Y. pestis T3SS

One of the advantages of this work is that it allows the comprehensive analysis of several components of the *Y. pestis* T3SS and multiple inflammasome pathways, side by side. This allowed us to cross-verify and disambiguate some of the confounding effects in this host-pathogen interaction, lending greater confidence in the final integrated model (Fig 4.1). A potential confounding factor in studying the *Y. pestis* T3SS is the effector YopJ, which kills cells, effectively suppresses signaling pathways on which other phenotypes rely, and may create the need for priming for some inflammasome studies.

Indeed, *in vivo* we saw little change in survival or most infection/immune related parameters unless both YopM and YopJ were deleted.

Previous studies have shown several immunosuppressive functions of YopJ[262, 264, 266, 267, 294, 324], and it has been puzzling that this effector does not appear to be needed for *Y. pestis* virulence [268, 269], Fig 3A), in contrast to *Y. pseudotuberculosis* and *Y. enterocolitica*. In contrast, immune stimulation induced by this effector molecule, such as the activation of caspase-1 and IL-1β/IL-18 release, has also been reported [124, 261]. One hypothesis is that the immunosuppressive and immune stimulatory actions of YopJ could balance each other, explaining why YopJ deletion has little effect on bacterial replication *in vivo*. Similarly, deletion of YopM has a modest effect on virulence via intradermal delivery of the CO92 strain[296], and no significant effect upon deletion in the KIM1001 strain upon s.c. delivery (Fig 2.3A). Notably, the CO92 strain carries a variant of YopJ which is less catalytically active than the KIM1001 variant[261, 325, 326]. It is possible that this variant of YopJ in CO92 partially unmasks the contribution of YopM to virulence.

Another aspect worth considering is that reduced activity or secretion of YopJ/YopP may not necessarily benefit the host[303, 327]. YopJ robustly suppresses IL-1β and IL-18 precursors as well as other NF-kB dependent cytokines[328, 329], but also triggers caspase-8 dependent activation of caspase-1, IL-1β and IL-18 at low levels[11, 124, 261]. Interestingly, the catalytic activity of YopJ positively correlates with its ability to induce caspase-8 dependent cytoxicity and IL-1β secretion. The increased catalytic activity may also cause slight attenuation in pneumonic plague[327, 330] Indeed, some

studies in non-microbial systems indicate that inhibition or lack of IKKB leads to a paradoxical increase in IL-1β secretion and caspase-8 activation despite an expected anti-inflammatory effect[331-333]. Could the non-canonical caspase-8 pathway be part of a host trapdoor mechanism for IL-1β/IL-18 production, designed to be triggered when pathogenic effectors such as Y opJ attempt to suppress the critical NF-kB or MAPK pathways after surface receptor activation?

Although YopJ has been proposed as a key *Yersinia* effector inducing caspase-1 cleavage[124, 261], our results suggest that YopJ is the weakest contributor to caspase-1 cleavage of the T3SS activators we describe. In macrophages, YopE-activated Pyrin and needle/translocon-activated NLRP3 and NLRC4 drive the majority of caspase-1 cleavage in the absence of YopM or YopK, respectively. Furthermore, YopJ may be particularly important in monocytes because unlike YopK or YopM it is able to prevent inflammasome-independent IL-1β production (Fig 3.9A). Finally, we have found that YopJ does not appear to directly induce cell death and IL-1β release in human PBMC, which could suggest that the YopJ-caspase-8 pathway to cell death and inflammasome activity could be of different significance in different cells and/or species (Fig 3.9A). In neutrophils, for example, YopJ does not seem to have the same pro-apoptotic function as in macrophages[278].

It is also notable that we observed that YopJ plays a significant role in suppressing inflammasome-independent IL-1β production in PBMCs (Fig 3.9A), while in a the macrophage system the importance of YopJ in controlling IL-1β production seemed secondary to YopM (Fig 2.1A, B). This diversity of function may indicate varying

importance of the effectors in different body compartments or route of infection; for example, YopJ may be more important in suppressing IL-1β and IL-18 production in septicemic plague, where PBMCs would be expected to play a central role in host defense. Furthermore, the ability of YopM to inhibit Pyrin in PBMCs does not require these cells to be primed. Thus, compared to the macrophage model, there does not appear to be any redundancy in the suppression of IL-1B production in PBMCs. This is interesting to consider, because deletion of YopM has been shown to reduce LD50 by 100,000 fold in a septicemic model of plague[295]. By contrast, PBMCs are not known to play a prominent role in the bubonic disease, where we observed redundant effects of YopM and YopJ on both IL-1B production and virulence (Fig 2.3).

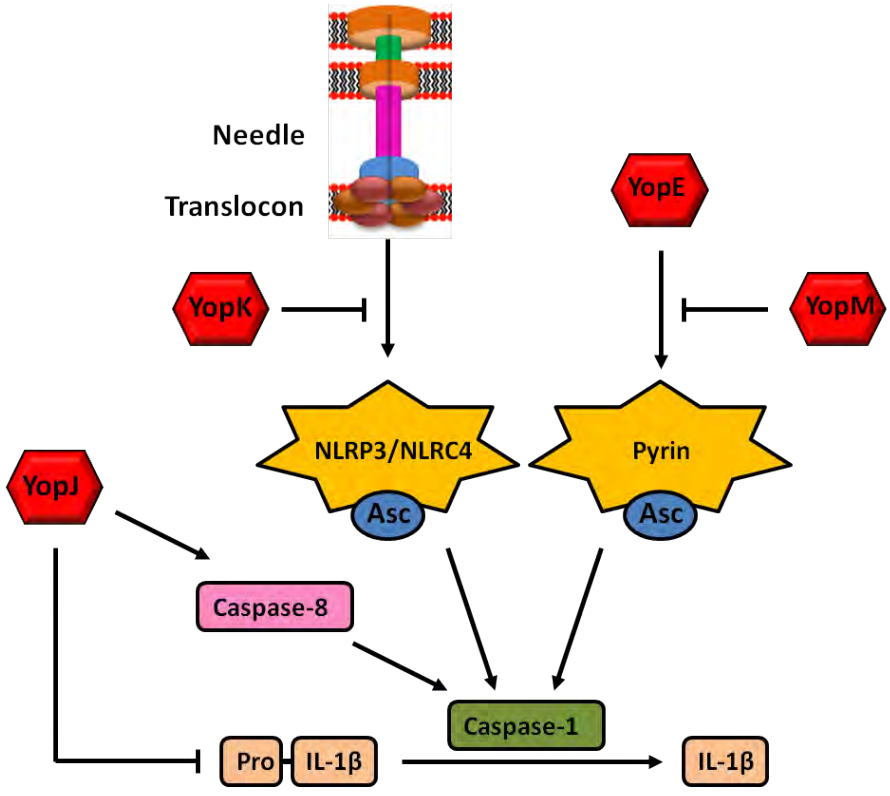


Figure 4.1. Proposed model integrating the major interactions of the *Y. pestis* T3SS with inflammasome pathways.

With regards to NLRP3 and NLRC4 by *Y. pestis*, activation of both inflammasomes depends on the presence of the functional T3SS apparatus[255, 256, 312]. Potential activators of NLRC4 include parts of the needle/translocon structure itself, such as YscI[334] or YscF[141]. This can be predicted because YscI and YscF have homologs in *Salmonella*, *Shigella*, *Burkholderia*, and *Pseudomonas* which activate NLRC4 via NAIP1 and NAIP2, respectively (Fig 1.2). Possible mechanisms for NLRP3 activation include hyper-translocation of the YopB/D translocon into the cell cytoplasm[256], and the destabilizing effects of a large pore in cell membrane or endosomes[335] perhaps affecting flux of ions or ROS[278]. The role of the translocon is also emphasized by data suggesting that increased expression of YopB increases inflammasome activation[256, 261]. It is also possible that in the presence of a functional needle, other molecules (e.g. LPS) pass from the bacterium into the host cytoplasm and activate inflammasome pathways; however, we did not observe a role for caspase-11 (Fig 3.2A), which would be expected in this scenario.

Very recent findings on cell death mediated by caspase-11 and caspase-1 via gasdermin D[96, 167] and by NEK7 via NLRP3/caspase-1[336, 337] suggest there are a number of additional players involved in regulation of inflammasome and pyroptosis pathways. Furthermore, bacterial components including YopK[255] could differently impact the various death pathways. Several molecules, in addition to those studied here, are likely involved in regulating cytotoxicity induced by the different *Yersinia* strains.

The identification of new cell-death mediating proteins could add to the understanding of why the various pathways induce different types and degrees of cell death.

With regards to the caspase-1/11/8-independent cell death induced by the *Y. pestis* needle/translocon that we observed in Fig 1C, one possible explanation is that pore-formation by the translocon triggers both caspase-1 dependent pyroptosis and caspase-1-independent apoptosis in parallel. Pore formation and membrane instability may trigger ROS and entry of K+ and/or calcium which triggers NLRP3, but which also induces sufficient mitochondrial damage to activate classical caspase-3-dependent apoptosis in the absence of caspase-1. While the details of the mechanism(s) are not clear, the majority of NLRP3 and NLRC4 activation and cytotoxicity by the needle/translocon is effectively prevented by YopK (Fig 3.3)[255, 256]. Brodsky and Marketon propose that YopK acts at the site of the translocon and regulates the delivery of other Yops[338, 339]. It is not clear if YopK may prevent the unintended entry of bacterial components other than Yops into the host cell. Potentially, YopK could also conceal inflammasome-activating motifs of a hyper-translocated needle/translocon, or stabilizes the pore to prevent membrane-damage associated inflammasome activation.

There is also evidence indicating that YopE, whose GAP activity inhibits RhoA/G, Rac1, and Cdc42, may also inhibit inflammasome activation by stabilizing the translocon pore [335, 340] or by regulating effector translocation[341]. An early report by Schotte and colleagues suggested that YopE inhibits caspase-1 activation in a manner dependent on Rac1[20]. This is entirely reconcilable with our evidence that YopE activates Pyrin because, first, YopE will not trigger Pyrin in cells where it is not expressed unless Pyrin is induced by

priming. Second, the inflammasome-activating effect of YopE is not apparent in the presence of YopM, because YopM inhibits the Pyrin pathway. Finally, as exemplified by the case of YopJ, a single effector can inhibit one inflammasome pathway while triggering another. The same appears to be true of YopE, where its inhibition of GAP activity may trigger one inflammasome (Fig 3.6E)[82, 342], while preventing activation of another (Fig 3.6E)[20, 335, 340, 341, 343].

It is also interesting that the stabilizing effect of YopE on translocation could be complemented by *P. aeruginosa* ExoS and ExoT, which are YopE homologues with GAP activity. It is not known whether ExoS and ExoT may also activate Pyrin. Our evidence also supports the idea that YopE can stabilize the translocon and reduce needle/translocon-triggered inflammasome activation (Fig3.5E). One possible implication of using YopK or YopE mutants is that they may both regulate translocation of effectors in some systems[255, 338, 339, 341]. While we have not directly tested the impact of this phenomenon, others have suggested that the use of a mutant YopE does not have a major impact on YopJ-influenced IL-1β release in the context of YopM deletion[315]. However, this may warrant further studies.

Other studies and ours suggest that in the absence of YopE GAP activity, the impact of other Yops, such as YopJ, on IL-1β release is not significantly impacted[315]. Indeed, the finding by Schotte and colleagues[20] points to the understudied role of cytoskeletal GEFs and GAPs (including perhaps Iqgap1) in inflammasome regulation. Many pathogens target host GTPases to inhibit motility and phagocytosis, and there is compelling evidence showing that these pathways play important roles both in negative and positive regulation of

inflammasomes[24, 82, 341, 344]. However, it is not at all clear what kind of GAP activity would be sufficient to trigger Pyrin, and which GAP effectors can be predicted to be candidate Pyrin activators.

Table 1. Summary of the functions of translocated Yops. References: Plano and Schesser 2013[345], Lee et al 2015, [346], Hentschke et al 2010[259], Brodsky et al 2010[255].

	Function
YopH	Inhibition of cytoskeleton & phagocytosis; tyrosine phosphatase, directly dephosphorylates and inhibits p130Cas and focal adhesion kinase (FAK).
YopE	Inhibition of cytoskeleton & phagocytosis; acts as a GAP for RhoA, Rac1, and Cdc42 resulting in GTP hydrolysis and subsequent inhibition of actin polymerization.
YopT	Inhibition of cytoskeleton & phagocytosis; cysteine protease, directly cleaves RhoA, Rac1, and Cdc42 at C-terminus, releasing them from plasma membrane and disrupting their function.
YopO/ YpkA	Inhibition of cytoskeleton & phagocytosis; GTPase-binding protein and/or PKA-like kinase; binds RhoA and Rac1; diverts and sequesters actin and cytoskeletal regulators VASP, EVL, WASP, and gelsolin to misregulate cytoskeletal activity.
YopK	Immunosuppression; thought to regulate the rate of effector delivery, and prevent hyper-translocation of the needle/translocon.
YopM	Immunosuppression; thought to inhibit caspase-1 activation; binds Rsk1 and Pkn1, with unknown significance; localized to the nucleus.
YopJ/ YopP	Immunosuppression; broadly inhibits NF-kB and MAPK pathways by targeting MEK2, IKKB, and TAK1; induces caspase-8 dependent apoptosis and caspase-1 activation.

Importantly, Yops H, O, and T also impact intracellular GTPase activity and have been reported to inhibit phagocytosis and cytoskeletal dynamics to varying degrees (see Table 1 for an overview of the functions of the seven translocated Yops). Thus, while in our mouse macrophage system we identified YopE to be the dominant activator of Pyrin (Fig 3.6), it is possible that other Rho GTPase inhibiting effectors may serve as alternative Pyrin

activators in other systems. Inhibition of phagocytosis by neutrophils is a critical aspect of *Y. pestis* pathogenesis, as the bacteria is unable to replicate within these cells. Because inhibition of phagocytosis (particularly by Yops E and H) is indispensible for the *Y. pestis* virulence[342, 345, 347, 348], it is plausible that *Y. pestis* also relies on a mechanism to silence the immune responses resulting from this activity. In this context, YopM may play an important role as a general Pyrin inhibitor to silence immune responses triggered by the anti-phagocytic Yops.

Control of Pyrin Inflammasome Activity

It has been proposed that the Pyrin inflammasome responds to inappropriate inhibition of Rho-GTPases by pathogens[36]. Pyrin has been shown to respond to a number of virulent pathogens and their secreted toxins, including *Francisella*, *Burkholderia*, *Vibrio*, and *Clostridium*[82, 128, 173, 218].

The mechanism by which inhibition of Rho GTPases could lead to Pyrin activation is an exciting question open to broad speculation. One possibility is that Pyrin reacts to excessive depolymerization of actin[81] as a direct consequence of RhoA inhibition by toxins such as TcdB[349]. Indeed, Pyrin is known to bind actin and colocalize with it at the leading edge together with Pstpip1[76, 80, 350]. Pyrin activation is also inhibited by the cytoskeletal stabilizer colchicine; however, in preliminary experiments we treated cells with inhibitors of actin polymerization - latrunculin B or cytochalasin D - and did not observe any change in Pyrin activation. Furthermore, alternative evaluation of the relationship between actin and Pyrin suggests that Pyrin activation may be associated not with actin

depolymerization but rather reorganization of Pstpip1 into reticulated fibers[350, 351]. Colchicine inhibits the formation of these structures and interestingly also leads to down-regulation of Pyrin expression.

Others have shown Pyrin to be activated in response to ribotoxic stress, which was attenuated by inhibition of p38 MAP Kinase[75]; inhibition of p38 also leads to increased activity of ERK[352], which is the direct activator of the YopM binding partner Rsk1. Rsk1 is known to play an important role in mediating cytoskeletal activity, including reducing actin stress fibers and impacting cell motility[353, 354]. Thus, increased Rsk1 activity may be associated with reduced Pyrin activity through cytoskeletal stabilization. Consistent with this, our data confirm the importance of the YopM C-terminus for inhibiting caspase-1 activation (Fig 3.8), which is also the region necessary for YopM to interact with Rsk1[258, 260]. Pkn1 may also play a role in this pathway, as it has been suggested to be directly activated by RhoA[322, 355, 356] and Rac1[357], and its downstream functions also include regulation of actin organization. Yet in general, both Pkn1 and Rsk1 are better studied in the context of cancer pathways[353, 354, 358-361], with little known about what roles they may play during infection.

Our immunoprecipitation experiments demonstrate that Pkn1 and Rsk1 interact with Pyrin during infection with *Y. pestis* even in the absence of YopM (Fig 3.9C), suggesting these proteins could have a role in regulating Pyrin activation in infectious and non-infectious situations. The ability of YopM to inhibit caspase-1 activation has been shown to depend on Iqgap1[258], which serves as a scaffolding protein for Rac1 and Cdc42[362], and has also been shown to interact with RhoA[363]. In our pull-down experiments we found

interaction between Pyrin and Iqgap1 as well. All of these proteins interact with and influence actin rearrangement, and it is fully conceivable that Pyrin regulation is indeed mediated by a cytoskeletal mechanism.

An alternative hypothesis stems from the fact that Pyrin is regulated by the cytosolic anchoring proteins of the 14-3-3 family, and has been shown to bind the isoforms 14-3-3ε, 14-3-3τ, and others [78, 79]. A large body of evidence indicates that 14-3-3 proteins directly and indirectly interact with RhoA[364, 365], Rac1[366-368], and Cdc42[369] to regulate a diverse variety of pathways. Interestingly, 14-3-3 proteins have been shown to bind Dengue viral protein NS3 and inhibit RIG-1 dependent responses to the pathogen[370]. Inhibition of Rho GTPases by YopE or similar toxins could lead to de-inhibition of Pyrin by 14-3-3ε and τ, with subsequent inflammasome formation. Pkn and Rsk1 are known to interact with and influence the function of 14-3-3 isoforms [319-321], with input from RhoA[322, 323]. In the absence of RhoA activity, the role of YopM may be to re-activate these kinases to either keep 14-3-3 bound to Pyrin, or possibly directly maintain S242 phosphorylation on Pyrin[78] to silence it directly.

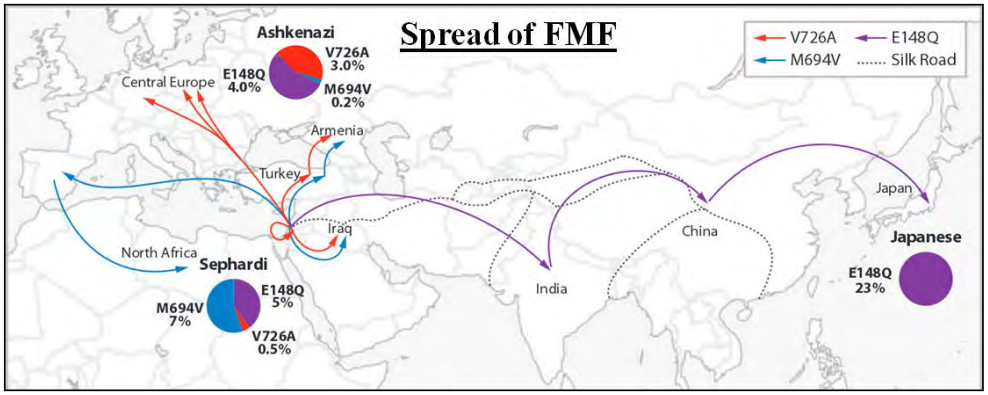
One potential caveat of this work is to what extent mouse and human Pyrin pathways overlap, since mouse Pyrin lacks the C-terminus B30.2 domain present in human Pyrin, and some important functional differences between mouse and human Pyrin have been reported[218]. However, our data in human PBMCs, THP-1 cells, and HEK293T cells suggests that the effects of YopM on the human pyrin pathway are comparable to the pathway in mice (Fig 3.9-3.10). Additionally, the phosphorylation sites on human Pyrin suggested to be important for binding and regulation by 14-3-3[78] are also present in mice,

and may have analogous function (S208, S209, and S242 in human Pyrin; S205, S206, S241 in mouse Pyrin). If 14-3-3 is indeed the mediator of YopM inhibition of Pyrin, then the fact that these phosphorylation sites are common to mouse and human Pyrin would be consistent with a similar mechanism of inhibition by YopM in both the mouse and human system.

Familial Mediterranean Fever and Plague

Mutations in the human Pyrin gene (Mefv) result in Familial Mediterranean Fever (FMF), the most common autoinflammatory disease in humans[74]. The majority of FMF cases involve mutations in the B30.2 domain causing spontaneous activation of the Pyrin inflammasome, and systemic inflammation[73]. This disease comes in waves of attacks accompanied by potentially severe abdominal pain, arthralgia, rashes, and progressive deposition of amyloid protein in kidneys and other organs (Fig 4.2). Treatment of choice is colchicine, which was described earlier.

A particularly exciting future direction of this work could be to test whether YopM is capable of suppressing Pyrin activation in FMF patients and heterozygous carriers. A tempting hypothesis is that FMF carriers may have had a selective advantage during plague epidemics due to enhanced activity or increased resistance to YopM. FMF carriers frequency is highest in Middle Eastern and Mediterranean populations, where approximately 1 in 4 individuals are carriers[371]. This is particularly interesting given that hotbeds of the first two plague epidemics were in the Mediterranean basin. Comparing a map of the spread of plague to a map of the distribution of mutant MEFV alleles suggests a pattern consistent with co-evolution (Fig 4.2).



Masters et al., Annu. Rev. Immunol. 2009. 27:621-68

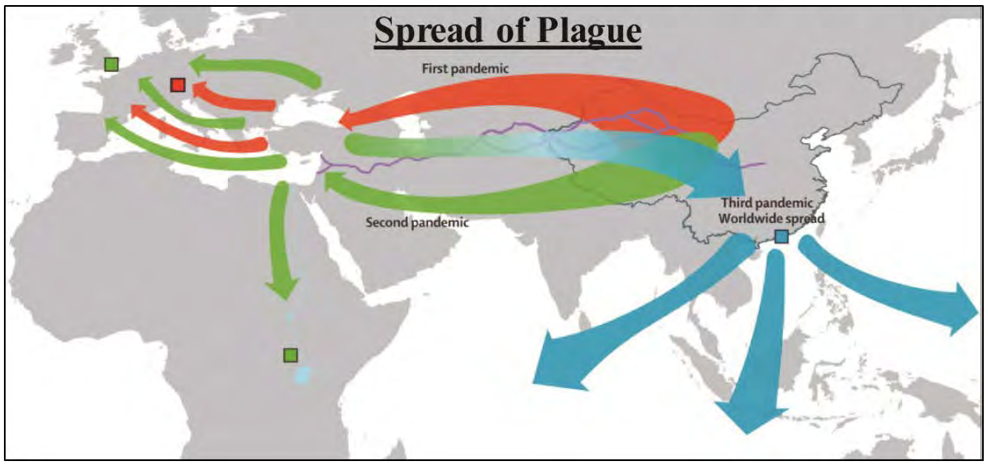


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Figure 4.2. Hotspots of plague and areas with high FMF carrier frequency significantly overlap [372, 373].

Conclusion

In conclusion, we have generated a comprehensive model describing the dynamics of the *Y. pestis* T3SS interactions with inflammasomes. The fact that so many bacterial components are involved in regulating IL-1β/IL-18 highlights the importance for the bacterium to keep these cytokines in check. In that respect, *Y. pestis* serves as a robust and fascinating model system for investigating immune evasion by pathogens and microbial manipulation of host IL-1β and IL-18 release.

The identification of YopE, YopM, Pkn1 and Rsk1 as potential novel exogenous and endogenous players in the regulation of the Pyrin inflammasome is a significant advance, both for the understanding of plague biology as well as for innate immunity. We hope that these findings will provide insight for the identification of better drug targets and improved therapeutics for Familial Mediterranean Fever and other diseases influenced by the Pyrin protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. The fully virulent KIM1001 strain of *Y. pestis*, the attenuated KIM5 (Δ*pgm*) BSL2 strain, and KIM5ΔYopJ were previously described[11, 225, 288]. The ΔYopM, and ΔYopM/J strains were generated both on the KIM5 and KIM1001 background as follows: An in-frame deletion removing amino acids 3 through 408 of 410 of the *yopM* gene was created via allelic exchange. PCR products made with primer sets yopM-A, yopM-B and yopM-C, yopM-D, respectively, were used to make a fused product by overlap PCR using primers A and D[374]. This product was cloned in the allelic exchange vector pRE107[375] in *E.coli* K12 strain β2155, a diamiopimelic acid auxotroph, and transferred to *Y. pestis* KIM1001 by conjugation. KIM1001 recombinants were selected on TB medium containing 100μg/ml ampicillin but no diaminopimelic acid. Following counter selection with 5% sucrose, deletion mutants were identified by P CR. The same procedure was followed to construct an in-frame deletion mutant of *yopJ*, *yopE,* and *yopK* in KIM1001, as well as *yopJ,* and *yopE* in KIM1001 Δ*yopM* using the respective gene specific A, B, C, and D primers shown in Table 2. Attenuated Δ*pgm* derivatives of each strain, bearing the designation KIM5 to indicate their altered chromosomal genotype, were derived from their respective KIM1001 parents by selection for loss of pigmentation on HIB Congo Red agar at 26°C. KIM5 Δ*pla* was also generated as described[288]. Loss of the pigmentation region (Δ*pgm*)/iron acquisition was confirmed by PCR with primers pgm-F, pgm-R; psn-F, psn-R; and hmsH-F, hmsH-R. Expression of YopM and YopJ was confirmed by RT-PCR.

A Type III secretion effector deficient strain (ΔT3SSe) was constructed by making sequential in-frame deletions, as described above, of *yopM* (amino acids 3-408 of 410), *yopE* (amino acids 40-197 of 220), *yopH* (amino acids of 3-467 of 469), *ypkA* (amino acids of 3-731 of 733), *yopJ* (amino acids 4-288 of 289), *yopK* (amino acids 4-181 of 183), and *yopT* (amino acids of 3-320 of 323) using the respective gene specific A, B, C, and D primers shown in Table 2. The deletions were made in *Y. pestis* KIM 1001 and a KIM5 derivative was generated as described above. This strain lacks Yops M/E/J/H/T/K and YpkA, but expresses Yops B/D and the machinery necessary to assemble a T3SS needle with a functional pore-forming translocon complex. To examine the contribution of individual Yops, the full-length genes of *yopK*, *yopM*, or *yopE* were restored to the ΔT3SE background. PCR products made with each respective yop's primer A and D (see Table 2), using wild-type KIM5 template, were cloned into pRE107 and allelic exchange carried out as described above. Strains expressing pairs of Yops (only YopM and YopE, and only YopK and YopE) were constructed by restoring the full-length *yopE* gene to the ΔT3SE *yopM*+ or ΔT3SE *yopK*+ backgrounds.

All *Y. pestis* strains were grown using TB media supplemented with 2.5mM $CaCl_2$. KIM1001 and derivative strains were plated on agar incubated at 37°C overnight and passed once before preparing inoculum for injection. Strains on the KIM5 background were plated overnight from frozen glycerol stocks and then grown at 26°C in liquid broth overnight; on the day of infection cultures were diluted 1:20 and grown for 2 hours at 26°C followed by a shift to 37°C for 2 hours. This transition is important to up-regulate expression of the T3SS while minimizing expression of F1 protein capsule[376],

which interferes with cell-based assays. Bacteria were then washed three times in RPMI (pre-warmed to 37°C), quantified by OD600, and added to cells at a multiplicity of infection (MOI) of 10 bacteria per cell in a 10uL volume within 1 hour of preparation.

Y. pseudotuberculosis IP2666ΔYopM and IP2666ΔYopM+recM mutant strains were a kind gift from Dr. Joan Mecsas and have been previously described[310]. *Y. pseudotuberculosis* strains were grown identically to the KIM5-background strains described above, except 2xYT media was used instead of TB (also supplemented with 2.5mM $CaCl_2$).

Heat-killed KIM5 for priming was prepared by growing KIM5 in liquid broth from freshly streaked plates overnight either at 26°C or 37°C. Bacteria were diluted 1:20, and grown for an additional three hours at the same temperature. Bacteria were then washed three times in endotoxin-free PBS, quantified by OD600, and incubated at 65°C for 1 hour to heat-kill. An aliquot of each temperature prep was streaked out to confirm 100% death, and the rest was frozen. For priming in subsequent experiments, an equivalent of MOI 10 of either prep was used.

Cell Culture and Preparation. Bone marrow derived macrophages (BMDMs) were differentiated in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 25mM HEPES, 10ug/mL ciprofloxacin, and 10% L929 conditioned medium containing M-CSF for 5 days. Bone marrow derived dendritic cells (BMDCs) were differentiated in R10 medium consisting of RPMI 1640, 10% FCS, 20mM HEPES, 2mM L-glutamine, 50μM β-mercaptoethanol, 100U/mL penicillin, 100μg/mL streptomycin, and 20ng/mL recombinant murine GM-CSF (Peprotech) for 9 days. BMDMs or BMDCs were

harvested and seeded at a density of 100,000 cells per well in a 96-well plate format overnight and stimulated the following day. HEK293T-Asc-YFP cells (a kind gift from Dr. Kate Fitzgerald) were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 25mM HEPES, and 10ug/mL ciprofloxacin. THP-1 YFP-Pyrin and siPyrin cell lines were a generous gift from Drs. Mark Wewers and Mikhail Gavrilin, and were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 25mM HEPES, and 10ug/mL ciprofloxacin. For stimulations, THP-1 cells were differentiated for 48-72 hours with 100nM Vitamin D3, harvested, and seeded for same day stimulation at either 100,000 cells in 96-well plate format, or at $1x10^7$ cells per 10cm dish for immunoprecipitation experiments. Human PBMCs were isolated from donor whole blood using Lymphoprep density gradient (#07851, StemCell), washed once, and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 25mM HEPES. PBMCs were seeded at a density of 100,000 c ells per well in a 96-well plate format and immediately used for infection.

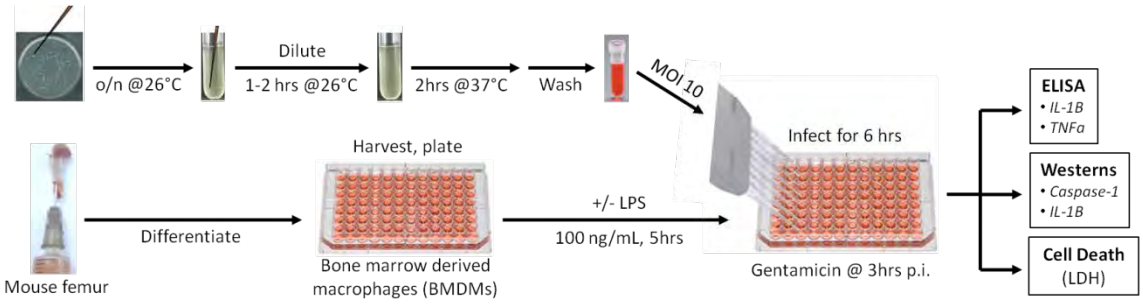

Figure 4.3. Outline of a standard experimental protocol and typical assay readouts.

Cell Stimulations. Cells were primed with 100 ng/mL LPS for 5 hours or allowed to rest in antibiotic-free RPMI with 10% FCS and 25mM HEPES without antibiotic

before addition of bacteria at an MOI of 10. Endpoints were as follows: 2 hours post-infection (p.i.) for RNA extraction, 3 hours p.i. for caspase-8 enzymatic assays (Caspase-Glo 8 Kit, Promega #G8200), 5 or 6 hours p.i. for LDH assay (CytoTox 96, Promega #G1780), and 6 hours for harvesting of supernatant and/or cells for analysis of cytokines by ELISA (R&D) or caspase-1 activation by SDS-PAGE and Western Blot (anti-caspase-1 #AG-20B-0042-C100, Adipogen; anti-IL-1β #DY401 840135, R&D; anti-pS380-Rsk1 #AF889, R&D). For time points exceeding 3 hours, 50ug/mL gentamicin was added at 3 hours p.i. In experiments with TcdB (#155, List), the inhibitor was directly added to cells at a final concentration of 0.4uM for 6 hours. Each graph represents results of two or more independent cell stimulations on separate days.

Immunoprecipitations. Cells were infected at MOI 10 as described above, and harvested either at 3 hours or 6 hours p.i., with 50ug/mL gentamicin added at 3 hours. Cells were washed once and lysed in co-IP buffer for 15 minutes (1% Triton X-100, 150mM NaCl, 5mM KCl, 2mM MgCl2, 1mM EDTA, 25mM Tris-HCl, pH 7.4) with protease and phosphatase inhibitor (#04693116001 and #04906845001, Roche). Lysate was cleared by centrifugation and an fraction was saved as loading input control. The remaining lysate was cleared once with protein G agarose beads (Thermo #20398) to remove non-specifically binding proteins, followed by incubation with fresh beads and pull-down antibody, either against GFP (#A11122, Life Technologies) or against Pkn1 (#MAB6100, R&D), for 2 hours at 4°C. Beads were then washed five times in co-IP buffer, and bound proteins were eluted by direct addition of SDS loading buffer buffer with 1mM DTT. Beads and saved lysate were analyzed by SDS-PAGE and analyzed by

Western Blot (anti-Iqgap1 #610611, BD Biosciences; anti-Rsk1 #sc-231, Santa Cruz; anti-Pyrin serum, a kind gift from Dr. Mark Wewers and Mikhail Gavrilin).

Cell death assays. The LDH assay was used to measure cell death at a fixed 5- or 6-hour endpoint (as indicated in figure legend); for this assay, regular media was replaced with X-vivo (#04-744Q, Lonza) supplemented with 3.5% FCS prior to infection. Additionally, a kinetic cell death assay using a DNA-binding dye was performed as follows: cells were incubated with 0.2uM ethidium homodimer (EthD-1, Sigma #46043) in regular media one hour before adding bacteria. Upon infection, the plate was placed in a Synergy H4 Plate Reader at 37°C and UV-induced fluorescence was measured every 10 minutes. Increased fluorescence correlates with DNA binding by EthD-1 upon entry through increasingly permeable cell membranes as cell death progresses.

RT-PCR. RNA was extracted using the QIAGEN RNEasy kit, followed by RT-PCR with the SYBR Green DNA probe (BioRad). C(t) values were normalized to GAPDH internal controls and the means were normalized to the unstimulated negative control group. For RT-PCR on RNA extracted from bacterial lysates, results were normalized to *Y. pestis* 16S rRNA internal control. Primers used for RT-PCR are listed in Table 3.

Cloning. pCDNA3-Pyrin and pCDNA3-14-3-3ε were kind gifts from Dr. Emad Alnemri and Dr. Michaela Gack, respectively. pRBH-YopM was constructed as follows: endogenous YopM was amplified out of pCD1 by direct PCR on KIM5 bacteria using primers YopM-F and YopM-R (see Table 2). Self-complementary oligos (Oligo 5.1 and Oligo 5.2) coding a his-tag sequence along with part of the YopM N-terminus were

designed and annealed to each other, resulting in 5' and 3' overhangs compatible for subsequent ligation. The YopM PCR product and pRBH vector were digested with BsrFI/XhoI and BamHI/XhoI respectively, gel-purified, and ligated together with the annealed Oligos 5.1 and 5.2. This resulted in YopM containing a His6 sequence immediately following the first methionine, inserted downstream of the CMV promoter and upstream of an mCherry reporter gene. The resulting pRBH-YopM construct was then cloned and maintained in TOP10 cells (Invitrogen).

Transfections. HEK293T cells stably expressing Asc-YFP were seeded at $1x10^6$ cells per well in 24-well format, and transfected with pcDNA3-Pyrin, pcDNA3-NLRP3, pcDNA3-14-3-3ε, pRBH-YopM, or empty control plasmids using Lipofectamine 2000 (Thermo #11668030). 250ng of each plasmid was transfected together with 250ng of either pRBH-YopM, pcDNA3-14-3-3ε, or empty control vector for a constant total of 500ng per well and no more than 250ng of any one plasmid. After 24 hours cells were stained with Hoechst 33342 and visualized using an epifluorescent microscope. Asc speckles were quantified and normalized to cell number in three separate fields per sample, using a standardized batch algorithm in Adobe Photoshop CS4.

Mice. All experiments involving mice were approved by the Institutional Animal Care and Use Committee. Mouse strains used in this study were described previously[118] and bred in-house. TCRβ-/-δ-/- (TCRβδ dKO, lacking TCRαδ and TCRγδ) were from Jackson Laboratories and provided by Ray Welsh. Pyrin-/- were from Jackson Laboratories on the 129S6/SvEvTac background, and for experiments with these mice littermates were used as controls. All other mice were on a C57Bl/6J background.

BMDMs were differentiated from bone marrow harvested from the femurs of 6-20 week old mice. Peritoneal macrophages were harvested by injecting mice intraperitoneally with1 ml of thioglycolate, and lavaging the peritoneal cavity 24 hours later with RPMI. Cells were seeded at 500,000 per well in 12-well format for subsequent stimulation.

To investigate a system where neutrophils are likely playing a major role in inflammatory signals, we conducted infections directly in the peritoneum of mice after stimulating heavy neutrophil recruitment with thioglycolate. Mice were injected intraperitoneally with 0.7mL 4% thioglycolate followed 3 hours later by same-side injection with $1x10^8$ CFU of KIM5, KIM5ΔYopM, KIM5ΔYopJ, KIM5ΔYopM/J, or mock in 0.3mL PBS. This approach gave a similar cellular composition of 90-98% neutrophils. The peritoneal cavity was lavaged with 3mL of Hank's Buffered Saline Solution (HBSS) 6 hours p.i. Cells were centrifuged and analyzed by flow cytometry with anti-Ly6G) and anti-CD11b (Becton Dickinson), and the cleared lavage fluid was assayed for IL-1β by ELISA. Flow cytometry data was analyzed by FlowJo software.

Subcutaneous (s.c.) inoculations were conducted by injecting mice with 50-140 CFU in 50μL of PBS in the nape of the neck behind the right shoulder blade; mice were then either monitored for survival for up to 40 days or sacrificed for collection of organs at 90 hours p.i. Intravenous (i.v.) inoculations were conducted by injecting mice with 40-50 CFU in 200μL PBS via the tail vein and harvesting serum, spleen and liver at 42 hours p.i. Upon dissection (s.c. infection), a single large lymph node on the side of infection was harvested from each mouse. Spleens and lymph nodes were collected in 0.7mL PBS and homogenized in the closed-system Miltenyi gentleMacs dissociater. 50μL of

homogenate was taken for serial dilutions and quantification of bacterial loads per organ. Excess spleen homogenate was treated immediately with protease and phosphatase inhibitor (Roche) and ciprofloxacin, and analyzed for cytokines by ELISA. Blood was harvested by cheek bleed and processed for collection of serum as previously described[118]. Livers were fixed in 10% formalin and stained by hematoxylin and eosin (H&E). Vaccination experiments were performed by infecting mice surviving infection with KIM1001ΔM/J on day 14 or 21 with virulent KIM1001 and monitoring survival for 25 days.

Statistical Analysis. *In vitro* assays were analyzed by two-way ANOVA followed by Bonferroni post-test. *In vivo* cytokines were tested for significance by one-way ANOVA followed by Bonferroni's multiple comparison. Non-parametric bacterial load data was analyzed by the Kruskal Wallis test followed by Dunn's post-test. Differences in survival were analyzed by Kaplan-Meyer analysis and logrank test. Values where $p < 0.05$ were considered significant.

Table 2. Primers and oligos used for generation of bacterial strains

Primer/Oligo Name	**Sequence 5' to 3'**
yopM-A	ATAGAGCTCTTCAAAAGGGGTACTGGATAC
yopM-B	GAACATATTGAATGCCTTTCT
yopM-C	AGAAAGGCATTCAATATGTTCGAGTAGTACGCAAGAGCGTTC
yopM-D	GGGTCTAGATTTACCAATTTTTTGATGGGG
yopJ-A	ATAGAGCTCCACTACTGATTCAACTTGGACG
yopJ-B	ACGGCAAATGCAGAGCAGTCCGATCATTTATTTATCCTTATTCA
yopJ-C	CTGCTCTGCATTTGCCGTTAATGTATTTTGGAAATCTTGCT
yopJ-D	GGGTCTAGACTGATGTCGTTTATTTCTGGGTAT
yopE-A	ATAGAGCTCAGCATTACACACTCCACAGTTGGGT
yopE-B	ACGCAGGCAGCAAATGAGATCAAA
yopE-C	CTCATTTGCTGCCTGCGTATATTGATCACTTGTTTG
yopE-D	ATATCTAGATATCCAGGCTGTTCAATGGTTGTCGAT
yopK-A	GGGGAGCTCTGTTAGCCATTATTTTGCTATAC
yopK-B	ACGGCAAATGCAGAGCAGAATAAACATAGTTACTACTCCCAAA
yopK-C	CTGCTCTGCATTTGCCGTGGATGAAGCTATATTAAAGAGTT
yopK-D	ATATCTAGACATTTAAAACAGGGCATGG
Pgm-F	CCGCAACAACATCATCCGTATTCA
Pgm-R	TTCGCTACCACTGAAATCCAAGAC
Psn-F	ATTGCTCCCCGCCATTGCTA
Psn-R	CATTGCTCTTACCCTGGTCGCCA
hmsH-F	CGTTTCAGTTGCCTGTGTGCTAAC
hmsH-R	CATCACTCGGTGTAGACATCGCT
YopM-F	CGCATAAAAATTCCCGGCG
YopM-R	GCACCTCGAGAATTATGAACGCTCTTGC
Oligo 5.1	GATCCATGCATCATCACCATCACCACTTCATAAATCCAAGAAATGTATCT AATACTTTTTTGCAAGAACCATTACGTCATTCTTCTAATTTAACTGAGATG
Oligo 5.2	CCGGCATCTCAGTTAAATTAGAAGAATGACGTAATGGTTCTTGCAAAAAA GTATTAGATACATTTCTTGGATTTATGAAGTGGTGATGGTGATGATGCATG

Table 3. Primers used for RT-PCR

Primer Name	**Sequence 5' to 3'**
GAPDH F	TGTGTCCGTCGTGGATCTGA
GAPDH R	CCTGCTTCACCACCTTCTTGA
Pro-IL-1β F	AGGCCACAGGTATTTTGTCG
Pro-IL-1β R	GCCCATCCTCTGTGACTCAT
Pro-IL-18 F	CAGGCTGTCTTTTGTCAACGA
Pro-IL-18 R	GACTCTTGCGTCAACTTCAAGG
Pyrin F	TCATCTGCTAAACACCCTGGA
Pyrin R	GGGATCTTAGAGTGGCCCTTC
IFN-β F	CTGTCTGCTGGTGGAGTTCA
IFN-β R	ATAAGCAGCTCCAGCTCCAA
IL-6 F	GAGCATTGGAAATTGGGGTA
IL-6 R	AACGATGATGCACTTGCAGA
YopM F	TTACCGCAGAGCCTGAAATC
YopM R	GCAACTCTGGCAATTCTTCC
YopJ F	TAGAAGTCATGCCCGCATTG
YopJ R	TGTCCTTATTGCCAGCATCG

References

1. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.

2. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.

3. Bell, J.K., et al., *Leucine-rich repeats and pathogen recognition in Toll-like receptors.* Trends Immunol, 2003. **24**(10): p. 528-33.

4. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.

5. Park, B.S., et al., *The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex.* Nature, 2009. **458**(7242): p. 1191-5.

6. Fritsch, J., et al., *Cell fate decisions regulated by K63 ubiquitination of tumor necrosis factor receptor 1.* Mol Cell Biol, 2014. **34**(17): p. 3214-28.

7. Legarda-Addison, D., et al., *NEMO/IKKgamma regulates an early NF-kappaB-independent cell-death checkpoint during TNF signaling.* Cell Death Differ, 2009. **16**(9): p. 1279-88.

8. Dondelinger, Y., et al., *RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition.* Cell Death Differ, 2013. **20**(10): p. 1381-92.

9. Dondelinger, Y., et al., *NF-kappaB-Independent Role of IKKalpha/IKKbeta in Preventing RIPK1 Kinase-Dependent Apoptotic and N ecroptotic Cell Death during TNF Signaling.* Mol Cell, 2015. **60**(1): p. 63-76.

10. Kim, S.J. and J. Li, *Caspase blockade induces RIP3-mediated programmed necrosis in Toll-like receptor-activated microglia.* Cell Death Dis, 2013. **4**: p. e716.

11. Weng, D., et al., *Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death.* Proc Natl Acad Sci U S A, 2014. **111**(20): p. 7391-6.

12. Wang, J.S., et al., *TAK1 inhibition-induced RIP1-dependent apoptosis in murine macrophages relies on constitutive TNF-alpha signaling and ROS production.* J Biomed Sci, 2015. **22**: p. 76.

13. Zak, D.E., V.C. Tam, and A. Aderem, *Systems-level analysis of innate immunity.* Annu Rev Immunol, 2014. **32**: p. 547-77.

14. Liu, Q., et al., *Cutting Edge: Synchronization of IRF1, JunB, and C/EBPbeta Activities during TLR3-TLR7 Cross-Talk Orchestrates Timely Cytokine Synergy in the Proinflammatory Response.* J Immunol, 2015. **195**(3): p. 801-5.

15. Tan, R.S., et al., *TLR cross-talk confers specificity to innate immunity.* Int Rev Immunol, 2014. **33**(6): p. 443-53.

16. Peroval, M.Y., et al., *A critical role for MAPK signalling pathways in the transcriptional regulation of toll like receptors.* PLoS One, 2013. **8**(2): p. e51243.

17. Vladimer, G.I., et al., *Inflammasomes and host defenses against bacterial infections.* Curr Opin Microbiol, 2013. **16**(1): p. 23-31.

18. Davis, B.K., H. Wen, and J.P. Ting, *The inflammasome NLRs in immunity, inflammation, and associated diseases.* Annu Rev Immunol, 2011. **29**: p. 707-35.

19. Maslanik, T., et al., *The inflammasome and danger associated molecular patterns (DAMPs) are implicated in cytokine and chemokine responses following stressor exposure.* Brain Behav Immun, 2013. **28**: p. 54-62.

20. Schotte, P., et al., *Targeting Rac1 by the Yersinia effector protein YopE inhibits caspase-1-mediated maturation and release of interleukin-1beta.* J Biol Chem, 2004. **279**(24): p. 25134-42.

21. Bergsbaken, T., S.L. Fink, and B.T. Cookson, *Pyroptosis: host cell death and inflammation.* Nat Rev Microbiol, 2009. **7**(2): p. 99-109.

22. Proell, M., et al., *The CARD plays a critical role in ASC foci formation and inflammasome signalling.* Biochem J, 2013. **449**(3): p. 613-21.

23. Fernandes-Alnemri, T., et al., *The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation.* Cell Death Differ, 2007. **14**(9): p. 1590-604.

24. Kayagaki, N., et al., *Non-canonical inflammasome activation targets caspase-11.* Nature, 2011. **479**(7371): p. 117-21.

25. Broz, P. and D.M. Monack, *Noncanonical inflammasomes: caspase-11 activation and effector mechanisms.* PLoS Pathog, 2013. **9**(2): p. e1003144.

26. Gurung, P., et al., *FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes.* J Immunol, 2014. **192**(4): p. 1835-46.

27. Gringhuis, S.I., et al., *Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome.* Nat Immunol, 2012. **13**(3): p. 246-54.

28. Maltez, V.I. and E.A. Miao, *Reassessing the Evolutionary Importance of Inflammasomes.* J Immunol, 2016. **196**(3): p. 956-62.

29. Miao, E.A., et al., *Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria.* Nat Immunol, 2010. **11**(12): p. 1136-42.

30. Tsuji, N.M., et al., *Roles of caspase-1 in Listeria infection in mice.* Int Immunol, 2004. **16**(2): p. 335-43.

31. Zheng, H., et al., *Resistance to fever induction and impaired acute-phase response in interleukin-1 beta-deficient mice.* Immunity, 1995. **3**(1): p. 9-19.

32. Case, C.L., S. Shin, and C.R. Roy, *Asc and Ipaf Inflammasomes direct distinct pathways for caspase-1 activation in response to Legionella pneumophila.* Infect Immun, 2009. **77**(5): p. 1981-91.

33. Sharp, F.A., et al., *Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome.* Proc Natl Acad Sci U S A, 2009. **106**(3): p. 870-5.

34. Li, H., S. Nookala, and F. Re, *Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release.* J Immunol, 2007. **178**(8): p. 5271-6.

35. Pisarev, V.M., et al., *Flt3 ligand and conjugation to IL-1beta peptide as adjuvants for a type 1, T-cell response to an HIV p17 gag v accine.* Vaccine, 2002. **20**(17-18): p. 2358-68.

36. Yang, J., H. Xu, and F. Shao, *Immunological function of familial Mediterranean fever disease protein Pyrin.* Sci China Life Sci, 2014. **57**(12): p. 1156-61.

37. Medlej-Hashim, M., et al., *[Familial Mediterranean Fever (FMF): from diagnosis to treatment]*. Sante, 2004. **14**(4): p. 261-6.

38. Stack, J.H., et al., *IL-converting enzyme/caspase-1 inhibitor VX-765 blocks the hypersensitive response to an inflammatory stimulus in monocytes from familial cold autoinflammatory syndrome patients.* J Immunol, 2005. **175**(4): p. 2630-4.

39. Hoffman, H.M., et al., *Role of the leucine-rich repeat domain of cryopyrin/NALP3 in monosodium urate crystal-induced inflammation in mice.* Arthritis Rheum, 2010. **62**(7): p. 2170-9.

40. Jha, S., et al., *The inflammasome sensor, NLRP3, regulates CNS inflammation and demyelination via caspase-1 and interleukin-18.* J Neurosci, 2010. **30**(47): p. 15811-20.

41. Gris, D., et al., *NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses.* J Immunol, 2010. **185**(2): p. 974-81.

42. Inoue, M., et al., *NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis.* Proc Natl Acad Sci U S A, 2012. **109**(26): p. 10480-5.

43. Halle, A., et al., *The NALP3 inflammasome is involved in the innate immune response to amyloid-beta.* Nat Immunol, 2008. **9**(8): p. 857-65.

44. Yan, Y., et al., *Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome.* Cell, 2015. **160**(1-2): p. 62-73.

45. Weinstock, J.V., et al., *IL-18 and IL-12 signal through the NF-kappa B pathway to induce NK-1R expression on T cells.* J Immunol, 2003. **170**(10): p. 5003-7.

46. Sahar, S., et al., *Angiotensin II enhances interleukin-18 mediated inflammatory gene expression in vascular smooth muscle cells: a novel cross-talk in the pathogenesis of atherosclerosis.* Circ Res, 2005. **96**(10): p. 1064-71.

47. Stylianou, E., et al., *Interleukin 1 induces NF-kappa B through its type I but not its type II receptor in lymphocytes.* J Biol Chem, 1992. **267**(22): p. 15836-41.

48. Lee, J.K., et al., *Differences in signaling pathways by IL-1beta and IL-18.* Proc Natl Acad Sci U S A, 2004. **101**(23): p. 8815-20.

49. Akira, S., *The role of IL-18 in innate immunity.* Curr Opin Immunol, 2000. **12**(1): p. 59-63.

50. Sims, J.E. and D.E. Smith, *The IL-1 family: regulators of immunity.* Nature Reviews Immunology, 2010. **10**(2): p. 89-102.

51. Nakae, S., et al., *IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response.* Int Immunol, 2003. **15**(4): p. 483-90.

52. Lebovic, D.I., et al., *IL-1beta induction of RANTES (regulated upon activation, normal T cell expressed and secreted) chemokine gene expression in*

endometriotic stromal cells depends on a nuc lear factor-kappaB site in the proximal promoter. J Clin Endocrinol Metab, 2001. **86**(10): p. 4759-64.

53. Saperstein, S., et al., *IL-1beta augments TNF-alpha-mediated inflammatory responses from lung epithelial cells.* J Interferon Cytokine Res, 2009. **29**(5): p. 273-84.

54. Saperstein, S., et al., *The effects of interleukin-1beta in tumor necrosis factor-alpha-induced acute pulmonary inflammation in mice.* Mediators Inflamm, 2009. **2009**: p. 958658.

55. Matsukawa, A., et al., *Production and regulation of monocyte chemoattractant protein-1 in lipopolysaccharide- or monosodium urate crystal-induced arthritis in rabbits: roles of tumor necrosis factor alpha, interleukin-1, and interleukin-8.* Lab Invest, 1998. **78**(8): p. 973-85.

56. Matsukawa, A., et al., *Involvement of growth-related protein in lipopolysaccharide-induced rabbit arthritis: cooperation between growth-related protein and IL-8, and interrelated regulation among TNFalpha, IL-1, IL-1 receptor antagonist, IL-8, and growth-related protein.* Lab Invest, 1999. **79**(5): p. 591-600.

57. Matsukawa, A., et al., *Neutrophil accumulation and activation by homologous IL-8 in rabbits. IL-8 induces destruction of cartilage and production of IL-1 and IL-1 receptor antagonist in vivo.* J Immunol, 1995. **154**(10): p. 5418-25.

58. Matsukawa, A., et al., *Analysis of the cytokine network among tumor necrosis factor alpha, interleukin-1beta, interleukin-8, and i nterleukin-1 receptor antagonist in monosodium urate crystal-induced rabbit arthritis.* Lab Invest, 1998. **78**(5): p. 559-69.

59. Matsukawa, A., et al., *Analysis of the inflammatory cytokine network among TNF alpha, IL-1 beta, IL-1 receptor antagonist, and IL-8 in LPS-induced rabbit arthritis.* Lab Invest, 1997. **76**(5): p. 629-38.

60. Muller, S., et al., *Poorly Cross-Linked Peptidoglycan in MRSA Due to mecA Induction Activates the Inflammasome and Exacerbates Immunopathology.* Cell Host Microbe, 2015. **18**(5): p. 604-12.

61. Miller, L.S., et al., *Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against Staphylococcus aureus in vivo.* J Immunol, 2007. **179**(10): p. 6933-42.

62. Ceballos-Olvera, I., et al., *Inflammasome-dependent pyroptosis and IL-18 protect against Burkholderia pseudomallei lung infection while IL-1beta is deleterious.* PLoS Pathog, 2011. **7**(12): p. e1002452.

63. Ketelut-Carneiro, N., et al., *IL-18 triggered by the Nlrp3 inflammasome induces host innate resistance in a pulmonary model of fungal infection.* J Immunol, 2015. **194**(9): p. 4507-17.

64. Sahoo, M., et al., *Role of the inflammasome, IL-1beta, and IL-18 in bacterial infections.* ScientificWorldJournal, 2011. **11**: p. 2037-50.

65. Zemans, R.L., S.P. Colgan, and G.P. Downey, *Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury.* Am J Respir Cell Mol Biol, 2009. **40**(5): p. 519-35.

66. Van Opdenbosch, N., et al., *Activation of the NLRP1b inflammasome independently of ASC-mediated caspase-1 autoproteolysis and speck formation.* Nat Commun, 2014. **5**: p. 3209.

67. Kavathas, P.B., et al., *Nod1, but not the ASC inflammasome, contributes to induction of IL-1beta secretion in human trophoblasts after sensing of Chlamydia trachomatis.* Mucosal Immunol, 2013. **6**(2): p. 235-43.

68. Hornung, V., et al., *AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC.* Nature, 2009. **458**(7237): p. 514-8.

69. Connolly, D.J. and A.G. Bowie, *The emerging role of human PYHIN proteins in innate immunity: implications for health and disease.* Biochem Pharmacol, 2014. **92**(3): p. 405-14.

70. Khare, S., et al., *The PYRIN domain-only protein POP3 inhibits ALR inflammasomes and regulates responses to infection with DNA viruses.* Nat Immunol, 2014. **15**(4): p. 343-53.

71. Rathinam, V.A., et al., *The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses.* Nat Immunol, 2010. **11**(5): p. 395-402.

72. Papin, S., et al., *The SPRY domain of Pyrin, mutated in familial Mediterranean fever patients, interacts with inflammasome components and inhibits proIL-1beta processing.* Cell Death Differ, 2007. **14**(8): p. 1457-66.

73. Chae, J.J., et al., *Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1beta activation and severe autoinflammation in mice.* Immunity, 2011. **34**(5): p. 755-68.

74. Manukyan, G. and R. Aminov, *Update on Pyrin Functions and Mechanisms of Familial Mediterranean Fever.* Front Microbiol, 2016. **7**: p. 456.

75. Yu, J.W., et al., *Ribotoxic stress through p38 mitogen-activated protein kinase activates in vitro the human pyrin inflammasome.* J Biol Chem, 2013. **288**(16): p. 11378-83.

76. Akkaya-Ulum, Y.Z., et al., *Pyrin-PSTPIP1 colocalises at the leading edge during cell migration.* Cell Biol Int, 2015. **39**(12): p. 1384-94.

77. Balci-Peynircioglu, B., et al., *Pyrin, product of the MEFV locus, interacts with the proapoptotic protein, Siva.* J Cell Physiol, 2008. **216**(3): p. 595-602.

78. Masters, S.L., et al., *Familial autoinflammation with neutrophilic dermatosis reveals a regulatory mechanism of pyrin activation.* Sci Transl Med, 2016. **8**(332): p. 332ra45.

79. Jeru, I., et al., *Interaction of pyrin with 14.3.3 in an isoform-specific and phosphorylation-dependent manner regulates its translocation to the nucleus.* Arthritis Rheum, 2005. **52**(6): p. 1848-57.

80. Waite, A.L., et al., *Pyrin and ASC co-localize to cellular sites that are rich in polymerizing actin.* Exp Biol Med (Maywood), 2009. **234**(1): p. 40-52.

81. Kim, M.L., et al., *Aberrant actin depolymerization triggers the pyrin inflammasome and autoinflammatory disease that is dependent on IL-18, not IL-1beta.* J Exp Med, 2015. **212**(6): p. 927-38.

82. Xu, H., et al., *Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome.* Nature, 2014. **513**(7517): p. 237-41.

83. Zhao, Y., et al., *The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus.* Nature, 2011. **477**(7366): p. 596-600.

84. Tenthorey, J.L., et al., *Molecular basis for specific recognition of bacterial ligands by NAIP/NLRC4 inflammasomes.* Mol Cell, 2014. **54**(1): p. 17-29.

85. Weng, L., et al., *The E3 ubiquitin ligase tripartite motif 33 is essential for cytosolic RNA-induced NLRP3 inflammasome activation.* J Immunol, 2014. **193**(7): p. 3676-82.

86. Mitoma, H., et al., *The DHX33 RNA helicase senses cytosolic RNA and activates the NLRP3 inflammasome.* Immunity, 2013. **39**(1): p. 123-35.

87. Latz, E., T.S. Xiao, and A. Stutz, *Activation and regulation of the inflammasomes.* Nat Rev Immunol, 2013. **13**(6): p. 397-411.

88. Saitoh, T., et al., *Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production.* Nature, 2008. **456**(7219): p. 264-8.

89. Harris, J., et al., *Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation.* J Biol Chem, 2011. **286**(11): p. 9587-97.

90. Shi, C.S., et al., *Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction.* Nat Immunol, 2012. **13**(3): p. 255-63.

91. Zhou, R., et al., *A role for mitochondria in NLRP3 inflammasome activation.* Nature, 2011. **469**(7329): p. 221-5.

92. Orlowski, G.M., et al., *Multiple Cathepsins Promote Pro-IL-1beta Synthesis and NLRP3-Mediated IL-1beta Activation.* J Immunol, 2015. **195**(4): p. 1685-97.

93. Cartledge, D.M., et al., *Inhibitors of cathepsins B and L induce autophagy and cell death in neuroblastoma cells.* Invest New Drugs, 2013. **31**(1): p. 20-9.

94. Repnik, U., et al., *Lysosomes and lysosomal cathepsins in cell death.* Biochim Biophys Acta, 2012. **1824**(1): p. 22-33.

95. Tatti, M., et al., *Reduced cathepsins B and D cause impaired autophagic degradation that can be almost completely restored by overexpression of these two proteases in Sap C-deficient fibroblasts.* Hum Mol Genet, 2012. **21**(23): p. 5159-73.

96. Kayagaki, N., et al., *Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling.* Nature, 2015. **526**(7575): p. 666-71.

97. Ruhl, S. and P. Broz, *Caspase-11 activates a canonical NLRP3 inflammasome by promoting K(+) efflux.* Eur J Immunol, 2015. **45**(10): p. 2927-36.

98. Rathinam, V.A., et al., *TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria.* Cell, 2012. **150**(3): p. 606-19.

99. Meunier, E. and P. Broz, *Interferon-induced guanylate-binding proteins promote cytosolic lipopolysaccharide detection by caspase-11.* DNA Cell Biol, 2015. **34**(1): p. 1-5.

100. Pilla, D.M., et al., *Guanylate binding proteins promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS.* Proc Natl Acad Sci U S A, 2014. **111**(16): p. 6046-51.

101. Kim, B.H., et al., *A family of IFN-gamma-inducible 65-kD GTPases protects against bacterial infection.* Science, 2011. **332**(6030): p. 717-21.

102. Yamamoto, M., et al., *A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii.* Immunity, 2012. **37**(2): p. 302-13.

103. Meunier, E., et al., *Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases.* Nature, 2014. **509**(7500): p. 366-70.

104. Aachoui, Y., et al., *Canonical Inflammasomes Drive IFN-gamma to Prime Caspase-11 in Defense against a Cytosol-Invasive Bacterium.* Cell Host Microbe, 2015. **18**(3): p. 320-32.

105. Aachoui, Y., et al., *Caspase-11 protects against bacteria that escape the vacuole.* Science, 2013. **339**(6122): p. 975-8.

106. Akhter, A., et al., *Caspase-11 promotes the fusion of phagosomes harboring pathogenic bacteria with lysosomes by modulating actin polymerization.* Immunity, 2012. **37**(1): p. 35-47.

107. Hagar, J.A., et al., *Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock.* Science, 2013. **341**(6151): p. 1250-3.

108. Tuncer, S., M.T. Fiorillo, and R. Sorrentino, *The multifaceted nature of NLRP12.* J Leukoc Biol, 2014. **96**(6): p. 991-1000.

109. Chen, G.Y., *Role of Nlrp6 and N lrp12 in the maintenance of intestinal homeostasis.* Eur J Immunol, 2014. **44**(2): p. 321-7.

110. Anand, P.K., et al., *NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens.* Nature, 2012. **488**(7411): p. 389-93.

111. Wlodarska, M., et al., *NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion.* Cell, 2014. **156**(5): p. 1045-59.

112. Ydens, E., et al., *Nlrp6 promotes recovery after peripheral nerve injury independently of inflammasomes.* J Neuroinflammation, 2015. **12**: p. 143.

113. Wang, P., et al., *Nlrp6 regulates intestinal antiviral innate immunity.* Science, 2015. **350**(6262): p. 826-30.

114. Zambetti, L.P. and A. Mortellaro, *NLRPs, microbiota, and gut homeostasis: unravelling the connection.* J Pathol, 2014. **233**(4): p. 321-30.

115. Gharagozloo, M., et al., *The nod-like receptor, Nlrp12, plays an ant i-inflammatory role in experimental autoimmune encephalomyelitis.* J Neuroinflammation, 2015. **12**: p. 198.

116. Lukens, J.R., et al., *The NLRP12 Sensor Negatively Regulates Autoinflammatory Disease by Modulating Interleukin-4 Production in T Cells.* Immunity, 2015. **42**(4): p. 654-64.

117. Ataide, M.A., et al., *Malaria-induced NLRP12/NLRP3-dependent caspase-1 activation mediates inflammation and hypersensitivity to bacterial superinfection.* PLoS Pathog, 2014. **10**(1): p. e1003885.

118. Vladimer, G.I., et al., *The NLRP12 inflammasome recognizes Yersinia pestis.* Immunity, 2012. **37**(1): p. 96-107.

119. Cai, S., et al., *NLRP12 modulates host defense through IL-17A-CXCL1 axis.* Mucosal Immunol, 2016. **9**(2): p. 503-14.

120. Kono, H., et al., *The IL-1-dependent sterile inflammatory response has a substantial caspase-1-independent component that requires cathepsin C.* J Immunol, 2012. **189**(7): p. 3734-40.

121. Provoost, S., et al., *NLRP3/caspase-1-independent IL-1beta production mediates diesel exhaust particle-induced pulmonary inflammation.* J Immunol, 2011. **187**(6): p. 3331-7.

122. Mayer-Barber, K.D., et al., *Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and do es not require TLR signaling in vivo.* J Immunol, 2010. **184**(7): p. 3326-30.

123. Guma, M., et al., *Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation.* Arthritis Rheum, 2009. **60**(12): p. 3642-50.

124. Philip, N.H., et al., *Caspase-8 mediates caspase-1 processing and innate immune defense in response to bacterial blockade of NF-kappaB and MAPK signaling.* Proc Natl Acad Sci U S A, 2014. **111**(20): p. 7385-90.

125. Ganesan, S., et al., *Caspase-8 modulates dectin-1 and complement receptor 3-driven IL-1beta production in response to beta-glucans and the fungal pathogen, Candida albicans.* J Immunol, 2014. **193**(5): p. 2519-30.

126. Antonopoulos, C., et al., *Proapoptotic chemotherapeutic drugs induce noncanonical processing and release of IL-1beta via caspase-8 in dendritic cells.* J Immunol, 2013. **191**(9): p. 4789-803.

127. Deng, Q., et al., *Pseudomonas aeruginosa Triggers Macrophage Autophagy To Escape Intracellular Killing by Activation of the NLRP3 Inflammasome.* Infect Immun, 2015. **84**(1): p. 56-66.

128. Gavrilin, M.A., et al., *Pyrin critical to macrophage IL-1beta response to Francisella challenge.* J Immunol, 2009. **182**(12): p. 7982-9.

129. Zamoshnikova, A., et al., *NLRP12 is a neutrophil-specific, negative regulator of in vitro cell migration but does not modulate LPS- or infection-induced NF-kappaB or ERK signalling.* Immunobiology, 2016. **221**(2): p. 341-6.

130. Boyden, E.D. and W.F. Dietrich, *Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin.* Nat Genet, 2006. **38**(2): p. 240-4.

131. Costa, T.R., et al., *Secretion systems in Gram-negative bacteria: structural and mechanistic insights.* Nat Rev Microbiol, 2015. **13**(6): p. 343-59.

132. Davis, A.J., D.A. Diaz, and J. Mecsas, *A dominant-negative needle mutant blocks type III secretion of early but not late substrates in Yersinia.* Mol Microbiol, 2010. **76**(1): p. 236-59.

133. Torruellas, J., et al., *The Yersinia pestis type III secretion needle plays a role in the regulation of Yop secretion.* Mol Microbiol, 2005. **57**(6): p. 1719-33.

134. Marlovits, T.C., et al., *Assembly of the inner rod determines needle length in the type III secretion injectisome.* Nature, 2006. **441**(7093): p. 637-40.

135. Wood, S.E., J. Jin, and S.A. Lloyd, *YscP and YscU switch the substrate specificity of the Yersinia type III secretion system by regulating export of the inner rod protein YscI.* J Bacteriol, 2008. **190**(12): p. 4252-62.

136. Bi, Y., et al., *Reduced apoptosis of mouse macrophages induced by yscW mutant of Yersinia pestis results from the reduced secretion of YopJ and relates to caspase-3 signal pathway.* Scand J Immunol, 2009. **70**(4): p. 358-67.

137. Gong, L., et al., *The Burkholderia pseudomallei type III secretion system and BopA are required for evasion of LC3-associated phagocytosis.* PLoS One, 2011. **6**(3): p. e17852.

138. Kubori, T. and J.E. Galan, *Salmonella type III secretion-associated protein InvE controls translocation of effector proteins into host cells.* J Bacteriol, 2002. **184**(17): p. 4699-708.

139. Kaniga, K., D. Trollinger, and J.E. Galan, *Identification of two targets of the type III protein secretion system encoded by the inv and s pa loci of Salmonella typhimurium that have homology to the Shigella IpaD and IpaA proteins.* J Bacteriol, 1995. **177**(24): p. 7078-85.

140. Holzer, S.U. and M. Hensel, *Functional dissection of translocon proteins of the Salmonella pathogenicity island 2-encoded type III secretion system.* BMC Microbiol, 2010. **10**: p. 104.

141. Jessen, D.L., et al., *Type III secretion needle proteins induce cell signaling and cytokine secretion via Toll-like receptors.* Infect Immun, 2014. **82**(6): p. 2300-9.

142. Osei-Owusu, P., et al., *The N terminus of type III secretion needle protein YscF from Yersinia pestis functions to modulate innate immune responses.* Infect Immun, 2015. **83**(4): p. 1507-22.

143. Yang, J., et al., *Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation.* Proc Natl Acad Sci U S A, 2013. **110**(35): p. 14408-13.

144. Sato, H. and D.W. Frank, *Multi-Functional Characteristics of the Pseudomonas aeruginosa Type III Needle-Tip Protein, PcrV; Comparison to Orthologs in other Gram-negative Bacteria.* Front Microbiol, 2011. **2**: p. 142.

145. Majowicz, S.E., et al., *The global burden of nontyphoidal Salmonella gastroenteritis.* Clin Infect Dis, 2010. **50**(6): p. 882-9.

146. McGhie, E.J., et al., *Salmonella takes control: effector-driven manipulation of the host.* Curr Opin Microbiol, 2009. **12**(1): p. 117-24.

147. Ao, T.T., et al., *Global burden of invasive nontyphoidal Salmonella disease, 2010(1).* Emerg Infect Dis, 2015. **21**(6).

148. Mogasale, V., et al., *Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment.* Lancet Glob Health, 2014. **2**(10): p. e570-80.

149. Kirk, M.D., et al., *World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis.* PLoS Med, 2015. **12**(12): p. e1001921.

150. Murray, C.J., et al., *Global, regional, and national disability-adjusted life years (DALYs) for 306 diseases and injuries and healthy life expectancy (HALE) for 188 countries, 1990-2013: quantifying the epidemiological transition.* Lancet, 2015. **386**(10009): p. 2145-91.

151. Groisman, E.A. and H. Ochman, *How Salmonella became a pathogen.* Trends Microbiol, 1997. **5**(9): p. 343-9.

152. Galan, J.E., *Salmonella interactions with host cells: type III secretion at work.* Annu Rev Cell Dev Biol, 2001. **17**: p. 53-86.

153. Figueira, R. and D.W. Holden, *Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors.* Microbiology, 2012. **158**(Pt 5): p. 1147-61.

154. Galan, J.E., *Molecular genetic bases of Salmonella entry into host cells.* Mol Microbiol, 1996. **20**(2): p. 263-71.

155. Hensel, M., et al., *Analysis of the boundaries of Salmonella pathogenicity island 2 and the corresponding chromosomal region of Escherichia coli K-12.* J Bacteriol, 1997. **179**(4): p. 1105-11.

156. Ochman, H. and E.A. Groisman, *Distribution of pathogenicity islands in Salmonella spp.* Infect Immun, 1996. **64**(12): p. 5410-2.

157. Ellermeier, J.R. and J.M. Slauch, *Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium.* Curr Opin Microbiol, 2007. **10**(1): p. 24-9.

158. Kimbrough, T.G. and S.I. Miller, *Assembly of the type III secretion needle complex of Salmonella typhimurium.* Microbes Infect, 2002. **4**(1): p. 75-82.

159. Mariathasan, S., et al., *Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf.* Nature, 2004. **430**(6996): p. 213-8.

160. Broz, P., et al., *Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella.* J Exp Med, 2010. **207**(8): p. 1745-55.

161. Kofoed, E.M. and R.E. Vance, *Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity.* Nature, 2011. **477**(7366): p. 592-5.

162. Rayamajhi, M., et al., *Cutting edge: Mouse NAIP1 detects the type III secretion system needle protein.* J Immunol, 2013. **191**(8): p. 3986-9.

163. Vinzing, M., et al., *NAIP and Ipaf control Legionella pneumophila replication in human cells.* J Immunol, 2008. **180**(10): p. 6808-15.

164. Broz, P., et al., *Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1.* Nature, 2012. **490**(7419): p. 288-91.

165. Kayagaki, N., et al., *Noncanonical inflammasome activation by intracellular LPS independent of TLR4.* Science, 2013. **341**(6151): p. 1246-9.

166. Shi, J., et al., *Inflammatory caspases are innate immune receptors for intracellular LPS.* Nature, 2014. **514**(7521): p. 187-92.

167. Shi, J., et al., *Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death.* Nature, 2015. **526**(7575): p. 660-5.

168. Zhao, W., et al., *The Salmonella effector protein SifA plays a dual role in virulence.* Sci Rep, 2015. **5**: p. 12979.

169. West, T.E., et al., *NLRC4 and TLR5 each contribute to host defense in respiratory melioidosis.* PLoS Negl Trop Dis, 2014. **8**(9): p. e3178.

170. Rosales-Reyes, R., et al., *Burkholderia cenocepacia type VI secretion system mediates escape of type II secreted proteins into the cytoplasm of infected macrophages.* PLoS One, 2012. **7**(7): p. e41726.

171. Bast, A., et al., *Caspase-1-dependent and -independent cell death pathways in Burkholderia pseudomallei infection of macrophages.* PLoS Pathog, 2014. **10**(3): p. e1003986.

172. Sahoo, M., et al., *Neutrophil elastase causes tissue damage that decreases host tolerance to lung infection with burkholderia species.* PLoS Pathog, 2014. **10**(8): p. e1004327.

173. Gavrilin, M.A., et al., *Activation of the pyrin inflammasome by intracellular Burkholderia cenocepacia.* J Immunol, 2012. **188**(7): p. 3469-77.

174. Aubert, D.F., S. Hu, and M.A. Valvano, *Quantification of type VI secretion system activity in macrophages infected with Burkholderia cenocepacia.* Microbiology, 2015. **161**(11): p. 2161-73.

175. Rosales-Reyes, R., et al., *The Type VI secretion system of Burkholderia cenocepacia affects multiple Rho family GTPases disrupting the actin cytoskeleton and the assembly of NADPH oxidase complex in macrophages.* Cell Microbiol, 2012. **14**(2): p. 255-73.

176. Flannagan, R.S., et al., *Burkholderia cenocepacia disrupts host cell actin cytoskeleton by inactivating Rac and Cdc42.* Cell Microbiol, 2012. **14**(2): p. 239-54.

177. de Zoete, M.R. and R.A. Flavell, *Detecting "different": Pyrin senses modified GTPases.* Cell Res, 2014. **24**(11): p. 1286-7.

178. Karmakar, M., et al., *Cutting edge: IL-1beta processing during Pseudomonas aeruginosa infection is mediated by neutrophil serine proteases and i s independent of NLRC4 and caspase-1.* J Immunol, 2012. **189**(9): p. 4231-5.

179. Faure, E., et al., *Pseudomonas aeruginosa type-3 secretion system dampens host defense by exploiting the NLRC4-coupled inflammasome.* Am J Respir Crit Care Med, 2014. **189**(7): p. 799-811.

180. Patankar, Y.R., R. Mabaera, and B. Berwin, *Differential ASC requirements reveal a key role for neutrophils and a noncanonical IL-1beta response to Pseudomonas aeruginosa.* Am J Physiol Lung Cell Mol Physiol, 2015. **309**(8): p. L902-13.

181. Diao, L., et al., *Burn plus lipopolysaccharide augments endoplasmic reticulum stress and NLRP3 inflammasome activation and reduces PGC-1alpha in liver.* Shock, 2014. **41**(2): p. 138-44.

182. Kung, V.L., et al., *An rhs gene of Pseudomonas aeruginosa encodes a virulence protein that activates the inflammasome.* Proc Natl Acad Sci U S A, 2012. **109**(4): p. 1275-80.

183. Cohen, T.S. and A.S. Prince, *Activation of inflammasome signaling mediates pathology of acute P. aeruginosa pneumonia.* J Clin Invest, 2013. **123**(4): p. 1630-7.

184. Jabir, M.S., et al., *Mitochondrial damage contributes to Pseudomonas aeruginosa activation of the inflammasome and is downregulated by autophagy.* Autophagy, 2015. **11**(1): p. 166-82.

185. Jabir, M.S., et al., *Caspase-1 cleavage of the TLR adaptor TRIF inhibits autophagy and beta-interferon production during Pseudomonas aeruginosa infection.* Cell Host Microbe, 2014. **15**(2): p. 214-27.

186. Pang, Z., et al., *AIM2 inflammasome is dispensable for the host defense against Pseudomonas aeruginosa infection.* Cell Mol Biol (Noisy-le-grand), 2015. **61**(3): p. 63-70.

187. Henriksson, M.L., et al., *Exoenzyme S shows selective ADP-ribosylation and GTPase-activating protein (GAP) activities towards small GTPases in vivo.* Biochem J, 2002. **367**(Pt 3): p. 617-28.

188. Ganter, M.T., et al., *Role of small GTPases and al phavbeta5 integrin in Pseudomonas aeruginosa-induced increase in lung endothelial permeability.* Am J Respir Cell Mol Biol, 2009. **40**(1): p. 108-18.

189. Litvak, Y., et al., *High catalytic efficiency and resistance to denaturing in bacterial Rho GTPase-activating proteins.* Biol Chem, 2011. **392**(6): p. 505-16.

190. Ince, D., F.S. Sutterwala, and T.L. Yahr, *Secretion of Flagellar Proteins by the Pseudomonas aeruginosa Type III Secretion-Injectisome System.* J Bacteriol, 2015. **197**(12): p. 2003-11.

191. Patankar, Y.R., et al., *Flagellar motility is a key determinant of the magnitude of the inflammasome response to Pseudomonas aeruginosa.* Infect Immun, 2013. **81**(6): p. 2043-52.

192. Huus, K.E., et al., *Clinical Isolates of Pseudomonas aeruginosa from Chronically Infected Cystic Fibrosis Patients Fail To Activate the Inflammasome during Both Stable Infection and Pulmonary Exacerbation.* J Immunol, 2016.

193. Ketko, A.K., et al., *Surfactant protein A binds flagellin enhancing phagocytosis and IL-1beta production.* PLoS One, 2013. **8**(12): p. e82680.

194. Anantharajah, A., et al., *Correlation between cytotoxicity induced by Pseudomonas aeruginosa clinical isolates from acute infections and I L-1beta secretion in a model of human THP-1 monocytes.* Pathog Dis, 2015. **73**(7).

195. Nakahira, K., et al., *Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome.* Nat Immunol, 2011. **12**(3): p. 222-30.

196. Torres, I.M., et al., *Acidosis potentiates the host proinflammatory interleukin-1beta response to Pseudomonas aeruginosa infection.* Infect Immun, 2014. **82**(11): p. 4689-97.

197. Namba, T., et al., *Autophagic clearance of mitochondria in the kidney copes with metabolic acidosis.* J Am Soc Nephrol, 2014. **25**(10): p. 2254-66.

198. Wojtkowiak, J.W. and R.J. Gillies, *Autophagy on acid.* Autophagy, 2012. **8**(11): p. 1688-9.

199. Marino, M.L., et al., *Autophagy is a protective mechanism for human melanoma cells under acidic stress.* J Biol Chem, 2012. **287**(36): p. 30664-76.

200. Tate, S., et al., *Airways in cystic fibrosis are acidified: detection by exhaled breath condensate.* Thorax, 2002. **57**(11): p. 926-9.

201. Tang, X.X., et al., *Acidic pH increases airway surface liquid viscosity in cystic fibrosis.* J Clin Invest, 2016. **126**(3): p. 879-91.

202. Pezzulo, A.A., et al., *Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung.* Nature, 2012. **487**(7405): p. 109-13.

203. Rimessi, A., et al., *Mitochondrial Ca2+-dependent NLRP3 activation exacerbates the Pseudomonas aeruginosa-driven inflammatory response in cystic fibrosis.* Nat Commun, 2015. **6**: p. 6201.

204. Wonnenberg, B., et al., *Probenecid reduces infection and inflammation in acute Pseudomonas aeruginosa pneumonia.* Int J Med Microbiol, 2014. **304**(5-6): p. 725-9.

205. Hanley, P.J., et al., *Transient P2X7 receptor activation triggers macrophage death independent of Toll-like receptors 2 and 4, c aspase-1, and panne xin-1 proteins.* J Biol Chem, 2012. **287**(13): p. 10650-63.

206. Sato, H. and D.W. Frank, *ExoU is a pot ent intracellular phospholipase.* Mol Microbiol, 2004. **53**(5): p. 1279-90.

207. Czechowska, K., et al., *Cheating by type 3 secretion system-negative Pseudomonas aeruginosa during pulmonary infection.* Proc Natl Acad Sci U S A, 2014. **111**(21): p. 7801-6.

208. Arlehamn, C.S. and T.J. Evans, *Pseudomonas aeruginosa pilin activates the inflammasome.* Cell Microbiol, 2011. **13**(3): p. 388-401.

209. Cassel, S.L., et al., *Inflammasome-independent IL-1beta mediates autoinflammatory disease in Pstpip2-deficient mice.* Proc Natl Acad Sci U S A, 2014. **111**(3): p. 1072-7.

210. Lauriano, C.M., et al., *MglA regulates transcription of virulence factors necessary for Francisella tularensis intraamoebae and intramacrophage survival.* Proc Natl Acad Sci U S A, 2004. **101**(12): p. 4246-9.

211. Santic, M., et al., *The Francisella tularensis pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm.* Cell Microbiol, 2005. **7**(7): p. 969-79.

212. Sjostedt, A., *Virulence determinants and pr otective antigens of Francisella tularensis.* Curr Opin Microbiol, 2003. **6**(1): p. 66-71.

213. Gavrilin, M.A., et al., *Internalization and phagos ome escape required for Francisella to induce human monocyte IL-1beta processing and release.* Proc Natl Acad Sci U S A, 2006. **103**(1): p. 141-6.

214. Huang, M.T., et al., *Deletion of ripA alleviates suppression of the inflammasome and MAPK by Francisella tularensis.* J Immunol, 2010. **185**(9): p. 5476-85.

215. Atianand, M.K., et al., *Francisella tularensis reveals a disparity between human and mouse NLRP3 inflammasome activation.* J Biol Chem, 2011. **286**(45): p. 39033-42.

216. Ghonime, M.G., et al., *Inflammasome priming is similar for francisella species that differentially induce inflammasome activation.* PLoS One, 2015. **10**(5): p. e0127278.

217. Gillette, D.D., et al., *Virulent Type A Francisella tularensis actively suppresses cytokine responses in human monocytes.* Front Cell Infect Microbiol, 2014. **4**: p. 45.

218. Gavrilin, M.A. and M.D. Wewers, *Francisella Recognition by Inflammasomes: Differences between Mice and Men.* Front Microbiol, 2011. **2**: p. 11.

219. Man, S.M., et al., *The transcription factor IRF1 and guanylate-binding proteins target activation of the AIM2 inflammasome by Francisella infection.* Nat Immunol, 2015. **16**(5): p. 467-75.

220. Burckstummer, T., et al., *An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome.* Nat Immunol, 2009. **10**(3): p. 266-72.

221. Fernandes-Alnemri, T., et al., *AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA.* Nature, 2009. **458**(7237): p. 509-13.

222. Roberts, T.L., et al., *HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA.* Science, 2009. **323**(5917): p. 1057-60.

223. Fernandes-Alnemri, T., et al., *The AIM2 inflammasome is critical for innate immunity to Francisella tularensis.* Nat Immunol, 2010. **11**(5): p. 385-93.

224. Woodward, J.J., A.T. Iavarone, and D.A. Portnoy, *c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response.* Science, 2010. **328**(5986): p. 1703-5.

225. Montminy, S.W., et al., *Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response.* Nat Immunol, 2006. **7**(10): p. 1066-73.

226. de Bruin, O.M., et al., *The biochemical properties of the Francisella pathogenicity island (FPI)-encoded proteins IglA, IglB, IglC, PdpB and DotU suggest roles in type VI secretion.* Microbiology, 2011. **157**(Pt 12): p. 3483-91.

227. Mougous, J.D., et al., *A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus.* Science, 2006. **312**(5779): p. 1526-30.

228. Leiman, P.G., et al., *Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin.* Proc Natl Acad Sci U S A, 2009. **106**(11): p. 4154-9.

229. Marra, A., et al., *Identification of a Legionella pneumophila locus required for intracellular multiplication in human macrophages.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9607-11.

230. Hubber, A. and C.R. Roy, *Modulation of host cell function by Legionella pneumophila type IV effectors.* Annu Rev Cell Dev Biol, 2010. **26**: p. 261-83.

231. Berger, K.H. and R.R. Isberg, *Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila.* Mol Microbiol, 1993. **7**(1): p. 7-19.

232. Roy, C.R., K.H. Berger, and R.R. Isberg, *Legionella pneumophila DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake.* Mol Microbiol, 1998. **28**(3): p. 663-74.

233. Rolando, M. and C. Buchrieser, *Legionella pneumophila type IV effectors hijack the transcription and translation machinery of the host cell.* Trends Cell Biol, 2014. **24**(12): p. 771-8.

234. Banga, S., et al., *Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family.* Proc Natl Acad Sci U S A, 2007. **104**(12): p. 5121-6.

235. Laguna, R.K., et al., *A Legionella pneumophila-translocated substrate that is required for growth within macrophages and protection from host cell death.* Proc Natl Acad Sci U S A, 2006. **103**(49): p. 18745-50.

236. Casson, C.N., et al., *Caspase-11 activation in response to bacterial secretion systems that access the host cytosol.* PLoS Pathog, 2013. **9**(6): p. e1003400.

237. Case, C.L., et al., *Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. 1851-6.

238. Amer, A., et al., *Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf.* J Biol Chem, 2006. **281**(46): p. 35217-23.

239. Molofsky, A.B., et al., *Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection.* J Exp Med, 2006. **203**(4): p. 1093-104.

240. Case, C.L. and C.R. Roy, *Asc modulates the function of NLRC4 in response to infection of macrophages by Legionella pneumophila.* MBio, 2011. **2**(4).

241. Cerqueira, D.M., et al., *Caspase-1 but Not Caspase-11 Is Required for NLRC4-Mediated Pyroptosis and Restriction of Infection by Flagellated Legionella Species in Mouse Macrophages and In Vivo.* J Immunol, 2015. **195**(5): p. 2303-11.

242. Suzuki, S., et al., *Shigella type III secretion protein MxiI is recognized by Naip2 to induce Nlrc4 inflammasome activation independently of Pkcdelta.* PLoS Pathog, 2014. **10**(2): p. e1003926.

243. Kobayashi, T., et al., *The Shigella OspC3 effector inhibits caspase-4, antagonizes inflammatory cell death, and promotes epithelial infection.* Cell Host Microbe, 2013. **13**(5): p. 570-83.

244. Suzuki, T., et al., *Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages.* PLoS Pathog, 2007. **3**(8): p. e111.

245. Paciello, I., et al., *Intracellular Shigella remodels its LPS to dampen the innate immune recognition and evade inflammasome activation.* Proc Natl Acad Sci U S A, 2013. **110**(46): p. E4345-54.

246. Butler, T., *Plague gives surprises in the first decade of the 21st century in the United States and worldwide.* Am J Trop Med Hyg, 2013. **89**(4): p. 788-93.

247. Viboud, G.I. and J.B. Bliska, *Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis.* Annu Rev Microbiol, 2005. **59**: p. 69-89.

248. Broz, P., et al., *Function and molecular architecture of the Yersinia injectisome tip complex.* Mol Microbiol, 2007. **65**(5): p. 1311-20.

249. Cornelis, G.R., et al., *The virulence plasmid of Yersinia, an antihost genome.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1315-52.

250. Auerbuch, V., D.T. Golenbock, and R.R. Isberg, *Innate immune recognition of Yersinia pseudotuberculosis type III secretion.* PLoS Pathog, 2009. **5**(12): p. e1000686.

251. Cornelis, G.R., *The Yersinia Ysc-Yop virulence apparatus.* Int J Med Microbiol, 2002. **291**(6-7): p. 455-62.

252. Gemski, P., et al., *Presence of a virulence-associated plasmid in Yersinia pseudotuberculosis.* Infect Immun, 1980. **28**(3): p. 1044-7.

253. Gemski, P., J.R. Lazere, and T. Casey, *Plasmid associated with pathogenicity and calcium dependency of Yersinia enterocolitica.* Infect Immun, 1980. **27**(2): p. 682-5.

254. Skrzypek, E. and S.C. Straley, *Differential effects of deletions in lcrV on secretion of V antigen, regulation of the low-Ca2+ response, and virulence of Yersinia pestis.* J Bacteriol, 1995. **177**(9): p. 2530-42.

255. Brodsky, I.E., et al., *A Yersinia effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system.* Cell Host Microbe, 2010. **7**(5): p. 376-87.

256. Zwack, E.E., et al., *Inflammasome activation in response to the Yersinia type III secretion system requires hyperinjection of translocon proteins YopB and YopD.* MBio, 2015. **6**(1): p. e02095-14.

257. LaRock, C.N. and B.T. Cookson, *The Yersinia virulence effector YopM binds caspase-1 to arrest inflammasome assembly and processing.* Cell Host Microbe, 2012. **12**(6): p. 799-805.

258. Chung, L.K., et al., *IQGAP1 is important for activation of caspase-1 in macrophages and i s targeted by Yersinia pestis type III effector YopM.* MBio, 2014. **5**(4): p. e01402-14.

259. Hentschke, M., et al., *Yersinia virulence factor YopM induces sustained RSK activation by interfering with dephosphorylation.* PLoS One, 2010. **5**(10).

260. McPhee, J.B., P. Mena, and J.B. Bliska, *Delineation of regions of the Yersinia YopM protein required for interaction with the RSK1 and PRK2 host kinases and their requirement for interleukin-10 production and v irulence.* Infect Immun, 2010. **78**(8): p. 3529-39.

261. Lilo, S., Y. Zheng, and J.B. Bliska, *Caspase-1 activation in macrophages infected with Yersinia pestis KIM requires the type III secretion system effector YopJ.* Infect Immun, 2008. **76**(9): p. 3911-23.

262. Zhou, H., et al., *Yersinia virulence factor YopJ acts as a deubiquitinase to inhibit NF-kappa B activation.* J Exp Med, 2005. **202**(10): p. 1327-32.

263. Zhou, L., A. Tan, and M.B. Hershenson, *Yersinia YopJ inhibits pro-inflammatory molecule expression in human bronchial epithelial cells.* Respir Physiol Neurobiol, 2004. **140**(1): p. 89-97.

264. Mittal, R., S.Y. Peak-Chew, and H.T. McMahon, *Acetylation of MEK2 and I kappa B kinase (IKK) activation loop residues by YopJ inhibits signaling.* Proc Natl Acad Sci U S A, 2006. **103**(49): p. 18574-9.

265. Mukherjee, S. and K. Orth, *In vitro signaling by MAPK and NFkappaB pathways inhibited by Yersinia YopJ.* Methods Enzymol, 2008. **438**: p. 343-53.

266. Paquette, N., et al., *Serine/threonine acetylation of TGFbeta-activated kinase (TAK1) by Yersinia pestis YopJ inhibits innate immune signaling.* Proc Natl Acad Sci U S A, 2012. **109**(31): p. 12710-5.

267. Meinzer, U., et al., *Yersinia pseudotuberculosis effector YopJ subverts the Nod2/RICK/TAK1 pathway and activates caspase-1 to induce intestinal barrier dysfunction.* Cell Host Microbe, 2012. **11**(4): p. 337-51.

268. Lemaitre, N., et al., *Yersinia pestis YopJ suppresses tumor necrosis factor alpha induction and contributes to apoptosis of immune cells in the lymph node but is not required for virulence in a rat model of bubonic plague.* Infect Immun, 2006. **74**(9): p. 5126-31.

269. Palace, S.G., et al., *Genome-wide mutant fitness profiling identifies nutritional requirements for optimal growth of Yersinia pestis in deep tissue.* MBio, 2014. **5**(4).

270. Szaba, F.M., et al., *TNFalpha and IFNgamma but not perforin are critical for CD8 T cell-mediated protection against pulmonary Yersinia pestis infection.* PLoS Pathog, 2014. **10**(5): p. e1004142.

271. Szaba, F.M., et al., *D27-pLpxL, an avirulent strain of Yersinia pestis, primes T cells that protect against pneumonic plague.* Infect Immun, 2009. **77**(10): p. 4295-304.

272. Do, Y., et al., *Targeting of LcrV virulence protein from Yersinia pestis to dendritic cells protects mice against pneumonic plague.* Eur J Immunol, 2010. **40**(10): p. 2791-6.

273. Do, Y., et al., *Broad T cell immunity to the LcrV virulence protein is induced by targeted delivery to DEC-205/CD205-positive mouse dendritic cells.* Eur J Immunol, 2008. **38**(1): p. 20-9.

274. Shannon, J.G., et al., *Yersinia pestis subverts the dermal neutrophil response in a mouse model of bubonic plague.* MBio, 2013. **4**(5): p. e00170-13.

275. Depaolo, R.W., et al., *Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis.* Cell Host Microbe, 2008. **4**(4): p. 350-61.

276. Robinson, R.T., et al., *Yersinia pestis evades TLR4-dependent induction of IL-12(p40)2 by dendritic cells and subsequent cell migration.* J Immunol, 2008. **181**(8): p. 5560-7.

277. Vagima, Y., et al., *Circumventing Y. pestis Virulence by Early Recruitment of Neutrophils to the Lungs during Pneumonic Plague.* PLoS Pathog, 2015. **11**(5): p. e1004893.

278. Spinner, J.L., et al., *Neutrophils are resistant to Yersinia YopJ/P-induced apoptosis and are protected from ROS-mediated cell death by the type III secretion system.* PLoS One, 2010. **5**(2): p. e9279.

279. Eisele, N.A., et al., *Chemokine receptor CXCR2 mediates bacterial clearance rather than neutrophil recruitment in a murine model of pneumonic plague.* Am J Pathol, 2011. **178**(3): p. 1190-200.

280. Pujol, C. and J.B. Bliska, *Turning Yersinia pathogenesis outside in: subversion of macrophage function by intracellular yersiniae.* Clin Immunol, 2005. **114**(3): p. 216-26.

281. Fredriksson-Ahomaa, M., A. Stolle, and H. Korkeala, *Molecular epidemiology of Yersinia enterocolitica infections.* FEMS Immunol Med Microbiol, 2006. **47**(3): p. 315-29.

282. Perry, R.D. and J.D. Fetherston, *Yersinia pestis--etiologic agent of plague.* Clin Microbiol Rev, 1997. **10**(1): p. 35-66.

283. Du, Y., R. Rosqvist, and A. Forsberg, *Role of fraction 1 antigen of Yersinia pestis in inhibition of phagocytosis.* Infect Immun, 2002. **70**(3): p. 1453-60.

284. Lahteenmaki, K., et al., *Yersinia pestis Pla has multiple virulence-associated functions.* Adv Exp Med Biol, 2003. **529**: p. 141-5.

285. Lahteenmaki, K., M. Kukkonen, and T.K. Korhonen, *The Pla surface protease/adhesin of Yersinia pestis mediates bacterial invasion into human endothelial cells.* FEBS Lett, 2001. **504**(1-2): p. 69-72.

286. Sebbane, F., et al., *Role of the Yersinia pestis plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague.* Proc Natl Acad Sci U S A, 2006. **103**(14): p. 5526-30.

287. Goguen, J.D., T. Bugge, and J.L. Degen, *Role of the pleiotropic effects of plasminogen deficiency in infection experiments with plasminogen-deficient mice.* Methods, 2000. **21**(2): p. 179-83.

288. Sodeinde, O.A., et al., *A surface protease and the invasive character of plague.* Science, 1992. **258**(5084): p. 1004-7.

289. Kolodziejek, A.M., C.J. Hovde, and S.A. Minnich, *Yersinia pestis Ail: multiple roles of a single protein.* Front Cell Infect Microbiol, 2012. **2**: p. 103.

290. Minnich, S.A. and H.N. Rohde, *A rationale for repression and/or loss of motility by pathogenic Yersinia in the mammalian host.* Adv Exp Med Biol, 2007. **603**: p. 298-310.

291. Vadyvaloo, V., et al., *Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in Yersinia pestis.* PLoS Pathog, 2010. **6**(2): p. e1000783.

292. Yother, J., T.W. Chamness, and J.D. Goguen, *Temperature-controlled plasmid regulon associated with low calcium response in Yersinia pestis.* J Bacteriol, 1986. **165**(2): p. 443-7.

293. Van Engelenburg, S.B. and A.E. Palmer, *Imaging type-III secretion reveals dynamics and spatial segregation of Salmonella effectors.* Nat Methods, 2010. **7**(4): p. 325-30.

294. Mukherjee, S., et al., *Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation.* Science, 2006. **312**(5777): p. 1211-4.

295. Kerschen, E.J., et al., *The plague virulence protein YopM targets the innate immune response by causing a global depletion of NK cells.* Infect Immun, 2004. **72**(8): p. 4589-602.

296. Ye, Z., et al., *Gr1+ cells control growth of YopM-negative yersinia pestis during systemic plague.* Infect Immun, 2009. **77**(9): p. 3791-806.

297. Leung, K.Y., B.S. Reisner, and S.C. Straley, *YopM inhibits platelet aggregation and is necessary for virulence of Yersinia pestis in mice.* Infect Immun, 1990. **58**(10): p. 3262-71.

298. Gurung, P. and T.D. Kanneganti, *Novel roles for caspase-8 in IL-1beta and inflammasome regulation.* Am J Pathol, 2015. **185**(1): p. 17-25.

299. Bossaller, L., et al., *Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner.* J Immunol, 2012. **189**(12): p. 5508-12.

300. Philipovskiy, A.V. and S.T. Smiley, *Vaccination with live Yersinia pestis primes CD4 and CD8 T cells that synergistically protect against lethal pulmonary Y. pestis infection.* Infect Immun, 2007. **75**(2): p. 878-85.

301. Mombaerts, P., *Lymphocyte development and function in T-cell receptor and RAG-1 mutant mice.* Int Rev Immunol, 1995. **13**(1): p. 43-63.

302. Marketon, M.M., et al., *Plague bacteria target immune cells during infection.* Science, 2005. **309**(5741): p. 1739-41.

303. Brodsky, I.E. and R. Medzhitov, *Reduced secretion of YopJ by Yersinia limits in vivo cell death but enhances bacterial virulence.* PLoS Pathog, 2008. **4**(5): p. e1000067.

304. Maelfait, J., et al., *Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta maturation by caspase-8.* J Exp Med, 2008. **205**(9): p. 1967-73.

305. Uittenbogaard, A.M., et al., *Toward a molecular pathogenic pathway for Yersinia pestis YopM.* Front Cell Infect Microbiol, 2012. **2**: p. 155.

306. Ye, Z., et al., *Distinct CCR2(+) Gr1(+) cells control growth of the Yersinia pestis DeltayopM mutant in liver and spleen during systemic plague.* Infect Immun, 2011. **79**(2): p. 674-87.

307. Lindner, I., et al., *Modulation of dendritic cell differentiation and function by YopJ of Yersinia pestis.* Eur J Immunol, 2007. **37**(9): p. 2450-62.

308. Rosadini, C.V., et al., *A Single Bacterial Immune Evasion Strategy Dismantles Both MyD88 and TRIF Signaling Pathways Downstream of TLR4.* Cell Host Microbe, 2015. **18**(6): p. 682-93.

309. Autenrieth, S.E., et al., *Immune evasion by Yersinia enterocolitica: differential targeting of dendritic cell subpopulations in vivo.* PLoS Pathog, 2010. **6**(11): p. e1001212.

310. McCoy, M.W., et al., *The C-terminal tail of Yersinia pseudotuberculosis YopM is critical for interacting with RSK1 and for virulence.* Infect Immun, 2010. **78**(6): p. 2584-98.

311. Caulfield, A.J., et al., *The Pla protease of Yersinia pestis degrades fas ligand to manipulate host cell death and inflammation.* Cell Host Microbe, 2014. **15**(4): p. 424-34.

312. Ratner, D., et al., *Manipulation of IL-1beta and IL-18 production by Yersinia pestis effectors YopJ and YopM and redundant impact on virulence.* J Biol Chem, 2016.

313. Brubaker, R.R., *Influence of Na(+), dicarboxylic amino acids, and pH in modulating the low-calcium response of Yersinia pestis.* Infect Immun, 2005. **73**(8): p. 4743-52.

314. Rajamaki, K., et al., *Extracellular acidosis is a nov el danger signal alerting innate immunity via the NLRP3 inflammasome.* J Biol Chem, 2013. **288**(19): p. 13410-9.

315. Schoberle, T.J., et al., *Uncovering an Important Role for YopJ in the Inhibition of Caspase-1 in Activated Macrophages and Promoting Yersinia pseudotuberculosis Virulence.* Infect Immun, 2016.

316. Papin, S., et al., *The tumor necrosis factor alpha-dependent activation of the human mediterranean fever (MEFV) promoter is mediated by a synergistic interaction between C/EBP beta and NF kappaB p65.* J Biol Chem, 2003. **278**(49): p. 48839-47.

317. Bradley, M.N., L. Zhou, and S.T. Smale, *C/EBPbeta regulation in lipopolysaccharide-stimulated macrophages.* Mol Cell Biol, 2003. **23**(14): p. 4841-58.

318. Ghonime, M.G., et al., *Inflammasome priming by lipopolysaccharide is dependent upon ERK signaling and proteasome function.* J Immunol, 2014. **192**(8): p. 3881-8.

319. Cavet, M.E., S. Lehoux, and B.C. Berk, *14-3-3beta is a p90 r ibosomal S6 kinase (RSK) isoform 1-binding protein that negatively regulates RSK kinase activity.* J Biol Chem, 2003. **278**(20): p. 18376-83.

320. Isagawa, T., et al., *Involvement of protein kinase PKN1 in G2/M delay caused by arsenite.* Mol Carcinog, 2005. **43**(1): p. 1-12.

321. Harrison, B.C., et al., *Protein kinase C-related kinase targets nuclear localization signals in a subset of class IIa histone deacetylases.* FEBS Lett, 2010. **584**(6): p. 1103-10.

322. Hutchinson, C.L., et al., *Differential binding of RhoA, RhoB, and RhoC to protein kinase C-related kinase (PRK) isoforms PRK1, PRK2, and PRK3: PRKs have the highest affinity for RhoB.* Biochemistry, 2013. **52**(45): p. 7999-8011.

323. Nam, H.J., et al., *p90 ribosomal S6 kinase 1 (RSK1) isoenzyme specifically regulates cytokinesis progression.* Cell Signal, 2014. **26**(2): p. 208-19.

324. Mukharjee, A. and A.K. Basu, *Autoerythrocytic sensitization.* J Assoc Physicians India, 1982. **30**(8): p. 547-8.

325. Zheng, Y., et al., *YopJ-induced caspase-1 activation in Yersinia-infected macrophages: independent of apoptosis, linked to necrosis, dispensable for innate host defense.* PLoS One, 2012. **7**(4): p. e36019.

326. Zauberman, A., et al., *Interaction of Yersinia pestis with macrophages: limitations in YopJ-dependent apoptosis.* Infect Immun, 2006. **74**(6): p. 3239-50.

327. Zauberman, A., et al., *Yersinia pestis endowed with increased cytotoxicity is avirulent in a bubonic plague model and induces rapid protection against pneumonic plague.* PLoS One, 2009. **4**(6): p. e5938.

328. Orth, K., *Function of the Yersinia effector YopJ.* Curr Opin Microbiol, 2002. **5**(1): p. 38-43.

329. Spinner, J.L., et al., *Role of the Yersinia YopJ protein in suppressing interleukin-8 secretion by human polymorphonuclear leukocytes.* Microbes Infect, 2016. **18**(1): p. 21-9.

330. Zheng, Y., et al., *A Yersinia effector with enhanced inhibitory activity on the NF-kappaB pathway activates the NLRP3/ASC/caspase-1 inflammasome in macrophages.* PLoS Pathog, 2011. **7**(4): p. e1002026.

331. Hsu, L.C., et al., *IL-1beta-driven neutrophilia preserves antibacterial defense in the absence of the kinase IKKbeta.* Nat Immunol, 2011. **12**(2): p. 144-50.

332. Greten, F.R., et al., *NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta.* Cell, 2007. **130**(5): p. 918-31.

333. Tilstra, J.S., et al., *Pharmacologic IKK/NF-kappaB inhibition causes antigen presenting cells to undergo TNFalpha dependent ROS-mediated programmed cell death.* Sci Rep, 2014. **4**: p. 3631.

334. Kwuan, L., W. Adams, and V. Auerbuch, *Impact of host membrane pore formation by the Yersinia pseudotuberculosis type III secretion system on the macrophage innate immune response.* Infect Immun, 2013. **81**(3): p. 905-14.

335. Viboud, G.I. and J.B. Bliska, *A bacterial type III secretion system inhibits actin polymerization to prevent pore formation in host cell membranes.* EMBO J, 2001. **20**(19): p. 5373-82.

336. Schmid-Burgk, J.L., et al., *A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component of NLRP3 Inflammasome Activation.* J Biol Chem, 2016. **291**(1): p. 103-9.

337. Shi, H., et al., *NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component.* Nat Immunol, 2015.

338. Dewoody, R., et al., *YopK regulates the Yersinia pestis type III secretion system from within host cells.* Mol Microbiol, 2011. **79**(6): p. 1445-61.

339. Dewoody, R., P.M. Merritt, and M.M. Marketon, *YopK controls both rate and fidelity of Yop translocation.* Mol Microbiol, 2013. **87**(2): p. 301-17.

340. Sheahan, K.L. and R.R. Isberg, *Identification of mammalian proteins that collaborate with type III secretion system function: involvement of a chemokine receptor in supporting translocon activity.* MBio, 2015. **6**(1): p. e02023-14.

341. Aili, M., et al., *Regulation of Yersinia Yop-effector delivery by translocated YopE.* Int J Med Microbiol, 2008. **298**(3-4): p. 183-92.

342. Wang, X., et al., *The GAP activity of type III effector YopE triggers killing of Yersinia in macrophages.* PLoS Pathog, 2014. **10**(8): p. e1004346.

343. Thinwa, J., et al., *Integrin-mediated first signal for inflammasome activation in intestinal epithelial cells.* J Immunol, 2014. **193**(3): p. 1373-82.

344. Basak, C., et al., *NF-kappaB- and C/EBPbeta-driven interleukin-1beta gene expression and PAK1-mediated caspase-1 activation play essential roles in interleukin-1beta release from Helicobacter pylori lipopolysaccharide-stimulated macrophages.* J Biol Chem, 2005. **280**(6): p. 4279-88.

345. Plano, G.V. and K. Schesser, *The Yersinia pestis type III secretion system: expression, assembly and role in the evasion of host defenses.* Immunol Res, 2013. **57**(1-3): p. 237-45.

346. Lee, W.L., J.M. Grimes, and R.C. Robinson, *Yersinia effector YopO uses actin as bait to phosphorylate proteins that regulate actin polymerization.* Nat Struct Mol Biol, 2015. **22**(3): p. 248-55.

347. Bubeck, S.S. and P.H. Dube, *Yersinia pestis CO92 delta yopH is a potent live, attenuated plague vaccine.* Clin Vaccine Immunol, 2007. **14**(9): p. 1235-8.

348. Straley, S.C. and M.L. Cibull, *Differential clearance and host-pathogen interactions of YopE- and YopK- YopL- Yersinia pestis in BALB/c mice.* Infect Immun, 1989. **57**(4): p. 1200-10.

349. May, M., et al., *Difference in F-actin depolymerization induced by toxin B from the Clostridium difficile strain VPI 10463 and toxin B from the variant Clostridium difficile serotype F strain 1470.* Toxins (Basel), 2013. **5**(1): p. 106-19.

350. Taskiran, E.Z., et al., *The effect of colchicine on pyrin and pyrin interacting proteins.* J Cell Biochem, 2012. **113**(11): p. 3536-46.

351. Yu, J.W., et al., *Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants.* Mol Cell, 2007. **28**(2): p. 214-27.

352. Hall, J.P. and R.J. Davis, *Inhibition of the p38 pathway upregulates macrophage JNK and ERK activities, and the ERK, JNK, and p38 MAP kinase pathways are reprogrammed during differentiation of the murine myeloid M1 cell line.* J Cell Biochem, 2002. **86**(1): p. 1-11.

353. Larrea, M.D., et al., *RSK1 drives p27Kip1 phosphorylation at T198 to promote RhoA inhibition and increase cell motility.* Proc Natl Acad Sci U S A, 2009. **106**(23): p. 9268-73.

354. Lara, R., et al., *An siRNA screen identifies RSK1 as a key modulator of lung cancer metastasis.* Oncogene, 2011. **30**(32): p. 3513-21.

355. Hutchinson, C.L., et al., *Mutational analysis reveals a single binding interface between RhoA and its effector, PRK1.* Biochemistry, 2011. **50**(14): p. 2860-9.

356. Lim, W.G., et al., *The very C-terminus of PRK1/PKN is essential for its activation by RhoA and downstream signaling.* Cell Signal, 2006. **18**(9): p. 1473-81.

357. Modha, R., et al., *The Rac1 polybasic region is required for interaction with its effector PRK1.* J Biol Chem, 2008. **283**(3): p. 1492-500.

358. Lommel, M.J., et al., *L-plastin Ser5 phosphorylation in breast cancer cells and in vitro is mediated by RSK downstream of the ERK/MAPK pathway.* FASEB J, 2016. **30**(3): p. 1218-33.

359. Jilg, C.A., et al., *PRK1/PKN1 controls migration and metastasis of androgen-independent prostate cancer cells.* Oncotarget, 2014. **5**(24): p. 12646-64.

360. Torbett, N.E., A. Casamassima, and P.J. Parker, *Hyperosmotic-induced protein kinase N 1 activation in a vesicular compartment is dependent upon Rac1 and 3-phosphoinositide-dependent kinase 1.* J Biol Chem, 2003. **278**(34): p. 32344-51.

361. Wang, L., et al., *Ganglioside GD1a suppresses TNFalpha expression via Pkn1 at the transcriptional level in mouse osteosarcoma-derived FBJ cells.* Biochem Biophys Res Commun, 2008. **371**(2): p. 230-5.

362. Zhang, B., J. Chernoff, and Y. Zheng, *Interaction of Rac1 with GTPase-activating proteins and putative effectors. A comparison with Cdc42 and R hoA.* J Biol Chem, 1998. **273**(15): p. 8776-82.

363. Uezu, A., et al., *Modified SH2 domain to phototrap and identify phosphotyrosine proteins from subcellular sites within cells.* Proc Natl Acad Sci U S A, 2012. **109**(43): p. E2929-38.

364. Kakinuma, N., et al., *Kank regulates RhoA-dependent formation of actin stress fibers and c ell migration via 14-3-3 in PI3K-Akt signaling.* J Cell Biol, 2008. **181**(3): p. 537-49.

365. Ye, Y., et al., *Down-regulation of 14-3-3 Zeta Inhibits TGF-beta1-Induced Actomyosin Contraction in Human Trabecular Meshwork Cells Through RhoA Signaling Pathway.* Invest Ophthalmol Vis Sci, 2016. **57**(2): p. 719-30.

366. Kobayashi, H., et al., *Involvement of 14-3-3 proteins in the second epidermal growth factor-induced wave of Rac1 activation in the process of cell migration.* J Biol Chem, 2011. **286**(45): p. 39259-68.

367. Xiao, Y., et al., *14-3-3tau promotes breast cancer invasion and metastasis by inhibiting RhoGDIalpha.* Mol Cell Biol, 2014. **34**(14): p. 2635-49.

368. Doppler, H., et al., *Neuregulin mediates F-actin-driven cell migration through inhibition of protein kinase D1 via Rac1 protein.* J Biol Chem, 2013. **288**(1): p. 455-65.

369. Das, M., et al., *Phosphorylation-dependent inhibition of Cdc42 GEF Gef1 by 14-3-3 protein Rad24 spatially regulates Cdc42 GTPase activity and oscillatory dynamics during cell morphogenesis.* Mol Biol Cell, 2015. **26**(19): p. 3520-34.

370. Gack, M.U., *Mechanisms of RIG-I-like receptor activation and manipulation by viral pathogens.* J Virol, 2014. **88**(10): p. 5213-6.

371. Gershoni-Baruch, R., et al., *Familial Mediterranean fever: prevalence, penetrance and genetic drift.* Eur J Hum Genet, 2001. **9**(8): p. 634-7.

372. Masters, S.L., et al., *Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (*).* Annu Rev Immunol, 2009. **27**: p. 621-68.

373. Vizcarra, J., *Mapping the spread of a pandemic*.

374. Horton, R.M., et al., *Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension.* Gene, 1989. **77**(1): p. 61-8.

375. Edwards, R.A., L.H. Keller, and D.M. Schifferli, *Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression.* Gene, 1998. **207**(2): p. 149-57.

376. Lenz, J.D., et al., *Expression during host infection and localization of Yersinia pestis autotransporter proteins.* J Bacteriol, 2011. **193**(21): p. 5936-49.