ACTIVATION AND EVASION OF THE INFLAMMASOME BY *YERSINIA*

Erin E. Zwack

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Supervisor of Dissertation

____ _____

Igor E. Brodsky

Assistant Professor of Pathobiology

Graduate Group Chairperson

_____ _____

Daniel S. Kessler, Associate Professor of Cell and Developmental Biology

Dissertation Committee

Bruce D. Freedman, Associate Professor of Pathobiology
Michael Povelones, Assistant Professor of Pathobiology
Dieter M. Schifferli, Professor of Microbiology
Jun (Jay) Zhu, Associate Professor of Microbiology

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Abstract

Activation and Evasion of the Inflammasome by *Yersinia*

Erin E. Zwack

Igor E. Brodsky

Multicellular organisms constantly encounter microbes, ranging from beneficial to pathogenic. In order to mount appropriate immune responses that allow the host to clear pathogens while maintaining a balance with nonpathogenic microbes, the innate immune system must discriminate between pathogens and commensals. Through the recognition of virulence structures and activities, innate immune cells can distinguish pathogens from commensals. One such virulence structure, the type III secretion system (T3SS), translocates effector proteins into target cells in order to disrupt or modulate host cell signaling pathways and establish replicative niches. Over 25 species of pathogenic gram-negative bacteria depend upon T3SSs to cause productive infection. However, recognition of T3SS activity by cytosolic Pattern Recognition Receptors (PRRs) of the Nucleotide-Binding Domain Leucine Rich Repeat (NLR) family, either through detection of translocated products or membrane disruption, induces assembly of multiprotein complexes known as inflammasomes. *Yersinia pseudotuberculosis* (*Yptb*) is an ideal model for inflammasome recognition of the T3SS as *Yptb* expresses an archetypal T3SS and is a genetically tractable, natural rodent pathogen. Investigation of the interaction between the inflammasomes and the T3SS could reveal important mechanistic and cell biological information about the inflammasomes themselves as well as a potential target for treating T3SS expressing bacteria. Although effectors of *Yptb* has been shown to actively inhibit inflammasome activation, until this work very little was known about what inflammasome is actually activated by the T3SS, what activity of the T3SS is recognized, and how *Yersinia*'s YopK protein inhibits inflammasome activation. Therefore, we investigated the bacterial and host interactions required for inflammasome activation and the mechanism by which YopK inhibits inflammasome activation. To dissect the contribution of the different consequences of T3SSs, pore-formation and translocation, to inflammasome activation, we took advantage of variants of YopD and LcrH that separate these functions. Our findings indicated that inflammasome activation required hyper-translocation of YopB/D. Using macrophages deficient in caspase-1, caspase-11, or certain guanylate binding proteins, we characterized the host pathways activated by hyper-translocation of YopD/B. Finally, using mutations in YopK, we characterized how YopK prevents inflammasome activation. Overall, our findings help define how bacterial virulence activities activate innate immune responses.

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LIST OF ABBREVIATIONS

ASC:	Apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain
ATP:	Adenosine triphosphate
B6:	C57Bl/6 mouse background
BMDMs:	Bone-marrow-derived macrophages
CARD:	Caspase activation and recruitment domain
DAMPs:	Damage-associated molecular patterns
DCs:	Dendritic cells
ExoS:	Exoenzyme S
FBS:	Fetal Bovine Serum
GAP:	GTPase-activating protein
Gbp:	Guanylate binding proteins
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gsdmd:	gasderminD
HIN:	Hematopoietic interferon-inducible nuclear domain
IFNγ:	Interferon gamma
ICE:	Interleukin-1β-converting enzyme
IM:	Inner Membrane
IQGAP1:	IQ motif- containing GTPase-activating protein 1
LDH:	Lactate dehydrogenase
LLO:	Listeriolysin O
LPS:	Lipopolysaccharide
LukAB:	Leukocidin A/B
MS ring:	Membrane and supramembrane ring
NAIP:	Neuronal apoptosis inhibitory protein
NLR:	Nucleotide-binding domain, leucine rich repeat protein
OM:	Outer Membrane
PAMPS:	Pathogen-associated molecular patterns
PI:	Propidium iodide
PP:	Periplasm
PRRs:	Pattern recognition receptors
PYD:	Pyrin domain
RACK1:	Receptor for Activated C Kinase 1
RBCs:	Red blood cells, sheep erythrocytes
ROS:	Reactive oxygen species
T3SS:	Type three secretion system
TLR:	Toll-like receptor
Ye:	*Yersinia enterocolitica*
Yops:	*Yersinia* outer proteins
Yp:	*Yersinia pestis*
Yptb:	*Yersinia pseudotuberculosis*

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CHAPTER 1

INTRODUCTION

A. Microbes and Pathogens

Microbes constantly colonize the human body. The whole contingent of colonizing microbes comprises the microbiota. Humans depend on our microbiota for functions ranging from nutrient acquisition to immune function (Molloy et al., 2012; Sansonetti et al., 2011). In addition to the beneficial and neutral microbes that constantly colonize eukaryotic surfaces and tissues, humans encounter pathogenic microbes, which cause damage to the host upon infection. For an infection to be productive, the microbe needs to replicate inside the host and then spread to other hosts or environments where it can successfully multiply. The harm caused to the host as part of the pathogens life cycle distinguishes pathogens from other microbes. Virulence structures and factors allow for the pathogen to set-up a productive infection, which will damage the host. Different virulence structures play important roles in varying aspects of infection. For example, modulation of host cell processes to prevent clearance or obtain nutrients can be driven by delivery of effectors into cells by specialized secretion systems (Coburn et al., 2007). Additionally appropriate adhesion of the pathogen to host surfaces to both prevent clearance and allow for dissemination can be modulated by various virulence structures such as pili (Gerlach and Hensel, 2007). Virulence factors such as toxins can also play multiple roles such as allowing for nutrient acquisition, killing cell types that could allow for clearance of the pathogen, and promoting environments that allow for increased transmission to new hosts (Chaussee et al., 1997; do Vale et al., 2016; Spaan et al., 2015). The immune response to pathogens often requires robust innate and adaptive responses that try to eradicate the pathogen even at the expense of tissue damage. This is in contrast to the responses to the microbiota, which maintain the balance between host and microbe with limited and tightly controlled inflammation (Sansonetti, 2011). Inappropriate immune responses to commensals often lead to chronic inflammatory

diseases (Belkaid and Hand, 2014). Because of the disparate natures of the responses required to manage pathogens and commensals, a critical function of the immune system is distinguishing pathogens from commensals.

B. Innate immune recognition of pathogens

Once pathogens breach epithelial barriers, innate immune cells including neutrophils, macrophages, and dendritic cells often are the first cells to encounter pathogens. The signals produced by innate cells play a key role in restricting pathogen replication as well as instructing the subsequent immune responses. The immune cells recognize both virulence structures and activities of pathogens. How an innate immune cell recognizes a microbe determines the type and robustness of the response. Surface associated pattern recognition receptors such as the Toll-like receptors (TLRs) recognize conserved molecular patterns such as bacterial lipopolysaccharide (LPS), flagellin, and peptidoglycan (Chow et al., 1999; Hayashi et al., 2001; Janeway and Medzhitov, 2002; Poltorak et al., 1998; Schwandner et al. 1999). These molecules are conserved among nonpathogenic and pathogenic bacteria. The response downstream of recognition consists mainly of a transcriptional response with the up-regulation of many proinflammatory cytokines. Some of these cytokines are secreted to trigger similar transcriptional changes in neighboring cells while other cytokines are produced in a pro-form that require post-translational modification (Vance et al., 2009). Additionally, post-translational modifications can be required to induce activity of certain enzymes. Detection of a second signal that is specific to pathogens normally controls these post-translational modifications. Pathogen specific secondary signals range from detection of conserved microbial products in the cytosol to inhibition of normal cell processes such as transcription and translation to formation of pores in membranes by toxins or other microbial proteins (Vance et al., 2009). Different classes of pathogen-associated signals can engage different signaling pathways resulting in divergent outcomes. For example,

pathogen-induced blockade of transcription or translation after initial priming by surface receptor signaling often leads to apoptotic cell death (Monack et al., 1997; Palmer et al., 1998; Gil and Esteban, 2000), whereas sensing pore-forming toxins or introduction of bacterial products such as flagellin into the cytosol results in a pro-inflammatory form of cell death termed pyroptosis (Bergsbaken et al., 2009; Miao et al., 2006; Miao et al., 2010a; Craven et al., 2009). Pyroptosis involves the activation of a multiprotein complex termed the inflammasome (Martinon et al., 2002).

C. The canonical inflammasomes

The inflammasomes are multi-protein complexes that typically consist of a nucleotide binding domain leucine rich repeat protein (NLR), the apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain (ASC), and the cysteine protease caspase-1 (Fig. 1-1; Martinon et al., 2002). Recent studies using super-resolution microscopy revealed that the inflammasome complex forms a spiral tube with the NLRs on the outside and caspase-1 on the inside of the structure (Man et al., 2014). Upon oligomerization in the complex, caspase-1 undergoes autocleavage into the active p10 and p20 subunits from its 45 kDa proform (Martinon et al., 2002).

Figure 1-1.

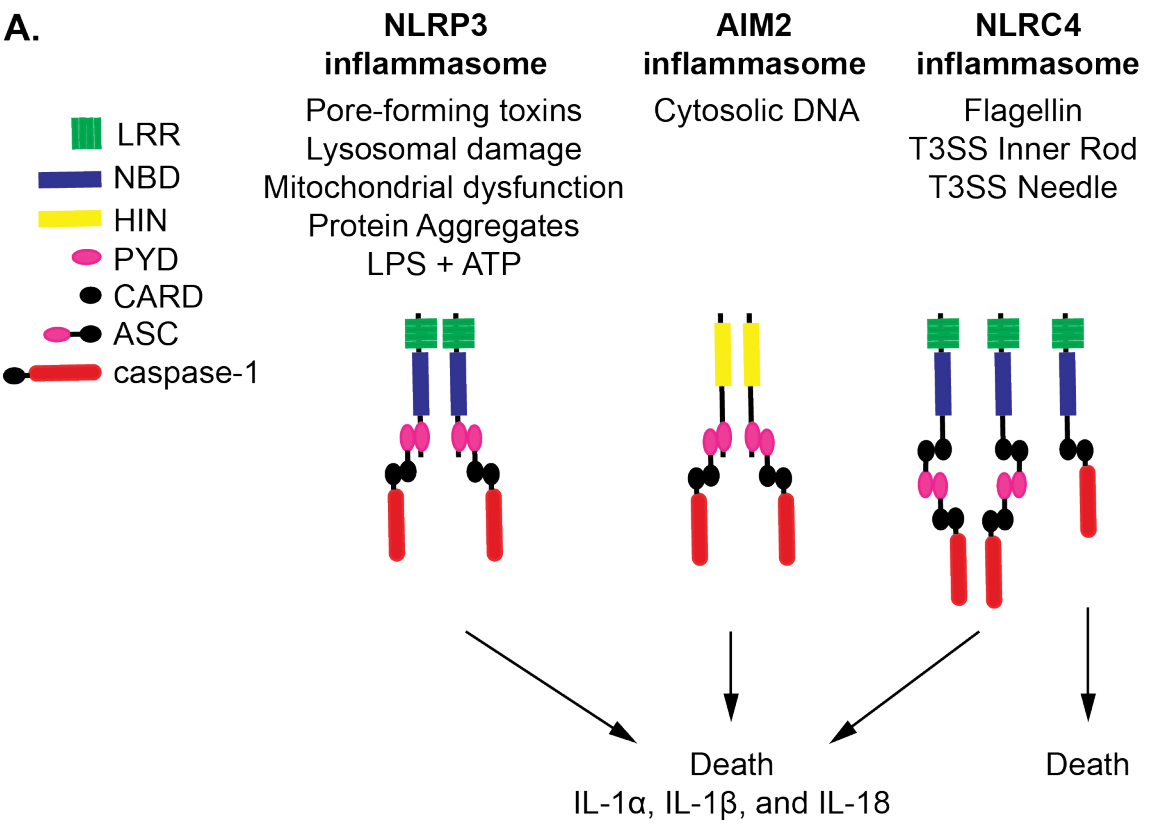

Figure 1-1: Model of Canonical Inflammasome activation. Three inflammasomes (NLRP3, NLRC4, and AIM2) that demonstrate the paradigm of canonical inflammasomes are depicted. The NLRP3 inflammasome (Left) uses the NLR protein NLRP3, which contains a leucine rich repeat (LRR) domain, a nucleotide binding domain (NBD) and a pyrin domain (PYD). Upon activation by one of its many stimuli, NLRP3 recruits the adapter protein ASC, which contains both a PYD and caspase activation and recruitment domain (CARD). ASC allows for the cysteine protease caspase-1 to be recruited. The AIM2 inflammasome (middle) requires the protein AIM2, which has a Hematopoietic interferon-inducible nuclear domain (HIN) and a PYD, to sense cytosolic DNA. Upon activation, recruitment of ASC and caspase-1 occurs. The NLRC4 inflammasome (right) is named for the use of the protein NLRC4, which consists of an LRR, NBD, and CARD. As NLRC4 contains a CARD, it can directly recruit caspase-1. Alternatively, NLRC4

can interact with caspase-1 through ASC. Downstream of formation of the ASC-containing inflammasome, IL-1α, IL-1β, and IL-18 secretion occurs along with death. Direct recruitment of caspase-1 by NLRC4 leads only to death.

The activation of capase-1 in the inflammasome complex leads to the processing of IL-1 family cytokines IL-1β and IL-18 (Martinon et al., 2002). Caspase-1 activation is additionally required for secretion of IL-1β and IL-18 as well as IL-1α, which is thought to be active in both its pro and processed form and does not use caspase-1 for processing (Mosley et al., 1987). Processing and secretion of these cytokines require oligomerization of the complex using the adaptor protein ASC. In addition to the processing and secretion of IL-1 family cytokines, caspase-1 activation leads to a type of death called pyroptosis (Bergsbaken et al., 2009). Caspase-1 mediated death does not require this oligomerization. NLRC4, which can bind directly to caspase-1 via its caspase-1 activation and recruitment domain (CARD), has been shown to allow for cell death in the absence of ASC foci (Fig. 1-1; Case et al., 2009; Case and Roy, 2011). Recently, this death has been shown to require cleavage of GasderminD (encoded by *Gsdmd*) by caspase-1 (Shi et al*.,* 2015). Cleavage frees the N-terminus of GSDMD, which is sufficient to cause pyroptotic-like death although the precise mechanism is unknown (Shi J et al., 2015; Kayagaki et al., 2015).

The various stimuli that trigger inflammasome activation require different NLRs. Direct binding of any stimuli to an NLR has yet to be demonstrated. NLRC4 was initially thought to bind directly to its stimuli of flagellin, but it has since been shown that neuronal apoptosis inhibitory protein (NAIP) 5 binds flagellin and then activates NLRC4 (Lightfield et al., 2008). NLRC4 also works in conjunction with NAIP2 and NAIP1 to detect the type three secretion system (T3SS) inner rod and needle proteins, respectively (Miao et al.

2010b; Yang et al., 2013). The NLRC4 stimuli are structurally and conceptually related as they make up the rod portions of the evolutionarily related flagellar apparatus and type three secretion system. NLRP3 recognizes a wide range of stimuli including pore-forming toxins, uric acid and silica crystals, lysosomal disruption, and extracellular adenosine triphosphate (ATP) with LPS priming (Fig. 1-1; Craven et al., 2009; Hornung et al., 2008; Mariathasan et al., 2006; Martinon et al. 2006). How all of these various NLRP3 stimuli lead to inflammasome activation is unknown although potassium efflux is required for NLRP3 inflammasome activation by all described stimuli to date (Munoz-Planillo et al., 2013). In addition to NLRs, absent in melanoma 2 (AIM2), which contains a hematopoietic interferon-inducible nuclear domain (HIN) and a pyrin domain (PYD), activates the inflammasome downstream of recognition of double stranded cytosolic DNA (Fig. 1-1; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). If multiple stimuli are acting upon the cell, all activated NLRs go to the same structure generating a single caspase-1 puncta (Man et al., 2014). While typically caspase-1 is required for all aspects of pyroptosis, caspase-11 can lead to caspase-1-independent cell death and IL-1α secretion with caspase-1-dependent IL-1β and IL-18 secretion under certain circumstances (Kayagaki et al., 2011).

D. The non-canonical inflammasome

In 1998 before the inflammasome was defined, Wang S *et al.* determined that caspase-11 was required for IL-1α and IL-1β secretion after injection of LPS into mice. In 2011, it was determined that *Casp1*$^{-/-}$ mice, which were supposed to be missing only caspase-1, produced a nonfunctional, truncated form of caspase-11. The caspase-1 deletion was initially generated on a 129 mouse background. In 129 mice, caspase-11 contains a mutation that results in a non-functional truncated caspase-11. Caspase-1 and caspase-11 are too close on the chromosome to allow for segregation. Therefore, the nonfunctional caspase-11 could not be swapped with the functional caspase-11 of

C57Bl/6 mice (Kayagaki et al., 2011). Until this point, all experiments to determine a role for the inflammasome used the *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice. Generation of *Casp1* and *Casp11* single knockout mice determined that while caspase-11 was dispensable for certain stimuli such as extracellular ATP with priming and monosodium urate crystals, caspase-11 and not caspase-1 was required for cell death and IL-1α release in response to infection with nonpathogenic gram-negative bacteria (Kayagaki et al., 2011). Interestingly, both caspase-1 and caspase-11 were required for IL-1β processing and secretion. This combination of caspase-11-dependent, caspase-1-independent cell death and IL-1α secretion, and caspase-1/11-dependent IL-1β secretion was termed the non-canonical inflammasome response (Fig. 1-2, Kayagaki et al., 2011). The actual trigger from infection with gram-negative nonpathogenic bacteria is the release of hexa-acylated or penta-acylated lipid A of LPS into the cytosol (Hagar et al., 2013; Kayagaki et al., 2013; Shi et al., 2014). Hexa-acylated lipid A was found to bind directly to caspase-11 in the cytosol. Intracellular LPS is currently the only known activator of the non-canonical inflammasome (Shi et al., 2014).

Figure 1-2.

A.

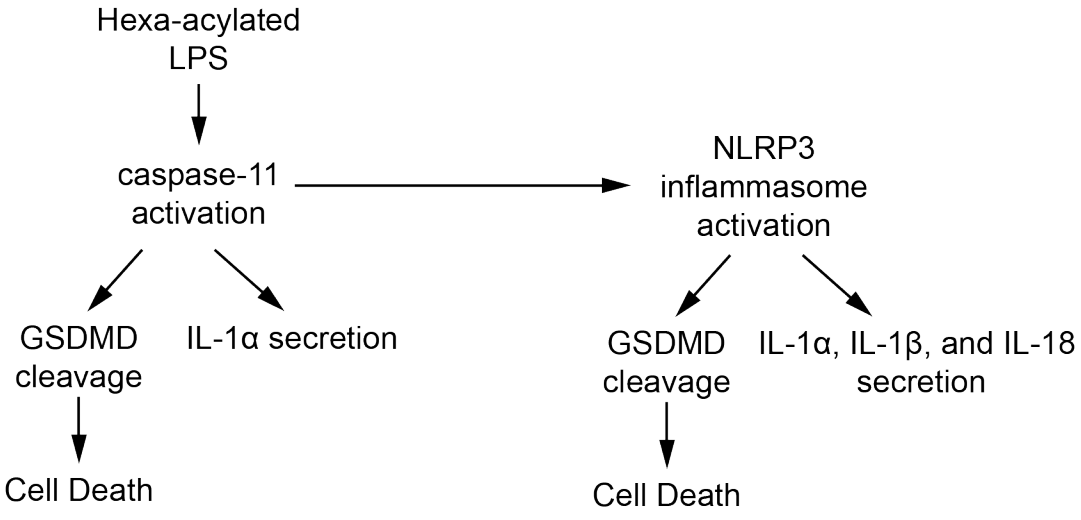

Figure 1-2: Non-canonical inflammasome activation. Intracellular hexa-acylated LPS activates caspase-11 leading directly to cell death and IL-1α secretion. Caspase-11 activation also leads to activation of the canonical NLRP3 inflammasome, which allows for the processing and secretion of IL-1β and IL-18 as well as cell death and IL-1α secretion.

Interestingly, a subset of interferon inducible GTPases called guanylate binding proteins (Gbps) was found to be important for the activation of the non-canonical inflammasome (Meunier et al., 2014; Pilla et al., 2014). Gbps were initially characterized to aid in protection against vacuolar pathogens such as intracellular parasites and bacteria. After being recruited to the vacuole, Gbps recruit nitric oxide synthase machinery and autophagy machinery to control these pathogens (MacMicking, 2012). During non-canonical inflammasome activation, Gbps play a role both when lipid A is directly transfected into the cytosol as well as during infection with gram-negative bacteria (Pilla et al., 2014; Meunier et al., 2014). In the case of gram-negative bacterial infection, Gbps are thought to lyse the lysosome to release the lipid A, as one group has shown to be the case (Meunier et al., 2014). The role of Gbps in the cytosol is less well defined. The Coers group showed that transfected LPS from *Legionella pneumophila*, *Escherichia coli*, and *Salmonella* Minnesota had significantly lower non-canonical inflammasome activation in the absence of the Gbps from chromosome 3 (Pilla et al., 2014). Additionally, *Chlamydia muridarum*, which successfully excludes Gbps from its vacuole, activates both the non-canonical inflammasome and canonical inflammasome. Inflammasome activation by *Chlamydia muridarum* is dependent on the Gbps on chromosome 3 (Finethy et al., 2015). In both of these models, the individual contributions of the Gbps have yet to be determined. It has been suggested that Gbps may act as a platform to facilitate formation of the non-canonical inflammasome (Pilla et al., 2014). This hypothesis is based on Gbps being shown to both bind NLRP3 and be required for higher levels of NLRP3-ASC complex oligomerization (Shenoy et al., 2012).

E. The Role of inflammasome activation in immune response

Both inflammasome-dependent cell death and cytokine release play important roles in the immune response. Triggering death of infected cells destroys the replicative niche of obligate intracellular pathogens and exposes intracellular pathogens to extracellular defenses such as complement, antibody, and neutrophils (Chow et al., 2016). It has also been speculated that extracellular bacteria that attach to cells are cleared when macrophages clear the now dead cell to which they are attached (Bergman et al., 2009). In addition to causing immediate harm to the pathogen, the nature of the death allows for the release of damage-associated molecular patterns (DAMPs) such as HMGB1 and ATP. These DAMPs can then signal through pattern recognition receptors (PRRs) and other receptors on surrounding cells to stimulate cytokine production and recruitment of immune cells (Bergsbaken et al., 2009).

In addition to signaling by DAMPs, the inflammasome actively secretes the pro-inflammatory cytokines IL-1α, IL-1β, and IL-18. IL-1α and IL-1β signal through the same receptor IL-1R (Dower et al., 1986) while IL-18 signals through the IL-18R (Debets et al., 2000). Signaling by IL-1α, IL-1β, and potentially IL-18 leads to upregulation of the chemokine CXCL1, which triggers neutrophil recruitment (Lee et al., 2015; He et al., 2008). Additionally, IL-1β and IL-18 lead to activation of macrophages and dendritic cells (DCs) by stimulating noncognate production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon gamma (IFNγ) respectively by T-cells (Lukens et al., 2012; Kupz et al., 2012). The enhancement of innate responses can play a critical role in bacterial clearance.

Signaling by IL-1α and IL-1β also play important roles in generating adaptive responses both directly and indirectly. IL-1α and IL-1β signaling act directly on T-cells to differentiate them into Th17 cells in an antigen-specific manner (Sutton et al., 2006). More frequently,

we observe indirect effects of IL-1α and IL-1β on T-cells through interactions with DCs that have experienced IL-1R signaling. In West Nile Virus infection IL-1R signaling in subsets of DCs was critical for effector T-cell reactivation in the central nervous system (Durrant et al., 2013). Activation of DCs by IL-1R signaling was required for pathologic CD4 T-cell response in autoimmune myocarditis (Eriksson et al., 2003). Additionally, IL-1R signaling was required for migration of DC cells to the draining lymph node during flu infection. Failure of IL-1R$^{-/-}$ DCs to migrate to the draining lymph node resulted in decreased CD8 T-cell function (Pang et al., 2013). The complex, multi-armed response generated by the inflammasome combined with the wide range of stimuli recognized makes the inflammasome a guardian against many types of pathogenic threats. In my thesis work, I focused on understanding how the inflammasome recognizes a conserved virulence structure that allows for bacterial pathogens to subvert the host in unique ways to promote infection.

F. The Type Three Secretion System of gram-negative bacteria

There are various mechanisms by which bacteria secrete protein products from within the bacteria to extracellular spaces. These systems are termed type 1 – type VII secretion depending on their structures and evolutionary relationships (Green and Mecsas, 2016). The type III secretion system (T3SS) is found exclusively in gram-negatives and is utilized to translocate bacterial proteins into host cells. The name type three secretion first appeared in a review describing type 1 and type 2 secretion in gram-negative bacteria (Salmond and Reeves, 1993). The secretion of *Yersinia* outer proteins (Yops) by *Yersinia enterocolitica* did not match either pathway. Additionally, proteins found in *Pseudomonas solanacearum* were homologous to *Yersinia* proteins required for Yop secretion (Salmond and Reeves, 1993). Thus, it was posited that a novel pathway existed for secretion. Many of these secreted proteins such as YopE of *Yersinia* and Exoenzyme S (ExoS) of *Pseudomonas* were found to only affect cells when inside the

host cell (Rosqvist et al., 1990; Frithz-Lindsten et al., 1997). The proteins YopD and YopB of *Yersinia* were found to be necessary for delivery of YopE into the host cell (Rosqvist et al., 1995; Sory and Cornelis, 1994). Further characterization of T3SSs determined a general structure (Fig. 1-3), which includes two membrane spanning rings. A cytosolic ATPase provides the power for pumping out effectors (Diepold et al., 2012). Connected to the cytosolic components is the inner membrane export apparatus, which is inside the membrane and supramembrane ring (MS ring). The MS ring spans the inner membrane and connects with the outer membrane secretin ring in the periplasm (Diepold et al., 2012). Lining the channel inside the bacterium is the inner rod. The needle follows the rod and spans the space between the bacterium and host cell. At the tip of the needle is the translocon, which interacts with the host cell membrane and allows for entry of effectors into the cell (Diepold et al., 2012). The structure of the T3SS described above is highly similar to the structure of the bacterial flagellar apparatus. Phylogenetic studies determined that they share a common ancestor (Gophna et al., 2003). The similarity in structure allows for accidental translocation of flagellin monomers by the T3SS, which leads to potent activation of the NLRC4 inflammasome (Badea et al., 2009; Franchi et al., 2006, Lightfield et al., 2008; Miao et al., 2006; Miao et al., 2008). Even though accidental translocation of flagellin allows for immune detection of certain T3SS expressing bacteria, the T3SS is still essential to the survival or virulence of these bacteria (Galan and Curtiss, 1989; Galle et al., 2012).

Figure 1-3.

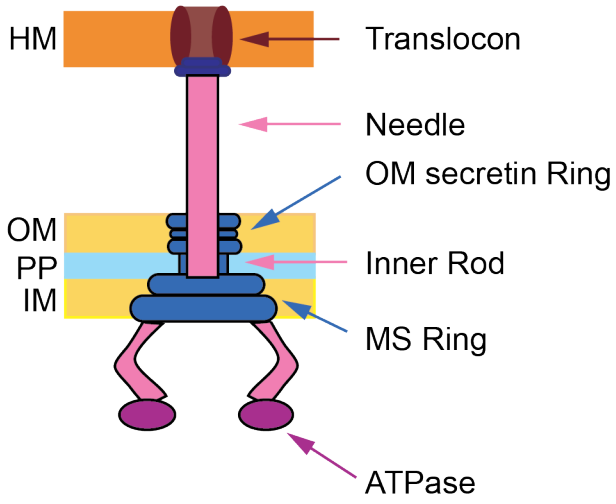

Figure 1-3: Schematic of T3SS. IM - inner membrane, PP - periplasm, OM – outer membrane, HM - host membrane, MS - membrane and supramembrane.

In general, deletion or inhibition of the T3SS leads to attenuation of infection by the bacteria (Bailey et al. 2007; Coburn et al., 2007; Gemski et al., 1980a, Gemski et al., 1980b; Galan and Curtiss, 1989; Shea et al., 1996). Small molecule inhibitors have been found that can inhibit translocation of effectors (Muschiol et al., 2006; Harmon et al., 2010). Many of these small molecule inhibitors affect translocation in multiple species. For example, an inhibitor originally identified to inhibit translocation by *Yersinia pseudotuberculosis* was also capable of inhibiting translocation in *Chlamydia trachomatis* and *Chlamydia pneumoniae* (Muschiol et al., 2006; Bailey et al., 2007). With increasing incidences of multi-drug resistant infections, new approaches to combating multiple groups of bacteria are becoming essential.

Over 25 species of gram-negative bacteria express T3SSs including *Chlamydia trachomatis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Shigella flexneri*, and *Yersinia pestis*. T3SS expressing bacteria infect a variety of hosts including plants,

insects, and mammals. Some are obligate intracellular bacteria while others are facultative intracellular bacteria and extracellular bacteria. The diverse niches that they occupy require different types of effectors to establish productive infection. However, the general structure of the T3SS as observed by electron microscopy remains well conserved among the different species (Cornelis, 2006). Such a well-conserved, essential virulence structure would make an excellent target for the various inflammasomes. Here we investigate the archetypal T3SS of *Yersinia pseudotuberculosis* to determine how the virulence activities of the T3SS such as pore-formation and translocation may be recognized by the inflammasomes.

G. Human pathogenic *Yersinia*

Yersinia species are gram-negative coccobacillus bacteria of the family *Enterobacteriaceae*. The genus *Yersinia* consists of 18 different species to date. Only three species of *Yersinia* are pathogenic to mammals including humans: *Yersinia pestis* (*Yp*), *Yersinia pseudotuberculosis* (*Yptb*), and *Yersinia enterocolitica* (*Ye*) (McNally et al., 2016). *Yersinia pestis*, the most well known species of *Yersinia*, was identified as the causative agent of plague in 1894 (Rosenberg, 1968). Transmission typically occurs either through the bite of an infected flea or the inhalation of *Yp* containing droplets from an individual infected with the pneumonic form of plague (Wren, 2003). While often thought of as exclusively a scourge of the past, plague is still around today. The prairie dog population of the Western United States provides a reservoir for *Yp*. The appearance of multi-drug resistant cases and its potential as a bioterrorism agent continue to draw interest to understanding *Yersinia pestis*. Inside the human host, *Yp* has a preference for leukocytes (Balada-Llasat and Mecsas, 2006; Maldonado-Arocho et al., 2013; Marketon et al., 2005). During the bubonic form of the plague, bacteria traffics and grows in the lymph node resulting in inflammation and swelling of the lymph node. *Yp* infection can also spread to the lungs (pneumonic plague) or systemically (septicemic plague) (Wren,

2003). *Yp* directly evolved from *Yptb* through a combination of gene acquisition and gene loss that allowed for transmission by fleas, a change in virulence, and restriction of lifestyle (Achtman et al., 1999; Chain et al., 2004; Hinnebusch et al., 2002; Zimbler et al., 2015)

While *Yp* is a vector-borne disease that often goes systemic, both *Yptb* (its direct ancestor) and *Ye* are transmitted through the oral-fecal route (Wren, 2003). They share the same tropism for lymph nodes and leukocytes as *Yp* (Durand 2010, Koberle 2009). However, the diseases caused by these infection are usually self-limiting gastroenteritis and do not often go systemic in immunocompetent individuals. *Ye* is the most common cause of food-borne *Yersinia* infection (FDA, 2012). Although the route of transmission and actual disease caused by the three *Yersinia* species are distinct, the ability to infect mammals is conferred by a conserved 70 kb virulence plasmid that encodes a type III secretion system (T3SS) and associated effectors (Gemski et al., 1980a, Gemski et al., 1980b; Portnoy et al., 1981, Cornelis and Wolf-Watz, 1997). Thus, we employ the less virulent *Yersinia pseudotuberculosis* to study the T3SS. *Yersinia pseudotuberculosis* is an ideal model system as it is a natural pathogen of rodents and provides a tractable host and bacterial genetic model to study natural systemic *Yersinia* infection.

H. *Yersinia* type three secretion system and effectors

As mentioned above, the *Yersinia* T3SS is an archetypal T3SS that was used to determine much of the structure and function of T3SSs in general (Cornelis and Wolf-Watz, 1997). The T3SS allows for effectors to be translocated from the bacteria into the host cell (Cornelis and Wolf-Watz, 1997). The translocon of *Yersinia* is comprised of the pore proteins YopB and YopD as well as the tip platform protein LcrV (Diepold et al., 2012). In general, *Yersinia pseudotuberculosis* encodes six to seven effectors depending on strain: YopE, YopH, YopJ, YopK, YopM, YopO/YpkA, and YopT. The different

effectors influence host cell processes and structures in various ways that support *Yersinia* survival (Viboud and Bliska, 2005; Bliska et al., 2013). YopJ, an acetylase, inhibits NFκB and MAPK (Mukherjee et al., 2006; Palmer et al., 1998). In the absence of priming, YopJ activates a caspase-8-dependent apoptosis and inflammasome-independent caspase-1 processing (Monack et al., 1997). When cells are primed prior to infection, this YopJ death pathway is circumvented, and YopJ in the absence of YopM seems to play a role in the inhibition of caspase-1 (Schoberle et al., 2016).

In addition to working by themselves, several effectors may work in concert to affect the same host process. For example the effectors YopE, YopH, YopO, and YopT all target various cellular components that are important for phagocytosis (Rosqvist et al., 1990; Andersson et al., 1996; and Grosdent et al., 2002). While it has been shown that *Yersinia* can survive and replicate at some level in both macrophages and neutrophils (Cavanaugh and Randall, 1959, Pujol et al., 2003, Spinner et al., 2014), its preferred lifestyle seems to be extracellular. Through injection of this cohort of effectors, *Yersinia* both increases the likelihood that it will remain extracellular and decreases the ability of neutrophils to respond to *Yersinia* (Songsungthong et al., 2010, Rolan et al., 2013). The subversion of host defense such as neutrophil oxidative burst and phagocytosis seem to be a common theme among *Yersinia*'s effectors and most likely provides the ability to productively infect mammalian hosts. Interestingly, the same mechanism that allows for *Yptb* to infect mammals leaves *Yersinia* vulnerable to cytosolic immune pathways that otherwise would not have encountered *Yersinia*.

I. The role of inflammasome activation in *Yersinia* infection

As mentioned above YopJ can trigger caspase-1 activation through a caspase-8-dependent pathway (Philip et al., 2014; Weng et al., 2014). However, when macrophages are primed with TLR ligands before infection with wild-type *Yersinia in vitro*,

death of macrophages becomes caspase-1-dependent apoptosis-independent (Bergsbaken and Cookson, 2007). Intriguingly, the deletion of all known effectors severely attenuates *Yersinia* infection in C57BL/6 (B6) mice. This attenuation is relieved in caspase-1/11 deficient mice (Brodsky et al., 2010). As PrgJ (T3SS inner rod) and PrgI (T3SS needle subunit) of *Salmonella* activate the NLRC4 inflammasome (Miao et al., 2010b; Yang et al., 2013), it seemed plausible that *Yersinia* inner rod (YscI) and needle (YscF) proteins caused the inflammasome activation. Although there is some NLRC4-dependent inflammasome activation during *in vitro* infection with effectorless *Yersinia*, the majority of caspase-1 processing is dependent on NLRP3 (Brodsky et al., 2010). Additionally at the time these experiments were performed, it was not known that the caspase-1-deficient mice were actually *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice. Therefore, it was unclear whether the NLRP3-dependent caspase-1 processing was through the canonical or non-canonical pathway. As both pathogens and commensals shed LPS and other molecules that stimulate TLR signaling, innate immune cells *in vivo* can have up-regulated pro-IL-1β and other inflammasome associated proteins. Therefore, wild-type *Yersinia* should activate the inflammasome *in vivo* and would be susceptible to caspase-1/11-dependent clearance unless other effectors were capable of inhibiting inflammasome activation.

J. Evasion of the inflammasome by *Yersinia*

With the ability of the inflammasome to effectively control *Yersinia*, it is not surprising that *Yersinia* has multiple effectors that inhibit activation of the inflammasome. Interestingly, neither YopM or YopK, the two effectors that have been demonstrated to inhibit the inflammasome, employ enzymatic activities for this function. YopM's mechanism of inflammasome inhibition is better defined than YopK's. Evdokimov and colleagues initially crystalized YopM and determined that it contained leucine rich repeats (LRR) with very little additional secondary structure (2001). LRR domains typically are involved in protein-protein interactions and binding. In the case of *Yersinia pseudotuberculosis* YPIII strain, a

capase-1 cleavage motif consisting of Tyrosine-Leucine-Threonine-Aspartic Acid (YLTD) in the LRR domain was shown to allow YopM to directly bind to caspase-1 (LaRock and Cookson, 2012). The ability of YopM to bind caspase-1 was required to inhibit activation in this strain. Different strains of *Yersinia pseudotuberculosis* and *Yersinia pestis* express different isoforms of YopM (Boland et al., 1998). Interestingly while these isoforms also inhibit caspase-1 activation, they do not require binding to caspase-1 (Chung et al., 2014). In the case of *Yptb* strain 32777, YopM is unable to bind caspase-1. *Yp* strain Kim5 has the same YLTD motif as *Yptb* YPIII; however, the aspartic acid residue and caspase-1 binding were not required for caspase-1 inhibition. Instead, the scaffolding protein IQGAP1, which interacts with YopM's LRR, was required for caspase-1 inhibition (Chung et al., 2014). Interestingly, deletion of the c-terminus of YopM also abrogated the ability of YopM to inhibit caspase-1 activation (Chung et al., 2014). It has previously been shown that the c-terminus of YopM interacts with the kinase ribosomal S6 protein kinase 1 (RSK1) (McPhee et al., 2010) and that the interaction with YopM increases the activity of RSK1 (McDonald et al., 2003). Whether this activation of RSK1 or some other protein interaction with the c-terminus of YopM is responsible for caspase-1 inhibition is still unknown. Additionally, it had been questioned whether YopM cooperated with any other effectors to inhibit inflammasome activation.

To test whether YopM coordinated with other effectors, the ability of *Yersinia* to activate the inflammasome in primed macrophages was tested with strains that lacked both YopM and another effector (Schoberle et al., 2016). While single deletion of YopJ has no detectable effect on inflammasome activation in primed macrophages, deletion of YopJ and YopM together allows for greater inflammasome activation than YopM deletion alone (Schoberle et al., 2016). In unprimed macrophages, the effect of YopJ on the magnitude of inflammasome output in response to infection with *Yersinia* lacking YopM seems to be

a consequence of the suppression of pro-IL-1β and pro-IL-18 expression (Ratner et al., 2016). However, in primed macrophages the effect of YopJ working in concert with YopM to inhibit the inflammasome seems to be independent of YopJ's suppression of pro-IL-1β and pro-IL-18 expression (Schoberle et al., 2016). How YopJ and YopM are working together is still undefined. While YopM plays an important role in the inhibition of the inflammasome, the effector YopK has also been shown to be important for inhibition of the inflammasome by *Yersinia*.

YopK was the first *Yersinia* effector described to inhibit inflammasome activation (Brodsky et al., 2010) and has long been demonstrated to be important for virulence. Although YopK performs important functions during *Yersinia* infection, no sequence homolog exists. YopK knockouts were shown to cause attenuated infection in Balb/c mice (Straley and Cibull, 1989; Holmstrom et al., 1995), C57BL/6 mice (Brodsky et al., 2010) and rabbits (Najdenski et al., 2003). While the mechanism for YopK inhibition of the inflammasome remains unknown, YopK was found to interact in several ways with *Yersinia'*s Type Three Secretion System, which is required for inflammasome activation. Initially, YopK was determined to negatively regulate translocation of other effectors and affect the lytic ability of the translocon pore potentially by decreasing pore size (Holmstrom et al., 1997). Originally, YopK was not thought to be translocated as it could not be visualized inside cells by confocal microscopy (Holmstrom et al., 1997). This result could be an effect of YopK being translocated at low levels or the epitope(s) recognized being obscured as more recent data employing GSK-tagged YopK and the YopK-β-lactamase fusion system detected translocation of YopK into host cells (Brodsky et al., 2010; Thorslund et al., 2011). It was then determined that to regulate translocation rate YopK acts inside the host cell (Dewoody et al., 2011). Furthermore, translocated YopK interacts with both YopD and YopB (Brodsky et al., 2010; Thorslund et al., 2011) as well

as host proteins (Thorslund et al., 2011). Initially Thorslund suggested interaction with the host protein RACK1 was important for YopK regulation of translocation; however, subsequent data suggest that while RACK1 interacts with YopK, this interaction may not be responsible for regulation of translocation (Dewoody et al., 2013).

Determining how YopK inhibits the inflammasome can provide insights into potential functional homologs in other T3SS expressing bacteria. Additionally, understanding how YopK inhibits the inflammasome during *Yersinia* infection reveals potential targets for clinical intervention to reactivate an effective, natural pathway for *Yersinia* clearance.

K. Dissertation Aims

Over 25 species of pathogenic gram-negative bacteria depend upon the T3SS to cause productive infection. Their T3SSs appear structurally similar by electron microscopy and chemical inhibitors can effectively target T3SS from different families (Cornelis, 2006; Harmon et al., 2010; Muschiol et al., 2006) Outside of NLRC4 recognition of flagellin, the T3SS inner rod of *Salmonella*, and the T3SS needle subunit of *Salmonella*, not much is known about how recognition can occur and if pathogen subversion of the inflammasome is necessary for infection. Previous studies have implicated NLRC4-independent inflammasome activation in the recognition of T3SS expressing *Yersinia* (Brodsky et al., 2010); potentially other T3SS expressing gram-negative bacteria can trigger similar responses. *Yersinia pseudotuberculosis* is an ideal model for inflammasome recognition of the T3SS as *Yptb* expresses an archetypal T3SS and provides a genetically tractable, natural rodent pathogen. Investigation into the interaction between the inflammasomes and the T3SS could reveal important mechanistic and cell biological information about the inflammasomes themselves as well as a potential target for treating T3SS expressing

bacteria. Although *Yersinia pseudotuberculosis* has been shown to actively inhibit inflammasome activation and NLRC4-independent inflammasomes recognize the T3SS (Brodsky et al., 2010), until this work very little was known about what inflammasome is actually activated by the T3SS, what activity of the T3SS is recognized, and how *Yersinia*'s YopK protein inhibits inflammasome activation. Therefore, the aims of the work presented in this dissertation are as follows:

1) To determine the essential activities of the *Yersinia* T3SS that trigger inflammasome activation.

Previous studies have shown that the T3SS of *Yersinia* activates the inflammasome in the absence of all of its known effectors. However, these mutants only lacked the six known effectors and still formed functional T3SSs. The recognition of the T3SS could potentially occur by either detection of the translocon pore or detection of a translocated molecule that is not one of the six known effectors. In Chapter 3, we used in-frame deletion mutations in the pore protein YopD to separate pore-formation from translocation in order to determine if pore-formation was sufficient to activate the inflammasome. We found that inflammasome activation by the T3SS required translocation. We further identified hyper-translocation of the translocon pore proteins YopD and YopB to be required for this activation. Delivery of membrane active proteins into the cytosol of host cells is not limited to T3SS expressing bacteria. Further understanding of how YopD and YopB activate the inflammasome may add insight into how other pathogens should or could be detected and cleared.

2) To determine the role of the non-canonical inflammasome in recognition of the T3SS.

Previous studies identified a role for inflammasomes in the recognition of *Yersinia* expressing a T3SS. However, these studies were done using mice or bone-marrow-derived macrophages (BMDMs) that functionally lacked both caspase-1 and caspase-11. As NLRC4 plays virtually no role in recognizing the *Yersinia* T3SS, we wished to determine whether the non-canonical or canonical inflammasomes recognize the T3SS. In chapter 4, we infected single *Casp11*$^{-/-}$ or *Casp1*$^{-/-}$ BMDMs to determine the contribution of the non-canonical inflammasome to recognition of the T3SS. Our findings indicate that the non-canonical inflammasome plays the dominant role in T3SS recognition, but there is a canonical inflammasome component.

3) To dissect the molecular mechanisms that lead to inflammasome activation downstream of the initial trigger.

The inflammasomes recognize a wide range of fairly divergent stimuli. By determining a molecular mechanism for activation of the inflammasomes downstream of the T3SS, we can potentially gain insights on how the inflammasome is activated in response to similar stimuli from other pathogen systems. In Chapter 4, we demonstrate that translocated YopD can accumulate at lysosomes. Additionally, deletion of the five Gbps found on chromosome 3 prevents activation of the inflammasome in response to *Yersinia*'s T3SS in a manner similar to caspase-11 deletion. Our findings suggest a cooperative role for YopD and Gbps. However, it is still unknown whether YopD is the direct signal or if YopD and Gbps work together to release a different *Yersinia* molecule such as Lipid A into the cytosol from the lysosome.

4) To investigate how the T3SS secreted effectors and structural components work together to evade the inflammasome and bacterial clearance.

It has been shown that deletion of YopK is sufficient to allow for caspase-1/11 mediated attenuation of infection *in vivo*; however, the mechanism by which YopK prevents inflammasome activation has not been directly determined. Work described previously in this dissertation as well as by Kwuan et al. 2013 demonstrated that pore-formation is insufficient to activate the inflammasome. This suggests a model where YopK inhibits inflammasome activation by inhibiting translocation of the pore-proteins YopD and YopB. In Chapter 5, we use a combination of non-functional YopK mutants as well as T3SS chaperone protein mutants to investigate whether the ability of YopK to inhibit the inflammasome is solely dependent upon its ability to limit YopD and YopB translocation. Interestingly, we learned that the T3SS and its related proteins make up a finally tuned system that is kept in delicate balance. Small changes destroy the balance and cause attenuation of the infection by triggering various immune responses depending on how the balance changed.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Yersinia strains are described in Table 3-1 and Table 5-1. For infection of cultured cells, *Yersinia* were grown overnight with aeration in 2xYT broth at 26 °C. The bacteria were diluted into fresh 2XYT containing 20 mM sodium oxalate and 20 mM $MgCl_2$. Bacteria were grown with aeration for 1 hour at 26 °C followed by 2 hours at 37 °C prior to infection. Wild-type *Salmonella* Typhimurium strain SL1344 was grown overnight with aeration in LB broth at 37 °C. The bacteria were diluted into fresh LB containing 300 mM NaCl. Bacteria were grown standing to induce SPI-1 expression. For infection of mice, *Yersinia* were grown overnight with aeration in 2xYT broth at 26 °C and diluted in PBS.

Cell Culture and Infection Conditions

Mice were housed in accordance with the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee (IACUC). All studies involving mice were performed in accordance with University of Pennsylvania IACUC approved protocols. Bone marrow (BM) cells were grown for 7-8 days in DMEM containing Hepes, 10% Fetal Bovine Serum (FBS), and 30% L929 supernatant in a humidified incubator. Differentiated BMDMs were replated overnight into 24-, 48-, or 96-well dishes. IFNγ primed macrophages were treated with 100 Units/mL recombinant IFNγ. *Gbp*$^{chr3-/-}$ BM was provided by Eric Feeley and Jörn Coers (Duke University, Durham, NC). *Casp1*$^{-/-}$ BM was provided by Till Strowig and Richard Flavell (Yale University, New Haven, CT). *Casp11*$^{-/-}$ was initially made by Junying Yuan (Harvard University) and kindly provided to us by Tiffany Horng (Harvard University).

Mouse infections

Mouse infections were performed as previously described (Brodsky et al., 2010) with minor changes. In brief, Eight- to ten- week-old age-and sex-matched mice were infected intraperitoneally with 2 x 10^4 bacteria. Mice were euthanized and the tissues harvested at 3-4 days post-infection. Bacterial load was determined by plating dilutions of tissue homogenates.

Hemolysis Assay

Pore-formation was determined by hemolysis assay as previously described with minor modifications (Olsson et al., 2004). Briefly: sheep erythrocytes (RBCs) were infected with T3SS induced *Yersinia* at an MOI of 0.05 - 0.1 for 3 hours at 37 °C. RBCs were then resuspended in 150 μL of cold PBS and spun down at 4°C for 15 minutes. 100 μL of supernatant was transferred to a flat bottom plate and read for absorbance at 545 nm. Percent hemolysis was normalized to the parent strain.

PI Uptake Assay

HeLa cells were seeded 1x10^4/well overnight in a 12-well chamber slide. Cells were loaded for 45 minutes with CellTracker Green (1:2000, ThermoFisher Scientific) and then washed with PBS. PI was added to media (1:200), and cells were infected at an MOI of 100. Gentamicin was added 2 hours post infection. Cells were imaged 4 hours post infection using Leica DMI4000 with Yokagawa CSU-X1 Spinning Disk Confocal Attachment (10x objective). Images were then analyzed using Metamorph 7.6 to quantify PI positive cells.

Translocation Assay

HeLa cells were plated in a clear bottom, black 96-well tissue culture plate (Greiner Bio one) overnight. Cells were infected at a MOI of 5 with *Yersinia* expressing beta-lactamase fused to YopE (Brodsky and Medzhitov, 2008), YopM, YopJ, or YopD (Dewoody et al., 2013) for 2 hours. Gentamicin was added. CCF4-AM was loaded into cells using LiveBLAzer-FRET B/G Loading Kit (ThermoFisher Scientific) for 1.5 - 2 hours. The fluorescence was read using Biotek Synergy plate

reader. The response ratio was then calculated using the formula (blue:green ratio)/(average negative blue:green ratio).

Cell Death Assay

Cytotoxicity was assayed using a lactate dehydrogenase (LDH) release assay kit (Clontech). In brief, BMDMs were differentiated from bone marrow with 30% L929 supernatant media for seven days before being plated. They were then infected with an MOI of 10, 20, or 100 for 4 hours. In some cases, cells were primed for 3 – 4 hours with 500 ng/mL of LPS. 2.5mM ATP was added to primed cells as a control for NLRP3 inflammasome activation. After 1 hour gentamicin was added. Four hours post infection, the plate was spun down, and assayed for LDH release.

Cytokine Production

Cells were primed for 3 – 4 hours with 500 ng/mL of LPS. The cells were then infected as in the cytotoxicity assay. The supernatants and recombinant cytokine standards (R&D Systems) were collected and added to ELISA plates that had been coated overnight with purified IL-1α, IL-1β, or IL-6 purified antibodies (eBioscience). The cytokines were then detected with the corresponding biotin antibodies (eBioscience) for 1 hour at room temp. Streptavidin-horseradish peroxidase (HRP) (Fisher Scientific) was then added. The plates were developed with citric acid buffer with O-Phenylenediamine (Sigma Aldrich). 3 M sulfuric acid was used to stop the reaction. Absorbance was read at 490 nm.

Western Blotting

Western blots were performed as described previously (Brodsky et al., 2010). In brief, cell lysates were run on a 4%-12% gradient gel (caspase-1 blots, ThermoFisher Scientific) and transferred to a PVDF membrane. Membranes were blotted with the primary antibodies rabbit anti-mouse caspase-1 p10 antibody (SC-514, Santa Cruz), rat anti-mouse caspase-1 p20 (Genentech), and mouse anti-actin (Sigma) or anti-YopD, anti-YopB {Chung, 2014 #4;Noel, 2009 #66}, anti-β-lactamase (QED Bioscience Inc), and anti-LcrV antibodies (kind a gift of Matthew Nilles,

University of North Dakota). Secondary antibodies were goat anti-rabbit (Jackson Immunoresearch), goat anti-rat (Jackson Immunoresearch), or horse anti-mouse HRP (Cell Signaling). Blots were developed with Pierce ECL Western Blotting Substrate (Fisher Scientific).

Immunofluorescence staining

BMDMs were plated 1.5-2x10^5/well on glass coverslips in 24-well plates overnight. Two hours prior to infection, cells were treated with z-YVAD-fmk (R&D Systems or SM Biochemicals LLC) to a final concentration of 25 μM. BMDMs were then infected with an MOI of 5 for 2 hours. mCherry expressing *Yersinia* were used instead of anti-*Yersinia* antibody for infections that required anti-Gbp2 antibody. Cells were fixed in 4% paraformaldehyde prepared from 16% paraformaldehyde (Electron Microscopy Sciences) for 20 minutes at room temperature and then washed twice in PBS. Cells were permeabilized for 10 minutes at room temperature in 0.2% triton X-100 (Sigma) and washed once in PBS. Cells were blocked for 1 hour at room temperature or overnight at 4°C in 10% BSA-PBS. Coverslips were then inverted onto primary antibody cocktails on parafilm and incubated for 1 hour at 37°C. Mouse monoclonal antibodies against YopD (1:500) and YopB (1:500) and rabbit polyclonal antibody against total *Yersinia* have been described (Chung et al., 2014; Grabenstein et al., 2004; Noel et al., 2009). Rabbit anti-caspase-1 p10 antibody (1:100) was from Santa Cruz. Hybridoma rat anti-LAMP1 (neat, Developmental Studies Hybridoma Bank at the University of Iowa) was used to distinguish lysosomes. Staining with rabbit anti-Gbp2 (Coers Lab Duke University, Haldar et al., 2013) was performed either overnight at 4°C prior to the other stains or for 1 hour at 37°C with the other primary antibodies if also staining with FAM-FLICA. Coverslips were washed and stained with appropriate combinations of goat anti-mouse Alex488 (ThermoFisher Scientific), goat anti-rabbit Alexa568 (ThermoFisher Scientific), goat anti-rabbit Alexa488 (ThermoFisher Scientific), goat anti-mouse Alexa647 (ThermoFisher Scientific), and goat anti-rat Alexa647 (Jackson Immunoresearch). Coverslips were washed 4 times. Coverslips were then stained with either a cocktail of Hoechst (1:1000, ThermoFisher Scientific)

and Phalloidin Alexa647 (1:40, ThermoFisher Scientific) for 1 hour at 37°C or HCS CellMask Blue stain (ThermoFisher Scientific) for 1 hr at room temperature. Coverslips were then washed 4 times. Coverslips were mounted on glass slides with FluoromountG (Southern Biotech). Cells were imaged with a Leica SP5 inverted confocal microscope and analyzed using Metamorph 7.6 or Volocity 6.3.

For FAM-FLICA staining for active caspase-1, BMDMs were plated 1.5-2x10^5/well on glass coverslips in 24-well plates overnight. FAM-FLICA (Immunochemistry Technologies) was added 1 hour post infection. The rest of the protocol proceeded as above described.

Metamorph Analysis

Analysis of translocation in single cells was performed by first compressing all individual *z*-plane stacks into a single 'maximum projection'. This enables analysis of the total area in the cell occupied by translocated protein. The final image was segmented into individual cells based on analysis of nuclear and actin staining. Each defined cell was converted into a region and transferred onto images of YopD and caspase-1 staining. Thresholds for positive and negative signal were set based on no primary antibody, uninfected, and Δ*yopB* or Δ*yopD*-infected control samples. The images were then analyzed for the intensity and area of staining in the regions. To quantify the amount of YopD not associated with bacteria, the area defined by YopD and/or bacterial staining was separated into whether pixels in that specific region were positive or negative for staining, and areas of YopD and bacterial overlap were determined. Areas of the cell determined to contain both YopD and bacterial staining were subtracted from total YopD staining. The degree of YopD staining was determined in pre- and post-subtraction images, and percent of bacteria-free YopD was calculated on a per cell basis by the area occupied by YopD post-subtraction by the area occupied by YopD before subtraction.

Volocity Analysis

Analysis of microscopy images was performed on 3D reconstructions of the z-stacks. Single cells were identified with the find object function based on CellMask Blue staining. The images were then analyzed for staining of *Yersinia*, YopD, LAMP1, Gbp2, caspase-1 using the find objects function to generate populations for each stain. Thresholds for positive and negative signals were based on no primary, uninfected, and Gbp2 knockout control samples. To determine the amount of translocated YopD, the pixels in the regions defined by YopD and/or bacterial staining were separated into whether pixels in that specific region were positive or negative for each stain. Regions determined to be positive for both YopD and *Yersinia* staining were subtracted from the total YopD staining using the subtract function. The remaining pixels were assigned to the population translocated YopD. To determine the amount of colocalization between YopD and Gbp2, YopD, or LAMP1, or the colocalization between Gbp2 and caspase-1, the regions defined by translocated YopD, Gbp-2, Lamp1, or caspase-1 puncta staining were separated into whether pixels in that specific region were positive or negative for each stain of interest. Regions that contained both stains of interest were assigned to the new population using the intersect function. The different populations were then compartmentalized into the cells. A measurement table was made for each of the compartmentalized populations. Using the analysis feature, the volume of each population per cell was calculated. Percent translocated or colocalized YopD was determined by dividing the volume of translocated or colocalized YopD in a cell by the volume of total YopD in that cell.

Statistical Analysis

Statistical analysis was performed using Prism 6 (Graphpad Software, Inc). One way ANOVA followed by Tukey's Multiple Comparison test was used for the analysis of data requiring multiple

comparisons. Otherwise, Unpaired Student's *t* tests were used for the analysis with Welch's correction in cases of unequal standard deviation.

CHAPTER 3

Inflammasome activation in response to the *Yersinia* Type III Secretion System requires hyperinjection of translocon proteins YopB and YopD

This chapter appeared as a published peer-reviewed article titled “Inflammasome activation in response to the *Yersinia* Type III Secretion System requires hyperinjection of translocon proteins YopB and YopD” by Erin E. Zwack, Annelise G. Snyder, Meghan A. Wynosky-Dolfi, Gordon Ruthel, Naomi M. Philip, Melanie M. Marketon, Matthew S. Francis, James B. Bliska, and Igor E. Brodsky. mBio, 2015.

Abstract:

Type III Secretion Systems (T3SS) translocate effector proteins into target cells in order to disrupt or modulate host cell signaling pathways and establish replicative niches. However, recognition of T3SS activity by cytosolic Pattern Recognition Receptors (PRRs) of the Nucleotide-Binding Domain Leucine Rich Repeat (NLR) family, either through detection of translocated products or membrane disruption, induces assembly of multiprotein complexes known as inflammasomes. Macrophages infected with *Yersinia pseudotuberculosis* strains lacking all known effectors or lacking the translocation regulator YopK induce rapid activation of both the canonical NLRP3 and non-canonical caspase-11 inflammasomes. While this inflammasome activation requires a functional T3SS, the precise signal that triggers inflammasome activation in response to *Yersinia* T3SS activity remains unclear. Effectorless strains of *Yersinia* as well as Δ*yopK* strains translocate elevated levels of T3SS substrates into infected cells. To dissect the contribution of pore-formation and translocation to inflammasome activation, we took advantage of variants of YopD and LcrH that separate these functions of the T3SS. Notably, YopD variants that abrogated translocation but not pore-forming activity failed to induce inflammasome activation. Furthermore, analysis of individual infected cells revealed that inflammasome activation at the single cell level correlated with translocated levels of YopB and YopD themselves. Intriguingly, LcrH mutants that are fully competent for effector translocation but produce and translocate lower levels of YopB and YopD also fail to trigger inflammasome activation. Our findings therefore suggest that hyper-translocation of YopD and YopB is linked to inflammasome activation in response to the *Yersinia* T3SS.

Importance

The innate immune response is critical to effective clearance of pathogens. Recognition of conserved virulence structures and activities by innate immune receptors such as NLRs

constitute one of the first steps in mounting the innate immune response. However, pathogens, such as *Yersinia* actively evade or subvert components of host defense, such as inflammasomes. The T3SS secreted protein YopK is an essential virulence factor that limits translocation of other Yops, thereby limiting T3SS-induced inflammasome activation. However, what triggers inflammasome activation in cells infected by YopK-deficient *Yersinia* is not clear. Our findings indicate that hyper-translocation of pore complex proteins promotes inflammasome activation and that YopK prevents inflammasome activation by the T3SS by limiting translocation of YopD and YopB themselves.

Introduction

The innate immune system plays a crucial role in host defense against pathogens. Pattern recognition receptors (PRRs) recognize conserved microbial structures expressed by both pathogenic and nonpathogenic bacteria, commonly termed pathogen associated molecular patterns (PAMPs) (Janeway, 1989; Janeway and Medzhitov, 2002). Cytosolic receptors that detect both virulence activities as well as bacterial molecules within the cytosol, which generally occurs as a direct consequence of pathogen virulence machinery activities, provide an additional layer of sensing of pathogenic bacteria (Vance et al., 2009).

Virulence activities such as pore-formation by toxins or secretion systems, and delivery of bacterial products into the cytosol of target cells trigger the activation of a cytosolic immune surveillance pathway that culminates in assembly of multiprotein complexes termed inflammasomes (Brodsky and Monack, 2009; Lamkanfi and Dixit, 2009). Inflammasome assembly typically requires a nucleotide binding domain leucine-rich repeat protein (NLR), which responds to particular pathogen-associated stimuli, and induces oligomerization of apoptosis-associated speck-like protein (ASC) and the cysteine protease caspase-1 (Lamkanfi and Dixit,

2009; Martinon et al., 2002). Inflammasome activation results in pyroptosis, a programmed cell death characterized by caspase-1 processing and the release of IL-1 family cytokines, IL-1α, IL-1β, and IL-18 (Cookson and Brennan, 2001). Additionally, a recently described non-canonical inflammasome also engages caspase-11 to induce cytotoxicity and IL-1α and contributes to maximal IL-1β processing and secretion by caspase-1 (Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012).

Different inflammasomes respond to distinct signals. The NLRP3 inflammasome recognizes endogenous indicators of tissue damage and tissue stress, such as extracellular ATP and sodium urate crystals as well as microbial products such as viral and bacterial pore-forming proteins {Hornung et al., 2008; Kanneganti et al., 2006; Lamkanfi and Dixit, 2009; Mariathasan et al., 2006; Martinon et al., 2006), whereas NLRC4 in conjunction with the NLR family apoptosis inhibitory protein (NAIP) proteins detects the presence of cytosolic flagellin and structurally similar products of the type III secretion system (T3SS) itself, such as PrgJ (Gong and Shao, 2012; Miao et al., 2006; Miao et al., 2008; Miao et al., 2010; Roy and Zamboni, 2006; Tenthorey et al., 2014; Yang et al., 2013). Recognition of pathogen-specific signals by inflammasomes promote anti-microbial defense either via the immunological effects of IL-1 family cytokines (Casson et al., 2013; Ichinohe et al., 2009) or through induction of pyroptosis (Miao et al., 2010a).

The pathogenic Yersiniae*, Yersinia pseudotuberculosis, Yersinia enterocolitica,* and *Yersinia pestis* express a conserved T3SS that injects *Yersinia* Outer Proteins (Yops) into target cells (Cornelis and Wolf-Watz, 1997). Once inside the cell, the Yops disrupt a number of cellular processes including actin cytoskeleton rearrangement, protein phosphorylation, and NF-κB and MAPK signaling (Viboud and Bliska, 2005; Palmer et al., 1998). Two Yops, YopK and YopM, modulate inflammasome activation, thereby interfering with host innate immune defense and

promoting *Yersinia* infection (Brodsky et al., 2010; Chung et al., 2014; LaRock and Cookson, 2012).

Once translocated into the cell, YopK interacts with integral membrane components of the translocation complex, YopD and YopB, to prevent hyper-translocation of other Yops (Brodsky et al., 2010; Holmstrom et al., 1997; Thorslund et al., 2011). YopK may therefore limit translocation of other Yops by physically blocking or modulating the size of the translocon pore. YopK may also limit translocation by interacting with the host scaffolding protein RACK1 or potentially other cellular factors (Dewoody et al., 2013; Thorslund et al., 2011). In addition to a hyper-translocation of effectors, *yopK* mutants exhibit inflammasome-dependent attenuation in animal infections, indicating that inappropriate translocation of other Yops or additional factors triggers inflammasome activation, and leads to bacterial clearance *in vivo* (Brodsky et al., 2010). Interestingly, NLRP3, not NLRC4 is the primary driver of inflammasome activation in response to the *Yersinia* T3SS, in contrast to several other bacterial pathogens, such as *Salmonella*, likely due to inverse regulation of flagellin and T3SS expression in *Yersinia* (Brodsky et al., 2010; Miao et al., 2006; Minnich and Rohde, 2007).

The YopB and YopD proteins are integral membrane proteins that form the translocon pore through which other Yops are delivered into the host cell. Thus, complete deletion of either YopB or YopD results in a nonfunctional T3SS (Neyt and Cornelis, 1999; Nordfelth and Wolf-Watz, 2001; Rosqvist et al., 1995; Sory and Cornelis, 1994). However, specific in-frame deletions in YopD result in loss of translocation ability while maintaining pore-forming activity (Olsson et al., 2004). Conversely, mutations in the YopB and YopD chaperone, LcrH, impact pore-forming capacity due to reduced steady-state expression levels of YopB and YopD, yet maintain wild-type

levels of translocation, implying that pore-formation and translocation are separable biological features of the T3SS (Edqvist et al., 2007).

Intriguingly, in addition to forming the translocation pore through which the classical effector Yops are injected into host cells, YopB and YopD themselves are also translocated into the host cell, albeit at relatively low levels in WT *Yersinia* (Dewoody et al., 2013; Francis and Wolf-Watz, 1998). The functional consequences of this translocation of components of the T3SS pore complex are not well understood but appear to be a general feature of the T3SS as *Salmonella* SipC and SipB pore complex proteins are also translocated (Collazo and Galan, 1997).

Critically, the mechanism by which the *Yersinia* T3SS induces NLRP3 inflammasome activation remains unclear. As both pore-forming toxins and translocated bacterial products can cause inflammasome activation (Kanneganti et al., 2006; McNeela et al., 2010; Mitoma et al., 2013; Craven et al., 2009}, inflammasome activation could potentially be the result of pore-formation generated by the insertion of the pore complex or due to translocation of a molecule by the T3SS.

Here we employed the YopD and LcrH mutants that separate pore-formation and translocation activities, and find that pore-formation is not sufficient to activate the inflammasome, consistent with recent observations (Kwuan et al., 2013). Strikingly, we also note that while translocation competence is required for inflammasome activation, it is not sufficient, as LcrH mutants with a functional translocon that display wild-type levels of effector Yop translocation but do not translocate detectable levels of YopB or YopD fail to trigger inflammasome activation. Moreover, hyper-translocation of YopB and YopD correlated with an increased likelihood of caspase-1 activation at the single-cell level. These findings suggest that the translocation of YopB and/or

YopD may mediate inflammasome activation in response to the *Yersinia* T3SS, and that YopK functions to prevent excessive translocation of these proteins.

Results

Translocation is required for inflammasome activation in response to *Yersinia* T3SS

Translocation of currently undefined T3SS client proteins may be responsible for NLRP3 inflammasome activation in macrophages by the *Yersinia* T3SS (Brodsky et al., 2010; Kwuan et al., 2013). Alternatively, alteration in the stoichiometry, conformation, or pore-forming properties of the translocon components YopB and YopD could be responsible. In order to dissect the relative contributions of translocation and pore-formation of the T3SS to inflammasome activation, we generated in-frame *yopD* deletion mutants in a *Y. pseudotuberculosis* strain lacking Yops E, J, and K (Δ*yopEJK*), which induces robust T3SS-dependent NLRP3 inflammasome activation in murine macrophages. These in-frame deletion mutations in *yopD* separate the pore-forming and translocation activities of YopD in wild-type *Yersinia* (Olsson et al., 2004).

To accurately determine bacterially induced pore-formation and differences in translocation, we performed translocation and pore-formation assays with HeLa cells and sheep red blood cells that do not undergo inflammasome-mediated cell death in response to infection. We observed that deletion of amino acids **4-20 or 53-68** (termed YopDΔ1 and YopDΔ3 respectively) completely abrogated translocation of a YopE-β-lactamase reporter construct similarly to what is seen with Δ*yopB* or YopDΔ4 (**aa 73-90**), which have been previously demonstrated to generate nonfunctional T3SS. In contrast, YopDΔ7 (**150-170)** maintained translocation although at a reduced level compared to $YopD^{WT}$ (Fig. 3-1A). Pore-formation as measured by hemolysis in

sheep red blood cells was significantly reduced in YopDΔ1, YopDΔ3, and YopDΔ7 in comparison to $YopD^{WT}$. However, these variants still caused significant levels of pore-formation relative to Δ*yopB* and YopDΔ4, which eliminated the hemolytic capacity of the T3SS (Fig. 3-1B). To address potential threshold effects resulting from the reduced pore-forming activity of YopDΔ1 and YopDΔ3, we performed our macrophage infections at both a MOI of 20 and a MOI of 100. Critically, at both MOIs, YopDΔ1 and YopDΔ3 failed to induce IL-1β secretion, LDH release, or cleavage of caspase-1, suggesting that eliminating translocation abrogated the ability of the *Yersinia* T3SS to induce inflammasome activation (Fig. 3-1C-E). Differential secretion of IL-1β in response to different YopD variants was not due to effects of YopD on TLR signaling per se, as secretion of IL-6 was unaffected in response to infection with different YopD variants (Fig. 3-1E). Interestingly, while YopDΔ1, Δ3 and Δ7 were all capable of inducing hemolysis in RBCs, they did not induce propidium iodide (PI) uptake in HeLa cells, another measure of "pore formation", implying that this pore formation, at least in nucleated cells, is distinct from the T3SS pore itself (Fig. 3-2) Together, these data suggest that pore-forming capacity of the *Yersinia* T3SS is insufficient to induce inflammasome activation; rather, that translocation of an unknown molecule induces NLRP3 inflammasome activation.

Deletion of *yopK* results in hyper-translocation of YopD and YopB

Our data and previously published work (Brodsky et al., 2010; Dewoody et al., 2011; Dewoody et al., 2013; Holmstrom et al., 1997; Olsson et al., 2004) suggest that YopK normally functions to regulate translocation by the T3SS, and that the hyper-translocation taking place in the absence of YopK triggers inflammasome activation. The integral translocon components YopB and YopD are also translocated during infection, raising the possibility that excess translocation of YopD or YopB themselves might be responsible for T3SS-induced inflammasome activation (Dewoody et al., 2013; Francis and Wolf-Watz, 1998). To determine whether YopB and YopD translocation

might be correlated with inflammasome activation, we performed confocal microscopy on *Yersinia*-infected macrophages and analyzed both the translocation of T3SS pore complex proteins and caspase-1 puncta formation. Macrophages were treated with YVAD-fmk to enhance puncta visualization by preventing death of the macrophages or escape of caspase-1 from the puncta. Formation of caspase-1 puncta indicates oligomerization of caspase-1, and is an indicator of inflammasome formation (Broz et al., 2010). Confocal fluorescence microscopy analysis of YopD translocation revealed a significant correlation between the degree of YopD translocation, defined by intensity and area of anti-YopD staining, and the formation of caspase-1 puncta (Fig. 3-3). A similar correlation was found for YopB (Fig. 3-4). Infection with Δ*yopEJK* resulted in a significantly elevated percentage of puncta positive cells than infection with Δ*yopEJ,* consistent with previous studies that showed YopK prevents inflammasome activation (Brodsky et al., 2010). Interestingly, YopB and YopD accumulated in the cytosol in large punctate or vesicular structures that were devoid of bacteria (Fig. 3-3D-E). Both the extent (area) and percent of cytosolic YopD were significantly elevated in the absence of YopK. We did not observe co-localization of either YopD or YopB with the caspase-1 puncta themselves, implying that any role of YopB or YopD in inflammasome activation was not the result of direct recognition by NLRP3.

Translocation is not sufficient for T3SS-induced inflammasome activation

To further test the possibility that YopD or YopB translocation might be responsible for inflammasome activation, we generated two separate LcrH mutants designated *lcrHmut1* (encoding $LcrH^{Y40C, I101M, K102R}$) and *lcrHmut2* (encoding $LcrH^{Q46R, F72S, Q81R, H162R}$) as well as the *lcrH* null mutant (Δ*lcrH*) in the Δ*yopEJK* strain background. LcrH is a chaperone for both YopB and YopD, and specific amino acid mutations in LcrH lead to reduced steady-state levels of stable YopB and YopD without affecting the translocation of other Yops (Edqvist et al., 2007). Consistent with established work that these amino acid changes in LcrH affect the T3SS, both

lcrHmut1 and *lcrHmut2* expressed and secreted lower levels of YopB and YopD (Fig. 3-5A and B), but did not affect the translocation of a YopE-β-lactamase reporter (Fig. 3-5D). Furthermore, *lcrHmut1* also had no effect on translocation of a YopM-β-lactamase reporter, demonstrating that these LcrH mutants do not impact translocation of secreted effector Yops. Importantly, another structural component of the T3SS, LcrV, as well as YopE-β-lactamase, were expressed to an equal extent in the bacteria themselves, but exhibited a hypersecretory phenotype in the *lcrH* mutant strain backgrounds, consistent with previous observations that LcrH mutations or translocon mutations that reduce expression of YopD or YopB result in hypersecretion (Anderson et al., 2002; Edqvist et al., 2006; Wattiau et al., 1994; Williams and Straley, 1998)(Fig. 3-5A and B). Critically, the *lcrHmut1* and Δ*lcrH* strains were unable to translocate YopD-β-lactamase (Fig. 3-5E). Despite translocating effector Yops in a manner that was indistinguishable from wild-type bacteria, both *lcrHmut1* and *lcrHmut2* strains failed to trigger macrophage cytotoxicity and IL-1β secretion (Fig. 3-5E and F). Collectively these data suggest that hyper-translocation of LcrH-dependent pore-complex proteins is required for inflammasome activation.

Hyper-translocation of YopD is necessary for caspase-1 puncta formation

To test the contribution of translocated YopD and YopB to inflammasome activation, we examined inflammasome activation in the context of *lcrHmut1* as this mutant had reduced steady-state levels of YopB and YopD but the reduction was not as great as in *lcrHmut2* (Fig. 3-5A). Notably, confocal microscopy analysis of individual *Yersinia*-infected cells demonstrated that *lcrHmut1 Yersinia* strains translocated significantly lower levels of YopD than wild-type *Yersinia*, as quantified by intensity and area of antibody staining, in accordance with our findings using the YopD-β-lactamase reporter (Fig. 3-6A-C). Critically, this reduced translocation of YopD was linked to loss of inflammasome activation, as a significantly higher percentage of Δ*yopEJK* *Yersinia*-infected cells exhibited evidence of caspase-1 puncta in comparison with *lcrHmut1*-infected cells (Fig. 3-6D). Translocation of YopB was similarly reduced in *lcrHmut1*-infected cells, and no caspase-1 puncta were observed (Fig. 3-4). Altogether, our data support a model in which

excess translocation of YopD and/or YopB triggers NLRP3 inflammasome activation. Although an additional T3SS-translocated product that is inappropriately translocated in the absence of YopK may also lead to NLRP3 inflammasome activation, the lack of inflammasome activation by specific LcrH mutants that limit translocation of Yops D and B without impacting translocation of other T3SS substrates indicates that YopD and/or B play a critical role in triggering this response.

Discussion

Inflammasome activation plays a key role in inflammatory responses during infection or disruption of tissue homeostasis, and distinct NLR proteins respond to different inflammatory triggers such as the presence of microbial products within the cytosol of target cells. The NLRP3 inflammasome is triggered by structurally diverse microbial and stress-associated signals (Allen et al., 2009; Ichinohe et al., 2009; Iyer et al., 2013; Kanneganti et al., 2006; Kim et al., 2013; Mariathasan et al., 2006; McNeela et al., 2010; Menu et al., 2012; Shimada et al., 2012; Shio et al., 2009; Zhou et al., 2011), and previous studies demonstrated that the *Yersinia* type III secretion system can trigger NLRP3 inflammasome activation in the absence of all known secreted effectors (Brodsky et al., 2010). Several studies have demonstrated that in the absence of YopK, a secreted *Yersinia* effector protein that negatively regulates the translocation activity of the T3SS, NLRP3 inflammasome activation is significantly enhanced (Brodsky et al., 2010; Kwuan et al., 2013; LaRock and Cookson, 2012). However, the mechanism by which the NLRP3 inflammasome is triggered by the *Yersinia* T3SS is unknown.

Interactions between the *Yersinia* virulence machinery and inflammasome activation involve an intricate interplay between *Yersinia* effector proteins, TLR signaling, and innate immune pathways. In the context of NF-κB and MAPK blockade by YopJ, cells such as naïve macrophages trigger a distinct form of caspase-1 activation that engages the cell extrinsic death

pathway and promotes anti-*Yersinia* immune defense independently of known inflammasome components (Monack et al., 1997; Philip et al., 2014). Interestingly, LPS-primed macrophages do not undergo YopJ-induced caspase-1 activation, possibly as a result of inhibition of caspase-1 activation by YopM (Chung et al., 2014; LaRock and Cookson, 2012). However, in the absence of all known effector Yops or in the absence of Yops J and K alone, the *Yersinia* T3SS triggers a robust NLRP3 inflammasome activation that involves both canonical and non-canonical inflammasomes in both LPS-primed and unprimed macrophages (Bergsbaken and Cookson, 2007; Brodsky et al., 2010; Casson et al., 2013; Shin and Cornelis, 2007). The mechanism by which the *Yersinia* T3SS induces this NLRP3 inflammasome activation in the absence of all known secreted effectors remains unclear.

The various activities of the T3SS have been extensively probed by taking advantage of bacterial genetic tools to generate mutants deficient in key components of the translocon and associated proteins (Edqvist et al., 2007; Olsson et al., 2004). In particular, in-frame deletions in the integral translocon component YopD make it possible to separate the pore-forming ability of the translocon from the capacity of the T3SS to translocated effector proteins (Olsson et al., 2004). Specifically, deletion of amino acids 4-20 or 53-68 in YopD compromises the ability of the T3SS to translocate effectors, but has a minimal impact on the ability of the T3SS to induce hemolysis in sheep RBCs, one measure of pore-forming ability ((Olsson et al., 2004) and Fig. 3-1A). Interestingly, we found that these YopD deletion mutants failed to induce inflammasome activation, despite being able to lyse RBCs, consistent with recent findings by Kwuan et al., that pore formation is insufficient to induce inflammasome activation (2013). Thus, some structural feature of the translocation process and/or a particular translocated molecule(s) is the cue for inflammasome activation.

Indeed we found that translocation is necessary but insufficient for NLRP3 inflammasome activation, as mutations in the T3SS chaperone gene *lcrH* that do not affect YopE or YopM translocation, but are not capable of forming pores in RBCs, are also incapable of triggering inflammasome activation. Critically, our data show that LcrH mutant *Yersinia* that produce a functional translocon in the presence of limited YopB and YopD proteins, have a specific defect in translocation of YopD and YopB. Single-cell-based analyses of YopD and YopB translocation revealed a significant correlation between the extent of translocation of these proteins and the formation of an active inflammasome complex.

Taken together, these studies suggest that hyper-translocation of YopD or YopB in the absence of YopK is responsible for NLRP3 inflammasome activation. An alternative possibility is that structural differences in the translocon resulting from mutation of LcrH or YopD alters the membrane-perturbing properties of the translocon. While the YopDΔ1, Δ3, and Δ7 mutants are all capable of inducing RBC lysis, we did not detect measurable PI uptake in HeLa cells infected with these mutants (Fig. 3-2). These data suggest that membrane perturbation or lysis in different cell types involves distinct features of the translocon. Indeed, neither of the LcrH mutants used in this study induce RBC lysis, despite being fully competent for translocation ((Edqvist et al., 2007) and Fig. 3-5). These data suggest that pore formation measured by leakage of dyes across eukaryotic cell membranes during *Yersinia* infection detects the presence of a host cell-derived pore that is assembled or activated in response to the activity of the T3SS. Whether this pore is itself dependent on caspase-1 activation (Fink and Cookson, 2006) or is responsible for mediating caspase-1 activation, or whether this system constitutes a self-amplifying positive-feedback loop, remains to be determined.

How translocation of YopD or YopB is linked to NLRP3 inflammasome activation is currently unclear. YopD and YopB are both amphipathic and membrane-active, raising the possibility that at elevated concentrations in the target cell, insertion of one or both of these proteins into membranes of cellular organelles may induce inflammasome activation. Disruption of lysosomal membranes or Golgi membranes has been linked to NLRP3 inflammasome activation (Hornung et al., 2008; Ichinohe et al., 2010; Ito et al., 2012) raising the possibility that insertion of YopD or YopB into these membranes triggers NLRP3. We observed large aggregates or superstructures of YopD and YopB in cells infected by YopK-deficient *Yersinia* (Fig. 3-3 and 3-6 and Fig. 3-4). These structures were not simply the presence of YopD or YopB staining around the *Yersinia*, as a significant percentage of these structures were not associated with intact bacteria (Fig. 3-3). Protein aggregates or crystals are known to induce NLRP3 inflammasome activation; large aggregates of YopB or YopD could therefore lead to NLRP3 inflammasome activation. Future studies will dissect the molecular mechanism for NLRP3 activation by translocated YopD and/or YopB.

It is possible that YopD and YopB translocation serves as a surrogate for elevated translocation of an unknown factor or protein that is more directly responsible for T3SS-induced inflammasome activation. While it remains possible that another protein or bacterial molecule is translocated by the *Yersinia* T3SS and induces NLRP3 activation, our data suggest that YopD and/or YopB translocation into the cytosol is responsible, and that YopK prevents the excessive translocation of YopD and YopB into target cells in order to avoid triggering this innate inflammatory response. Interestingly, initial studies of inflammasome activation by *Salmonella* suggested that microinjected SipB, the *Salmonella* homolog of YopB, induced caspase-1 activation (Hersh et al., 1999). Thus, activation of the NLRP3 inflammasome by the bacterial T3SS machinery may be a broadly conserved innate response to the cytosolic presence of T3SS pore complex proteins.

Acknowledgments

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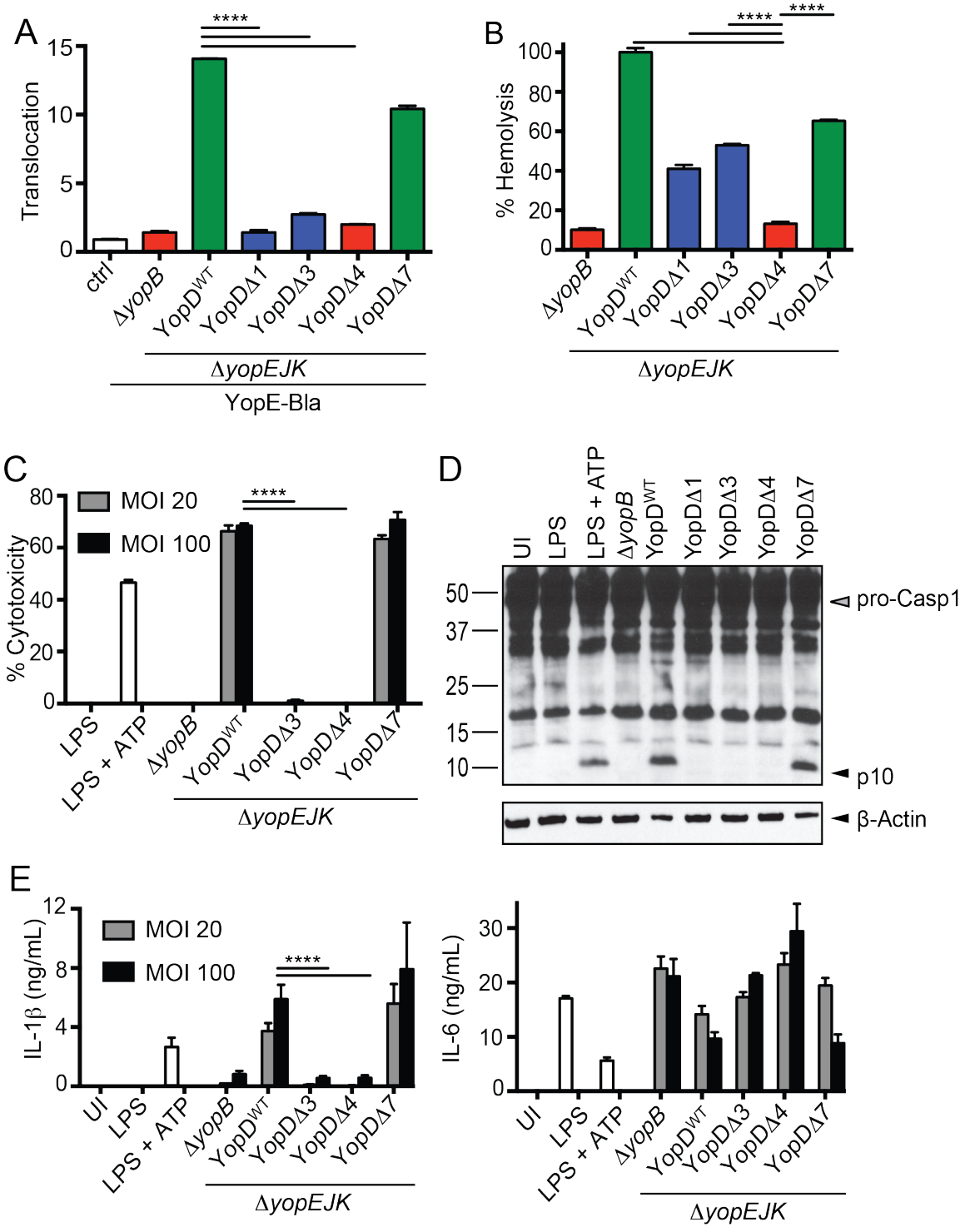
Figure 3-1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YopDΔ7
ΔyopEJK
YopE-Bla
B
% Hemolysis
100
80
60
40
20
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ΔyopB
YopD^WT
YopDΔ1
YopDΔ3
YopDΔ4
YopDΔ7
ΔyopEJK
C
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60
40
20
0
MOI 20
MOI 100
****
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LPS + ATP
ΔyopB
YopD^WT
YopDΔ3
YopDΔ4
YopDΔ7
ΔyopEJK
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YopDΔ7
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LPS
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ΔyopB
YopD^WT
YopDΔ3
YopDΔ4
YopDΔ7
ΔyopEJK

Figure 3-1. Translocation is required for inflammasome activation by *Yersinia* T3SS. **A.** HeLa cells were infected with indicated strains expressing a YopE-β-lactamase fusion protein (YopE-Bla) or Δ*yopEJK* lacking the β-lactamase construct (ctrl). Cells were loaded with CCF4-AM dye, and the ratio of blue:green signal (translocation) was calculated as described in Materials and Methods. Results are representative of 3 independent experiments. **B.** Sheep red blood cells were infected with indicated YopD deletion mutants or the controls Δ*yopB* and *ΔyopEJK*. Supernatants were assayed for release of hemoglobin as described in Materials and Methods. The graph is representative of two to four independent experiments with independent 6 replicates per sample. **C.** BMDMs were infected with indicated YopD deletion mutants, Δ*yopB*, or Δ*yopEJK* or treated with LPS or LPS + ATP, and cytotoxicity was determined by LDH release. The graph is of a representative experiment from one of five independent experiments (MOI 20) or 2 independent experiments (MOI 100) with 3 replicates per sample. **D.** BMDMs were infected with the indicated bacterial strains or treated with LPS or LPS + ATP. Cell lysates were assayed for pro- and processed caspase-1 and actin as indicated. **E.** Supernatants from BMDMs infected with indicated bacterial strains were assayed for levels of secreted IL-1β and IL-6 by ELISA as described in Materials and Methods. **** $p<0.0001$.

Figure 3-2

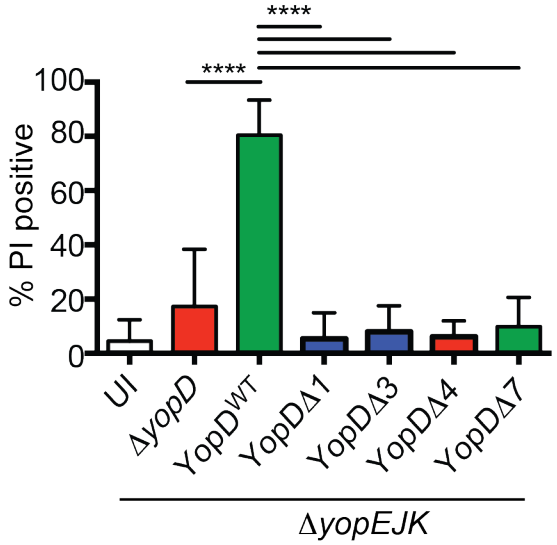


Figure 3-2. In-frame YopD deletions eliminate pore formation in HeLa cells independent of translocation and hemolysis. HeLa cells were infected at an MOI of 100 with the indicated YopD mutants or the controls Δ*yopEJKD* and Δ*yopEJK* in the presence of PI for 2 hours prior to the addition of gentamicin. Images were taken 2 hours post gentamicin addition. Images were analyzed for the percent of HeLa cells containing PI. Data pooled from three independent experiments. **** $p<0.0001$.

Figure 3-3

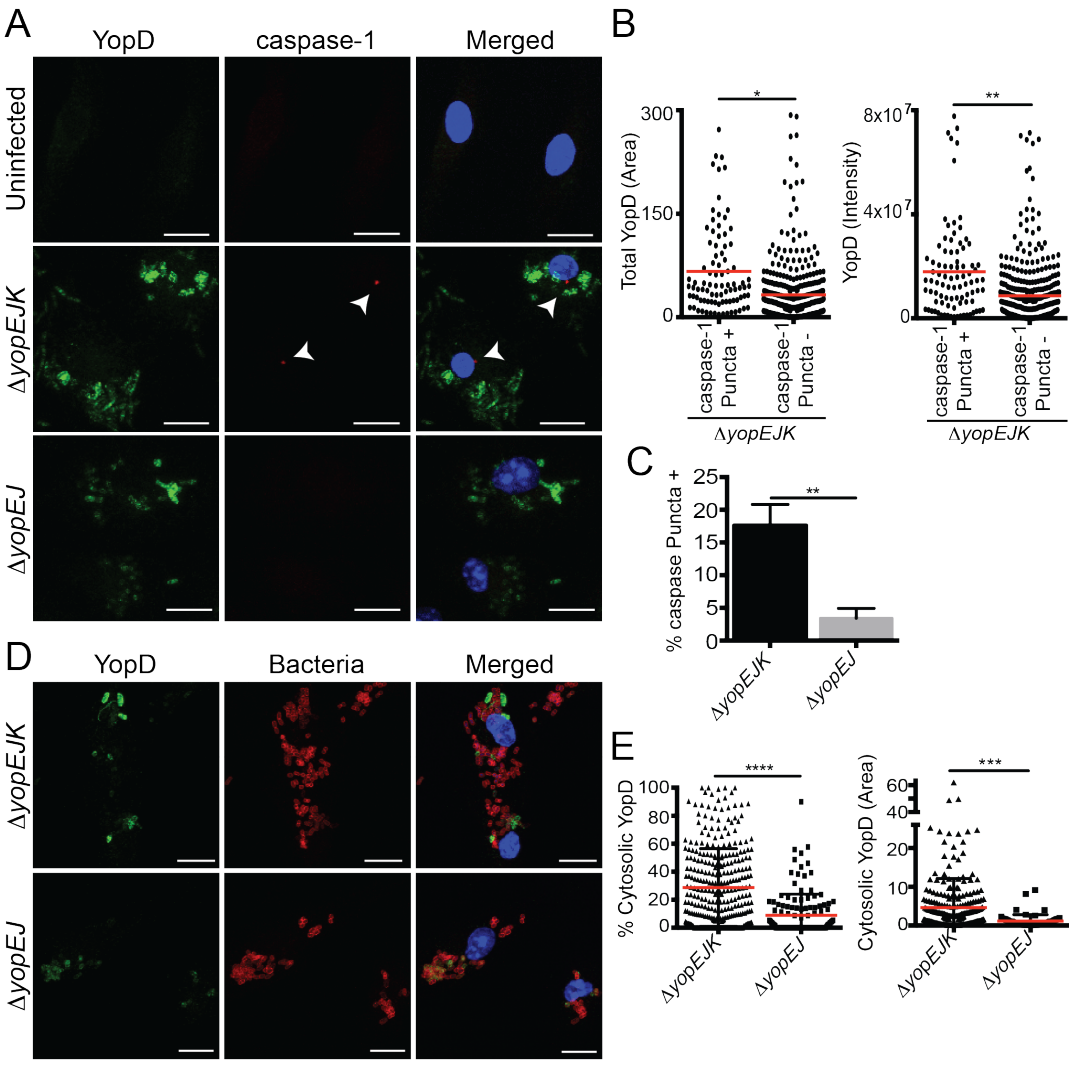
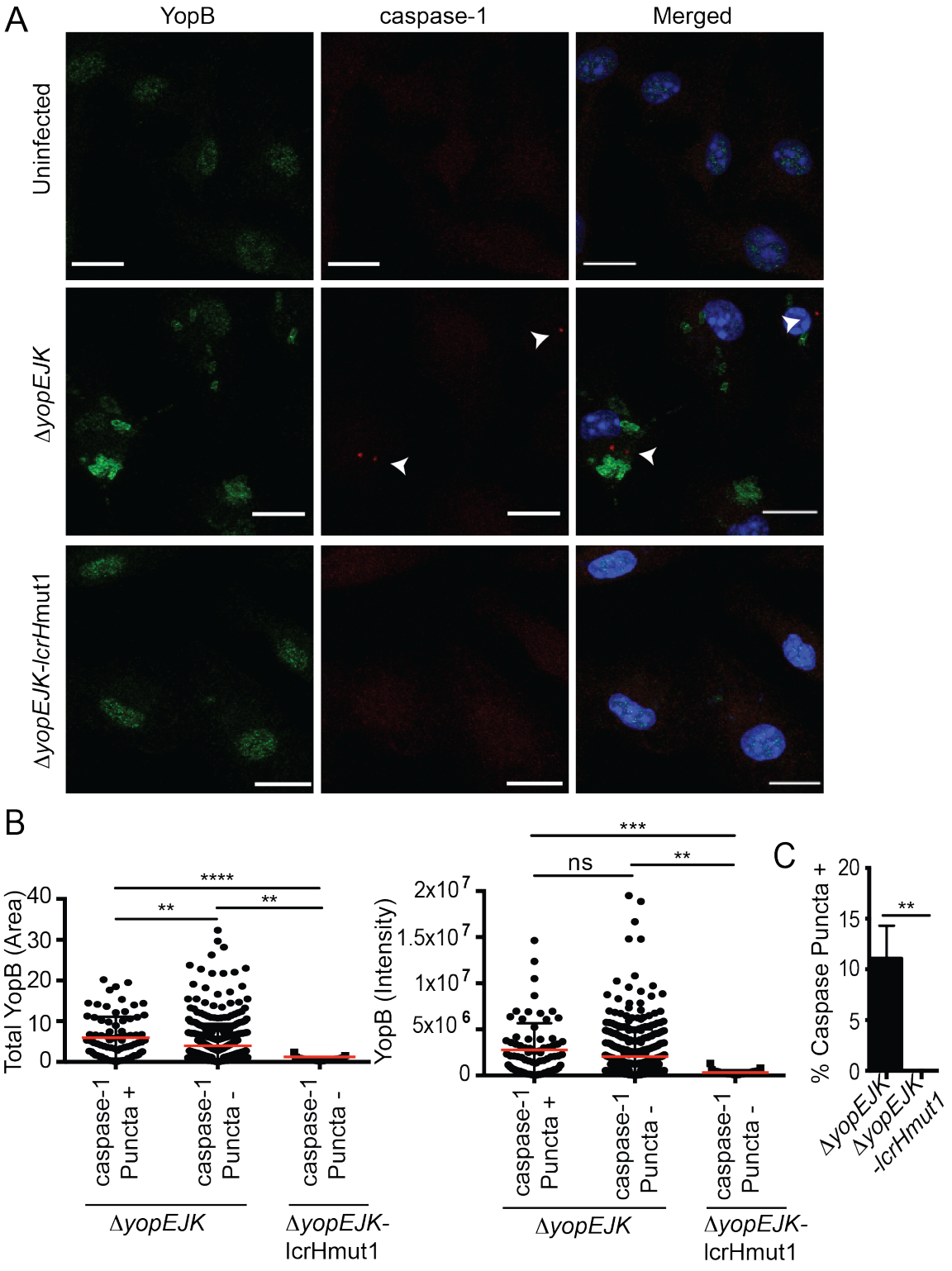


Figure 3-3. Increased YopD translocation correlates with activation of caspase-1. A. Combined z-stacks of confocal microscopy images of BMDMs that were pretreated with YVAD and left uninfected or infected with Δ*yopEJK* or Δ*yopEJ* for 2 hrs. Cells were stained for YopD (green), caspase-1 (red), and DNA (blue). Arrows denote caspase-1 puncta. **B.** Total area of YopD (μm^2) in caspase-1 puncta positive cells versus caspase-1 puncta negative cells in Δ*yopEJK* infection. Total intensity of YopD staining for caspase-1 puncta positive cells versus caspase-1 puncta negative cells infected with Δ*yopEJK*. Each point represents a single cell. **C.**

Percent of YopD staining cells that were positive for caspase-1 puncta. **D.** Combined z-stacks of confocal microscopy images of cells that were uninfected or infected with Δ*yopEJK* or Δ*yopEJ* for 2 hrs. Cells were stained with antibodies against YopD (green), *Yersinia* (red), and Hoechst to visualize DNA (blue). Amount of cytosolic YopD was determined as described in Materials and Methods. **E.** Percent and total area of cytosolic YopD (μm^2) obtained from analyzing microscopy images as in D. (B,C,E) Each point represents a single cell, data are pooled from 3 independent experiments. Red lines represent mean of data. Scale bars are 10 μm. (B,C) 477 individual cells were analyzed. (E) 321 individual cells were analyzed. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Figure 3-4


A
YopB
caspase-1
Merged
Uninfected
ΔyopEJK
ΔyopEJK-IcrHmut1
B
Total YopB (Area)
****
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**
caspase-1 Puncta +
caspase-1 Puncta -
caspase-1 Puncta -
ΔyopEJK
ΔyopEJK-IcrHmut1
YopB (Intensity)
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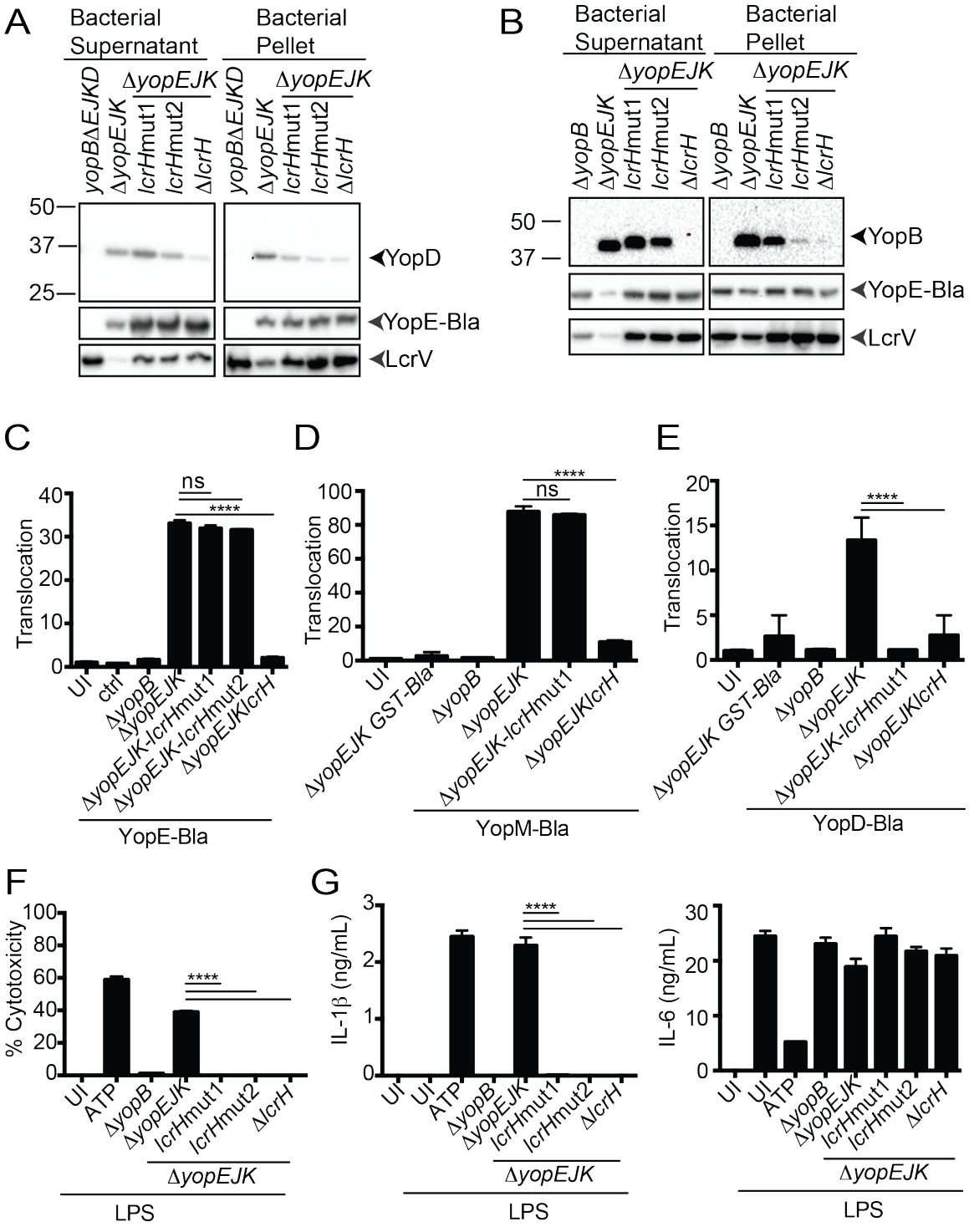
C
% Caspase Puncta +
**
ΔyopEJK
ΔyopEJK -IcrHmut1

Figure 3-4. Increased translocation of YopB correlates with caspase-1 activation. **A.** Combined z-stacks of confocal microscopy images of YVAD-pretreated BMDMs that were left uninfected or infected with Δ*yopEJK* or Δ*yopEJK-lcrHmut1* for 2 hrs. Cells were stained for YopB (green), caspase-1 (red), and DNA (blue). White arrows indicate active caspase-1 puncta. **B.** Total area (μm^2) and total intensity of YopB staining in cells infected with Δ*yopEJK* (circles) or Δ*yopEJK-lcrHmut1* (squares). Data pooled from at least three independent experiments respectively. Each point represents a single cell. 646 individual cells were analyzed. Red bars indicate mean of data. **C.** Percent of YopB staining cells that contain active caspase-1 puncta. Scale bars are 10 μm. (B) One-way ANOVA with Tukey Multiple Comparison tests ** p< 0.005, *** $p < 0.0005$ **** p< 0.0001 (C) Student's t-test **** p< 0.0001.

Figure 3-5


A
Bacterial Supernatant
Bacterial Pellet
ΔyopEJK
yopBΔEJKD
ΔyopEJK
IcrHmut1
IcrHmut2
ΔIcrH
50
37
25
YopD
YopE-Bla
LcrV
B
Bacterial Supernatant
Bacterial Pellet
ΔyopEJK
ΔyopB
ΔyopEJK
IcrHmut1
IcrHmut2
ΔIcrH
50
37
YopB
YopE-Bla
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C
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YopD-Bla
F
% Cytotoxicity
****
UI
ATP
ΔyopB
ΔyopEJK
IcrHmut1
IcrHmut2
ΔIcrH
ΔyopEJK
LPS
G
IL-1β (ng/mL)
****
UI
UI
ATP
ΔyopB
ΔyopEJK
IcrHmut1
IcrHmut2
ΔIcrH
ΔyopEJK
LPS
IL-6 (ng/mL)
UI
UI
ATP
ΔyopB
ΔyopEJK
IcrHmut1
IcrHmut2
ΔIcrH
ΔyopEJK
LPS

Figure 3-5. Minimal translocon is insufficient for inflammasome activation by *Yersinia* T3SS A. TCA precipitated bacterial supernatants and bacteria pellet from inducing cultures were probed for YopD, YopE-β-lactamase, and LcrV. Δ*yopEJKBD* strain lacking YopE-Bla construct was used as a control. **B.** YopB and LcrV. **C**. HeLa cells were infected with lcrH wild-type, point mutant, or deletion strains, as indicated, or Δ*yopB* control strains expressing YopE-Bla. Δ*yopEJK* lacking the YopE-Bla plasmid was used as a negative control (ctrl). Translocation was determined as described in materials and methods. Graph is of a representative experiment from one of three independent experiments with 3 replicates per treatment per experiment. **D.** HeLa cells were infected with *lcrH* wild-type, point mutant, or deletion strains, as indicated, or Δ*yopB* control strains expressing YopM-Bla and YopD-Bla. Translocation was determined as described in Materials and Methods. Graph is a representative experiment from one of three independent experiments with 3 replicates per treatment per experiment. **E.** Percent cytotoxicity was determined by assaying LDH in supernatants following infection of BMDMs with indicated bacterial strains, as in C, or treatment with LPS or LPS+ATP. F. ELISA for IL-1β or IL-6 from LPS primed BMDMs infected with indicated bacterial mutant strains, Δ*yopB*, or Δ*yopEJK*, or treated with ATP or media control alone. Representative graph from one of three independent experiments with 3 replicates per treatment. * $p < 0.05$, **** $p < 0.0001$

Figure 3-6

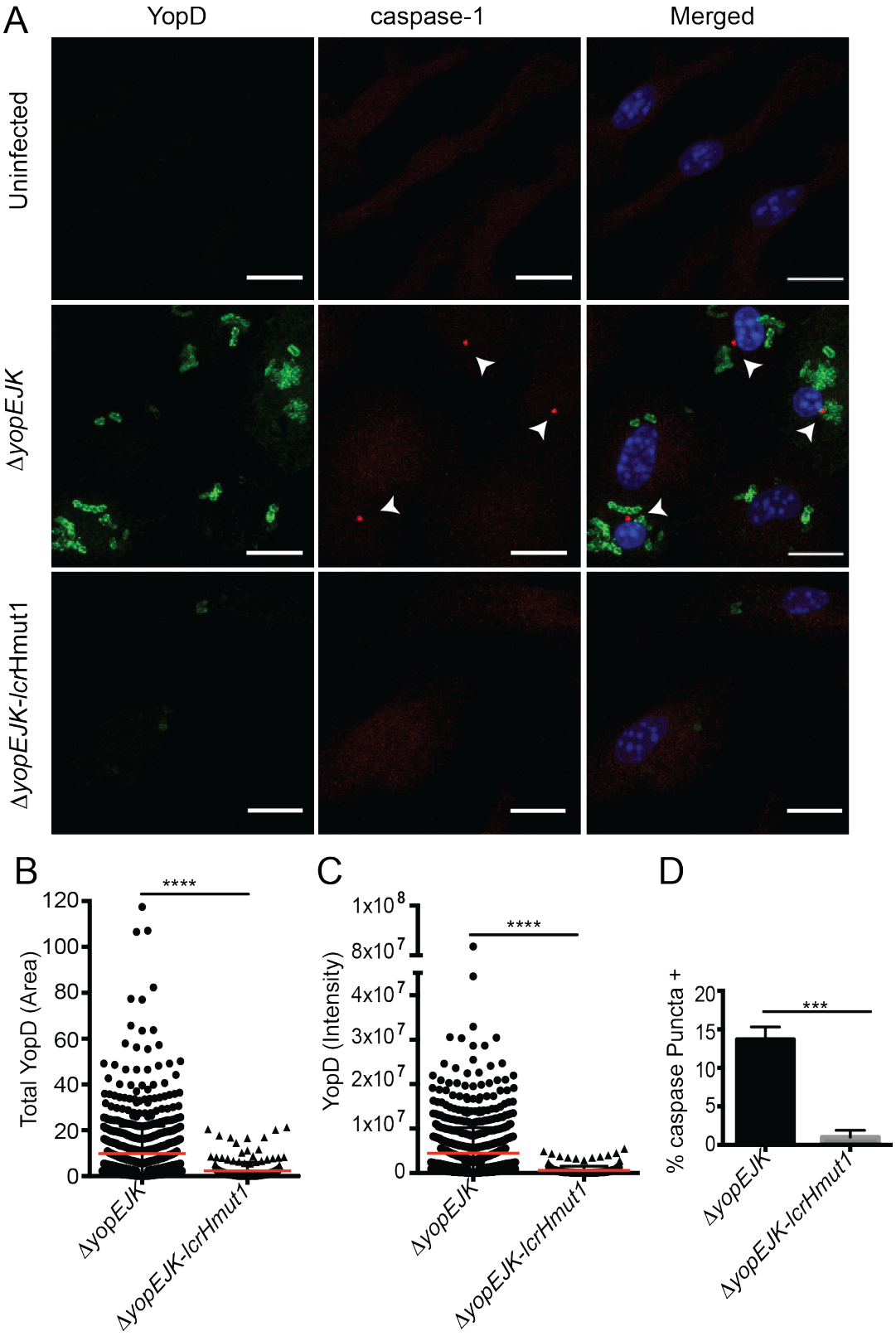

A
YopD
caspase-1
Merged
Uninfected
ΔyopEJK
ΔyopEJK-lcrHmut1
B
Total YopD (Area)
120
100
80
60
40
20
0
****
ΔyopEJK
ΔyopEJK-lcrHmut1
C
YopD (Intensity)
1x10 8
8x10 7
4x10 7
3x10 7
2x10 7
1x10 7
0
****
ΔyopEJK
ΔyopEJK-lcrHmut1
D
% caspase Puncta +
20
15
10
5
0
***
ΔyopEJK
ΔyopEJK-lcrHmut1

Figure 3-6. Hyper-translocation of pore complex proteins is necessary for inflammasome activation. **A.** Combined z-stacks of confocal microscopy images of YVAD-pretreated BMDMs that were left uninfected or infected with Δ*yopEJK* or Δ*yopEJ* for 2 hrs. Cells were stained for YopD (green), caspase-1 (red), and DNA (blue). White arrows denote active caspase-1 puncta. **B.** Total area of YopD staining (μm^2) in cells infected with Δ*yopEJK* (circles) or Δ*yopEJK-lcrHmut1* (triangles). **C.** Total intensity of YopD staining in cells infected with Δ*yopEJK* (circles) or Δ*yopEJK-lcrHmut1* (triangles). Each point represents a single cell. 1242 individual cells were analyzed. All data pooled from 3 independent experiments. Scale bars are 10 μm. **D.** Percent of cells with YopD staining that were positive for caspase-1 puncta. *** $p < 0.0005$, **** $p<0.0001$.

Table 3-1. *Yersinia* strains used in this study

Strain Name	Relevant Characteristics	Reference or Source
Y. pseudotuberculosis		
IP2666 (WT)	Wild-type 0:3 strain	Black and Bliska, 1997
ΔyopB	Full deletion of translocon protein YopB on IP2666 background	Palmer et al., 1998
ΔyopEJ	Full deletion of Yops E and J on IP2666 background	This work
ΔyopEJK	Full deletion of Yops E, J, and K on IP2666 background	This work
yopBΔyopEJKD	Point mutation causing a non-functional, truncated YopB on *ΔyopEJKD* background	This work
YopDΔ1	In frame deletion of amino acids 4-20 on the *ΔyopEJK* background	Olsson et al., 2004, This work
YopDΔ3	In frame deletion of amino acids 53-68 on the *ΔyopEJK* background	Olsson et al., 2004, This work
YopDΔ4	In frame deletion of amino acids 73-90 on the *ΔyopEJK* background	Olsson et al., 2004, This work
YopDΔ7	In frame deletion of amino acids 150-170 on the *ΔyopEJK* background	Olsson et al., 2004, This work

*lcrH*mut1	LcrH$^{Y40C, I101M, K102R}$ on *ΔyopEJK* background	Edqvist et al. 2007, This work
*lcrH*mut2	LcrH$^{Q46R, F72S, Q81R, H162R}$	Edqvist et al. 2007, This work
ΔlcrH	Deletion of *lcrH* on *ΔyopEJK* background	Edqvist et al. 2007, This work

CHAPTER 4

A role for guanylate binding proteins in activation of the inflammasome downstream of YopD hyperinjection

Abstract:

The T3SS plays essential roles in the virulence of many bacteria by allowing for the delivery of bacterial effectors into the host cell. These effectors manipulate host cell processes to allow for enhanced survival of the bacteria. However, NLRs, a class of internal immune sensors, recognize a variety of stimuli such as translocated bacterial products and disruption of internal membranes and induce the formation of a multiprotein complex called the inflammasome. Additionally, a separate caspase-11-dependent inflammasome can also recognize infection. We recently determined that hyper-translocation of the *Yersinia* T3SS translocon pore proteins YopD and YopB is required for inflammasome activation in response to infection by T3SS expressing *Yersinia* in the absence of YopK (Zwack et al., 2015). However, the mechanism(s) through which YopD and YopB activate the inflammasome are unclear. Intriguingly, *Yersinia* infection is recognized predominantly by the non-canonical inflammasome with some contribution by the canonical inflammasome. We used confocal microscopy to determine that YopD can associate with lysosomes. Translocated YopD and translocation-competent *Yersinia* recruited Gbp2; suggesting translocated YopD damages lysosomes. Although recruitment of Gbp2 does not correlate with inflammasome activation on its own, deletion of the five Gbps found on chromosome 3 resulted in a decrease in inflammasome activation in response to *Yersinia* of a similar extent as caspase-11 deletion. Our findings suggest that YopD interacts with Gbps to activate the inflammasome either directly or indirectly through the release of another bacterial product.

Introduction:

Innate immune recognition of pathogens plays a vital role in host defense. Surface and endosomal TLRs recognized conserved structures of pathogens. Signaling through TLRs leads to gene expression downstream of NFκB and MAPK. Intracellular receptors such as the NLRs recognize a combination of conserved virulence structures and virulence activities such as translocated flagellin and pore-formation. Activation of NLRs results in the formation of a multi-

protein complex called the inflammasome. Assembly of the inflammasome is necessary for the activation of caspase-1 in response to certain infections. Activation of caspase-1 leads to the processing of IL-1β and IL-18, the secretion of IL-1α, IL-1β, and IL-18, and cell death through cleavage of GSDMD (Shi et al., 2015).

In addition to the canonical inflammasome described above, there also exists a non-canonical inflammasome that causes GSDMD-mediated cell death and IL-1α secretion independently of caspase-1 (Fig. 1-2) (Kayagaki et al., 2015; Shi et al., 2015). The non-canonical inflammasome requires the activation of caspase-11. Caspase-11 then causes GSDMD cleavage and IL-1α secretion. Caspase-11 activation also leads to activation of the canonical NLRP3 inflammasome, which results in the processing and secretion of IL-1β and IL-18. In response to infection with gram-negative bacteria, Gbps have been demonstrated to play an important role in activating the caspase-11 inflammasome. Gbps have been described to function both at the lysosome as well as in the cytosol during non-canonical inflammasome activation by gram-negative bacteria and transfection of LPS (Meunier et al., 2014; Pilla et al., 2014).

Bacterial secretion systems play an essential role during infection for many gram-negative bacteria. However, certain types of these systems such as the type III and type IV secretion systems introduce bacterial products that can interfere with host processes directly into the cytosol of cells. It has previously been demonstrated that the T3SS of *Yersinia* can be recognized by the inflammasome (Bergsbaken and Cookson, 2007; Brodsky et al., 2010). The human pathogenic *Yersiniae*, *Yersinia pestis* (*Yp*), *Yersinia pseudotuberculosis* (*Yptb*), and *Yersinia enterocolitica* (*Ye*), all encode what is considered an archetypal T3SS as well as associated effectors. The T3SS and its effectors are essential for *Yersinia* infection. In the absence of all known effectors, or minimally both the effectors YopJ and YopK, the T3SS of *Yersinia* activates

the inflammasome. We recently demonstrated that translocation of the *Yersinia* T3SS translocon pore proteins YopD and YopB is required for this inflammasome activation downstream of *Yersinia pseudotuberculosis* infection in the absence of the T3SS effectors YopK and YopJ (Zwack et al, 2015). However, the mechanism(s) through which translocation of YopD and YopB activate the inflammasome are unclear. In this study we attempt to define a mechanism for inflammasome recognition of type III secretion system translocon proteins. As initial studies on activation of the inflammasome by the T3SS used *Casp1Casp11* $^{-/-}$ bone marrow, we first considered the possibility that translocation of YopD and YopB were activating the non-canonical inflammasome. Here we demonstrate that the non-canonical inflammasome is the main but not sole inflammasome contributing to T3SS recognition. We also hypothesize that translocation of YopD leads to the insertion of YopD into internal membranes as YopD contains a transmembrane domain. Interestingly, we observed that YopD can accumulate at lysosomes. As Gbps are recruited to lysosomes that contain pathogens and have been described to function both at and downstream of lysosomes containing gram-negative bacteria to activate the inflammasome (Meunier et al., 2014; Pilla et al., 2014), we wanted to test whether Gbps would recognize YopD at lysosomes or the consequences of YopD colocalizing with lysosomes to activate the inflammasomes. We determined that Gbps play a role in the caspase-11 inflammasome activation downstream of YopD translocation. Based on our data, we propose a model for inflammasome activation during *Yersinia* infection where translocated YopD and YopB associate with lysosomes and release both host and bacterial products from the lysosomes. The Gbps act downstream of the YopD/B induced initial damage to the lysosomes to activate the non-canonical inflammasome.

Results:

Yersinia T3SS predominantly activates the caspase-11 non-canonical inflammasome

Different stimuli activate the canonical and non-canonical NLRP3 inflammasome. Certain mechanisms can potentially be used in both pathways such as disruption of the lysosome while other mechanisms such as potassium efflux result only in activation of the canonical inflammasome activation pathway (Kayagaki et al., 2011; Meunier et al. 2014). To determine whether infection with *Yersinia* expressing a T3SS triggered non-canonical or canonical inflammasome activation, we infected BMDMs from B6, *Casp1*$^{-/-}$, *Casp11*$^{-/-}$, and *Casp1Casp11*$^{-/-}$ mice. As expected, treatment with LPS + ATP, a canonical NLRP3 inflammasome activator, caused robust death and IL-1β and IL-1α secretion in B6 and *Casp11*$^{-/-}$ BMDMs but not *Casp1*$^{-/-}$ or *Casp1Casp11*$^{-/-}$ BMDMs. BMDMs infected with Δ*yopB Yersinia,* which do not express a functional T3SS, did not die or secrete inflammasome dependent cytokines (Fig. 4-1 A-C). Interestingly, in comparison to B6 BMDMs, infection with Δ*yopEJK Yersinia*, which express a functional T3SS but are missing a subset of secreted effector proteins, caused a drastic decrease in cell death in *Casp11*$^{-/-}$ as well as *Casp1Casp11*$^{-/-}$. A smaller but statistically significant decrease in death was observed in the *Casp1*$^{-/-}$ (Fig. 4-1A). As IL-1β processing and secretion require caspase-1 in both the canonical and non-canonical inflammasomes it was unsurprising to see complete abrogation of secretion in the *Casp1*$^{-/-}$ and *Casp1Casp11*$^{-/-}$ in response to Δ*yopEJK Yersinia* infections. While IL-1β secretion was drastically reduced in *Casp11*$^{-/-}$ BMDMs during infection, a substantial amount of IL-1β was still secreted (Fig. 4-1B). In contrast, *Casp1*$^{-/-}$ did not have a significant effect on IL-1α secretion during infection by Δ*yopEJK* (Fig. 4-1C). These data suggest that the non-canonical inflammasome plays the predominant role in recognition of T3SS expressing *Yersinia* but is not solely responsible for recognition of *Yersinia*.

YopD can associate with the late endosome/lysosome marker LAMP1

We previously determined that translocation of the pore proteins YopD and YopB induces inflammasome activation in response to T3SS expressing *Yersinia* (Zwack et al., 2015). Confocal microscopy of Δ*yopEJK* infected BMDMs showed that YopD forms larger structures in the cell (Zwack et al., 2015). As YopD is membrane active and damage to lysosomes has been demonstrated to be important for both canonical and non-canonical inflammasome activation (Meunier et al., 2014; Hornung et al., 2008), we examined whether YopD colocalized with lysosomes. We performed confocal microscopy on BMDMs infected with mCherry expressing *Yersinia* to determine the extent of translocated YopD colocalization with LAMP1 expressing lysosomes (Fig. 4-2A). YopD did colocalize with lysosomes in both Δ*yopEJK* and Δ*yopEJ* infection as determined by the volume of translocated YopD that intersected with LAMP1 staining (Fig. 4-2B). Although the volume of translocated YopD colocalized with lysosomes was not significantly different between Δ*yopEJK* and Δ*yopEJ* infection (Fig. 4-2B), it is possible that the amount of YopD contained in that volume may differ. Interestingly, in both Δ*yopEJK* and Δ*yopEJ* infection, the percent of translocated YopD that colocalizes with lysosomes in a cell predominantly falls in two jackpot populations (100% colocalized and 0% colocalized) with a spread of cells in between the two main populations (Fig. 4-2C). Potentially, these cells with a higher percent of YopD colocalization may be responsible for inflammasome activation.

Gbps contribute to inflammasome activation by *Yersinia's* T3SS

As YopD can colocalize with lysosomes and Gbps have been demonstrated to help lyse lysosomes and recognize cytosolic LPS that would be released from lysosomes during non-canonical inflammasome activation (Meunier et al., 2014, Pilla et al., 2014, Finethy et al., 2015), we tested whether the Gbps found on chromosome 3 contributed to inflammasome activation in response to T3SS expressing *Yersinia* by infecting B6 and $Gbp^{chr3-/-}$ BMDMs. Δ*yopEJK* infection had significantly decreased cytotoxicity and IL-1β and IL-1α secretion in $Gbp^{chr3-/-}$ BMDMs (Fig. 4-

3 A-C). Importantly, the inflammasome-independent cytokine IL-6 was not affected in *Gbp*$^{chr3-/-}$ BMDMs (Fig. 4-3D). Caspase-1 processing was undetectable in IFNγ primed cell lysates (Fig. 4-3E).

As cell lysates from Δ*yopEJK i*nfected *Gbp*$^{chr3-/-}$ BMDMs had no detectable caspase-1 processing and Gbps have been found to contribute to the canonical inflammasome (Shenoy et al., 2012), we wanted to see if Gbps were acting in both the canonical and non-canonical inflammasome pathways. If Gbps were working in both pathways, formation of caspase-1 puncta should be significantly lower in Δ*yopEJK* infected *Gbp*$^{chr3-/-}$ BMDMs in comparison to *Casp11*$^{-/-}$ BMDMs. We used confocal microscopy to look for caspase-1 activation as signified by caspase-1 puncta in *Casp11*$^{-/-}$, *Gbp*$^{chr3-/-}$, and B6 BMDMs (Fig. 4-4A). The percent of cells with active caspase-1 was significantly decreased in both *Casp11*$^{-/-}$ and *Gbp*$^{chr3-/-}$ BMDMs in comparison to B6 BMDMs after Δ*yopEJK* infection (Fig. 4-3B). There was not a significant difference in the percent cells with active caspase-1 between *Casp11*$^{-/-}$ and *Gbp*$^{chr3-/-}$ BMDMs. In conjunction with caspase-11 being the predominant inflammasome activated by hyper-translocation of YopD/B, these data suggest that the chromosome 3 Gbps play a role in non-canonical inflammasome activation in response to hyper-translocation.

Gbp2 colocalizes with both YopD and *Yersinia*

Chromosome 3 encodes 5 different Gbps: Gbps 1, 2, 3, 5, and 7. Gbp2 was found to be involved in non-canonical inflammasome activation for both the destruction of lysosomes and the recognition of LPS in the cytosol (Meunier et al., 2014; Pilla et al., 2014). Additionally, Gbp2 was found to colocalize with Galectin-8, which is recruited to lysed lysosomes (Meunier et al., 2014). Based on this data, we hypothesized that Gbp2 could colocalize with YopD containing lysosomes

and assist in inflammasome activation by potentially increasing lysosomal damage. Using confocal microscopy, we were able to look for recruitment of Gbp2 with translocated YopD as well as *Yersinia* (Fig. 4-5A). A percentage of the translocated YopD in the cell colocalizes with Gbp2. This percentage is significantly higher in Δ*yopEJK* infected BMDMs in comparison to Δ*yopEJ* infected BMDMs (Fig. 4-5B). Interestingly, Gbp2 colocalizes highly to Δ*yopEJK Yersinia* but not Δ*yopEJ Yersinia* (Fig. 4-5C). In data not shown, Gbp2 only colocalized to *Yersinia* that expressed T3SSs that were capable of translocation. This suggests that *Yersinia* can translocate Yops from inside the lysosome. Due to the limits of the microscope's resolution, we could potentially miss translocated YopD that inserts back into the *Yersinia* containing lysosome. Additionally, Gbp2 recruitment is dependent on Galectin-3, which is a marker of lysosomal membrane damage (Feeley et al., in submission). This observation suggests that Gbp2 is recruited after damage occurs to the lysosome. It is possible that Gbp2 recruitment leads to exacerbation of this damage. It is also possible that in this system, Gbp2 is not playing the main role in Gbp-dependent non-canonical inflammasome activation.

Gbp2 colocalization with YopD is insufficient for inflammasome activation

To determine the contribution of Gbp2 colocalization with YopD to inflammasome activation, we examined whether colocalization of Gbp2 correlated with caspase-1 activation in Δ*yopEJK* infected BMDMs using confocal microscopy (Fig. 4-6A). As Gbps have been suggested to act as platforms for inflammasome assembly in both the non-canonical and canonical inflammasomes (Shenoy et al., 2012; Pilla et al., 2014), we checked to see if Gbp2 colocalized with caspase-1 puncta. No Gbp2 staining colocalized with active caspase-1 puncta (Fig. 4-6A). Additionally, there was no difference in colocalization of Gbp2 to YopD in cells that contained active caspase-1 in comparison to cells that did not contain active caspase-1 (Fig. 4-6B). These data suggest that Gbp2 is not the main Gbp responsible for inflammasome activation in response to *Yersinia*

infection. While Meunier and colleagues (2014) observed Gbp2 functioning as the main player in non-canonical inflammasome activation in response to vacuolar gram-negative bacterial infection, other Gbps also contributed to the observed non-canonical inflammasome activation. Additionally, individual shRNA knockdown of Gbp-1, Gbp2, Gbp-3, and Gbp-5 all significantly decreased non-canonical inflammasome activation in response to cytosolic LPS (Pilla et al., 2014). As $Gbp^{chr3-/-}$ has such a large effect on inflammasome activation in response to *Yersinia*, we hypothesize that multiple Gbps each contribute partially to inflammasome activation. Alternatively, it is possible that a single Gbp on chromosome 3 is responsible for most of the non-canonical inflammasome activation.

Discussion:

Common bacterial virulence factors like specialized secretion systems and pore-forming toxins play important roles in establishing infection. Many bacteria employ various strategies to hide these key virulence factors from host immune detection. In the absence of the evasion strategies of the bacteria, the immune pathways activated through recognition of the virulence factor leads to clearance or decreased severity of the infection. By understanding which pathway is activated, to what extent the pathway is activated, and the precise mechanism used to activate the pathway, therapeutic agents can be developed that will allow for precise activation of that pathway even when the bacteria employ evasion strategies. *Yersinia*, which expresses a T3SS, inhibits inflammasome activation through translocation of YopK, YopM, and YopJ (Brodsky et al. 2010; Chung et al., 2014; LaRock and Cookson, 2012; Schoberle et al., 2016). It has been demonstrated that in the absence of YopK *in vivo Yersinia* infection is highly attenuated (Brodsky et al., 2010; Holmstrom et al., 1995; Najdenski et al., 2003; Straley and Cibull, 1989). This attenuation depends on caspase-1/11 activity (Brodsky et al., 2010). We have recently demonstrated that hyper-translocation of YopD and YopB was required for inflammasome activation in response to *Yersinia* infection (Zwack et al., 2015). However, the downstream

mechanism(s) remained unclear. Here we demonstrate that *Yersinia* infection activates predominantly the non-canonical inflammasome but also activates the canonical inflammasome (Figure 4-1; Casson et al., 2013). Currently only one known stimuli, intracellular LPS, has been demonstrated to activate the non-canonical inflammasome (Hagar et al. 2013; Kayagaki et al., 2013; Shi et al. 2014). By determining how *Yersinia* hyper-translocation of YopD and YopB result in non-canonical inflammasome activation, we can reveal basic biology of the non-canonical inflammasome. Additionally, translocation of YopD and YopB represents a class of virulence activities in which membrane active proteins of pathogens are delivered into the host cytosol. The mechanism by which translocated YopD and YopB interact with and are recognized by the host may elucidate broadly applicable features and rules about this class of virulence factors.

Lysosomal damage has been shown to play a role in both the canonical and non-canonical inflammasome (Hornung et al., 2008; Meunier et al., 2014). Here we demonstrate that translocated YopD can associate with Lamp1 containing lysosomes (Fig. 4-2) and that Gbp2 is recruited to both translocated YopD and *Yersinia* (Fig. 4-5). This suggests that YopD is causing damage to these lysosomes because recruitment of Gbp2 is dependent on the presence of Galectin-3, a known marker of lysosomal damage (Feeley et al., in submission; Paz et al., 2010). While Gbp2 recruitment does not correlate with caspase-1 activation during *Yersinia* infection (Fig. 4-6), knocking out all five of the Gbps on chromosome 3 significantly reduces inflammasome activation in response to *Yersinia* infection (Fig. 4-3). Interestingly, the degree of reduction in inflammasome activation in response to *Yersinia* is similar to caspase-11 deficiency (Fig. 4-4). Combined these data suggest that at least one Gbp found on chromosome three is important for non-canonical inflammasome activation during *Yersinia* infection. Our data suggest that Gbp2 is not the major Gbp involved in non-canonical inflammasome activation in response to hyper-translocation of YopD and YopB. However, we cannot discount that the Gbps are working in

combination and that Gbp2 could play a role in conjunction with one or more of the other Gbps found on chromosome 3.

As these data suggest that translocated YopD causes lysosomal damage to both *Yersinia* containing and *Yersinia* free lysosomes (Fig. 4-5), it is possible that release of a bacterial product from a *Yersinia* containing lysosome is responsible for activating the non-canonical inflammasome. While LPS from *Yersinia pestis* grown at 37 °C does not activate caspase-11, it is unknown whether LPS from *Yersinia pseudotuberculosis* grown at 37 °C can activate the non-canonical inflammasome. It was previously demonstrated that LPS from 37 °C grown *Yersinia pseudotuberculosis* can stimulate TLR4 but the stimulation is drastically weaker than LPS from 21 °C grown *Yersinia pseudotuberculosis* (Rebeil et al., 2004). To date, the non-canonical inflammasome seems to be stimulated by various LPS species similarly to how TLR4 is. Further experiments are needed to determine whether it is release of LPS or a different bacterial product that is important for non-canonical inflammasome activation by *Yersinia*.

Although YopD can associate with the lysosome, it is possible that activation of the inflammasome is separate from this association. As work by the Coers lab has demonstrated that Gbps can act in the cytosol to aid in activation of the non-canonical inflammasome (Finethy et al., 2015; Pilla et al., 2014), it is possible that the translocated YopD and YopB superstructures, which we previously reported (Zwack et al., 2015), may be separate from membranes and interact with Gbps in the cytosol. In the case of hexa-acylated LPS, activation of the non-canonical inflammasome occurs through direct binding of LPS to caspase-11. Interestingly, SipB, a translocon protein of the *Salmonella* SPI-1 T3SS, was initially found to bind to caspase-1. Because caspase-11 is closely related to caspase-1 and YopB is similar to SipB, these YopB and YopD superstructures could potentially interact directly with caspase-11. Further experiments are required to determine these interactions. The work described in this chapter provides a strong foundation for dissecting the complex mechanism by which *Yersinia* activates the non-canonical

inflammasome. The structure of T3SSs is highly conserved. Here we have identified players involved in the recognition of T3SS expressing *Yersinia*. These components possibly represent both a broader mechanism for recognizing T3SS expressing bacteria and points in the pathway that the bacteria potentially target to prevent recognition.

Figure 4-1

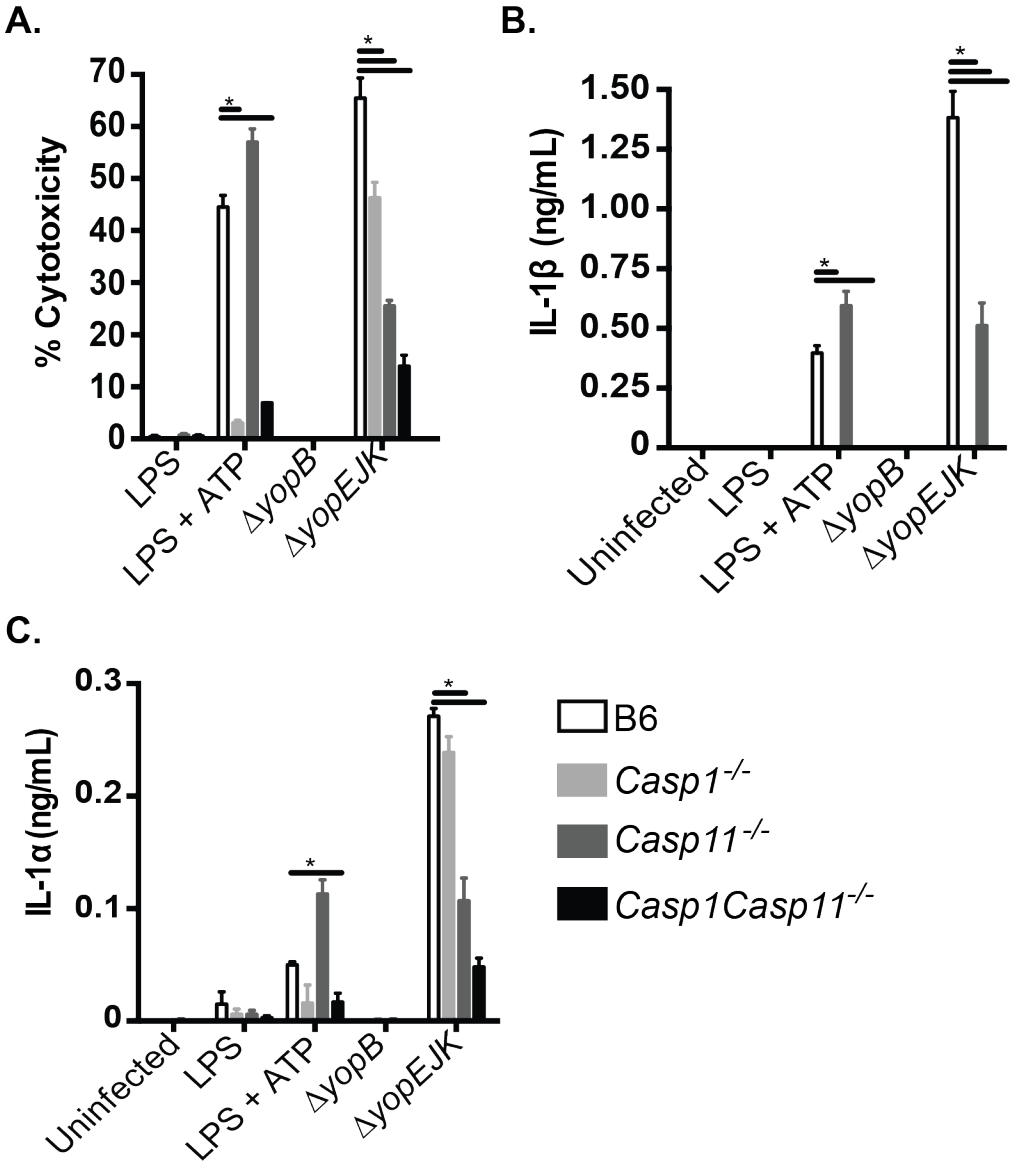


Figure 4-1: *Yersinia* infection primarily activates the non-canonical inflammasome.

A. B6, *Casp1*$^{-/-}$, *Casp11*$^{-/-}$, and *Casp1Casp11*$^{-/-}$ BMDMs were infected with indicated *Yersinia* strains or treated with LPS or LPS + ATP. Cytotoxicity was determined by LDH release. Graph is representative of three independent experiments. *$p < 0.05$.

B-C. Supernatants from B6, *Casp1*$^{-/-}$, *Casp11*$^{-/-}$, and *Casp-1Casp11*$^{-/-}$ BMDMs infected with indicated bacterial strains or treated with LPS or LPS + ATP were assayed for levels of secreted IL-1β and IL-1α by ELISA as described in materials and methods. Graph is representative of three independent experiments.*$p < 0.05$.

Figure 4-2

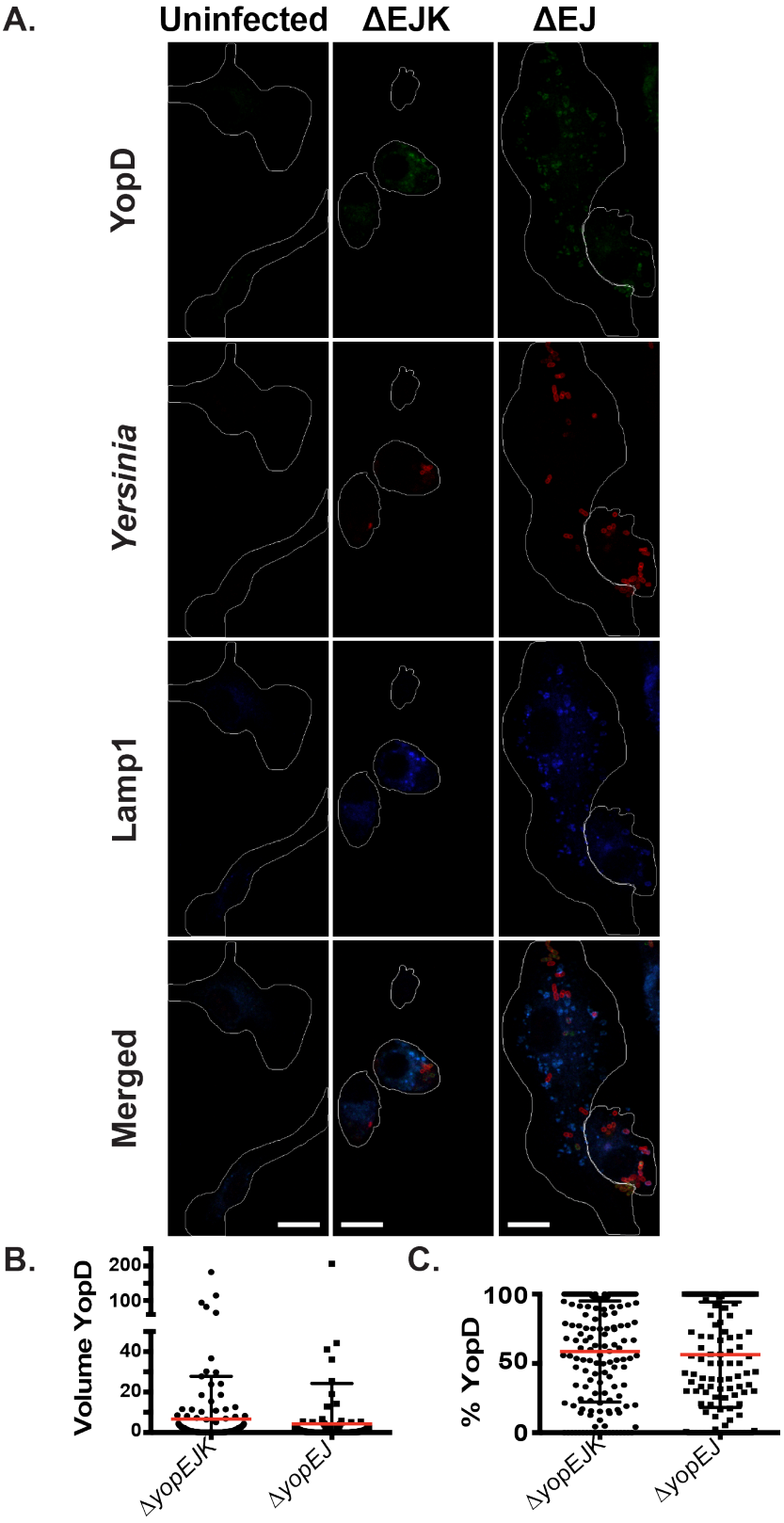

A.
Uninfected
ΔEJK
ΔEJ
YopD
Yersinia
Lamp1
Merged
B.
Volume YopD
200
100
40
20
0
ΔyopEJK
ΔyopEJ
C.
% YopD
100
50
0
ΔyopEJK
ΔyopEJ

Figure 4-2: Translocated YopD colocalizes with Lamp1 containing lysosomes. A. Single z-stacks of confocal microscopy images of BMDMs that were pretreated with YVAD and left uninfected or infected with Δ*yopEJK* or Δ*yopEJ* for 2 hrs. Cells were stained for YopD (green), *Yersinia* (red), and Lamp1 (blue). White outlines represent cells as determined by CellMask blue staining. White scale bar represents 10 μm. **B.** Volume of YopD colocalized with Lamp1 (μm^3) in Δ*yopEJK* and Δ*yopEJ.* Each dot represents a single cell. **C.** Percent of total translocated YopD colocalized with Lamp1 in Δ*yopEJK* and Δ*yopEJ.* Each dot represents a single cell. Graphs representative of 2 independent experiments.

Figure 4-3

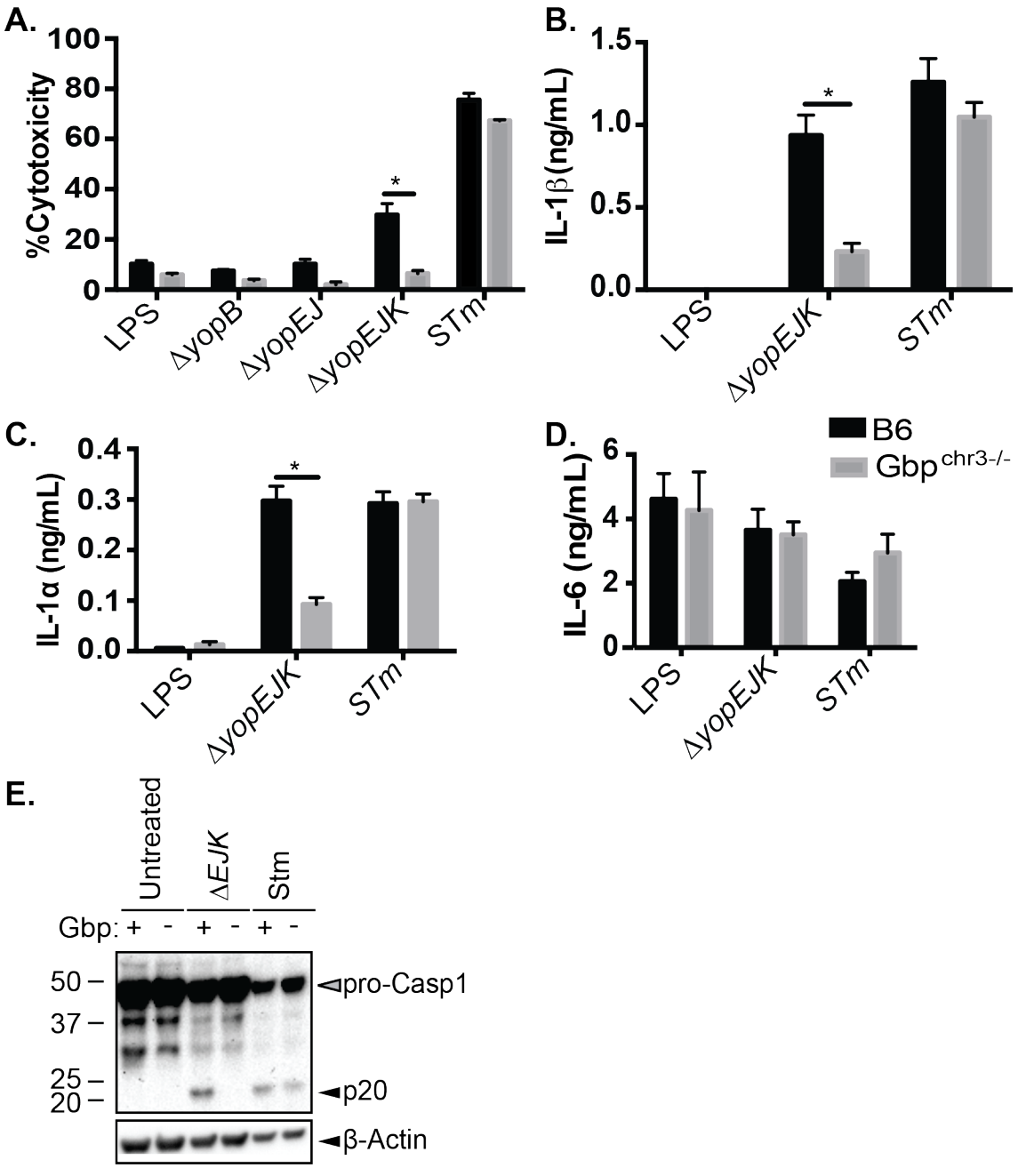


Figure 4-3: Guanylate Binding Proteins are important for *Yersinia* activation of the inflammasome. A. B6 and *Gbp*$^{chr3-/-}$ BMDMs were infected with indicated *Yersinia* strains or *Salmonella* or treated with LPS. Cytotoxicity was determined by LDH release. Graph is

representative of three independent experiments. *p < 0.05. **B-D.** Supernatants from B6 and *Gbp*$^{chr3-/-}$ BMDMs infected with indicated bacterial strains or treated with LPS were assayed for levels of secreted IL-1β, IL-1α, and IL-6 by ELISA as described in materials and methods. Graph is representative of three independent experiments.*p < 0.05. **E.** B6 and *Gbp*$^{chr3-/-}$ BMDMs infected with indicated bacterial strains. Cell lysates were assayed for pro- and processed caspase-1 and actin as indicated.

Figure 4-4

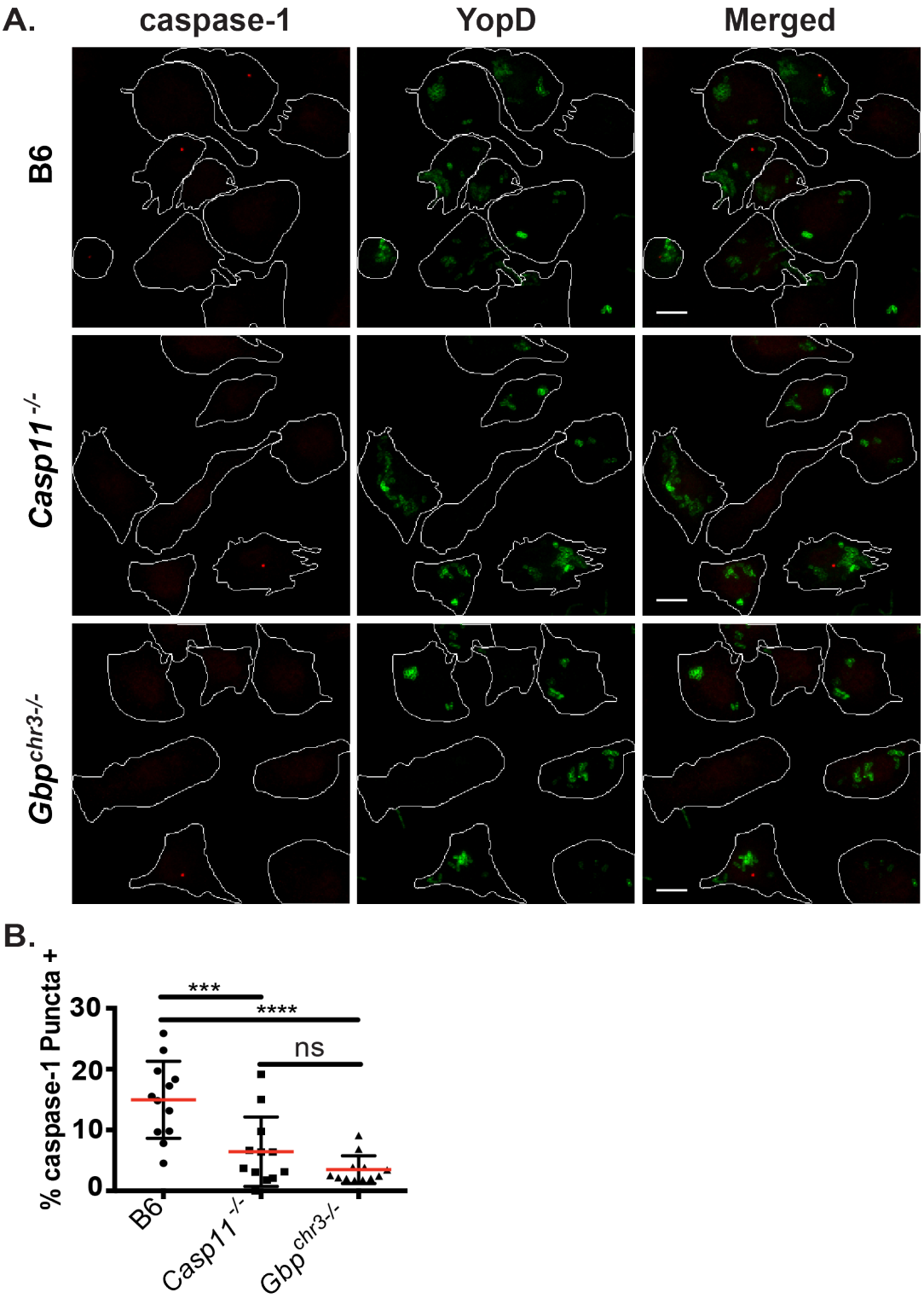
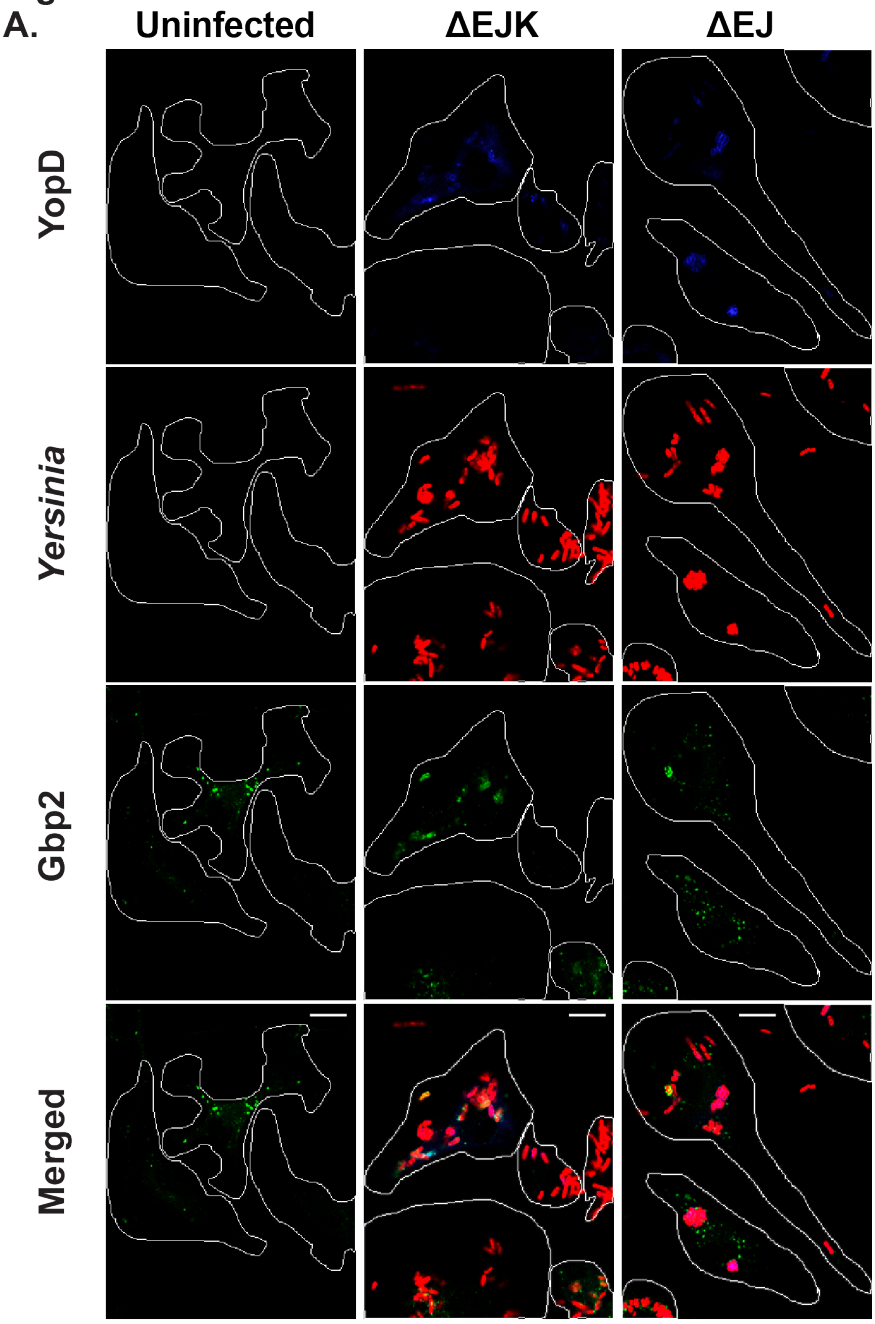
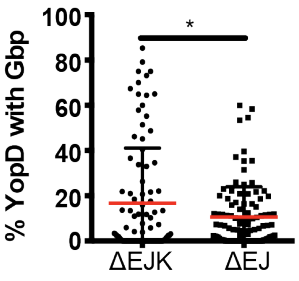

A.
caspase-1
YopD
Merged
B6
Casp11 -/-
Gbpchr3-/-
B.
% caspase-1 Puncta +
30
20
10
0
***
****
ns
B6
Casp11 -/-
Gbpchr3-/-

Figure 4-4: Guanylate binding proteins are involved in *Yersinia* activation of the non-canonical inflammasome. A. Collapsed z-stacks of confocal microscopy images of B6, *Casp11*$^{-/-}$, and *Gbp*$^{chr3-/-}$ BMDMs that were pretreated with YVAD and infected with Δ*yopEJK* for 2 hrs. Cells were stained for YopD (green) and caspase-1 p10 (red). White outlines represent cells as determined by CellMask blue staining. White scale bar represents 10 μm. **B.** Percent of total cells that are caspase-1 puncta positive. Each dot is representative of a single field. Data are pooled from 3 independent experiments. *** $p < 0.0005$. **** $p<0.0001$.

Figure 4-5


A.
Uninfected
ΔEJK
ΔEJ
YopD
Yersinia
Gbp2
Merged


B.
% YopD with Gbp
100
80
60
40
20
0
*
ΔEJK
ΔEJ

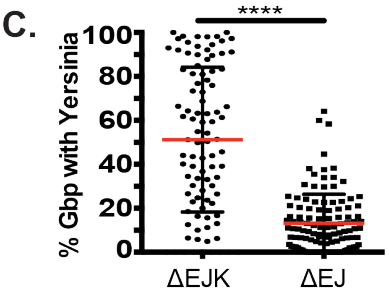

C.
% Gbp with Yersinia
100
80
60
40
20
0
****
ΔEJK
ΔEJ

Figure 4-5: Translocated YopD colocalizes with Gbp2. A. Single z-stacks of confocal microscopy images of BMDMs that were pretreated with YVAD and left uninfected or infected with Δ*yopEJK* or Δ*yopEJ* for 2 hrs. Cells were stained for YopD (blue), *Yersinia* (red), and Gbp2 (green). White outlines represent cells as determined by CellMask blue staining. White scale bar represents 10 μm. **B.** % of total translocated YopD that colocalizes with Gbp2 in Δ*yopEJK* and Δ*yopEJ.* Each dot represents a single cell. **C.** Percent of total Gbp2 that colocalizes with *Yersinia* in Δ*yopEJK* and Δ*yopEJ.* Each dot represents a single cell.

Figure 4-6

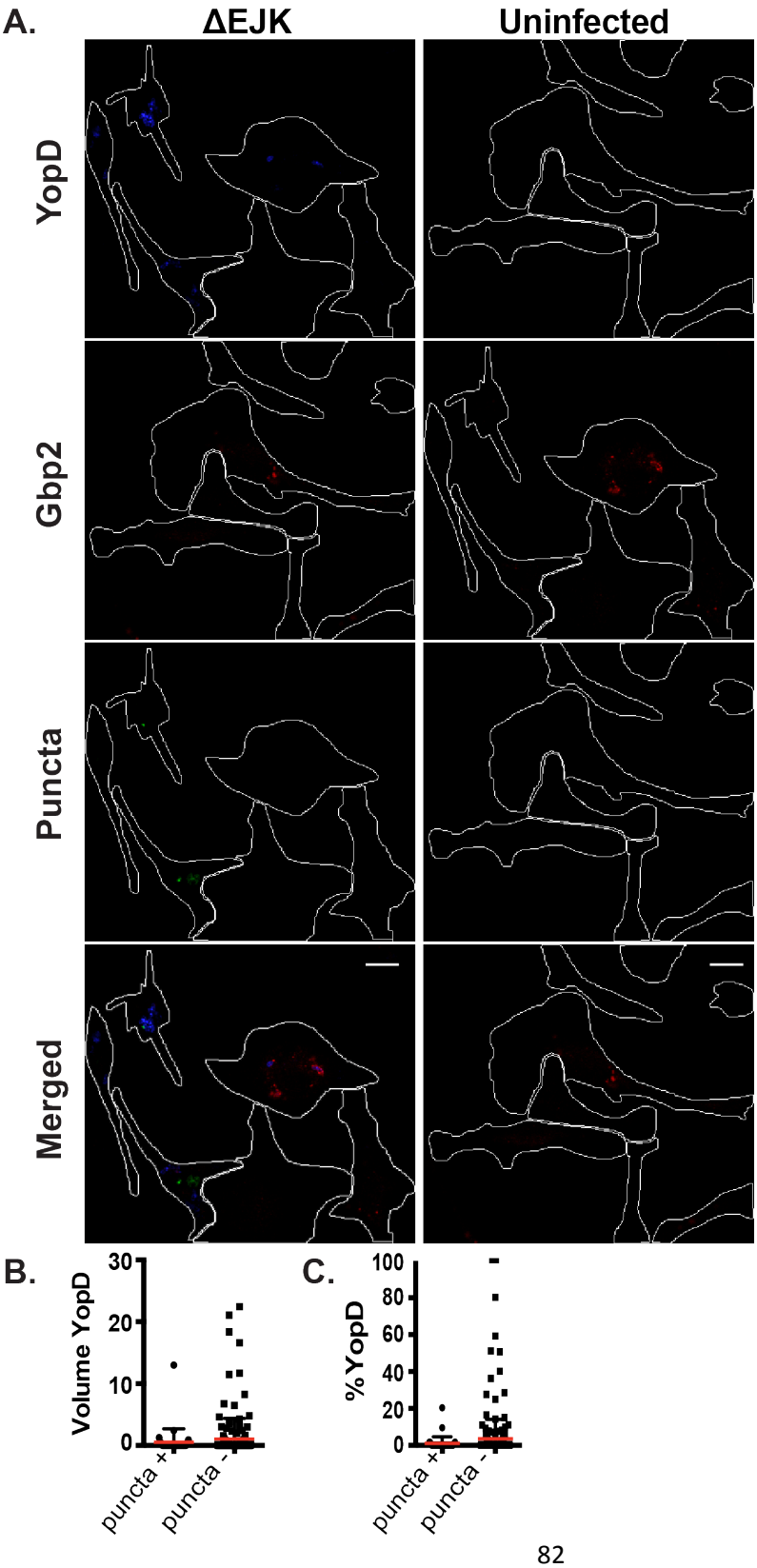

A.
ΔEJK
Uninfected
YopD
Gbp2
Puncta
Merged
B.
Volume YopD
30
20
10
0
puncta +
puncta -
C.
% YopD
100
80
60
40
20
0
puncta +
puncta -

Figure 4-6: Caspase-1 activation does not correlate with colocalization of Gbp2 with YopD. **A.** Single z-stacks of confocal microscopy images of BMDMs were left uninfected or infected with Δ*yopEJK* for 2 hrs. One hour post-infection FAM-FLICA (green) was added to detect active caspase-1. Cells were stained for YopD (blue), Gbp2 (red). White outlines represent cells as determined by CellMask blue staining. White scale bar represents 10 μm. **B.** Volume of YopD colocalized with Gbp2 (μm^3) in caspase-1 puncta positive and puncta negative cells. Each dot represents a single cell. **C.** Percent of total YopD colocalized with Gbp2 in caspase-1 puncta positive and puncta negative cells. Each dot represents a single cell. Graphs representative of 2 independent experiments.

CHAPTER 5

A role for YopK and balance of translocation in evading innate immune responses

Abstract:

Inflammasome mediated immune responses play a vital role in clearance of *Yersinia*. Recognition of *Yersinia* by the inflammasome requires expression of the T3SS. Because *Yersinia* requires translocation of effectors by the T3SS to establish infection in mammals, *Yersinia* acquired multiple mechanisms to avoid activation of the inflammasome. YopK was the first effector identified to prevent inflammasome activation during *Yersinia* infection, but how YopK prevents inflammasome activation remains unclear. As hyper-translocation of YopD and YopB are required for inflammasome activation, we tested whether mutations in YopK that allowed for increased translocation fail to prevent inflammasome activation. We determined that these mutants both fail to prevent inflammasome activation and are attenuated *in vivo*. To determine if YopK functions by preventing translocation of YopD/B, we expressed LcrHmut1, a mutated chaperone protein for YopB/D, in both WT and YopK-deficient *Yersinia*. Intriguingly, instead of having restored virulence, infections with these strains were fully attenuated. We determined that the presence of all the Yops combines with LcrHmut1 to generate a larger effect on translocation. Interestingly, deletion of YopK restored the level of translocation of both YopJ and YopM but not YopE. These data suggest that different effectors have more stringent requirements for proper translocation. Understanding the fundamental nature of the T3SS and the mechanisms which prevent immune recognition of the T3SS provide a strong foundation for finding therapeutics that help the immune system combat T3SS expressing bacteria.

Introduction:

Innate immune responses play critical roles in pathogen clearance. Combinations of surface and endosomal receptors like the TLRs and intracellular receptors such as the NLRs recognize conserved virulence structures and activities to generate an effective response to the pathogen (Janeway and Medzhitov, 2002; Vance et al., 2009). These responses include recruitment of neutrophils, activation of macrophages and dendritic cells, T cell activation, and many others (Chow et al., 2016; Lee et al., 2015; He et al., 2008; Lukens et al., 2012; Kupz et al., 2012). As

the structures and activities recognized by the immune system are often essential to the ability of the pathogen to survive in its host, pathogens have adapted ways to interfere with both the initial recognition and the downstream responses.

Yersinia requires expression of its T3SS and effectors in order to infect mammalian hosts. However, expression of the T3SS leads to activation of the inflammasome in part because of hyper-translocation of the pore proteins YopD and YopB (Zwack et al., 2015). *Yersinia* employs multiple mechanisms to prevent inflammasome activation. The effector YopM sequesters caspase-1 thus preventing its recruitment to the inflammasome (Chung et al., 2014; LaRock and Cookson, 2012). It has recently been found that YopJ also contributes to the inhibition of the inflammasome in primed macrophages although the mechanism remains unknown (Schoberle et al., 2016). YopK was the first effector identified to inhibit inflammasome activation but the mechanism remains unknown (Brodsky et al., 2010). Prior to determining that YopK inhibits the inflammasome, YopK was described to both decrease the size of the translocon pore and negatively regulate the translocation of other effectors into the cell (Holmstrom et al., 1997). Based on the previous observation that inflammasome activation in response to *Yersinia* requires hyper-translocation of YopD and YopB (Zwack et al., 2015), we hypothesize that YopK inhibits inflammasome activation by negatively regulating translocation of YopD and YopB. We further considered that preventing YopD and YopB translocation by alternative methods could complement YopK deletion. Here we define mutations in YopK that fail to inhibit inflammasome activation. Our data further demonstrates that *Yersinia*'s T3SS and effectors make a balanced system. Small changes to optimize one portion of the system can cause clearance of the bacteria by altering regulation of another effector.

Results:

YopK mutations that allow for increased translocation fail to prevent inflammasome activation

Thorslund and colleagues (2011) performed an alanine substitution screen spanning the full length of YopK. A query of these mutations by yeast two-hybrid indicated that RACK1 interacted with all YopK mutations except when the threonine and aspartic acid were substituted with alanines at position 45 and 46, respectively (Thorslund et al., 2011). Additionally they determined that this mutation conferred increased translocation in comparison to wild-type YopK. We hypothesized that interaction between YopK and RACK1 mediated translocation and that the regulation of translocation prevented inflammasome activation. In order to test whether interaction with RACK1 and the negative regulation of translocation was necessary for YopK inhibition of the inflammasome, we generated control mutations that interacted with RACK1 by yeast two-hybrid that were distal (SK28-29AA) or adjacent (LR43-44AA) to the mutation identified by Thorslund as well as the identified mutation (TD45-46AA)(Fig. 5-1A). As described previously, $YopK_{TD45-46AA}$ had increased translocation in comparison to wild-type YopK (Fig. 5-1B). Interestingly, while the control mutation $YopK_{SK28-29AA}$ allowed for translocation of YopE similarly to WT YopK, $YopK_{LR43-44AA}$ conferred significantly higher translocation in comparison to WT YopK (Fig. 5-1B). This data initially suggested that RACK1 was not required for regulation of translocation by YopK. Work by Dewoody and colleagues (2013) showed that $YopK_{TD45-46AA}$ was able to interact with RACK1 by using immunoprecipitation, thus further supporting that YopK mediates translocation independently of RACK1.

Although RACK1 does not seem necessary for YopK regulation of translocation, we still hypothesize that YopK regulation of translocation is required to prevent inflammasome activation. Using our YopK mutants that allow for increased translocation as well as our mutant that confers

normal regulation of translocation, we infected B6 BMDMs to determine if mutants that fail to regulate translocation allow for inflammasome activation. *Yersinia* expressing either $YopK_{LR43\text{-}44AA}$ or $YopK_{TD45\text{-}46AA}$ failed to prevent cell death, and IL-1β and IL-1α secretion (Fig. 5-1 C-E). Both mutations functioned like the full deletion. Conversely, *Yersinia* expressing either WT YopK or $YopK_{SK28\text{-}29AA}$ drastically decreased both cell death, and IL-1β and IL-1α secretion in comparison to full YopK deletion. As mutants that allowed for increased translocation activated the inflammasome and it has been demonstrated that inflammasome activation plays a key role in clearance of *Yersinia* that lack YopK (Fig. 5-1B; Brodsky et al., 2010), we next examined whether *Yersinia* expressing $YopK_{LR43\text{-}44AA}$ would be attenuated *in vivo*. B6 mice infected by intraperitoneal (i.p.) injection with $YopK_{LR43\text{-}44AA}$ had a lower bacterial burden in the spleen in comparison to WT and $YopK_{SK28\text{-}29AA}$ (Fig. 5-1F). Our data on these YopK mutations in combination with our previous observation that hyper-translocation of YopD and YopB is required for inflammasome activation support a model where YopK limits inflammasome activation by negatively regulating the translocation of YopD and YopB.

LcrH point mutants fully attenuate *Yersinia* infection *in vivo*

We previously demonstrated that certain point mutations in LcrH, the chaperone protein for both YopD and YopB, prevented the translocation of YopD and YopB while allowing for the translocation of other effectors when expressed in a Δ*yopEJK* background strain (Zwack et al., 2015). To test whether preventing translocation of YopD and YopB would restore virulence during *in vivo* infection by a Δ*yopK* strain of *Yersinia*, we generated the LcrH point mutant *lcrH*mut1 in both WT and Δ*yopK* strains of *Yersinia.* Infection with either WT-*lcrH*mut1 or Δ*yopK*-*lcrH*mut1 resulted in complete attenuation of the infection instead of enhanced virulence (Fig. 5-2 and data not shown). Interestingly, the bacterial burden observed phenocopied burdens observed when mice were infected with Δ*yopBD Yersinia*, which fail to make a functional T3SS as they lack the

translocon proteins (Fig. 5-2). This observation lead us to the hypothesis that interactions among the full complement of effectors and LcrH mutants affect translocation differently than the interactions among LcrH mutants and the effectors in the absence of YopE and YopJ.

In both the presence and absence of YopK, LcrH point mutants fail to properly translocate YopE

We next examined the effect of background strain on the translocation phenotype of our *lcrH* mutant. It has been demonstrated that YopE negatively regulates translocation of the Yops. YopE potentially induces this phenomenon by affecting actin cytoskeleton near the pore complex (Mejia et al., 2008). As WT-*lcrH*mut1 *Yersinia* express YopE, interactions between YopE activity and the translocon could result in reduced translocation that would not be seen with YopE-deficient strains. As previously demonstrated, Δ*yopEJK*-*lcrH*mut1 translocated normally YopE and YopM (Fig. 5-3). WT-*lcrH*mut1 had decreased translocation of YopE, YopM, and YopJ in comparison to WT and Δ*yopK*. Intriguingly, Δ*yopK*-*lcrH*mut1 allowed for translocation of YopM and YopJ at normal levels but failed to return YopE translocation to normal levels (Fig. 5-3). YopE inhibits actin rearrangement and plays a crucial role in both preventing neutrophil oxidative burst and phagocytosis (Grosdent et al., 2002; Songsungthong et al., 2010). While deletion of YopE has been demonstrated to cause partial attenuation *in vivo*, YopE deletion does not result in complete clearance (Songsungthong et al., 2010). Therefore, it is probable that another effector Yop's translocation is affected by the mutations in LcrH in the presence of YopE. The T3SS has no easily defined signal sequence that is common to its effectors. The differential effect of LcrHmut1 on effector translocation could reveal interesting information about translocation hierarchy and effector-T3SS apparatus interactions.

Discussion:

The human pathogenic *Yersiniae* require a T3SS and its associated effectors to cause a productive infection in mammalian hosts. These effectors target host cell processes that would otherwise aid in the clearance of *Yersinia*. YopJ targets NFκB and MAPK signaling to prevent up-regulation of cytokines and possibly aids in bypassing the gut barrier (Monack et al., 1997; Palmer et al., 1998). YopE inhibits actin rearrangement, which prevents both neutrophil oxidative burst and phagocytosis (Grosdent et al., 2002; Songsungthong et al., 2010). Yops H, O, and T also target cellular components important for phagocytosis (Grosdent et al., 2002). By targeting these innate immune defenses, *Yersinia* can survive in mammalian hosts. Incidentally, the machinery needed to deliver these effectors into the cell provides an excellent target for immune recognition. Hyper-translocation of the T3SS translocon proteins YopD and YopB leads to activation of the inflammasome (Zwack et al., 2015). In order to balance the necessity of translocating the effectors with the inevitability of translocating the translocon components, *Yersinia* evolved effectors that prevent activation of the inflammasome in response to infection by T3SS expressing *Yersinia*. Interestingly, both YopM and YopK, effectors that prevent inflammasome activation, do not use enzymatic activity to inhibit inflammasome activation. YopM either directly sequesters caspase-1 or indirectly acts on the inflammasome through IQGAP1 depending on the strain of *Yersinia* (Chung et al., 2014; LaRock and Cookson, 2012). The mechanism by which YopK inhibits the inflammasome has been somewhat elusive.

Here we demonstrate that mutations in YopK that decrease its ability to negatively regulate translocation also prevent inhibition of the inflammasome (Fig. 5-1A-E). These mutations are located immediately adjacent to each other and replace charged residues (Fig. 5-1A). It has been demonstrated that it is the change of the aspartic acid to an alanine and not the threonine to alanine substitution that abrogates YopK's ability to regulate translocation (Dewoody et al., 2013).

Based on these data, we hypothesize that this region plays an important part in the interactions between YopK and other proteins needed to negatively regulate translocation.

In the process of trying to determine how YopK inhibits inflammasome activation, we have also uncovered some basic information on how the components of the T3SS and its effectors interact. LcrHmut1 has previously been demonstrated to have a decreased ability to lyse red blood cells (Edqvist et al., 2007). This indicates that either the size or number of translocons produced by *Yersinia* is reduced. In the absence of fully functional YopE, translocation of effectors occurs at normal levels by *Yersinia* expressing LcrHmut1 (Fig. 5-3A-B, Zwack et al., 2015). As YopE's ability to inhibit actin rearrangement has been implicated in the negative regulation of translocation, it is possible that the absence of YopE allows for a more optimal configuration of the translocon in the host membrane. Thus, translocation occurs at a similar level to WT *Yersinia* even though the translocons are either smaller in size or fewer in number. In the presence of all the effectors, translocation of YopJ, YopM, and YopE is decreased when *Yersinia* express LcrHmut1 (Fig. 5-3). The combination of YopE's effect on the actin cytoskeleton (Mejia et al. 2008), YopK's dual functions of decreasing pore size and negatively regulating translocation (Holmstrom et al., 1997), and LcrHmut1's effect on translocons could act in concert to decrease the efficiency of effectors leaving the T3SS by reducing the size of the pore (Edqvist et al. 2007). In the absence of YopK, expression of LcrHmut1 in the presence of YopE does not affect the ability of the T3SS to translocate YopJ and YopM (Fig 5-3B-C). Intriguingly, the absence of YopK does not restore YopE translocation to normal levels (Fig. 5-3A). As deletion of YopE alone does not result in complete clearance of *Yersinia in vivo* (Songsungthong et al., 2010), YopH or possibly YopO translocation is likely similar to what we observe with YopE. YopE is the smallest of the three effectors that we tested for translocation; therefore, it is unlikely that pore size is responsible for the reduction in YopE translocation by Δ*yopK-lcrH*mut1. Instead, these data suggest that different Yops are more sensitive to the translocon pore configuration. Initial low-

level translocation of YopE could induce a conformation change in the translocon that could expose or hide different residues necessary for further YopE translocation. As failure of YopE to pass through the translocon should block other Yops from exiting the T3SS, this model suggests a hierarchy of effector translocation where YopM and YopJ are translocated prior to YopE. Another possibility is that sufficient T3SSs are produced by the bacteria that enough T3SSs are available to translocate the other Yops even if some are blocked by YopE. While translocation of YopE may be regulated by the conformation of the translocon, it is possible that interactions in the bacteria between YopE and either the translocon proteins or LcrH regulate YopE translocation. In the presence of endogenous YopE, the YopE-β-lactamase reporter may be competing for a limited supply of YopD or YopB. This competition could result in decreased translocation of the reporter construct. To test whether translocation of YopE is dependent on the conformation of the translocon or an interaction within the bacteria, we will use antibodies against YopE to look for translocation of endogenous YopE. Our findings enhance our current understanding of an important virulence structure of pathogens.

While the human immune system has evolved effective ways to identify and respond to pathogens, pathogens continue to evolve ways to circumvent these defenses. By understanding the mechanisms that pathogens use to bypass our defenses, we can develop therapeutic strategies that reactivate that arm of defense. The T3SS is an obvious target for immune recognition of many gram-negative bacteria. *Yersinia* employs multiple strategies to shut down activation of the inflammasome in response to infection with T3SS expressing *Yersinia*. There is no sequence homolog to YopK. Work done here and by others to determine how YopK functions and inhibits inflammasome activation will allow us to not only find ways to bypass YopK's inhibition of the inflammasome but also determine if there are functional homologs or other changes in different gram-negative bacteria that control accidental recognition of the pore proteins. Additionally, the new insights provided here on the balance maintained by the T3SS and

its effectors to allow for function could offer potential targets for destabilizing the T3SS. Acquiring a fundamental understanding of the virulence structures and effectors of pathogens provides us with the necessary foundation to develop new treatments in this age of multi-drug resistance.

Figure 5-1

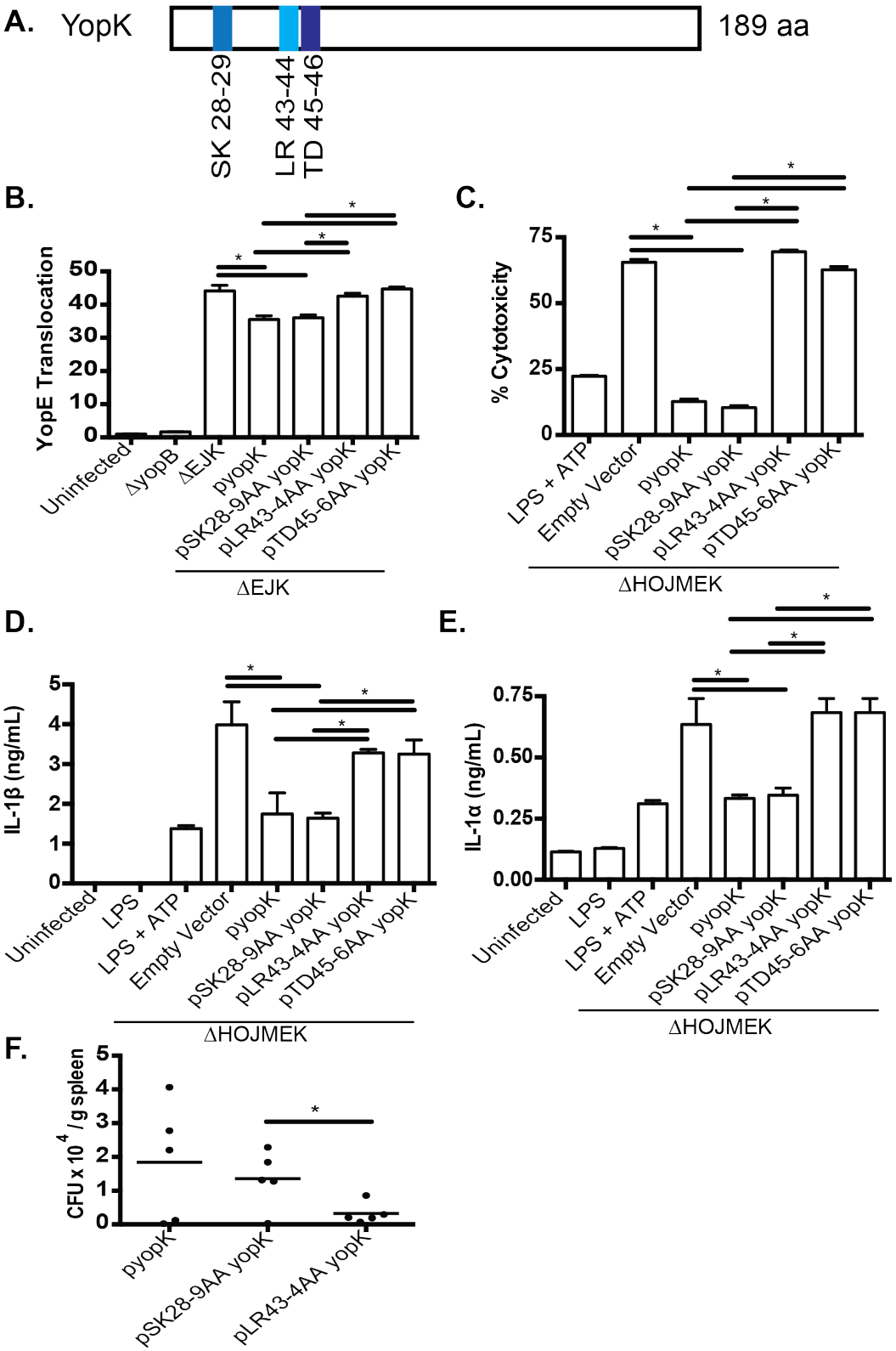

A.
YopK
189 aa
SK 28-29
LR 43-44
TD 45-46
B.
YopE Translocation
Uninfected
ΔyopB
ΔEJK
pyopK
pSK28-9AA yopK
pLR43-4AA yopK
pTD45-6AA yopK
ΔEJK
C.
% Cytotoxicity
LPS + ATP
Empty Vector
pyopK
pSK28-9AA yopK
pLR43-4AA yopK
pTD45-6AA yopK
ΔHOJMEK
D.
IL-1β (ng/mL)
Uninfected
LPS
LPS + ATP
Empty Vector
pyopK
pSK28-9AA yopK
pLR43-4AA yopK
pTD45-6AA yopK
ΔHOJMEK
E.
IL-1α (ng/mL)
Uninfected
LPS
LPS + ATP
Empty Vector
pyopK
pSK28-9AA yopK
pLR43-4AA yopK
pTD45-6AA yopK
ΔHOJMEK
F.
CFU x 10^4 / g spleen
pyopK
pSK28-9AA yopK
pLR43-4AA yopK

Figure 5-1: YopK mutants that allow for increased translocation fail to prevent inflammasome activation. A. Schematic of three mutations generated in YopK. **B.** HeLa cells were infected with indicated strains expressing a YopE-β-lactamase fusion protein (YopE-Bla). Cells were loaded with CCF4-AM dye and ratio of blue:green signal (translocation) was calculated as described in materials and methods. Representative of 3 independent experiments. **C.** BMDMs were infected with Δ*yopHOJMEK* expressing either an empty vector, WT YopK, or the indicated YopK mutants or treated with LPS + ATP. Cytotoxicity was determined by LDH release. Graph is representative of three independent experiments. *$p < 0.05$. **E-F.** Supernatants from BMDMs infected with indicated bacterial strains were assayed for levels of secreted IL-1β and IL-6 by ELISA as described in materials and methods. Graph is representative of three independent experiments. *$p < 0.05$. **G.** Mice were infected with indicated *Yersinia* strain by i.p. injection. Spleens were harvested and homogenized day 4 post infection. Homogenates were plated on LB irgasan plates. Colonies were counted and CFU/g spleen was calculated. *$p < 0.05$.

Figure 5-2

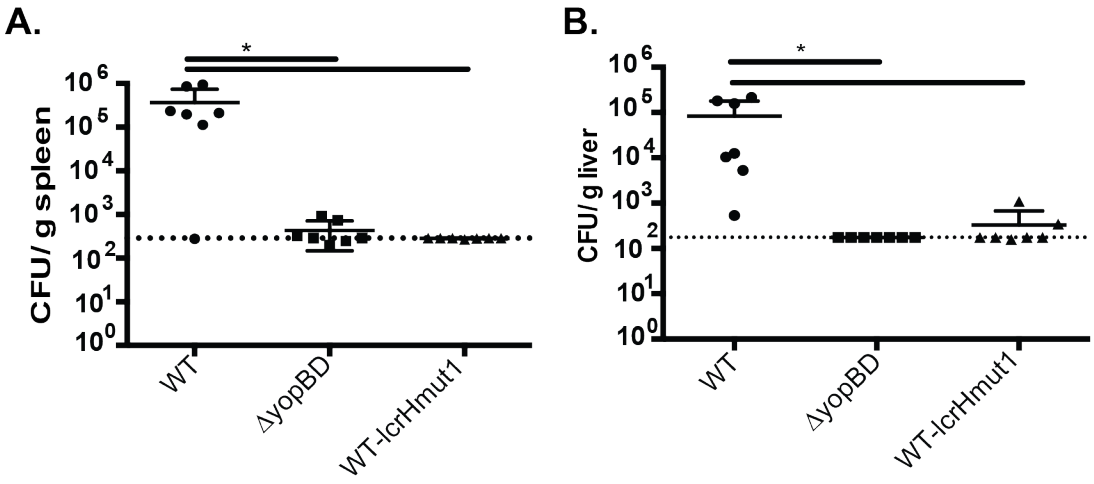


Figure 5-2: *Yersinia* expressing LcrH point mutants are attenuated *in vivo*. A. Spleens and **B. Livers** were harvested and homogenized day 3 post i.p. infection. Homogenates were plated on LB irgasan plates. Colonies were counted and CFU/g spleen was calculated. * $p < 0.05$. Dashed line indicates limit of detection. The graph is representative of 3 independent experiments.

Figure 5-3

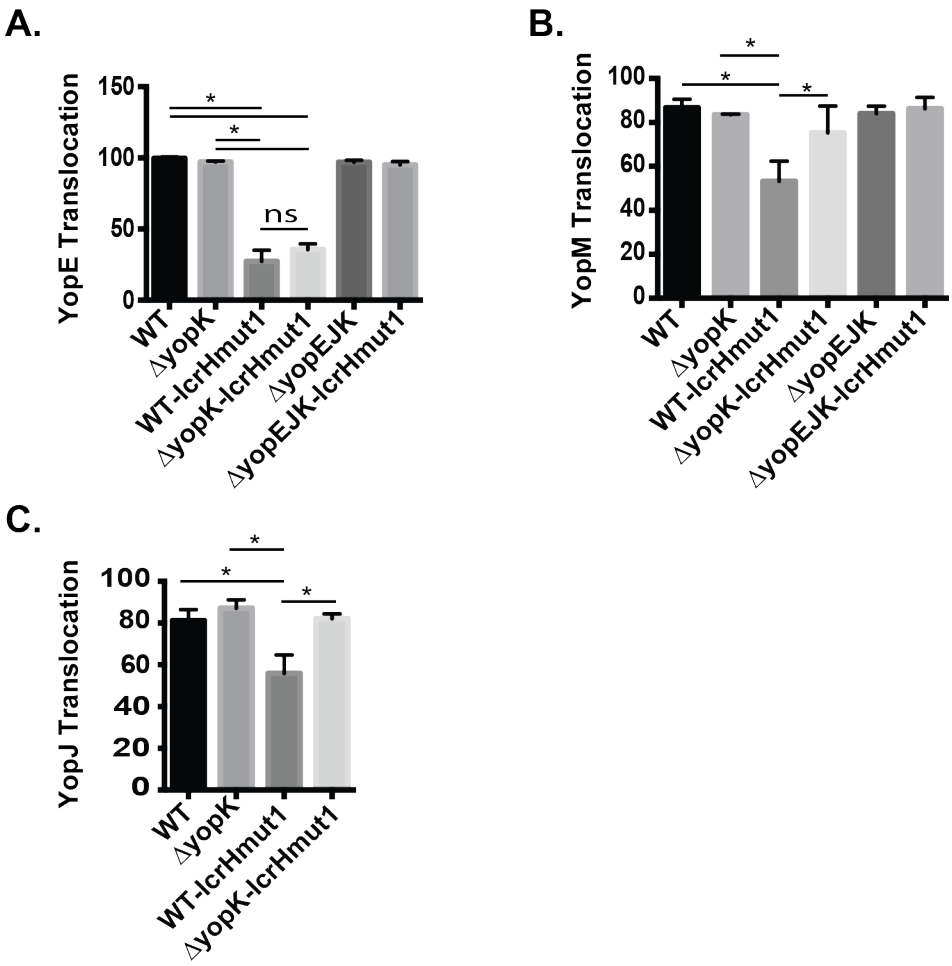


Figure 5-3: LcrHmut1 affects translocation of Yops in the presence of all the effectors.

HeLa cells were infected with indicated strains expressing **A.** YopE-β-lactamase fusion protein, **B.** YopM-β-lactamase fusion protein, or **C.** YopJ-β-lactamase fusion protein. Cells were loaded with CCF4-AM dye and ratio of blue:green signal (translocation) was calculated as described in materials and methods. * $p < 0.05$. The graph is representative of 3 independent experiments.

Table 5-1. *Yersinia* strains used in this study

Strain Name	Relevant Characteristics	Reference or Source
Y. pseudotuberculosis		
IP2666 (WT)	Wild-type 0:3 strain	Black and Bliska, 1997
ΔyopB	Full deletion of translocon protein YopB on IP2666 background	Palmer et al., 1998
ΔyopK	Full deletion of YopK on IP2666 background	Brodsky et al., 2010
ΔyopEJK	Full deletion of Yops E, J, and K on IP2666 background	Zwack et al., 2015
ΔyopHOJMEK	Full deletion of Yops H, O, J, M, E, and K on IP2666 background	Lilo et al., 2008
pyopK	YopK expressed on plasmid PACYC184	Brodsky et al., 2010
pSK28-29AA yopK	YopK expressed on plasmid PACYC184 encoding the amino acid mutation SK28-29AA	This work
pLR43-44AA yopK	YopK expressed on plasmid PACYC184 encoding the amino acid mutation LR43-44AA	This work
pTD45-46AA yopK	YopK expressed on plasmid PACYC184 encoding the amino acid mutation TD45-46AA	Thorslund et al., 2011 This work
ΔyopBD	Full deletion of translocon proteins YopB and YopD on IP2666 background	Zwack et al., 2015

*lcrH*mut1	LcrH$^{Y40C, I101M, K102R}$	Edqvist et al., 2007,

CHAPTER 6

DISCUSSION

In the work presented in this dissertation, I dissected mechanisms through which the T3SS of *Yersinia* activates the inflammasome. I demonstrated that hyper-translocation of the pore proteins YopD and YopB is required for inflammasome activation in response to the *Yersinia* T3SS. I also showed that this hyper-translocation leads mainly to non-canonical inflammasome activation through a process requiring guanylate binding proteins (Gbps) from chromosome 3. Furthermore, our data demonstrate that translocated YopD traffics to lysosomes and is associated with lysosomal damage. Our work also revealed new insights into how YopK, an effector protein that prevents inflammasome activation, functions to exert this activity.

A. Internal delivery of pore-forming proteins are a trigger for innate immune responses

In this study, we determined the necessary functions of the *Yersinia* T3SS for activation of the inflammasome. Earlier studies had determined that in the presence of LPS priming or the absence of YopK, *Yersinia* infection leads to inflammasome activation (Bergsbaken and Cookson, 2007; Brodsky et al., 2010); however, it remained unclear how the inflammasome was activated. As external delivery of pore-forming proteins such as α-hemolysin and listeriolysin O (LLO) activates the inflammasome (Craven et al., 2009; Meixenberger et al., 2010) and effectorless strains of *Yersinia* activate the inflammasome (Brodsky et al., 2010), we initially hypothesized that pore-formation caused by insertion of the T3SS translocon into the plasma membrane triggered the inflammasome. Surprisingly, analysis of a series of YopD mutants that separate pore-formation from translocation demonstrated that pore-formation caused by the translocon pore was insufficient to trigger inflammasome activation (Fig. 3-1). These data show that translocation is required to activate the inflammasome. As none of the six *Yersinia* effectors are needed for inflammasome activation, we investigated whether the translocon pore proteins YopD and YopB activated the inflammasome. While the T3SS can translocate YopB and YopD (Francis and Wolf-Watz, 1998; Dewoody et al., 2013), the effects of this translocation were

unknown. We determined that translocation of YopD/B correlated with activation of caspase-1 (Fig. 3-3 and Fig. 3-4). However, this result suggested two possible models for inflammasome activation: 1. translocation of YopD and YopB leads to activation of the inflammasome; or 2. translocation of YopD and YopB act as a surrogate reporter for the translocation of an unknown molecule, which activates the inflammasome. In order to test the hypothesis that translocation of YopD/B leads to inflammasome activation, we used mutants in the chaperone protein of YopD and YopB, which allowed for translocation of everything but YopD and YopB. Intriguingly, we observed that the specific hyper-translocation of YopD and YopB is the key signal for inflammasome activation (Fig. 3-4, Fig. 3-5, and Fig. 3-6).

YopD, a single pass transmembrane protein, and YopB, a double pass transmembrane protein, make up the translocon pore complex of the *Yersinia* T3SS along with the platform protein LcrV (Cornelis and Wolf-Watz, 1997; Hakansson et al., 1993). In the presence of YopK, *Yersinia* limits intracellular delivery of these proteins (Fig. 3-3D-E and Dewoody 2013), suggesting that YopK acts to inhibit inflammasome activation by negatively regulating the translocation of the pore proteins into the host cell. In the case of *Yersinia*, hyper-translocation of YopD and YopB lead to activation of the inflammasome by both the canonical and non-canonical inflammasome (Fig. 4-1) although the exact mechanism of inflammasome activation by hyper-translocation of the pore proteins is not fully defined.

Internally delivered pore proteins such as YopD and YopB are a general feature of bacterial pathogens that utilize T3SS (Dewoody et al., 2013; Francis and Wolf-Watz, 1998; Hersh et al. 1999). This is likely due to the mechanics of how the T3SS functions, as the translocon proteins themselves are secreted through the needle complex, and must assemble at the needle tip in order to generate a functional pore. The *Salmonella* homolog of YopB, known as SipB, is also injected by *Salmonella* into host cells, and initial studies describing activation of caspase-1 in response to *Salmonella* found that microinjection of SipB caused caspase-1 dependent cell death

(Hersh et al., 1999). Additionally, caspase-1 was co-immunoprecipitated from cells infected with *Shigella* expressing plasmid encoded SipB. These data demonstrated that SipB can associate with caspase-1 (Hersh et al., 1999). These observations suggest the potential for pore proteins to directly bind and possibly activate caspases (Fig. 6-1). In addition to the delivery of pore proteins into the cytosol by bacteria expressing T3SSs, many other pathogens employ virulence strategies that result in pore proteins being delivered or expressed inside the host cell. The internal delivery of these pore proteins often lead to insertion of the proteins into cytosolic membrane bound structures (Fig. 6-1). During influenza infection, the channel protein M2 inserts into the Golgi apparatus causing H^+ ion flux, the neutralization of the Trans Golgi Network, and the activation of the NLRP3 inflammasome (Ichinohe et al., 2010). The gram-positive bacteria *Listeria* express the cytolysin LLO when inside a host cell phagosome, allowing the *Listeria* to escape the vacuole (Moors et al., 1999). LLO expression is required for activation of both the NLRP3 inflammasome by disruption of the lysosome and the AIM2 inflammasome activation by releasing DNA from the lysosome into the cytosol (Kim et al., 2010). Similarly, release of pneumolysin from dying *Streptococcus pneumoniae* in the lysosome results in activation of both the AIM2 and NLRP3 inflammasomes (Fang et al., 2011). Interestingly, Leukocidin A/B (LukAB) of *Staphylococcus aureus* results in NLRP3-mediated cell death when inserted into the plasma membrane but triggers NLRP3-independent cell death with NLRP3-dependent caspase-1 activation and IL-1β secretion when delivered intracellularly (Melehani et al., 2015). Together these findings imply that host cells recognize disruption of normal functions by the insertion of pores into internal membrane structures as a feature of pathogenesis. Depending on the infection, intracellular delivery of pore proteins can either be an unintended consequence of or an important mechanism for establishing infection. No matter the reason for the presence of the pore protein inside the cell, intracellular pore-forming toxins are inherently foreign to the cytosol and always run the risk of damaging the cell. These features of pore-forming toxins make them ideal targets for intracellular immune recognition. Internal delivery of pore-forming proteins by pathogens allows for indirect activation of inflammasomes by damaging internal organelles as well as releasing bacterial

products from lysosomes and potentially allows for direct activation of caspases through binding by pore proteins.

Figure 6-1

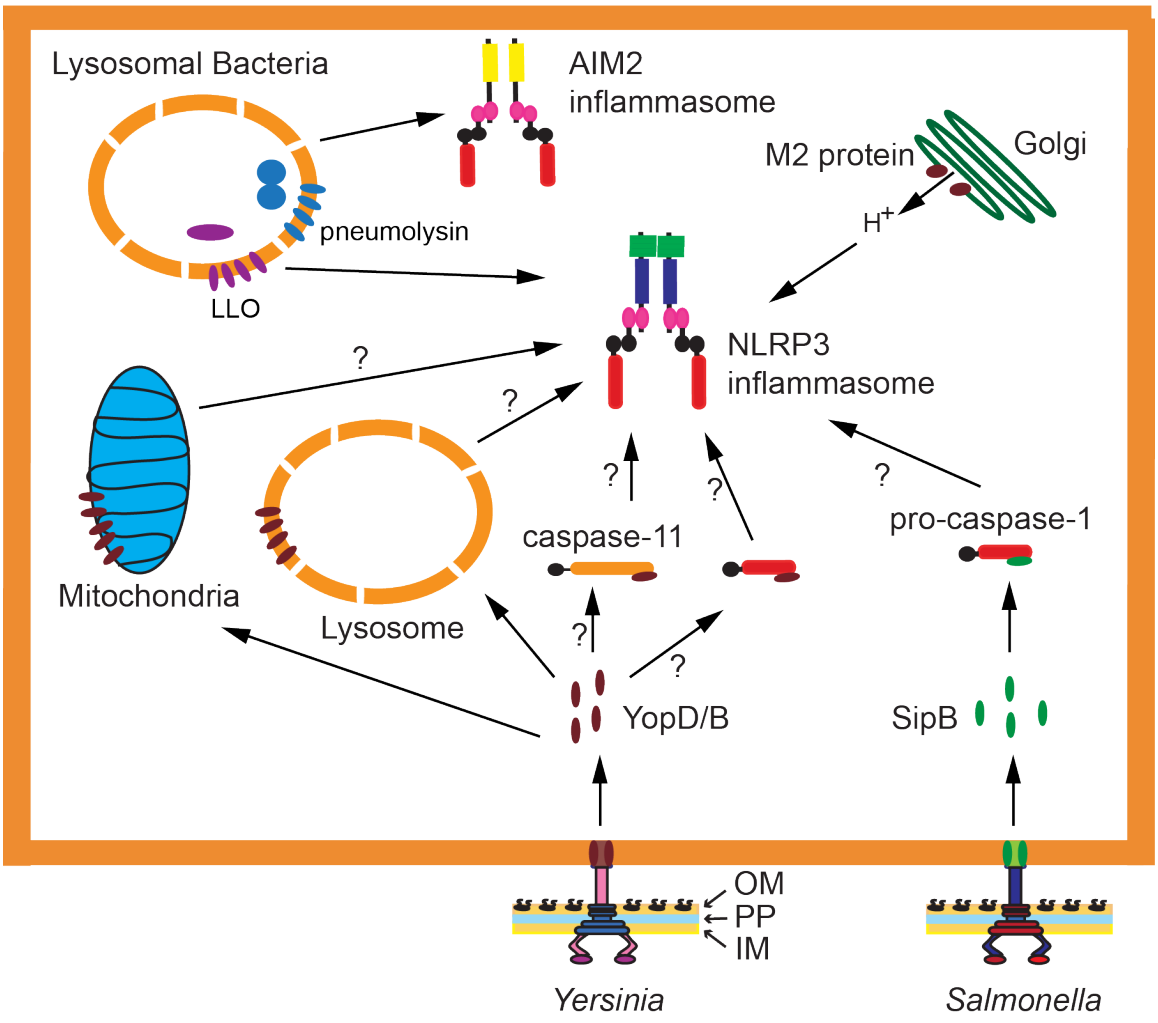


Figure 6-1: Schematic innate immune responses activated by internally delivered pore proteins. Bacterial and viral pore proteins can insert into a variety of internal membrane structures. Pore proteins can also interact with proteins in the cytosol.

Internal delivery of pore-forming proteins provides signals capable of activating multiple innate immune pathways. These proteins are spread across not just different bacterial species but different varieties of pathogens. As a result, they vary in sequence, which can make it difficult for direct recognition by one sensor. By recognizing the effects of virulence activities on the cell, the host cell can recognize classes of pathogenesis indicators that are employed by a broad range of pathogens. Additionally, the recognition of the activity of the proteins allows for both redundant and tunable responses. For example, many internally delivered pore-proteins damage lysosomes and release both lysosomal host contents and bacterial products. Release of host lysosomal contents leads to the activation of NLRP3, release of bacterial and viral DNA lead to AIM2 activation, and release of hexa-acylated LPS leads to non-canonical inflammasome activation (Fernandes-Alnemri et al., 2009; Hagar et al., 2013; Hornung et al., 2008; Kayagaki et al., 2013). In the case of the *Yersinia* proteins YopD/B, hyper-translocation leads to both canonical and non-canonical inflammasome activation (Fig. 4-1). These redundant mechanisms can compensate for evasion mechanisms instigated by pathogens such as the modification of LPS to a tetra-acylated form. Furthermore, recognition of downstream consequences of activities can make the response tunable. While certain signals elicit strong responses (flagellin in mouse model), other signals can be more modest. When an activity leads to multiple consequences, a cumulative effect can be observed and amplify the response. In the case of hyper-translocation of the *Yersinia* pore proteins, we observe both non-canonical and canonical inflammasome activation. Defining the mechanisms through which hyper-translocation of YopD/B activate these pathways will enhance our understanding of how these pathways interact to provide an optimal immune response.

B. A role for Guanylate Binding Proteins in non-canonical inflammasome activation downstream of YopD hyper-translocation

Interferon inducible GTPases have long been known to play roles in control of vacuolar pathogens. They recruit machinery such as nitric oxide synthases and autophagy proteins to

control these pathogens (Kim et al., 2011; MacMicking, 2012). Additionally, they can cause direct lysis of the lysosome leading to release of the pathogen into the cytosol as well as lysis of cytosolic bacteria (MacMicking, 2012). More recently a subset of these GTPases called the Gbps have been found to be involved in both canonical and non-canonical inflammasome activation (Shenoy et al., 2012; Meunier et al. 2014; Pilla et al., 2014; Finethy et al., 2015). Gbp5 can bind to NLRP3 and is thought to act as a platform or scaffolding protein that facilitates inflammasome formation (Shenoy et al., 2012). Gbps have been implicated in a number of roles in activation of the non-canonical inflammasome. For example, Gbps damage vacuolar integrity and allow for the release of LPS from the lysosome into the cytoplasmic compartment (Meunier et al., 2014). Additionally, it has been demonstrated that Gbps recognize cytosolic LPS independent from its release from the vacuole (Pilla et al., 2014). Here we demonstrated that the Gbps found on chromosome 3 play a critical role in inflammasome activation in response to *Yersinia* infection (Fig. 4-3 and 4-4).

Mechanistically, how Gbps contribute to *Yersinia*-induced inflammasome activation is currently unknown. However, Gbps may recognize the presence of damaged lysosomal membranes, or themselves be responsible for amplifying T3SS-induced membrane damage. Either or both of these mechanisms have the potential to link Gbp function to inflammasome activation. Whether Gbps act upstream of initial lysosomal damage potentially depends on the signal. We determined that YopD can colocalize with the lysosome (Fig. 4-2). Logically, colocalization of a membrane active protein with a membrane bound compartment could result in damage to the membrane. We found that Gbp2 colocalizes with both translocated YopD and Δ*yopEJK Yersinia* (Fig. 4-5). In collaboration with the Coers lab, we determined that colocalization of Gbp2 with *Yersinia* requires translocation activity of the T3SS. As recruitment of Gbp2 to *Yersinia* requires Galectin-3 and Galectin-3 recognizes damaged lysosomes, translocation-competent *Yersinia* potentially are capable of damaging the lysosome upstream of Gbp2 recruitment (Feeley et al., in submission;

Paz et al., 2010). This also suggests that translocated YopD, which colocalizes with lysosomes, triggers lysosomal membrane damage. Alternatively, it is possible that other Gbps from chromosome 3 are recruited to these lysosomes first and initiate the damage recognized by Galectin-3. Current technical limitations make it difficult to determine the temporal kinetics or recruitment of the other Gbps located on chromosome 3. While Gbp2 is recruited to YopD, its recruitment does not correlate with caspase-1 puncta formation (Fig. 4-6). This suggests that Gbp2 does not play a dominant role in the activation of the non-canonical inflammasome, or at the very least, that recruitment of Gbp2 to lysosomes is not the functional signal leading to inflammasome activation. These data may suggest that a single Gbp that is not Gbp2 plays the dominant role, multiple Gbps are playing redundant roles, or the Gbps individually have small cumulative effects. Future experiments using single knockout or siRNA knockdown of the Gbps located on chromosome 3 will help determine the contributions of the individual Gbps necessary for non-canonical inflammasome activation. Because different Gbps play distinct roles in other innate responses to vacuolar pathogens (Kim et al., 2011), identifying the responsible Gbps will provide important information not only on how hyper-translocation of YopD/B is recognized but also on the relationship between the different innate pathways that share the Gbps.

C. Potential models of inflammasome activation by hyper-translocation of YopD and YopB

We have demonstrated that *Yersinia* infection activates both the non-canonical and canonical inflammasomes (Fig. 4-1, Casson et al., 2013). Both the canonical and non-canonical inflammasome can potentially be activated downstream of the same virulence activity. Alternatively, multiple mechanisms could account for activation of the different inflammasomes, as well as activation of a single inflammasome. One model based on our data suggests that hyper-translocation of YopD mediates activation of both the non-canonical and canonical inflammasome through disruption of the lysosomal membrane (Fig. 6-2). We have shown that

YopD can colocalize with the lysosome (Fig. 4-2) and that Gbp2 is recruited to the lysosomes of *Yersinia*-infected cells, providing an indicator of lysosomal damage (Fig. 4-5). Notably, lysosomal disruption itself is a sufficient signal to activate the canonical NLRP3 inflammasome (Hornung et al., 2008). Potentially, the extent of host lysosomal content release modulates how much canonical inflammasome activation occurs. Damaged lysosomes that contain bacteria or bacterial PAMPs could also release bacterial products such as LPS. Currently, intracellular LPS is the only known non-canonical inflammasome activator (Hagar et al., 2013; Kayagaki et al., 2012; Shi et al., 2014). *Yersinia pseudotuberculosis* grown at 37 °C expresses a mix of tetra-acylated and penta-acylated LPS (Rebeil et al., 2004). Tetra-acylated LPS has been demonstrated to be non-stimulatory or antagonistic to the non-canonical inflammasome (Hagar et al., 2013; Kayagaki et al., 2013). However, transfection of penta-acylated LPS from *Francisella novicida* activated the non-canonical inflammasome (Hagar et al., 2013). In previous work with TLR4, LPS from *Yersinia pseudotuberculosis* that was grown at 37 °C was significantly less stimulatory than LPS from *Yptb* grown at 21 °C. However, it did allow for some stimulation as measured by cytokine production (Rebeil et al., 2004). As recognition of intracellular LPS has generally followed the same trend as TLR4 recognition of LPS, the release of LPS from the lysosome may be activating the non-canonical inflammasome.

Several experiments are still needed to test this model. To test whether canonical inflammasome activation is occurring by release of host lysosomal contents and not intracellular LPS alone, *Casp11*$^{-/-}$ macrophages would be pretreated with cathepsin B inhibitor or vehicle control, and then infected with *Yersinia*. Release of cathepsin B from the lysosome has previously been shown to be important for inflammasome activation downstream of lysosomal degradation (Hornung et al., 2008). Therefore, inhibition of cathepsin B should decrease canonical inflammasome activation if degradation of the lysosome by YopD/B is the trigger for canonical inflammasome activation. In the case of the non-canonical inflammasome, we need to test whether LPS is the signal. We are

currently trying two methods to test this portion of the model. First, we will express YopD and YopB in the gram-positive bacterium *Listeria monocytogenes* to see if we can deliver YopD and YopB internally in the absence of LPS. By fusing the signal sequence of *actA* from *Listeria* to the N-terminus of *yopD* and *yopB* and expressing the genes downstream of the *actA* promoter, YopD and YopB will only be expressed when *Listeria* is inside a host cell and should be exported out of the *Listeria* due to the ActA signal sequence. If *Listeria* can tolerate expressing YopD and YopB, this experiment will potentially determine whether YopD and YopB are sufficient to activate the non-canonical inflammasome in the absence of LPS. However, this experiment cannot exclude the possibility that LPS is also activating the inflammasome. To test whether LPS is activating the non-canonical inflammasome during *in vitro Yersinia* infection, we will grow *Yersinia pestis* at 37 °C overnight and for all three hours of the inducing culture. Using this method of growth will generate only tetra-acylated LPS and prevent any residual hexa-acylated LPS from the overnight or 1 hour of inducing culture growth at 26 °C from contaminating the infection. If the 37 °C grown *Yersinia pestis* fail to activate the non-canonical inflammasome, it suggests that LPS released from the lysosome is the signal during both *Yersinia pestis* and *Yersinia pseudotuberculosis* infection.

Figure 6-2

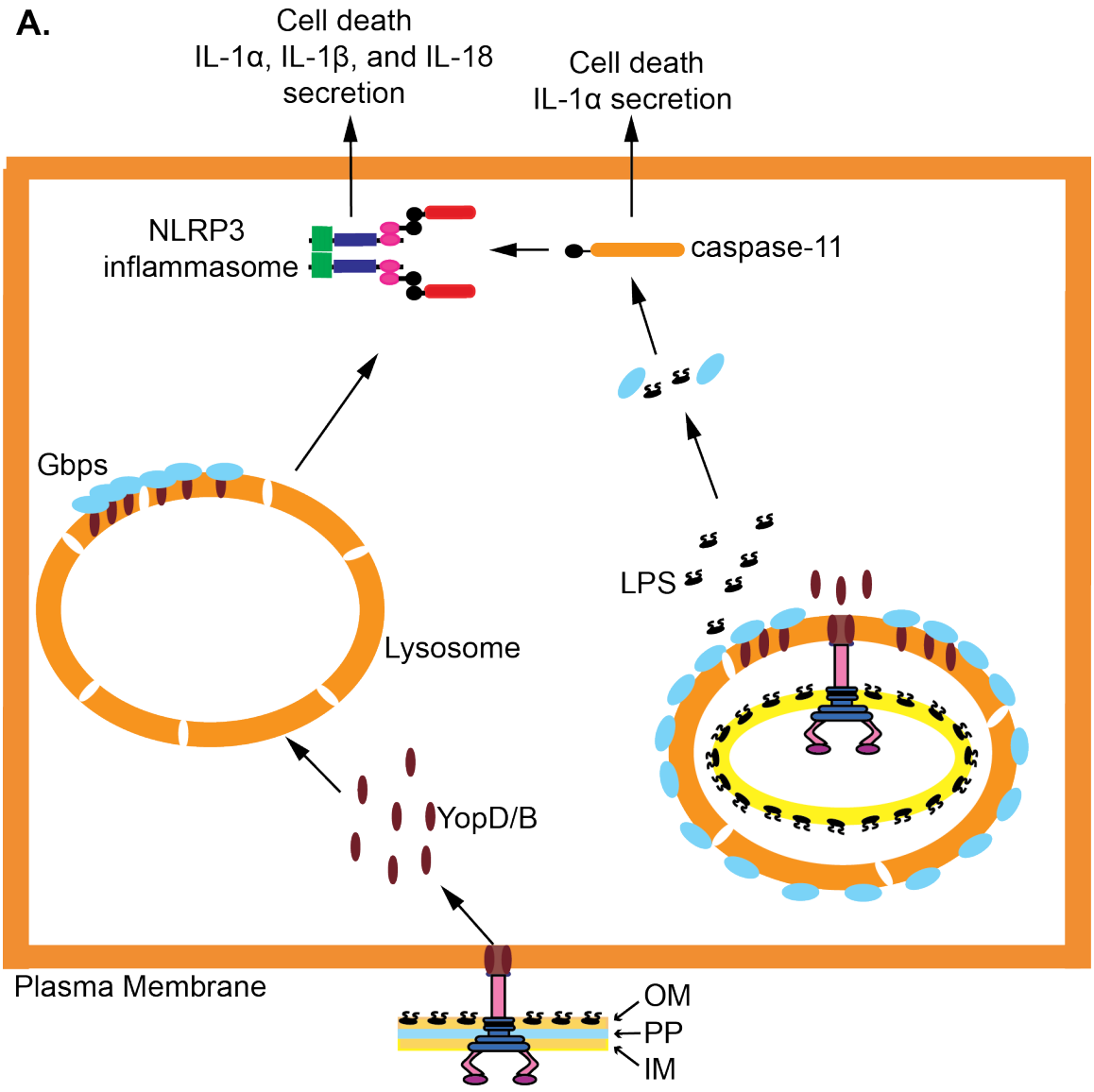


Figure 6-2: Model 1 – Release of lysosomal contents by YopD/B activates the canonical and non-canonical inflammasomes. *Yersinia* hyper-translocate YopD/B (burgundy) into the cell from either the plasma membrane or the lysosome. YopD/B associate with the membranes of both *Yersinia* containing and *Yersinia* free lysosome. This association causes damage and recruits Gbps (blue). Damage to the lysosomes allow for the release of both host and bacterial

products including LPS (black). Release of host proteins triggers NLRP3 canonical inflammasome activation while release of LPS leads to activation of the non-canonical caspase-11 inflammasome.

An alternative model for the activation of the non-canonical inflammasome is direct activation by YopD or YopB (Fig. 6-3). SipB, the *Salmonella* homolog of YopB, has previously been demonstrated to bind to the proform of caspase-1 (Hersh et al., 1999). As caspase-11 is the result of a gene duplication of caspase-1 (Sakamaki and Satou, 2009), YopB and potentially YopD may bind to caspase-11. If the *Listeria* experiment proposed above demonstrates that YopB and YopD delivery are sufficient for non-canonical inflammasome activation, immunoprecipitation of YopD and YopB will also be performed to test whether the activation occurs through direct binding. If YopD and YopB are sufficient to activate the non-canonical inflammasome either directly or indirectly, it would present new possibilities for how the non-canonical inflammasome interacts with the host cell environment. As caspase-1 can be activated through multiple NLR proteins as well as through a caspase-8 mediated pathway (Lamkanfi and Dixit, 2009; Philip et al., 2014; Weng et al., 2014), it is conceivable that caspase-11 is activated by more than just the direct binding of LPS.

Figure 6-3

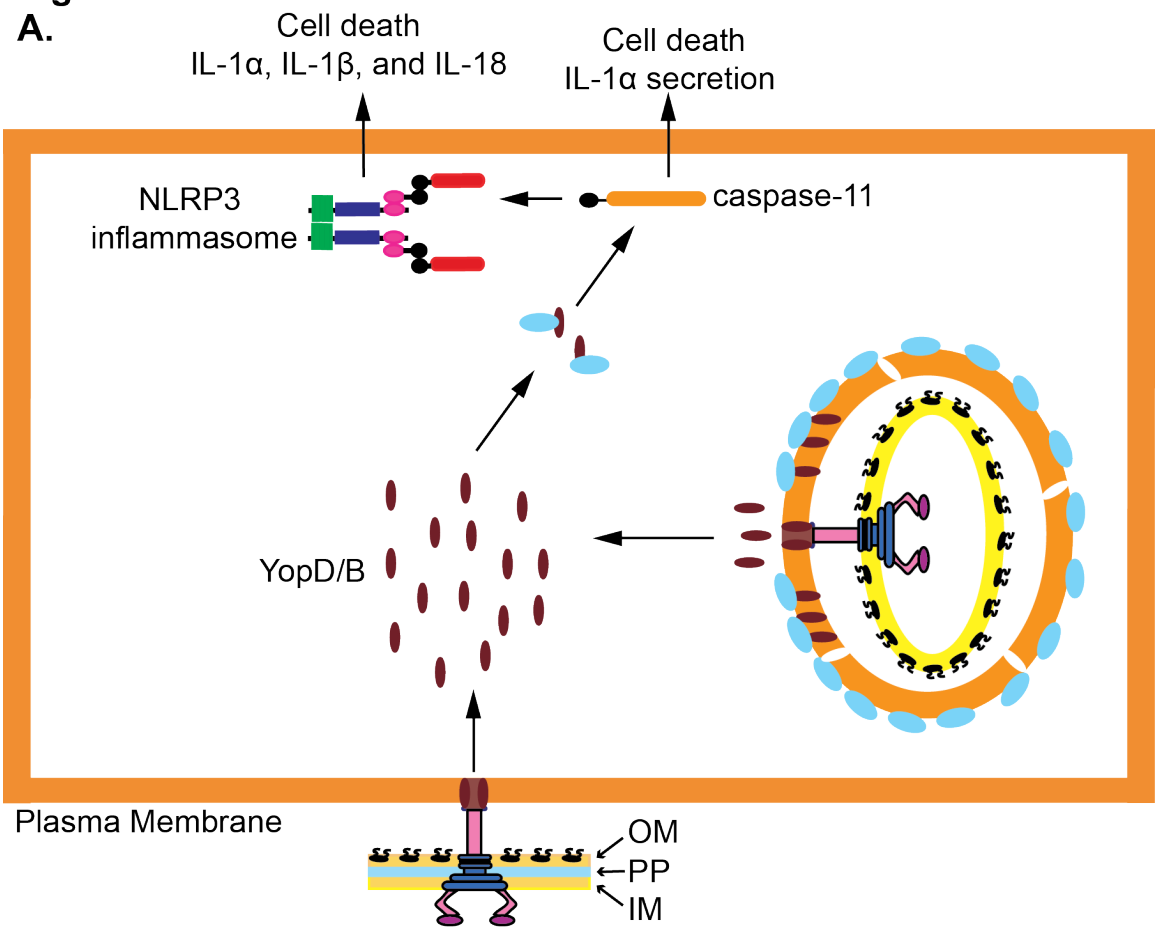


Figure 6-3: Model 2 - YopD/B activate caspase-11 non-canonical inflammasome. *Yersinia* hyper-translocate YopD/B (burgundy) into the cell from either the plasma membrane or the lysosome. YopD/B recruit Gbps (blue). YopD/B then activate the non-canonical inflammasome either by binding to capase-11 or to a currently unknown sensor.

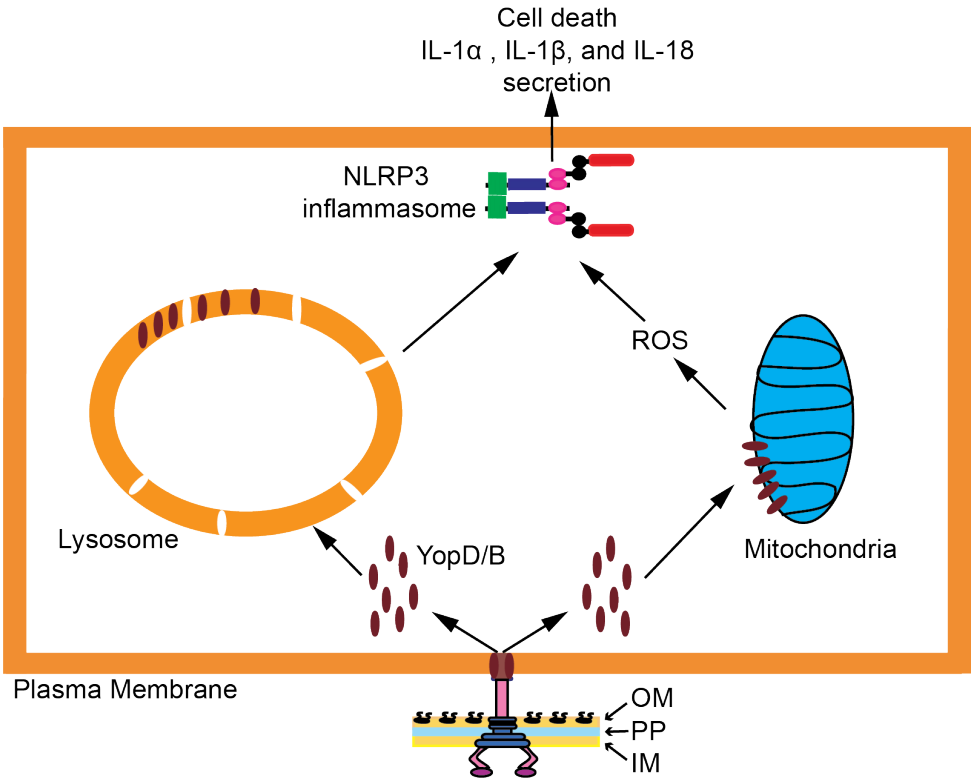
While lysosomal damage may result in canonical inflammasome activation, lysosomal damage is not the only possible stimulus for canonical inflammasome activation in response to hyper-translocation of YopD and YopB. It is possible that hyper-translocation of YopD/B activates the canonical inflammasome by multiple mechanisms including lysosomal damage (Fig. 6-4A).

Alternatively, hyper-translocation of YopD/B may activate the canonical inflammasome through a lysosome-independent mechanism (Fig 6-4B). As YopD and YopB are translocated into the host cell and there are other internal membranes beside lysosomes, YopD and YopB may insert into these other membranes. Preliminary data suggest that in addition to being recruited to lysosomal membranes, YopD can also colocalize with the mitochondrial membrane (data not shown). Mitochondrial dysfunction and ROS production are important activators of the NLPR3 inflammasome (Shimada et al., 2012; Zhou et al., 2011). In addition to the previously described cathepsin B inhibitor experiments to determine the contribution of lysosomal degradation to inflammasome activation, experiments are required to confirm if YopD and YopB actually colocalize with the mitochondria. Colocalization of YopD and YopB with mitochondria could cause increased production of mitochondrial ROS. Mitochondrial ROS has been demonstrated to participate in the activation of the NLRP3 inflammasome in response to various stimuli (Shimada et al., 2012; Zhou et al., 2011). One way to test if mitochondrial ROS is necessary for inflammasome activation in response to hyper-translocation of YopD/B is to over-express enzymes that break down ROS. Catalase converts the reactive oxygen species hydrogen peroxide into water and oxygen. A transgenic mouse strain called MCAT overexpresses human catalase and targets the enzyme to the mitochondria (Schriner et al., 2005). To test if YopD/B colocalization with mitochondria triggers inflammasome activation through increased mitochondrial ROS production, we will infect MCAT BMDMS with *Yersinia*. If mitochondrial hydrogen peroxide production is required for inflammasome activation, MCAT BMDMs should have decreased inflammasome activation as the catalase would quickly convert any hydrogen peroxide produced to water and oxygen. Alternatively, if a different species of reactive oxygen is required such as superoxide, we would not see a defect. Furthermore, as the non-canonical inflammasome is the dominant inflammasome, changes may be hard to see in the MCAT BMDMs. siRNA silencing of caspase-11 in MCAT BMDMs may be needed to see an effect. Additionally, colocalization of YopD or YopB with mitochondria could cause the release of cardiolipin from the mitochondria and activate the inflammasome in a ROS-independent manner

(Iyer et al., 2013). We will test for a role of cardiolipin in inflammasome activation by knocking down cardiolipin synthase and then infecting the macrophages. Understanding which mechanisms would recognize *Yersinia* infection in the absence of bacterial immune evasion provides insight into the key features of virulence for innate immune recognition. Additionally, knowing the steps in the activation of the inflammasome reveals the potential points in the process of immune recognition that must either be inhibited or evaded by the bacteria. In the case of *Yersinia*, determining the mechanism by which the inflammasome should be activated suggests models for how YopK as well as YopJ in the context of YopM prevent inflammasome activation.

Figure 6-4

A.


Cell death
IL-1α , IL-1β, and IL-18
secretion
NLRP3
inflammasome
ROS
Lysosome
YopD/B
Mitochondria
Plasma Membrane
OM
PP
IM

B.

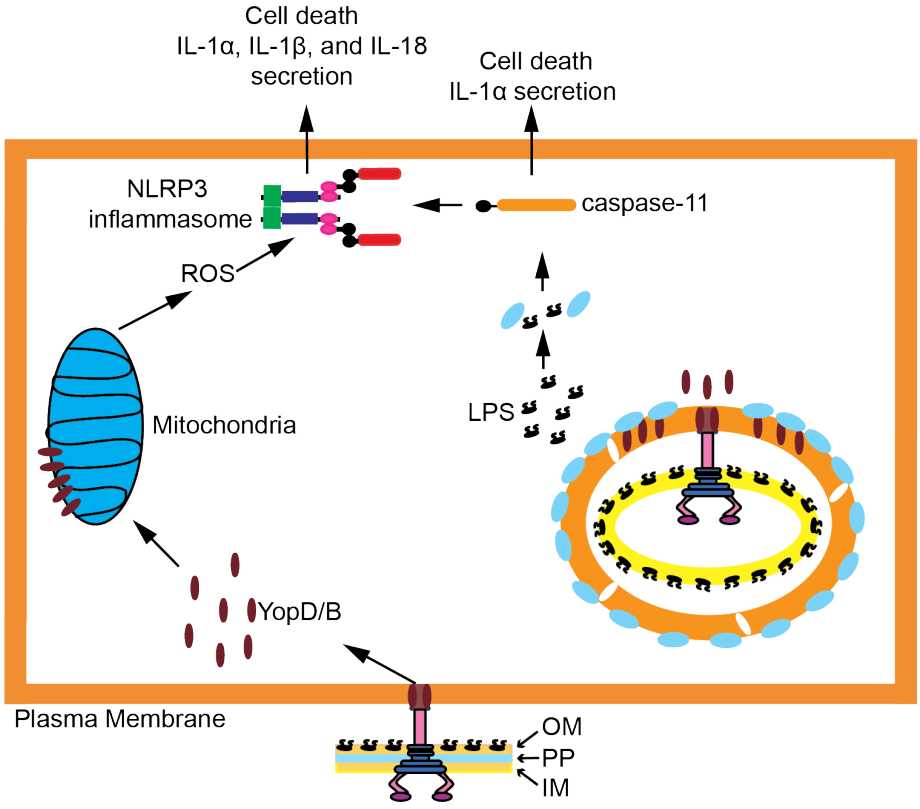

Cell death
IL-1α, IL-1β, and IL-18
secretion
Cell death
IL-1α secretion
NLRP3
inflammasome
caspase-11
ROS
Mitochondria
LPS
YopD/B
Plasma Membrane
OM
PP
IM

Figure 6-4: Model 3 - Multiple mechanisms for canonical inflammasome activation by *Yersinia*.

A. *Yersinia* hyper-translocate YopD/B (burgundy) into the cell. YopD/B translocated into the cell associate with lysosomal and mitochondrial membranes. Damage to the lysosomes allow for the release of host products. Release of host proteins triggers canonical NLRP3 inflammasome activation. Insertion of YopD/B into mitochondrial membranes leads to ROS production and activation of canonical NLRP3 inflammasome. **B.** *Yersinia* hyper-translocate YopD/B (burgundy) into the cell. YopD/B translocated into the cell associate with lysosomal and mitochondrial membranes. Release of bacterial products from the lysosome activates the non-canonical inflammasome while ROS production as a result of YopD/B's insertion into the mitochondria results in NLRP3 canonical inflammasome activation.

D. YopK evasion of the inflammasome

Yersinia lacking all of the known effectors had been demonstrated to activate the inflammasome *in vitro*. Bacterial burdens of the effectorless strain were lower than wild-type due to caspase-1/11-dependent mechanisms (Brodsky et al., 2010). This observation suggests that *Yersinia* encodes effectors that target the inflammasome pathways in order to allow *Yersinia* to utilize its T3SS without being detected and cleared by the host cell. Several Yops have since been identified to inhibit inflammasome activation during *Yersinia* infection. YopM has been identified to inhibit caspase-1 by either directly binding to caspase-1 or by recruiting the scaffolding protein IQ motif- containing GTPase-activating protein 1 (IQGAP1) (LaRock and Cookson, 2012; Chung et al., 2014). There is also the potential that interactions between YopM and the protein kinase RSK1 may be important for inhibition of inflammasome activation (Chung et al., 2014). Recently, YopJ has been shown to have a small role in inflammasome inhibition in primed macrophages although the mechanism is unclear (Schoberle et al., 2016). YopK was the first effector demonstrated to inhibit inflammasome activation (Brodsky et al., 2010). Deletion of YopK results

in significant caspase-1/11-dependent attenuation of infection *in vivo*. While YopK was the first *Yersinia* effector identified to inhibit inflammasome activation, the mechanism has been unclear. While the T3SS is well conserved among many gram-negative bacteria, YopK has no sequence homolog. Determining the mechanism by which YopK prevents inflammasome activation can potentially identify the presence or absence of functional homologs in other T3SS expressing bacteria. If no functional homologs exist, it would suggest that these bacteria evolved other changes that prevent recognition of the hyper-translocation of their translocon pore proteins.

In addition to preventing inflammasome activation, YopK functions to decrease the size of the translocon pore and to negatively regulate translocation (Holmstrom et al., 1997). Our data suggests a model where YopK prevents activation of the inflammasome by limiting the translocation of YopD and YopB into the cell. Thorslund and colleagues (2011) previously performed a yeast two-hybrid screen to determine if YopK function required interaction with any host proteins. They determined that RACK1 interacted with WT YopK and that only YopK with the mutation TD45-46AA failed to interact with RACK1 in the yeast two-hybrid screen. They further showed that expression of $YopK_{TD45\text{-}46AA}$ allowed for increased translocation compared to WT. We initially sought to test whether interaction between YopK and RACK1 is required to prevent inflammasome activation. We generated the original TD45-46AA mutation in YopK as well as two mutations that were determined to allow for interaction with RACK1 (SK28-29AA and LR43-44AA). We observed that both $YopK_{LR43\text{-}44AA}$ and $YopK_{TD45\text{-}46AA}$ allow for increased translocation of the effectors (Fig. 5-1B). This finding suggests that RACK1 interaction with YopK does not control translocation. Interestingly, both of these mutations failed to prevent inflammasome activation (Fig. 5-1C-E) and were located in the same region of the protein (Fig. 5-1A). Dewoody and colleagues (2013) demonstrated that the change of the charged aspartic acid residue to an alanine that was responsible for the increased translocation of effectors by $YopK_{TD45\text{-}46AA}$. Future work needs to examine whether this single amino acid change is also responsible for allowing

activation of the inflammasome. As the other mutation in the region that allows for increased translocation also changes a charged residue to an alanine (LR43-44AA), it would be beneficial to test whether it is mutation of the charged residue in $YopK_{LR43-44AA}$ that allows for both increased translocation and inflammasome activation. If changes to the charge residues in this region are responsible, it would suggest that this region is important for interactions with host or bacterial molecules that regulate translocation. Determining the regions of YopK that are important for this regulation could help identify the mechanism by which YopK controls translocation and therefore controls inhibition of the inflammasome. One way to determine if YopK actually prevents inflammasome activation by regulating translocation would be to enable *Yersinia* to limit YopB and YopD translocation in a YopK-deficient setting. While based on sound logic, current attempts have failed to complement the intended activity and instead elucidated just how interconnected and balanced the T3SS is.

E. The T3SS is tuned to deliver effector proteins that disrupt host signaling while limiting anti-pathogen innate responses by the target cell

Without the T3SS and its effectors, the three human pathogenic species of *Yersinia* are unable to infect mammals (Gemski et al., 1980a, Gemski et al., 1980b; Portnoy et al., 1981, Cornelis and Wolf-Watz, 1997). Like all bacteria that express T3SS, the effectors encoded by *Yersinia* play important roles in allowing the bacteria to invade, survive, and replicate in their chosen niche. Although essential to establishing a productive infection, the process of translocation must be highly regulated to avoid accidentally triggering innate immune pathways. One such example is the difference between YopJ and YopP, the *Yersinia enterocolitica* homolog of YopJ. While the two proteins are ninety-four percent identical, expression of YopP leads to higher cell death *in vitro* (Brodsky and Medzhitov, 2008). Interestingly, expressing YopP in *Yersinia pseudotuberculosis* during *in vivo* infection leads to an attenuated infection. The sequence difference in YopP allows for the protein to be translocated at a higher level resulting in an

increased inhibition of the NFκB and MAPK pathways (Brodsky and Medzhitov, 2008). While YopJ is thought to be important for penetrating the gut epithelial barrier during infection (Monack et al., 1998), the level of YopJ translocation must be tightly regulated to prevent triggering immune responses that can effectively clear the pathogen (Brodsky and Medzhitov, 2008). As described in our work, disregulated translocation and consequent hyper-translocation of YopD and YopB into the host cell leads to inflammasome activation (Fig. 3-3 – Fig. 3-7), which contributes to control of *Yersinia* infection (Brodsky et al., 2010; Zwack et al., 2015). Precise delivery of effectors and stringent control of T3SS components are required for effective function of the T3SS. Additionally, both effectors and structural components interact with each other to provide regulation (Holmstrom et al., 1997; Mejia et al., 2008; Williams and Straley, 1998). Therefore, modifications used to test one component of the system have the potential to detrimentally affect control of other components.

The balance required for appropriate function of the T3SS can thus make it difficult to dissect the true function of components that regulate T3SS activity. The nature of the T3SS became clear when we attempted to test the hypothesis that YopK prevents inflammasome activation by negatively regulating translocation of YopD and YopB into the host cell. As LcrHmut1 allowed for translocation of effector Yops at normal levels but not YopD or YopB when expressed in a Δ*yopEJK Yptb* strain (Fig. 3-5), we proposed that expression of LcrHmut1 in a Δ*yopK* strain would complement YopK's ability to prevent inflammasome activation and restore virulence. Interestingly, B6 mice completely cleared both WT and Δ*yopK* strains that expressed LcrHmut1 after i.p. infection (Fig. 5-2 and data not shown). Additionally, translocation of YopE, YopM, and YopJ was significantly decreased in WT-*lcrH*mut1 strains in comparison to WT while Δ*yopEJK* and Δ*yopEJK*-lcrHmut1 strains had similar levels of translocation (Fig. 5-3). Furthermore, Δ*yopK*-*lcrH*mut1 allowed for translocation of YopM and YopJ at equivalent levels to Δ*yopK*, but failed to translocate YopE at normal levels (Fig. 5-3). The only difference between Δ*yopK*-*lcrH*mut1 and

Δ*yopEJK*-lcrHmut1 is the presence of YopE and YopJ. YopE is a GTPase-activating protein (GAP) that downregulates Rho, Rac, and Cdc42 activity (Von Pawel-Rammingen et al., 2000). Translocation by the *Yersinia* T3SS has been shown to require activation of Rho downstream of *Yersinia* binding to β1 integrins (Mejia et al., 2008). Furthermore, YopE has previously been demonstrated to contribute to negatively regulate Yop delivery possibly by affecting actin structure at the membrane near the translocon pore (Mejia et al., 2008). This effect could result in a conformational change such as decreased pore size in the already altered translocon complex. Interestingly, YopE is smaller in size than both YopM and YopJ; thus, the decreased translocation is not caused by YopE being too large to fit through the pore. Instead, our findings indicate that a conformational shape change that exposes or hides different epitopes in the translocon is likely responsible for drastically reduced YopE translocation. Additionally, these findings may indicate an order to Yop effector secretion. If YopE is unable to exit the T3SS through the translocon, YopE would most likely block the T3SS and prevent translocation of other Yops. However, YopJ and YopM are translocated at normal levels in the YopK-deficient LcrHmut1 expressing strain. This finding could suggest that YopJ and YopM are translocated first. Alternatively, YopJ and YopM may be translocated normally as enough T3SSs are expressed that not every structure would be blocked by YopE. Thus, YopJ and YopM could be translocated through T3SSs that were not blocked by YopE. Furthermore, these findings imply that Yops have different sensitivities to changes in the T3SS structure (Fig. 6-5). These complex relationships between the T3SS, its effectors, and host cell processes should make changes to the T3SS difficult for bacteria. Understanding these relationships could present targets to render the T3SS ineffective and allow for natural clearance of the bacteria by the host.

Figure 6-5

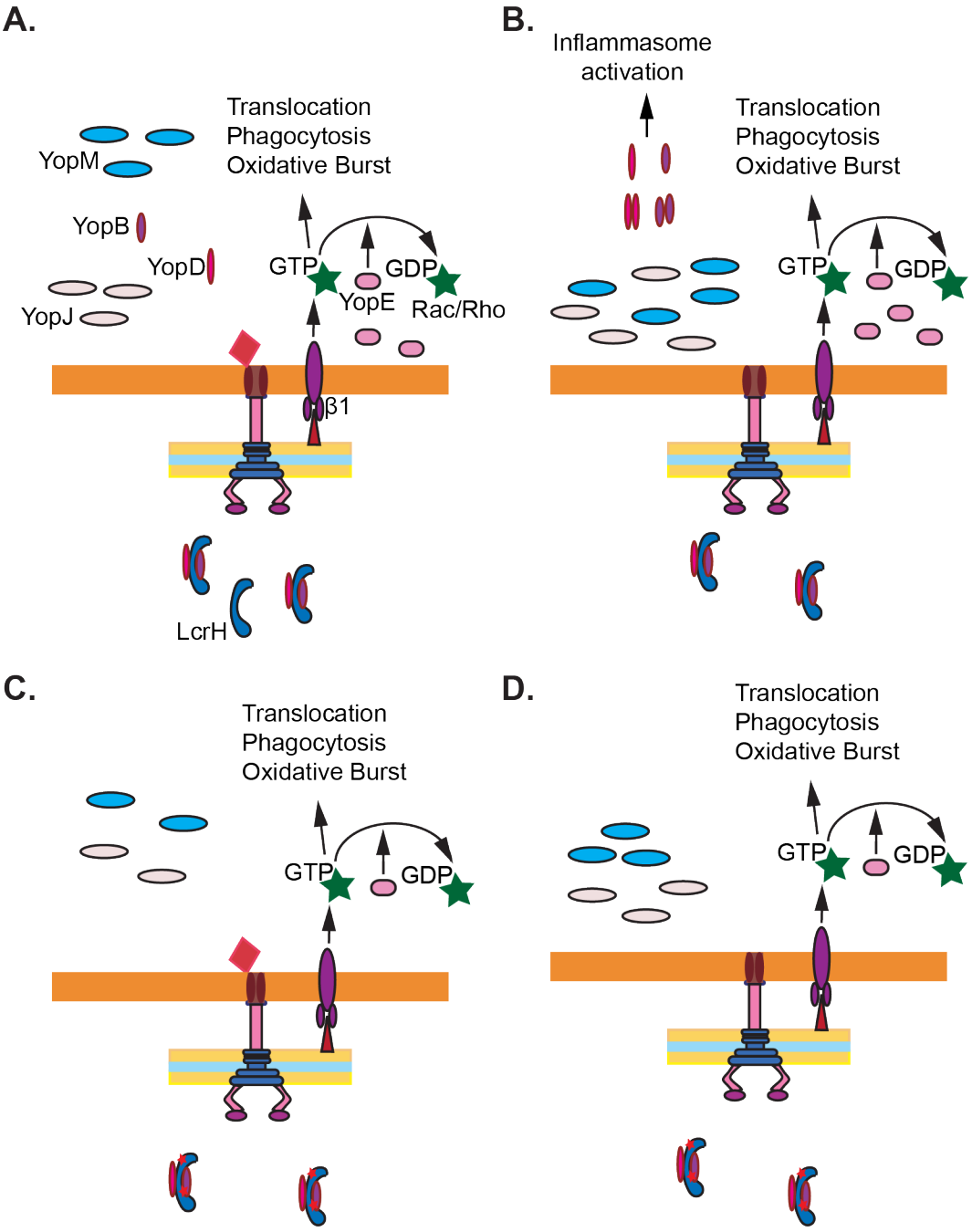


Figure 6-5: Schematic of T3SS translocon and effector interactions. A. WT *Yersinia* allows for sufficient translocation of effector Yops to modulate host cell processes while limiting translocation of the translocon pore proteins. **B.** YopK-deficient *Yersinia* allows for increased

translocation of Yops including the pore proteins YopD and YopB. The increased translocation of YopD and YopB leads to inflammasome activation. **C.** WT *Yersinia* expressing LcrHmut1 translocate smaller quantities of Yops. Mutations in LcrH, the chaperone protein of YopB and YopD, result in smaller translocons. In combination with YopK expression, the translocon pore may be too small to allow for efficient translocation. **D.** YopK-deficient *Yersinia* expressing LcrHmut1 translocate YopJ and YopM at normal levels but demonstrate significantly decreased YopE translocation. As YopE is smaller than both YopJ and YopM, the decrease in YopE translocation is most likely not a result of pore size. Expression of LcrHmut1 may result in a conformational change of the pore that affects a subset of Yops including YopE.

F. A role for alternative therapies in the treatment of bacterial pathogens

With first the discovery of penicillin and then more antibiotics, our ability to combat previously lethal and debilitating bacterial infections increased dramatically. Most of our current antibiotics come from the environment where one microbial organism produces an antibiotic to kill another microbe either to protect its niche or itself (Davies and Davies, 2010). The same organisms often also need to protect from self-intoxication with the antibiotic; therefore, antibiotic resistance normally already exists in the environment (Davies, 1994). Additionally, it has been suggested that certain antibiotic resistance genes originally evolved to play physiological roles separate from antibiotic resistance (Alonso et al., 2001). Often times these antibiotic resistance genes are encoded on mobile genetic elements such as plasmids and thus can be transferred to other microbes under the correct circumstances (Davies, 1994; Martinez and Baquero, 2002). Antibiotic resistance is becoming more of a clinical issue. While most antibiotic resistant bacteria are still susceptible to some antibiotics, multi-drug resistance has emerged in a small number of bacteria that often cause nosocomial infections. One group often referred to as the ESKAPE bugs consist of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Rice, 2008). Alternative therapies to our current antibiotics are needed to combat these infections. By understanding the

pathways that could clear the bacteria, the pathways that bacteria activate to increase pathogenesis, and the mechanics of virulence structures and factors required for survival of the bacteria, we can develop strategies to clear bacteria with the help of the immune system instead of directly killing the bacteria. Identifying pathways such as the inflammasome that would clear the pathogen in the absence of pathogen immune evasion provides targets for therapeutic intervention. Either by inactivating the pathogen's mechanism of evasion or activating a parallel pathway that will generate the same downstream consequences, we could potentially promote pathogen clearance by the immune system. Finally, we need to find ways to counteract virulence structures, factors, and activities that are essential for productive infection. The T3SS is conserved among many bacteria. In fact, the T3SS of *Yersinia* and *Pseudomonas aeruginosa* (one of the ESKAPE bugs) are extremely similar. Inhibitors of *Yersinia*'s T3SS have been found to also inhibit the T3SS of other bacteria (Bailey et al., 2007; Harmon et al., 2010). Through defining the necessary interactions of the T3SS in a model bacterium, we can potentially design therapies that are effective for multiple bacterial infections. Employing multiple strategies to enhance clearance of the bacteria by the immune system can provide alternatives to classical antibiotics.

G. Concluding Remarks

The work performed in this dissertation elucidated both mechanisms by which the host recognizes the presence of *Yersinia*'s T3SS and the potential mechanism by which the *Yersinia* effector YopK prevents inflammasome activation. The mechanics of recognizing internal delivery of pore proteins are potentially broadly applicable among both gram-negative and gram-positive bacteria as well as other pathogens. Additionally, we have revealed further connections between the *Yersinia* effectors and T3SS function. Although effectors differ among bacterial species, there may be certain general rules and interactions required for T3SS function that can be found by understanding the rules and interactions needed for the *Yersinia* T3SS to function. The effort and

energy expended by bacterial pathogens to subvert immune responses demonstrate the importance and efficacy of these responses to control pathogens. Work to understand both the host mechanism that is being subverted and the pathogen mechanism of subverting the host response plays a vital role in defining the pathogenesis of infection. Only by understanding pathogenesis can we develop effective treatments.

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