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**Mechanism of Caspase-1 Activation in Macrophages and its
Role in Pathogenesis by *Yersinia pestis***

A Dissertation Presented

by

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Abstract of the Dissertation

**Mechanism of Caspase-1 Activation in Macrophages and
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Pathogenic *Yersinia* species utilize a type III secretion system (T3SS) to translocate effectors called *Yersinia* outer proteins (Yops) into infected host cells. Previous studies demonstrated a role for effector Yops in the inhibition of caspase-1-mediated cell death and secretion of IL-1 β in naïve macrophages infected with *Y. enterocolitica*. Caspase-1 is a cysteine protease, which, upon activation by a multi-protein complex called the inflammasome, cleaves the precursor forms of the pro-inflammatory cytokines IL-1 β and IL-18 into their mature forms for secretion. Studies utilizing various pathogens, such as *Salmonella* and *Shigella*, have demonstrated a role for a secreted effector in the activation of caspase-1 during infection *in vitro* and *in vivo*.

Naïve murine macrophages were infected with a panel of different *Y. pestis* and *Y. pseudotuberculosis* strains to determine if Yops of these species inhibit caspase-1 activation. Additionally, strains of *Y. pestis* KIM8 (a KIM5 derivative) that lacked regions of the virulence plasmid, pCD1, were tested for the ability to activate caspase-1

in infected macrophages. Using these strains and a mutant expressing a catalytically inactive form of YopJ (KIM5 *yopJ* C172A), I determined that YopJ was required for the activation of caspase-1 during KIM5 infection. Additional experiments utilizing macrophages deficient for various inflammasome components were carried out to determine the possible role of these complexes in caspase-1 activation during KIM5 infection. Another *Y. pestis* strain expressing a variant of YopJ (KIM5 *yopJ*^{L177F E206K}) was tested for virulence and the ability to activate caspase-1 *in vivo*.

I determined that *Y. pestis* KIM5 displays an unusual ability to activate caspase-1 and kill infected macrophages as compared to other *Y. pestis* and *Y. pseudotuberculosis* strains tested. My results indicate that YopJ, as well as the inflammasome components NALP3 and ASC, are required for the activation of caspase-1 and the processing and release of IL-1 β from KIM5 infected macrophages. Additionally, I have shown that KIM5, but not KIM5 *yopJ*^{L177F E206K}, activates caspase-1 *in vivo*. These data demonstrate a novel role for YopJ, as well as the NALP3/ASC inflammasome complex, in the activation of caspase-1 and the production and release of IL-1 β from macrophages infected with *Y. pestis* KIM5.

Table of Contents

List of Tables.....	viii
List of Figures.....	ix
Acknowledgements.....	xii
Chapter 1. Introduction.....	1
1.1. <i>Yersinia</i> pathogenesis.....	1
1.1.1. <i>Yersinia</i> species classification.....	1
1.1.2. <i>Y. pestis</i> pathogenesis.....	1
1.1.3. <i>Y. pestis</i> virulence factors.....	2
1.1.4. Type III Secretion System (T3SS) and <i>Yersinia</i> outer proteins.....	3
1.1.5. <i>Y. pseudotuberculosis</i> pathogenesis.....	5
1.2. Innate immune response to infection.....	6
1.2.1. Toll-like receptors (TLRs).....	6
1.2.2. NOD-like receptors (NLRs).....	7
1.2.3. Nod1 and Nod2.....	8
1.3. The inflammasome and its role in caspase-1 activation and IL-1 β secretion.....	9
1.3.1. Caspase-1.....	10
1.3.2. Interleukin-1 β	10
1.3.3. Interleukin-18.....	11

1.3.4. ICE protein activating factor (Ipaf) and Neuronal apoptosis inhibitory protein 5 (Naip5) inflammasome complexes.....	12
1.3.5. NACHT-LRR-PYD-containing protein 3 (NALP3) inflammasome complex.....	13
1.4. Caspase-1 activation and its role during infection.....	16
1.5. Caspase-1 activation and <i>Yersinia</i> species.....	18
1.6. Hypothesis and rationale.....	20

**Chapter 2. The role of the *Yersinia* effector protein, YopJ, in the activation of
caspase-1 during infection of macrophages with *Y. pestis* strain KIM5.....**

2.1. Summary.....	28
2.2. Introduction.....	28
2.3. Experimental Methods.....	29
2.4. Results.....	37
2.5. Discussion.....	50

**Chapter 3. The NALP3/ASC inflammasome complex and potassium efflux are
important for caspase-1 activation and IL-1 β processing and release from KIM5-
infected macrophages.....**

3.1. Summary.....	77
3.2. Introduction.....	78
3.3. Experimental Methods.....	79
3.4. Results.....	82

3.5. Discussion.....	87
Chapter 4. <i>Yersinia pestis</i> KIM5 activates caspase-1 in CD11b+ cells <i>in vivo</i>.....	98
4.1. Summary.....	98
4.2. Introduction.....	99
4.3. Experimental Methods.....	101
4.4. Results.....	104
4.5. Discussion.....	107
Chapter 5. Concluding remarks and future directions.....	116
References.....	120

List of Tables

Table 2.1. <i>Yersinia</i> strains used in this Chapter and Chapter 3.....	58
Table 2.2. KIM8 pCD1 deletion mutant strains used in this Chapter.....	60
Table 4.1. <i>Yersinia</i> strains used in this Chapter.....	110

List of Figures

Figure 1.1. The <i>Yersinia</i> Type III Secretion System (T3SS).....	22
Figure 1.2. Modulation of host cell signaling by <i>Yersinia</i> outer proteins (Yops)	23
Figure 1.3. Toll-like receptors and NOD-like receptors structure and function.....	24
Figure 1.4. Inflammasome complex activation.....	26
Figure 1.5. Caspase-1 activation through the inflammasome.....	27
Figure 2.1. Determination of levels of IL-1 β secreted from macrophages infected with different <i>Y. pestis</i> or <i>Y. pseudotuberculosis</i> strains.....	61
Figure 2.2. Determination of factors required for IL-1 β secretion in macrophages infected with <i>Y. pestis</i> or <i>Y. pseudotuberculosis</i>	62
Figure 2.3. <i>Y. pestis</i> KIM5 grown at 37°C, but not at 28°C, induces maximal IL-1 β secretion during infection.....	64
Figure 2.4. Bacterial protein synthesis and intracellular survival are important for IL-1 β secretion.....	65
Figure 2.5. Time course analysis of cytokine secretion from macrophages infected with <i>Y.</i> <i>pestis</i>	66
Figure 2.6. Determination of factors required for cell death in macrophages infected with <i>Y. pestis</i>	67
Figure 2.7. Substitution of pIB102 for pCD1 in KIM5 does not stimulate IL-1 β secretion in infected macrophages.....	69
Figure 2.8. Identification of a region of pCD1 required for IL-1 β secretion and cell death in macrophages infected with <i>Y. pestis</i>	70

Figure 2.9. Detection of active caspase-1 in macrophages infected with <i>Y. pestis</i> by fluorescence microscopy.....	71
Figure 2.10. Analysis of bacterial association and intracellular survival in macrophages infected with <i>Y. pestis</i>	72
Figure 2.11. Determination of the role of bacterial phagocytosis for IL-1 β secretion and cell death in macrophages infected with <i>Y. pestis</i>	73
Figure 2.12. Determination of the role of YopH and YopO for IL-1 β secretion in macrophages infected with <i>Y. pestis</i> mutants.....	74
Figure 2.13. Determination of the role of YopJ for IL-1 β secretion, TNF- α secretion and cell death in macrophages infected with <i>Y. pestis</i> or <i>Y. pseudotuberculosis</i>	75
Figure 2.14. Determination of the importance of caspase-1 for IL-1 β secretion and cell death by infection of <i>Casp</i> ^{-/-} macrophages with <i>Y. pestis</i>	76
Figure 3.1. Determination of the importance of the inflammasome for IL-1 β and IL-18 secretion by infection of macrophages deficient for NALP3, ASC and Ipaf with KIM5.....	91
Figure 3.2. Determination of the importance of the inflammasome for TNF- α secretion and induction of cell death by infection of macrophages deficient for NALP3, ASC and Ipaf with KIM5.....	92
Figure 3.3. Determination of the minimal concentration of potassium chloride (KCl) needed to inhibit IL-1 β secretion from macrophages infected with KIM5.....	93

Figure 3.4. IL-1 β secretion, but not TNF- α production, from KIM5-infected macrophages is significantly inhibited in the presence of 30 mM KCl at 8 hr and 24 hr post-infection.....	94
Figure 3.5. Determination of the importance of the P2X ₇ receptor (P2X ₇ R) for cytokine production and induction of cell death by infection of P2X ₇ R-deficient macrophages with KIM5.....	96
Figure 3.6. Proposed model for caspase-1 activation in response to KIM5.....	97
Figure 4.1. Susceptibility to KIM5 infection of mice deficient for caspase-1.....	111
Figure 4.2. Alignment of the amino acid sequence of YopJ from different <i>Yersinia</i> strains.....	112
Figure 4.3. Susceptibility of mice to KIM5 or to the double mutant KIM5yopJ ^{L177F E206K}	113
Figure 4.4. Analysis of bacterial organ burden and caspase-1 activation in macrophages isolated from infected spleens.....	114
Figure 4.5. Survival of mice infected with <i>Y. pestis</i> strains.....	115

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Chapter 1: Introduction

1.1 *Yersinia* Pathogenesis

1.1.1 *Yersinia* species classification. The genus *Yersinia* is composed of 11 species, of which three (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*) are pathogenic to humans. *Y. pestis* is a Gram-negative bacterium and the etiological agent of plague, an acute and often fatal disease of both humans and animals (103). *Y. enterocolitica* and *Y. pseudotuberculosis* are primarily enteropathogens that cause self-limiting infections of the gastrointestinal tract (9, 109). Twenty-one different serogroups of *Y. pseudotuberculosis* have been identified, while *Y. enterocolitica* encompasses a heterogeneous group of organisms that have been classified into six biogroups (1, 126). Minor phenotypic differences have been used to classify *Y. pestis* into three biovars (Antiqua, Mediaevalis and Orientalis), each of which was historically associated with causing a major pandemic (103). Recent studies have demonstrated that *Y. pestis* is closely related to *Y. pseudotuberculosis* as they are highly similar at the chromosomal level; *Y. enterocolitica* is less closely related (1, 17, 57, 132). The studies presented in this dissertation deal specifically with *Y. pestis* and *Y. pseudotuberculosis* and therefore *Y. enterocolitica* will not be discussed.

1.1.2 *Y. pestis* pathogenesis. *Y. pestis*, also known as the Black Death or plague, caused immense devastation in Europe during the Middle Ages, killing millions. The World Health Organization recognizes plague as a re-emerging infectious disease, although it is not a major public health issue today due to the advent of effective antibiotics and public

health measures (19, 103). *Y. pestis* is typically transmitted through the bite of an infected flea that has previously fed on an infected rodent. Upon entry into the human host, *Y. pestis* can establish itself in the local draining lymph nodes, causing the characteristic swelling and tenderness associated with bubonic plague called buboes. The infection can disseminate into the bloodstream causing septicemic plague, leading to the infection of internal organs such as the spleen, liver and lungs. Colonization of the lungs produces a secondary pneumonia, which is easily transmissible from person to person by aerosolized droplets released by coughing or sneezing (103). Inhalation of these droplets by a susceptible individual causes primary pneumonic plague. *Y. pestis* has been classified as a Category A agent by the Centers for Disease Control and Prevention, due to its ease of transmission by the pulmonary route as well as its potential use as a bioterrorism agent. In 1994, an outbreak of pneumonic plague in Surat, India demonstrated that plague has not been eradicated and that it is still one of the most feared infectious diseases (103, 132).

1.1.3 *Y. pestis* virulence factors

It has been concluded that *Y. pestis* is closely related to *Y. pseudotuberculosis* (1, 12, 132). However, *Y. pestis* does contain unique pathogenicity factors, including two additional plasmids not found in *Y. pseudotuberculosis*, pMT1 and pPCP1, which are required for its virulence (108). pMT1 (~100 kb) encodes for the production of the capsule fraction 1 (F1) protein at 37°C to hinder phagocytosis; pPCP1 (9.6kb) encodes for the plasminogen activator (Pla), which allows for dissemination of the bacteria into the host after subcutaneous infection (12, 108, 109). Another factor present in *Y. pestis*

that is important for virulence is ~102 kb of chromosomal DNA called the pigmentation locus (*pgm*). Two regions within the *pgm* locus play an important role in *Y. pestis* virulence, *hms* (hemin storage) and *ybt* (yersiniabactin). The *hms* locus is involved in hemin adsorption; *hms*⁺ bacteria plated on Congo Red and incubated at 28°C cause the formation of pigmented colonies. The expression of *hms* is essential for transmission of plague from the flea vector into the mammalian host (103, 110). The *ybt* locus encodes for a siderophore, a small compound synthesized and secreted by the bacterium for iron acquisition, and plays a critical role at an early stage of infection (110). The *pgm* locus undergoes spontaneous deletion at high frequency and the loss of virulence in Δ *pgm* mutants is due mainly to the loss of *ybt*. Other genes have been identified in the *pgm* locus by Pujol *et al.* designated *rip* (required for intracellular proliferation); when deleted, there is a greatly decreased ability for the bacteria to survive in macrophages activated with IFN- γ (110).

1.1.4 Type III Secretion System (T3SS) and *Yersinia* outer proteins

All pathogenic *Yersinia* harbor a ~70 kb virulence plasmid (called pCD1 in *Y. pestis*, and pYV in *Y. pseudotuberculosis* and *Y. enterocolitica*) which encodes a type III secretion system (T3SS) and effector proteins called *Yersinia* outer proteins (Yops) (Figure 1.1). The T3SS is a needle-like apparatus that is maximally expressed at 37°C and is downregulated at ambient growth temperatures. When *Yersinia* growing at 37°C contact a host cell, the T3SS is activated to secrete and translocate Yops into the target cell (19, 111). Additionally, induction of the T3SS can occur under low calcium conditions *in vitro* (111).

There are six effector Yops (YopE, YopJ/YopP, YopH, YopT, YopM, and YopO/YpkA) along with two translocator Yops (YopB and YopD) (Figure 1.2). The translocation of Yops into the host cell is dependent on YopB and YopD. Both YopB and YopD have hydrophobic domains, which are thought to insert into the plasma membrane to form a channel to allow the T3SS to inject effector Yops (20, 136). The effector Yops are involved in disrupting or inhibiting many cellular pathways, including phagocytosis, cytokine production and survival (19, 20, 136). YopE, YopT and YpkA/YopO all target Rho GTPases which are involved in regulating a number of cellular functions, including actin cytoskeleton rearrangement and gene expression (136). YopE acts as a GTPase-activating protein (GAP), which can inactivate the Rho GTPases. YopT is a cysteine protease that can cleave Rho GTPases at their membrane anchor, releasing them into the cytosol. YopO (YpkA in *Y. pseudotuberculosis*) is a Rho GTPase guanosine nucleotide dissociation inhibitor (GDI) and a serine/threonine kinase which is activated by actin binding; one specific target of YopO kinase activity within the cell is G α q (92). YopH is a protein tyrosine phosphatase that has an anti-phagocytic property; it can block signaling from β 1 integrins, inhibiting bacterial internalization (20). YopM is the only effector whose exact function still remains unknown. It contains a variable number (12-20) of ~20-amino acid leucine-rich repeats (LRRs); the variability in number contributes to the heterogeneity in size of YopM among the Yersiniae. It has been shown that YopM can localize to the nucleus, suggesting that it may be involved in regulating genes in the cell cycle (121), but no conclusive evidence has been found (56). Lastly, YopJ (YopP in *Y. enterocolitica*) is an acetyl transferase that counteracts the pro-inflammatory response in

the host cell by inhibiting the mitogen-activated protein kinase (MAPK) and NF- κ B pathways; induction of apoptosis by YopJ has also been demonstrated (20, 100, 144).

Some of the Yops previously mentioned may also play unexpected roles in counteracting the innate immune response. For instance, YopH inhibits the production of monocyte chemoattractant protein 1 (MCP-1), which is involved in recruitment of macrophages to the site of infection (120). YopM targets the innate immune response by depleting natural killer (NK) cells from mice infected with *Y. pestis* (65). Lastly, YopE, and to a certain extent YopT, inhibit caspase-1 mediated maturation and release of IL-1 β in infected macrophages by targeting Rac1 (122).

1.1.5 *Y. pseudotuberculosis* pathogenesis. *Y. pseudotuberculosis* is a food-borne pathogen, which generally causes a self-limiting gastroenteritis. The bacteria are transmitted by the fecal-oral route and enter the small intestine after ingestion. The bacteria initially invade M cells, which are specialized immune cells located above the Peyer's patches of the gut associated lymphoid tissues. *Y. pseudotuberculosis* crosses the epithelial barrier via transport through M cells and subsequently replicates within Peyer's patches and mesenteric lymph nodes. In some rare instances, *Y. pseudotuberculosis* can spread systemically, leading to septicemia (20, 109, 136).

1.2 Innate immune response to infection

1.2.1 Toll-like receptors (TLRs)

Innate immunity plays a crucial role in the recognition of pathogens. Professional antigen presenting cells (i.e., macrophages and dendritic cells) serve a number of functions, including pathogen detection, antimicrobial responses and activation of the adaptive immune response (23). Pathogen detection by macrophages and dendritic cells is mediated in part by a family of pathogen recognition receptors called Toll-like receptors (TLRs). Studies in *Drosophila melanogaster* demonstrated that the protein Toll was involved not only in embryonic development, but also in innate immunity. Flies lacking Toll were highly susceptible to fungal and bacterial infections as compared to wild-type flies. These results led to the discovery of the first Toll-like proteins in mammals (55). Approximately 10 TLRs have been identified in humans (11 *Tlr* genes in mice) and these can recognize specific bacterial and viral products, called pathogen associated molecular patterns or PAMPs. Examples of TLR ligands include lipopolysaccharide (LPS), a component of Gram-negative bacteria; flagellin, a component of bacterial flagella; and peptidoglycan (PGN), a component common to all bacteria, with the exception of *Mycoplasma* and *Chlamydia* spp. (28, 105).

TLRs are integral membrane proteins composed of an extracellular LRR motif and an intracellular signaling domain called Toll-interleukin receptor (TIR), which interacts with an adaptor molecule called MyD88 (Figure 1.3A) (28, 105). Upon recognition of a specific PAMP by a specific TLR, a signaling cascade occurs and leads to the activation of kinases in the interleukin-1 receptor associated kinase (IRAK) family.

This leads to the recruitment of TRAF6, activation of the IKK complex and subsequent phosphorylation of I κ B α . Phosphorylation of I κ B α allows the transcription factor NF- κ B to translocate into the nucleus and induce the transcription of pro-inflammatory mediators, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) (23, 105).

1.2.2 NOD-like Receptors (NLRs)

The NOD-like receptors (NLRs) are a family of cytosolic pathogen recognition receptors, which play similar roles as TLRs, except they recognize PAMPs intracellularly (Figure 1.3B). As many as 23 NLR genes have been identified in humans and approximately 34 NLR genes in mice (62). NLRs have a tripartite structure consisting of an N-terminal effector region comprised of either a caspase activation and recruitment domain (CARD), PYRIN (PYD) or baculovirus inhibitor of apoptosis repeat domain (BIR), involved in protein-protein interactions; a centralized NACHT or NOD region required for nucleotide binding and self-oligomerization; and a C-terminal LRR involved in pattern recognition (40, 41, 62, 68, 131). It is thought that the C-terminal end of the NLR folds back over the NOD region, inhibiting self-oligomerization and activation of the NLR. When the LRR recognizes a PAMP, a conformational change occurs, exposing the NOD region and allowing for oligomerization as well as recruitment of effector molecules to the N-terminal region of the NLR for subsequent downstream signaling (27, 32, 62, 131). There are a number of members of the NLR family, which are classified into subfamilies called NODs, NAIPs and NALPs based on the N-terminal domains they contain.

1.2.3 Nod1 and Nod2

Nod1 and Nod2 are members of the NOD subfamily that were the first NLRs to be characterized (95). These NLRs are structurally similar to plant R proteins, which are involved in resistance against plant pathogens (31, 105). Recognition of intracellular pathogens by either Nod1 or Nod2 occurs through the recognition of meso-diaminopimelic acid (meso-DAP), a PGN degradation product, or muramyl dipeptide (MDP), the minimal motif found in all PGN, respectively (5). Sensing these specific motifs in PGN allows for the detection of Gram-negative or both Gram-negative and Gram-positive bacteria by Nod 1 and Nod2, respectively (28, 40, 41, 95, 105). Nod1 is ubiquitously expressed, while Nod2 expression is restricted to macrophages, dendritic cells, neutrophils and Paneth cells of the small intestine (23, 28). Activated Nod proteins can interact with RIP2, a serine/threonine kinase, through CARD-CARD interactions. Once RIP2 is activated, it can activate the IKK complex, leading to the phosphorylation of I κ B α and subsequent translocation of NF- κ B to the nucleus to activate transcription of pro-inflammatory cytokines (40, 133). Additionally, it has been demonstrated that the MAP kinase (MAPK) pathway can also be activated, specifically p38, JNK and ERK (62). Mutations in Nod1 and Nod2 have been associated with a number of inflammatory diseases such as asthma, atopic eczema, Crohn's disease and Blau syndrome (23, 44, 94). Nod-1 dependent NF- κ B activation has been observed in response to infection by *Helicobacter pylori*, *Shigella flexneri* and enteroinvasive *E. coli* (10, 45, 66, 106, 135). Since PGN components must be present intracellularly for the Nod proteins to be activated, it is hypothesized that the delivery of meso-DAP occurs either by invasion of the cytosol by intracellular bacteria or by the delivery of these products through bacterial

secretion systems, such as the Type IV secretion system of *H. pylori* (45, 135). Nod2 has been shown to play an important role in the clearance of *Listeria monocytogenes* after oral, but not intraperitoneal or intravenous, challenge (67). In contrast to NF- κ B activation by Nod1 and Nod2, the NLRs Ipaf/Naip5 and NALP3 are involved in the activation and assembly of a multi-protein complex called the inflammasome, which is responsible for the activation of the effector caspase, caspase-1 (discussed below).

1.3 The inflammasome and its role in caspase-1 activation and IL-1 β secretion

Tschopp and colleagues previously identified a multi-protein complex composed of the NLR NALP1, the adaptor protein ASC and the effector caspases, caspase-1 and caspase-5; this complex was named the inflammasome (Figure 1.4) (80). Their results were based on previous observations, which demonstrated that the activation of caspase-8 and caspase-9 involves procaspase recruitment to the oligomerized adaptors FADD and Apaf-1, respectively, forming an apoptosome (21, 75). Since then, many other inflammasome complexes have been identified and named after the NLR they contain (discussed further in sections 1.3.3 and 1.3.4) (76). ASC (apoptosis-associated speck-like protein containing a CARD) is a 22 kDa adaptor protein involved in recruiting other CARD-containing proteins to the inflammasome as well as serving as a bridge between NALP1, NALP3 or Ipaf and caspase-1. The inflammasome complex can recognize various stimuli and become activated, leading to the activation of caspase-1 and the subsequent processing and release of IL-1 β and IL-18.

1.3.1 Caspase-1

Caspases are a family of cysteine proteases, which upon activation can lead to apoptosis (programmed cell death) or to cytokine production. These proteases can be divided into two subfamilies: apoptotic caspases and inflammatory caspases. Apoptotic caspases can be further divided into initiator caspases (caspase-2, -8, -9, and -10) which are responsible for initiating the caspase activation cascade and effector caspases (caspase-3, -6, and -7) involved in the destruction of the cell during apoptosis (21). The remaining caspases (caspase-1, -4, -5, and mouse caspase-11 and -12) compose another family of caspases called the inflammatory caspases (84, 123). Of these, caspase-1 (also called ICE or IL-1 β converting enzyme) is involved in the processing and release of the pro-inflammatory cytokines IL-1 β and IL-18. Caspase-1 is synthesized as a 45kDa inactive zymogen and is self-activated by cleavage at internal aspartic acid residues to generate an active heterotetramer composed of two p10 and two p20 subunits (13, 21, 75). Activation of caspase-1 occurs through its recruitment to the inflammasome complex (70). Upon activation of caspase-1, cleavage of pro-IL-1 β and pro-IL-18 occurs and the mature forms of these cytokines are secreted.

1.3.2 Interleukin-1 β

Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine produced mainly from monocytes, macrophages and dendritic cells and is involved in the innate immune response. It is a potent endogenous pyrogen, causing fever, hypotension, synthesis of adhesion molecules, and production of the acute phase response, as well as a primary cause of acute and chronic inflammation (26, 34, 80, 81). Unregulated production of IL-1 β can be

deleterious to the host, leading to excessive inflammation and septic shock. The expression of IL-1 β is tightly regulated, in that distinct signals are required for its transcription and its processing and release (Figure 1.5) (13, 21, 34, 75, 96). The first signal can be transmitted via stimulation of a TLR signaling pathway (e.g., TLR4 recognition of LPS), leading to activation of the transcription factor NF- κ B and the expression of the 35 kDa precursor form of IL-1 β . A secondary signal is required to activate the inflammasome complex, leading to the activation of caspase-1 and subsequent processing and secretion of the 17 kDa mature form of IL-1 β . As IL-1 β lacks a signal sequence that would direct it to the Golgi for secretion, it is secreted by a non-conventional pathway, and there are a number of proposed models as to the mechanism of secretion (13, 29).

1.3.3 Interleukin-18

Interleukin-18 (IL-18, also known as interferon (IFN)- γ inducing factor) is a pro-inflammatory and immunoregulatory cytokine of the IL-1 cytokine family and is produced mainly by macrophages and dendritic cells (7, 26). Similar to IL-1 β , IL-18 is produced as a precursor (24 kDa) that is cleaved into a mature form (18 kDa) by active caspase-1. IL-18 drives the Th1 response in conjunction with IL-12 and IL-15 and upregulates the production of nitric oxide synthase, cell adhesion molecules and chemokines (7, 26, 81). Most notably, IL-18 is involved in stimulating the production of IFN- γ and TNF- α . Previous studies utilizing extracellular and intracellular bacteria have demonstrated that IL-18 can enhance host defenses and participate in resistance to

infection (7). Just as excessive IL-1 β is deleterious to the host, excess IL-18 has a similar effect.

1.3.4 ICE protease activating factor (Ipaf) and Neuronal apoptosis inhibitory protein 5 (Naip5) inflammasome complexes

ICE protease activating factor (Ipaf) and Neuronal apoptosis inhibitory protein 5 (Naip5) are members of the NAIP family of NLRs. Naip5 structurally resembles Ipaf except for differences in the N-terminal effector region, which contains three BIR regions instead of a CARD domain. The Ipaf inflammasome has been shown to recognize bacterial flagellin from *Pseudomonas aeruginosa* and *Salmonella typhimurium*, while Ipaf and Naip5 recognize flagellin from *Legionella pneumophila* (38, 40, 76, 87, 113, 114, 118, 127, 129). Ren *et al.* initially identified flagellin as the microbial product that elicited a rapid caspase-1-dependent cell death, which led to the restriction of *Legionella* growth intracellularly (113). Naip5⁺ C57BL/6 macrophages were able to restrict *Legionella* growth, while *naip5* mutant A/J macrophages, which are susceptible for *Legionella* growth, or C57BL/6 macrophages infected with a *flaA* mutant, which lacks flagellin, allowed the bacteria to replicate (89, 113). In the absence of Ipaf or caspase-1 activation, *Legionella* were able to replicate in phagosomes that avoided fusion with lysosomes (3). Additional studies demonstrated that the recognition of flagellin occurred in a TLR5-independent manner and required the Type IV secretion system of *Legionella* (T4SS) (89, 113). It has been proposed that both Ipaf mediated caspase-1 activation and Naip5 signaling are essential to inhibit *Legionella* replication within macrophages (69, 142). When caspase-1 activation was observed to occur in response to *Salmonella* infection,

Ipaf was shown to be required for caspase-1 activation, but it was unknown as to what component of *Salmonella* was being recognized for this response (77). Franchi *et al.* and Miao *et al.* independently identified Ipaf as the NLR required for caspase-1 activation and IL-1 β secretion from macrophages in response to cytoplasmic flagellin from *Salmonella* (38, 87). *Salmonella* strains lacking flagellin or expressing mutant flagellin were unable to activate caspase-1 in infected macrophages (38). Additionally, Ipaf activation was observed in macrophages where purified flagellin was introduced directly into the cytoplasm (87). The activation of Ipaf was dependent on an intact *Salmonella* T3SS as well as independent of TLR5 (38, 87). Several mechanisms have been proposed as to how caspase-1 activation occurs through Ipaf. Both Franchi *et al.* and Miao *et al.* have suggested that flagellin may enter the macrophage cytosol by way of the T3SS apparatus during delivery of effectors or through pores formed in the phagosome membrane. As for how Ipaf senses flagellin, flagellin may directly bind to Ipaf or flagellin may indirectly activate Ipaf by altering a cellular protein or activating an alternate cellular process that recognizes flagellin (38, 87). Recent studies utilizing *Pseudomonas aeruginosa* have paralleled previous studies with *Salmonella* and have demonstrated that the Ipaf inflammasome plays a key role in detection of flagellin from pathogens that utilize the T3SS (70, 88, 127).

1.3.5 NACHT-LRR-PYD-containing protein 3 (NALP3) inflammasome complex

NACHT-LRR-PYD-containing protein 3 (NALP3), a member of the NALP subfamily, in conjunction with the adaptor protein ASC, forms an inflammasome complex that is responsive to both microbial products and non-microbial “danger” signals (5, 60, 62, 63,

70, 82). Numerous studies have demonstrated that bacterial RNA, LPS, lipoprotein and PGN can activate NALP3, specifically in the presence of millimolar concentrations of adenosine triphosphate (ATP) (5, 60, 62, 70, 79, 128). ATP is released from damaged cells or cells undergoing death. Extracellular ATP activates the cation selective purinergic receptor P2X₇, which rapidly induces the collapse of normal ionic gradients, including the release of intracellular potassium (74, 102). Additionally, a larger non-selective channel opens, mediated by the hemichannel pannexin-1, which is recruited upon P2X₇ activation (61, 102). Pannexin-1 was found to be important for the activation of caspase-1 and IL-1 β secretion in LPS-stimulated macrophages treated with ATP (102, 107). Additionally the pannexin-1 pore may serve as a delivery system for microbial products into the host cytosol, leading to NALP3 activation (61).

Previous studies utilizing both human and mouse macrophages have shown that caspase-1 activation occurs due to potassium efflux, which is induced by P2X₇ receptor activation or through the use of pore-forming toxins. Walev *et al.* treated human monocytes with alpha-toxin and monitored the activation of caspase-1 by measuring IL-1 β secretion by ELISA. Caspase-1 activation was blocked in human monocytes through the use of potassium channel blockers or medium containing high concentrations (~150mM) of potassium (138). Their results indicated that potassium efflux might play a direct or indirect role in the activation of caspase-1. Fernandes-Alnemri *et al.* treated human macrophages with LPS and the TLR2 agonist Pam3CSK4 to induce caspase-1 activation and investigated the role of ASC in pyroptosis (33). They determined that under these conditions, a large supramolecular complex composed solely of ASC formed; this was termed a pyroptosome. Stimulation of human macrophage-like THP-1 cells with

potassium depleting agents induced the assembly of this complex, while blocking potassium efflux inhibited pyroptosome formation. Pyroptosis is a recently described form of cell death that is inherently pro-inflammatory and caspase-1 dependent (36); in macrophages, this form of cell death could be mediated by the pyroptosome. Petrilli *et al.* showed that the activation of the NALP3 inflammasome was triggered by low intracellular potassium concentrations in both mouse and human macrophages; inflammasome activation was blocked in the presence of 70 mM potassium chloride (104). Mariathasan *et al.* and Sutterwala *et al.* demonstrated through the use of NALP3- and ASC-deficient mice that NALP3 and ASC are required for inflammasome activation and subsequent caspase-1 activation and IL-1 β secretion in response to nigericin (a potassium ionophore), maitotoxin (shellfish toxin) and ATP, but not to *Salmonella* (79, 128). The NALP1b inflammasome, another member of the NALP subfamily, has been shown to recognize *Bacillus anthracis* (anthrax) lethal toxin (LT) and activate caspase-1 and induce pyroptosis (11, 35). Additionally, inflammasome formation was shown to be dependent on ion fluxes, such as potassium efflux, and proteasomal degradation of host proteins for initiation of cell death (141).

Certain host “danger” signals can be sensed by NALP3 and activate caspase-1. Both monosodium urate crystals and calcium pyrophosphate dihydrate crystals, from the conditions gout and pseudogout, respectively, have been shown to activate the NALP3 inflammasome in the absence of ATP (83). More recently, aluminum hydroxide adjuvants, widely used in human and animal vaccines, silica and asbestos have been shown to activate the NALP3 inflammasome and induce the production of IL-1 β and IL-18 (15, 30). Gram-positive, but not Gram-negative, bacteria have also been shown to

activate NALP3, specifically *Listeria monocytogenes* and *Staphylococcus aureus* (39, 79, 98). Listeriolysin O (LLO), a major virulence factor and pore-forming toxin, promotes the escape of *Listeria* from phagosomes into the cytosol. Macrophages infected with bacteria deficient for LLO were not able to activate caspase-1 or secrete IL-1 β , indicating that *Listeria* escape into the cytosol is important for detection by NALP3 (128). The toxins required for *Staphylococcus*-induced inflammasome activation remain unclear, since deletion of α -, β - or γ -toxins had no effect on caspase-1 activation. More research needs to be done to test other pathogens, Gram-positive or -negative, in order to determine if they too can activate caspase-1 through the NALP3 inflammasome.

1.4 Caspase-1 activation and its role during infection

Previous studies have shown that caspase-1 is important for the host innate immune response against a number of pathogens, including *Salmonella* and *Francisella* (71, 78, 91, 119). Earlier published reports had indicated that the *Salmonella* invasion protein, SipB, as well as the *Shigella* invasion plasmid antigen, IpaB, bound directly to and activated caspase-1 (53, 54, 130). These results have remained controversial; some have suggested the possibility that either the T3SS itself or an as yet unidentified protein may be the inducers of caspase-1 activation (71). SipB, and its homolog IpaB, insert into the host cell membrane to facilitate the translocation of effectors; *sipB* or *ipaB* mutants are defective for translocation as well as for caspase-1 activation (50, 85, 86). Others have demonstrated that cytosolic flagellin from *Salmonella* and an as yet unknown substrate from *Shigella* can activate caspase-1 and IL-1 β through Ipaf (discussed in section 1.3.4) (38, 87, 129).

In vivo studies have demonstrated an important role for caspase-1 in controlling infections by *Salmonella* and *Shigella*. It is suggested that by activating caspase-1 during infection, these bacteria induce inflammation and tissue destruction, leading to their dissemination to other organs and tissues of the host; eventually, however, the inflammatory response does eradicate the infection (71, 91, 112, 119). Activation of caspase-1 can actually help control the infection, as shown by the use of caspase-1-deficient mice, which are highly susceptible to infection and are unable to efficiently eradicate the bacteria (71, 119). Sansonetti *et al.* utilized caspase-1-, IL-18- and IL-1 β -deficient mice to determine the role of caspase-1 activation *in vivo* during infection with *Shigella*. Using the murine lung model of infection, they showed that caspase-1- and IL-18-deficient mice, but not IL-1 β -deficient mice, were unable to generate an effective inflammatory response or resolve the infection; these mice succumbed to infection significantly more often than wild-type mice (119). Recombinant IL-1 β enhanced the bacterial load and inflammatory response in caspase-1-deficient mice, but IL-1 β -deficient mice had more localized inflammation and restricted bacterial distribution than wild-type mice (119). These results suggested that caspase-1 activation and production of IL-1 β *in vivo* might lead to tissue destruction during infection, allowing *Shigella* to disseminate into other tissues of the host. This suggests that caspase-1 activation, which in turn cleaves pro-IL-1 β and pro-IL-18, induces an inflammatory response that helps control the infection (112, 119). Numerous animal studies utilizing *Salmonella* indicate that caspase-1, IL-1 β and IL-18 are all necessary for efficient colonization of the gastrointestinal tract in the mouse model of infection (58, 71, 90, 91, 112). Lara-Tejero *et al.* and Raupach *et al.* demonstrated that caspase-1 deficiency renders mice significantly more susceptible to

infection after oral challenge as compared to wild-type mice (71, 112). On the contrary, Monack *et al.* demonstrated that caspase-1-deficient mice were resistant to *Salmonella* colonization of Peyer's patches after oral infection as compared to wild-type mice (90). Wild-type or caspase-1-deficient mice inoculated intraperitoneally had no significant differences in colonization. It was suggested that the contrasting results between Monack *et al.* and Raupach *et al.* could be attributed to genetic differences in the mouse strains used (90, 112). Lastly, recent studies using *Francisella tularensis*, the causative agent of tularemia, have demonstrated that innate immunity against this pathogen is not only dependent on ASC and caspase-1, but that inflammasome activation occurs through type I interferon signaling (51, 52, 78). Caspase-1 and ASC-deficient mice were infected subcutaneously with *F. tularensis* subspecies *novicida*; these mice succumbed to infection more rapidly than wild-type animals and contained higher bacterial burdens (78). These results demonstrate an essential role for caspase-1 in the control and clearance of pathogens during *in vivo* infection.

1.5 Caspase-1 activation and *Yersinia* species

Previous studies have examined caspase-1 activation and IL-1 β secretion in macrophages infected with *Yersinia* species (6, 122, 124). Schotte *et al.* (122) examined these responses after they infected murine Mf4/4 macrophage-like cells with *Y. enterocolitica* E40 as well as different single or multiple *yop* mutants of this strain and measured IL-1 β secretion by Enzyme-linked immunosorbent assay (ELISA). Their results showed that a multi-effector mutant (*yopHOPEMT*) and to a certain extent a *yopPE* mutant could induce high amounts of IL-1 β secretion from infected macrophages. Additionally, their

results suggested that YopP (YopJ) could inhibit expression of pro-IL-1 β and that YopE could inhibit caspase-1 activation and IL-1 β secretion in *Y. enterocolitica*-infected Mf4/4 cells (122). Inhibition of caspase-1 activation was also observed when either YopE or YopT were overexpressed by transfection in cultured HEK293T cells. Overall, these findings implicated YopP as an inhibitor of pro-IL-1 β synthesis, and YopE and YopT as inhibitors of caspase-1 activation. Shin and Cornelis (124) investigated IL-1 β release from macrophages infected with *Y. enterocolitica*. They obtained evidence that pores generated by the T3SS translocation channel trigger activation of caspase-1 in Mf4/4 cells infected with a *Y. enterocolitica* E40 multi-*yop* effector mutant. Thus, in their model (46), YopE and YopT inhibit activation of caspase-1 by counteracting T3SS-dependent pore formation (136). More recently, Bergsbaken and Cookson obtained evidence that *Y. pseudotuberculosis* strain YPIII could induce caspase-1 activation in murine bone-marrow derived macrophages (BMDMs) that were activated by pre-exposure to LPS (6). They also observed that LPS-activated BMDMs infected with YPIII died of a caspase-1 mediated form of cell death termed pyroptosis (6, 36). It was hypothesized that the T3SS in YPIII transports an inflammasome-stimulating factor into the macrophage cytosol, and that priming macrophages with LPS overcomes the ability of translocated effector proteins to inhibit the activation of caspase-1 (6). Additionally, they observed caspase-1 activation in macrophages infected with a multi-effector mutant of *Y. pestis*, but not with *Y. pestis* lacking pCD1, suggesting the T3SS was required for this response. It is possible that a factor or factors encoded on pCD1 may be involved in activating caspase-1 during infection.

1.6 Hypothesis and Rationale

In this dissertation, the mechanism of caspase-1 activation and IL-1 β secretion during *Y. pestis* infection of macrophages was examined. Here, we aim to determine what factor or factors encoded by *Y. pestis* KIM5 activate caspase-1, the role of the inflammasome in this response and how caspase-1 acts in response to KIM5 infection *in vivo*. The results obtained during this study are broken down into three chapters to further elucidate the role of caspase-1 during KIM5 infection. Chapter 2 tackles the question of how *Y. pestis* KIM5 can activate caspase-1 in infected macrophages *in vitro*. We hypothesized that a factor encoded by KIM5 has the ability to activate caspase-1 and induce the production of mature IL-1 β from infected macrophages. This hypothesis was tested by using mutants of KIM5 that were deficient for translocation of effectors as well as deletion mutants of the virulence plasmid, pCD1. Chapter 3 deals with the role the inflammasome plays during KIM5 infection. Caspase-1 cleavage and activation occur through its recruitment to the inflammasome complex. Utilizing macrophages deficient for NALP3, ASC or Ipaf, we tested whether these specific complexes played a role during KIM5 infection. Chapter 4 discusses the results obtained from mice infected with KIM5. Previously, caspase-1 deficiency in mice infected with certain pathogens has been associated with higher bacterial loads as well as high mortality; thus, caspase-1 is important for the control and clearance of infection. Flow cytometry analysis of caspase-1 activation in leukocytes, quantification of bacterial organ burden and mouse survival studies were utilized to determine if KIM5 could activate caspase-1 *in vivo*. Lastly, Chapter 5 discusses future directions for this project. Taken as a whole, the results presented here demonstrate a

novel mechanism for caspase-1 activation by *Y. pestis* *in vitro* and *in vivo* not previously seen with other bacterial pathogens.

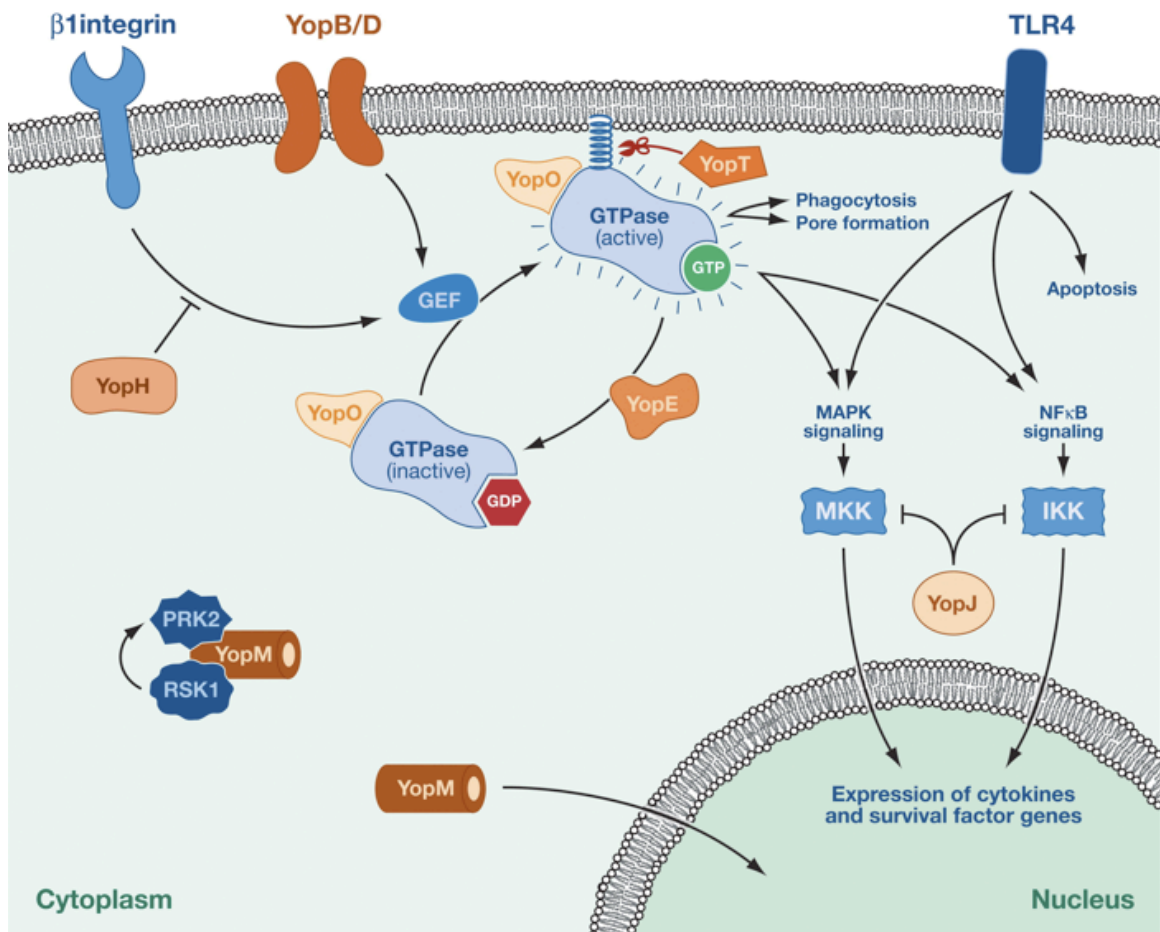


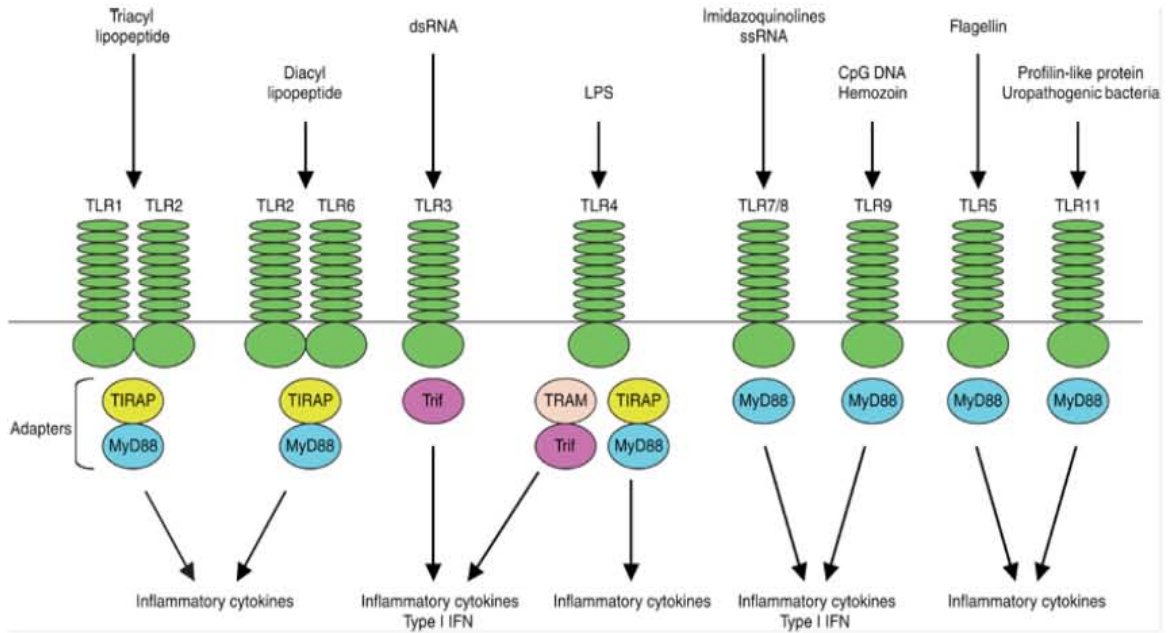
Figure 1.2. Modulation of host cell signaling by *Yersinia* outer proteins (Yops). Upon entry into the host cell, the effector Yops can inhibit a number of cellular processes, as illustrated above. Stimulation of $\beta 1$ integrin signaling can induce phagocytosis, which is inhibited by YopH. YopO, YopT and YopE can all block GTPase activation, inhibiting phagocytosis or pore formation. YopJ can block the production of cytokines and induce apoptosis by inhibiting both MAPK and NF- κ B pathways. YopM translocates to the nucleus; its exact function remains unknown. Adapted from Viboud and Bliska (136).

Figure 1.3. Toll-like receptors and NOD-like receptors: structure and function. (A)

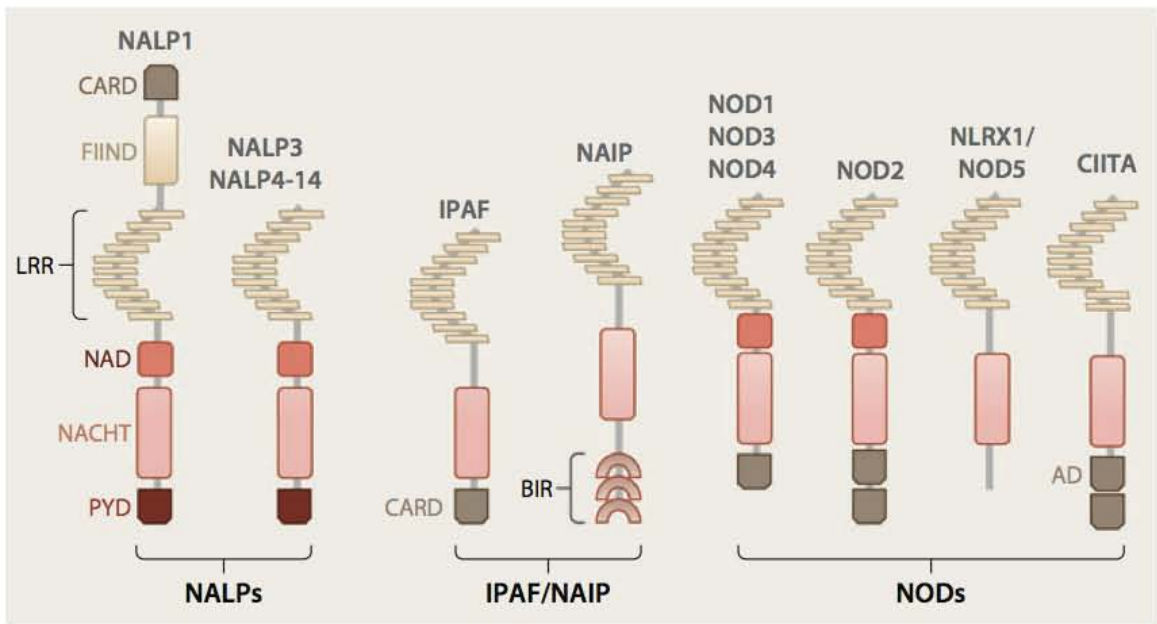
Toll-like receptors (TLRs) are composed of an extracellular leucine-rich repeat region (LRR) and intracellular Toll/IL-1 receptor (TIR) domain. TLRs can recognize various stimuli, such as LPS (TLR4) and flagellin (TLR5), through LRR regions (green ovals). Upon recognition, adaptor molecules (Trif, MyD88, TRAM, TIRAP) are recruited to TIR and propagate a signaling cascade to activate the production of pro-inflammatory cytokines and/or a type I interferon response. Adapted from Kawai and Akira (64). **(B)**

NOD-like receptors (NLRs) are similar to TLRs, except they are intracellular sensors of microbial products. NLRs are composed of an LRR region (yellow stacked rectangles), which functions similarly to the LRR of TLRs, an oligomerization domain (NOD or NACHT) and an effector domain (PYD, CARD or BIR). Activation of various NLRs through recognition of microbial products can result in the activation of the MAPK and NF- κ B pathways (NODs), or caspase-1 activation (NALPs, Ipaf/NAIP). Adapted from Martinon *et al.* (82).

A



B



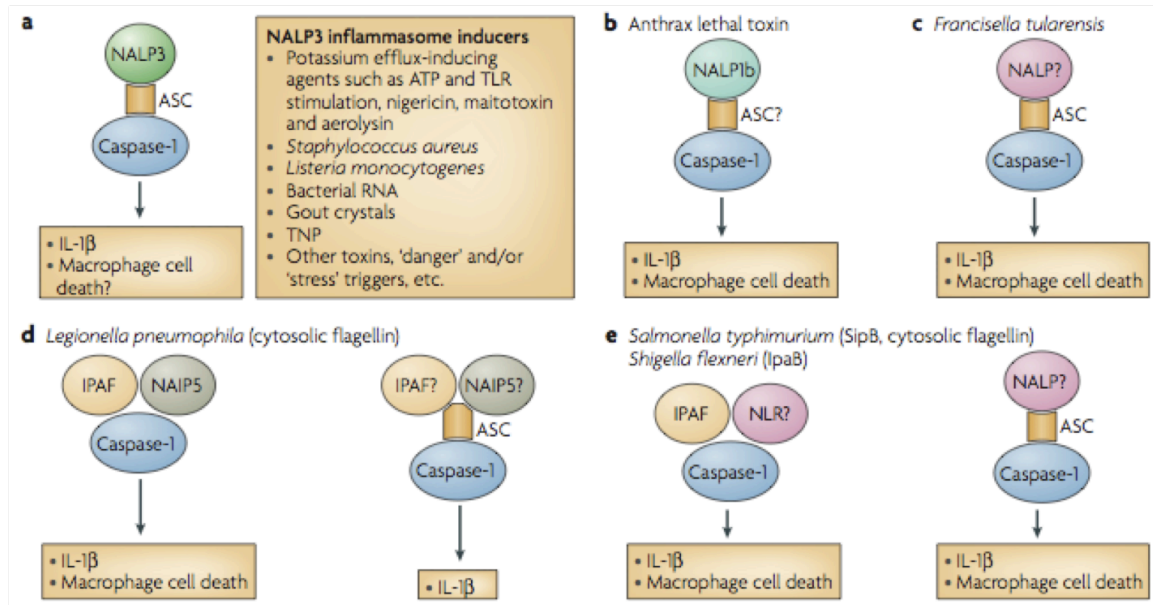


Figure 1.4. Inflammasome complex activation. (a) Upon activation of NALP3 by various stimuli, such as potassium efflux, the Gram-positive bacterium *Listeria monocytogenes* or bacterial RNA, an inflammasome complex composed of NALP3, the adaptor ASC and caspase-1 assembles, leading to caspase-1 activation. (b) Anthrax lethal toxin (LT) can activate the NALP1b inflammasome, leading to caspase-1 recruitment and activation. Gram-negative pathogens can be recognized through the IpaB/Naip5 inflammasome or through an as-yet-unidentified NLR (c-e). Adapted from Mariathasan *et al.* (76).

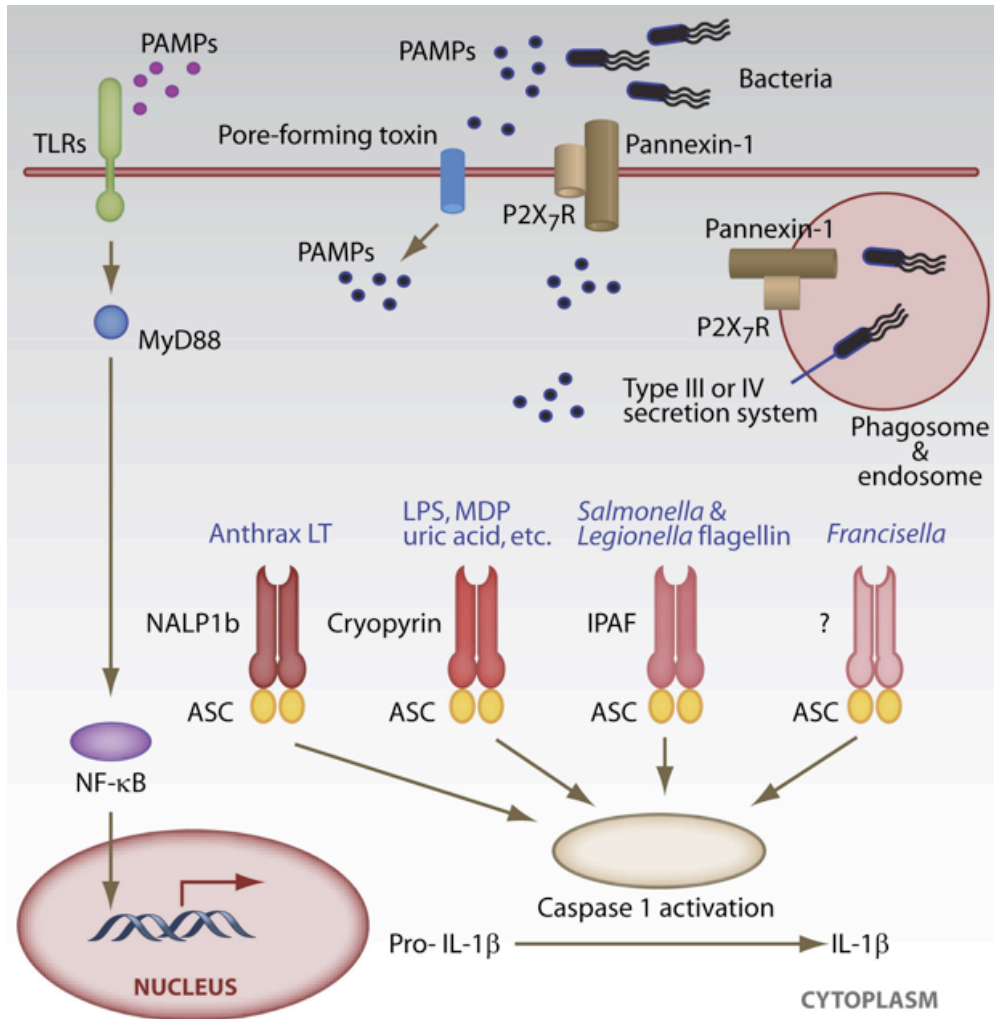


Figure 1.5. Caspase-1 activation through the inflammasome. IL-1 β processing and release is thought to occur in a two-step manner. First, TLRs recognize various stimuli (PAMPs) and activate the production of precursor IL-1 β (pro-IL-1 β) through activation of the transcription factor NF- κ B. Secondly, PAMPs can gain access to the cytosol either through pore-forming toxins, ATP-mediated pannexin-1 pore formation or type III or IV secretion systems. NLRs recognize these stimuli, are activated and induce the formation of caspase-1 activating inflammasome complexes. Caspase-1 activation leads to the processing of pro-IL-1 β into its mature form for secretion. Adapted from Kanneganti *et al.* (62).

Chapter 2: The role of the *Yersinia* effector protein YopJ in the activation of caspase-1 during infection of macrophages with *Y. pestis* strain KIM5

2.1 Summary

Previous studies have demonstrated that in macrophages infected with *Y. enterocolitica*, the T3SS and Yops can inhibit the production of mature IL-1 β by inhibiting the activation of caspase-1 (6, 122, 124). Here, we aimed to determine if effector Yops of *Y. pestis* and *Y. pseudotuberculosis* regulate caspase-1 activation and IL-1 β secretion in naïve BMDMs. Among a panel of different strains tested, *Y. pestis* isolates derived from the KIM background displayed an unusual ability to stimulate caspase-1 activation, IL-1 β secretion and cell death in infected macrophages. IL-1 β secretion from, but not death of, KIM5-infected macrophages required caspase-1 activity. Furthermore, caspase-1 activation, IL-1 β secretion, and death of macrophages infected with KIM5 required the type III secretion system and the enzymatic activity of the effector YopJ. These results suggest a novel role for YopJ in the activation of caspase-1 following infection of macrophages by *Y. pestis* KIM strains.

2.2 Introduction

A number of pathogenic bacteria, such as *Salmonella* and *Shigella*, can induce caspase-1 activation and processing and release of IL-1 β during infection of macrophages. On the

contrary, it has been observed that *Y. enterocolitica* does not activate these responses from infected macrophages. Studies performed by Schotte *et al.* demonstrated that YopE, and to a lesser extent, YopT could inhibit the activation of caspase-1 (122). Additionally, pore formation by the type III secretion system (T3SS) was shown to be required for caspase-1 activation from macrophages infected with *Y. enterocolitica*, but this response was blocked in the presence of Yops E and T (124). Recently, Bergsbaken and Cookson obtained evidence that the *Y. pseudotuberculosis* strain YPIII can activate caspase-1 in bone marrow derived macrophages (BMDMs) that have been pre-stimulated with LPS (6). It was hypothesized that the T3SS in YPIII transports an inflammasome-stimulating factor into the macrophage cytosol, and that priming macrophages with LPS overcomes the ability of translocated effector proteins to inhibit the activation of caspase-1 (6). In this chapter, we aim to determine if effector Yops from *Y. pestis* and *Y. pseudotuberculosis* can modulate caspase-1 activation and IL-1 β secretion during infection of naïve BMDMs.

2.3 Experimental Methods

Bacterial strains and growth conditions. *Y. pestis* and *Y. pseudotuberculosis* strains used in this study are listed in Table 2.1. The KIM5, KIM-D27 and KIM8 strains are related to a parental KIM strain and the pCD1 plasmid is the same for KIM5, KIM-D27 and KIM8, with the exception of the ampicillin (Ap) marker when used. CO92 contains its own unique pCD1. All pCD1⁺ *Y. pestis* strains used in this study lack the pigmentation locus (*pgm*) and therefore are conditionally virulent. Derivatives of KIM8 containing mutant pCD1 plasmids are listed in Tables 2.1 and 2.2. Derivatives of KIM6+ and CO92

in which the pigmentation (*pgm*) locus was spontaneously deleted (KIM6 and CO92 Δ *pgm*, respectively) were isolated on Congo red plates; the non-pigmented colonies (loss of *pgm*) were streaked on Congo red and *Yersinia* selective medium (Oxoid) plates. *pgm* deletion was verified by colony PCR as described (46). A pCD1 plasmid containing an ampicillin resistance cassette (pCD1Ap) was subsequently used to transform KIM6 by electroporation and selected for on Luria-Bertani (LB) agar plates containing 25 μ g/ml ampicillin, resulting in KIM5 (46). The KIM5*yopH::kan*, KIM5*yopkA::kan* and KIM5*caf1A::kan* mutants were constructed using the lamda red recombination method as described (22, 25, 117) to insert Km^r cassettes into the corresponding genes. Briefly, PCR products were generated by using primers containing 20 nucleotide homologous to the extremities of the kanamycin cassette and 50-nt extensions homologous to the *Yersinia* target sequence. PCR products were purified by ethanol precipitation and dialysis; electrocompetent KIM5 harboring pKOBEG, which encodes the λ phage $\text{red}\gamma\beta\alpha$ operon under the arabinose inducible pBAD promoter, were transformed with the appropriate PCR products. Transformed bacteria were plated on LB agar plates containing kanamycin; colony PCR was performed to verify insertion of the kanamycin cassette. The KIM5*yopB* mutant was constructed by allelic exchange using the plasmid pJB4 (99). The resulting mutation in pCD1Ap corresponds to the *yopB18* allele, which contains an in-frame deletion of nucleotides 496-774 (99). The KIM5*yopJC172A* mutant was constructed using allelic exchange, placing the C172A mutation into the *yopJ* gene in the pCD1Ap plasmid of KIM5, and verified as described (144). The C172A mutation corresponds to a change from TGTGGT, encoding Cys172 and Gly173, to GCCGGC, encoding Ala172 and Gly173. The KIM-D27 Δ *phoP* mutant was constructed by allelic

exchange using plasmid pSB890 as previously described (47). Briefly, an in-frame deletion mutation in *phoP* was created by removal of nucleotides 127-429. The restriction fragment containing this deletion was ligated to pSB890. The resulting plasmid, PSB890- Δ *phoP*, was electroporated into S17 λ pir and the resultant strain was selected for on LB agar plates containing tetracycline and used to transfer pSB890- Δ *phoP* to KIM-D27 by conjugation. Transconjugants were selected on *Yersinia* selective medium and LB containing tetracycline. Tetracycline resistant colonies were grown overnight in LB and plated on LB agar supplemented with 5% sucrose for negative selection against the *sacB* gene encoded on pSB890. Sucrose resistant colonies were tested for the presence of Δ *phoP* by colony PCR. A derivative of KIM5 in which pCD1 was replaced with pIB102 (KIM5 pIB102), a virulence plasmid from *Y. pseudotuberculosis* YPIII, was constructed by electroporation. The *Y. pseudotuberculosis yopJ* and *yopE yopJ* mutants of IP2666 (Table 2.1) were constructed by allelic exchange using the plasmid pLP13 (99) to delete the entire *yopJ* open reading frame (nucleotides 1-867), and IP2666 and IP6 *yopE* (8) as the recipient strains, respectively. The *yopJ yopE yopH yopM yopO yopK* mutant of IP2666 (Table 2.1) was generated from the pYV-cured strain IP2666c (125). The pYV plasmid purified from YP37 (137) was used to transform IP2666c by electroporation; the kanamycin resistance gene inserted in *yopE* (*yopE::kan*) was used to select for transformants. The arabinose-inducible plasmid encoding the open reading frame of KIM YopJ, pYopJ-GSK, was used to transform KIM8 Δ 2 and IP2666 J- by electroporation and selected for on ampicillin-containing plates. *Y. pestis* and *Y. pseudotuberculosis* strains were cultivated on heart infusion (HI) (Difco) or LB agar plates, respectively, for two days at 28°C. Cultures were grown overnight with aeration in HI (*Y. pestis*) or LB (*Y.*

pseudotuberculosis) broth at 28°C. The next day the cultures were diluted to an OD₆₀₀ of 0.1 in the same medium supplemented with 2.5 mM CaCl₂ and incubated for 2 hr at 37°C with aeration. Bacterial cultures were washed once with phosphate-buffered saline (PBS) then resuspended in PBS for OD₆₀₀ measurements. Bacterial growth medium was supplemented with ampicillin (25 µg/ml) and/or kanamycin (25 µg/ml) when appropriate. KIM5/GFP was cultured in the presence of 0.5 mM IPTG during growth at 37°C to induce expression of GFP. Where indicated, a final concentration of 0.02% arabinose was maintained in cell culture medium to induce expression of YopJ from the pYopJ-GSK plasmid during infection.

Bone marrow macrophage isolation and culture conditions. Bone marrow derived macrophages (BMDM) were isolated from the femurs of 6- to 8-week-old C57BL/6 female mice (Taconic Laboratories) or caspase-1-deficient (*Casp1*^{-/-}) mice (142) as previously described (16, 108). Briefly, bone marrow cells isolated from two femurs were suspended in tissue culture medium (Dulbecco's Modified Eagle Medium [DMEM] containing 20% fetal bovine serum, 30% L-cell-conditioned medium and 0.1 M sodium pyruvate [BMM-high]) and seeded into 100 mm non-tissue culture treated petri dishes (Nunc) at approximately 4 x 10⁶ cells per plate. After 5 days of incubation at 37°C plus 5% CO₂, macrophages were collected and used for infection assays. The *Casp1*^{-/-} mice were backcrossed to C57BL/6 mice for 7 generations.

Macrophage infections. Twenty-four hours before infection, BMDM were seeded into 24-well plates at a density of 1.5 x 10⁵ cells/ml as described (108), with the exception that the tissue culture medium (infection medium, BMM-low) contained 10% fetal bovine serum, 15% L-cell-conditioned medium and 1% 0.1 M sodium pyruvate. Bacteria were

grown as described above or the subculture was grown at 28°C for 2 hrs where indicated and used to infect macrophages at a multiplicity of infection (MOI) of ten bacteria per macrophage. After addition of bacteria, plates were centrifuged for 5 minutes at 95 x g to induce contact between bacteria and macrophages. After incubation at 37°C with 5% CO₂ for 15 minutes, macrophages were washed once with pre-warmed PBS to remove any bacteria that had not been taken up. Fresh infection medium containing 8 µg/ml of gentamicin was added for 1 hr at 37°C with 5% CO₂ to reduce viability of extracellular bacteria (>99%) (97, 108). After 1 hr, macrophages were washed once with PBS and a lower concentration of gentamicin (4.5 µg/ml) in fresh tissue culture medium was added for the remaining incubation times as indicated in figure legends to inhibit growth of extracellular bacteria. To inhibit bacterial protein synthesis, chloramphenicol (Cm) was added to wells concurrently with bacteria at a final concentration of 30 µg/ml for 24 hrs. To inhibit bacterial uptake, macrophages were exposed to 3.9 µM cytochalasin D (Sigma) for 2 hr prior to infection as well as during the 20 minutes of infection. To inhibit caspase-1 activity, macrophages were exposed to 100 µM of caspase-1 inhibitor Ac-YVAD-cmk (Calbiochem) for 1 hr prior to infection. Dimethyl sulfoxide (DMSO) was used as solvent for cytochalasin D and Ac-YVAD-cmk. The final concentrations of DMSO used as solvent control for cytochalasin D and Ac-YVAD-cmk treatment conditions were 28 mM and 140 mM, respectively. In some experiments macrophages were exposed to 50 ng/ml of *Escherichia coli* 026:B6 LPS (Sigma-Aldrich, cat. # L-2654) for 4 hr prior to infection.

Cytokine measurements. Levels of IL-1β and TNF-α secreted into tissue culture media during infection assays were measured by the Quantikine Mouse IL-1β or TNF-α

Immunoassay kits, respectively, as per manufacturer's instructions (R&D Systems). Levels of IL-18 secreted into tissue culture media were measured using the Mouse IL-18 ELISA kit as per the manufacturer's protocol (Medical & Biological Laboratories Co., LTD). Supernatants from two replicate wells per infection condition were collected, centrifuged to remove cellular debris and transferred to new tubes. Supernatants were diluted appropriately, and 50 µl of each diluted sample was analyzed. Standard curves were used to estimate levels of cytokines in each sample. The replicate values for each infection condition were averaged.

Lactate dehydrogenase (LDH) release. Supernatants from infected macrophages were collected and analyzed for LDH release using the CytoTox-96 non-radioactive cytotoxicity assay (Promega), following manufacturer's instructions. Supernatants from two replicate wells per infection condition were collected and centrifuged to remove cellular debris. LDH levels in each replicate sample were measured in triplicate. Spontaneous LDH release was measured from supernatants of uninfected cells while total LDH release was measured from uninfected cells that were lysed by freezing and thawing. OD values of the six measurements for each infection condition were averaged and the percentage of LDH per infection condition was calculated as follows: % LDH release = [(LDH infected - spontaneous LDH release) / (total LDH release - spontaneous LDH release)] x 100.

Detection of active caspase-1 using FAM-YVAD-FMK. To assess the presence of active caspase-1 by fluorescence microscopy, infected BMDM on coverslips were incubated with FAM-YVAD-fluoromethylketone (FAM-YVAD-FMK; Immunochemistry Technologies) as per manufacturers' protocol. After one hour,

macrophages were washed and fixed in 2.5% paraformaldehyde in PBS for 30 minutes at room temperature. The fixed cells were incubated in 0.1% Triton X-100 in PBS for 10 minutes to permeabilize the cell membrane and washed once with PBS and once with 3% BSA in PBS to prevent non-specific antibody binding. Bacteria were immunolabeled by incubating for 30 minutes with rabbit anti-*Yersinia* antiserum SB349 (8) diluted 1:1000 in 3% BSA in PBS. Cells were washed three times with PBS and incubated for 30 minutes with goat anti-rabbit secondary antibody conjugated to AlexaFluor594 (AF594; Molecular Probes) diluted at 1:1500. After washing, coverslips were mounted on glass microscope slides and visualized by fluorescence microscopy using a Nikon Eclipse E600 microscope equipped with a 40x objective. Images were captured using a Sony Progressive 3CCD camera and processed with Adobe Photoshop 6.0.

Colony forming unit (CFU) assay. At various time points post-infection, infected macrophages were washed three times with PBS and lysed with 0.5 ml of 0.1% Triton X-100. Lysates were then removed and an additional 0.5 ml of 0.1% Triton X-100 was used to wash the wells. Lysates and washes were collected into 2 ml microcentrifuge tubes and used for serial 10-fold dilutions. Dilutions were spread on HI plates and incubated for two days at 28°C, after which output CFU were counted. Two replicates of each infection condition were analyzed in each experiment and the results averaged. Averages presented (Log₁₀ CFU per ml) are derived from three independent experiments.

Bacterial phagocytosis assay. Macrophages on coverslips were infected with KIM5/GFP for 20 min, then washed and fixed in 2.5% paraformaldehyde in PBS for 30 minutes at room temperature. The fixed cells were washed once with PBS and once with 3% BSA in PBS to prevent non-specific antibody binding. Bacteria were immunolabeled

with rabbit anti-*Yersinia* antiserum SB349 and goat anti-rabbit secondary antibody conjugated to AlexaFluor594 as described above. After washing, coverslips were mounted on glass microscope slides and visualized by fluorescence microscopy using a Zeiss Axioplan2 microscope equipped with a 40X objective. A Spot camera (Diagnostic Instruments) was used to sequentially capture the AlexaFluor594 (red) and GFP (green) signals in four to five random fields from each coverslip. The red and green images were overlaid using Adobe Photoshop 6.0, and the percentage of internalized bacteria was quantified by counting the number of intracellular bacteria (green) and dividing this number by the sum of internalized and extracellular bacteria (red). Between 1200 and 2500 bacteria were counted for each condition.

Statistical analysis. In general, experiments analyzed for significance were performed three independent times. Probability (P) values were calculated by one-way ANOVA and Tukey's multiple comparisons post test and were considered significant if less than 0.05 (GraphPad Prism 4.0).

2.4 Results

***Yersinia pestis* KIM isolates stimulate infected macrophages to secrete higher levels of IL-1 β as compared to other *Yersinia* strains.** It has been previously shown that Yop effectors inhibit expression and secretion of IL-1 β in the macrophage-like cell line Mf4/4 infected with *Y. enterocolitica* (122, 124). BMDMs were infected with different *Y. pseudotuberculosis* and *Y. pestis* strains to determine if the T3SS and Yops of these species play a similar role in the inhibition of IL-1 β release. Initially, BMDMs were infected with the pCD1⁺ *Y. pestis* strains KIM5, KIM-D27, or CO92 Δ *pgm* (Table 2.1) pre-grown at 37°C to upregulate expression of the T3SS and Yops. An ELISA assay was utilized to measure the levels of IL-1 β present in tissue culture supernatants after 24 hr of infection. As shown in Figure 2.1, panel A, macrophages infected with KIM5 or KIM-D27 secreted much higher levels of IL-1 β as compared to macrophages infected with CO92 Δ *pgm*. This analysis was extended to pYV⁺ *Y. pseudotuberculosis* strains IP2777 and IP2666 (Table 2.1). Results showed that KIM5-infected macrophages secreted substantially greater levels of IL-1 β as compared to macrophages infected with IP2777 or IP2666 (Figure 2.1, panel B). Thus, strains derived from *Y. pestis* KIM displayed an unusual ability to stimulate IL-1 β secretion from macrophages as compared to other strains of *Y. pestis* (CO92) or *Y. pseudotuberculosis*. To determine if the phenotype observed for KIM5 would be similar to that of a *Y. pseudotuberculosis* multiple *yop* mutant, we next compared the response of BMDMs infected with KIM5 to macrophages infected with isogenic single (*yopE*, *yopJ*) or multiple (*yopEJ*, *yopJEHMOK*) *yop* mutants of IP2666 (Table 2.1) pre-grown at 37°C. Surprisingly, the amount of IL-1 β secreted from macrophages infected with KIM5 was ~40-fold higher than the IL-1 β secreted from

BMDMs infected with the *Y. pseudotuberculosis yopJEHMOK* mutant (Figure 2.1, panel C). The *yopJEHMOK* mutant did induce significantly higher amounts of IL-1 β to be secreted from infected BMDM as compared to wild-type IP2666 (Figure 2.1, panel D), as expected from previous studies performed with *Y. enterocolitica* (122). Macrophages infected with the single *yopE* mutant reproducibly secreted slightly higher amounts of IL-1 β as compared to macrophages infected with the wild-type bacteria, reflecting the proposed role of YopE as an inhibitor of caspase-1 activation (122), but this difference was not statistically significant (Figure 2.1, panel D). The other *Y. pseudotuberculosis* mutants tested (*yopJ* and *yopEJ*) did not induce higher levels of IL-1 β secretion from BMDMs as compared to the parental strain (Figure 2.1, panel D). Thus, although these results suggested that YopE and perhaps additional Yops inhibit IL-1 β secretion from macrophages infected with *Y. pseudotuberculosis* IP2666, and are similar to those reported by Schotte *et al.* for *Y. enterocolitica* E40 (122), we have demonstrated that *Y. pestis* KIM strains have a novel capacity to stimulate high-level secretion of IL-1 β from infected BMDMs.

Caspase-1 activity and the T3SS are important for IL-1 β secretion during KIM5 infection. The importance of caspase-1 activation for IL-1 β secretion from macrophages infected with various pathogenic bacteria has been previously demonstrated (58, 71, 78, 91, 112) (142). We obtained evidence that caspase-1 was activated in KIM5-infected macrophages using a fluorochrome inhibitor of caspases (FLICA) reagent specific for active caspase-1 (FAM-YVAD-FMK). By microscopic analysis, the FAM-YVAD-FMK fluorescent signal was associated with KIM5-infected macrophages beginning at 4 hr

post-infection (see Figure 2.9). To determine if caspase-1 activity was required for IL-1 β secretion from KIM5-infected macrophages, BMDMs were treated for 1 hr before infection with 100 μ M of the caspase-1 inhibitor Ac-YVAD-cmk (YVAD). As shown in Figure 2.2, panel A, a 1 hr pretreatment with YVAD significantly inhibited IL-1 β secretion from BMDMs infected with KIM5 for 24 hr, suggesting that caspase-1 activity was important for IL-1 β secretion under these infection conditions. Macrophages similarly treated with DMSO only as a solvent control showed no decrease in IL-1 β secretion (Figure 2.2, panel A). Because YVAD treatment only partially reduced IL-1 β secretion, it is possible that in the absence of caspase-1 activity (see also Figure 2.14), other caspases can mediate processing and secretion of IL-1 β in KIM5-infected BMDMs. Alternatively, because the ELISA used does not discriminate between pro and mature forms of IL-1 β , and KIM5-infected BMDMs are dying (see Figure 2.6), it is possible that the IL-1 β detected in the supernatant of KIM5-infected cells represents a mixture of mature IL-1 β secreted via caspase-1 and pro IL-1 β released upon cell lysis.

YopB is required for translocation of effector Yops into host cells infected with *Yersinia* (42). A KIM5 *yopB* mutant (Table 2.1) was used to test whether effector translocation is required for IL-1 β secretion during KIM5 infection of BMDMs. The *yopB* mutant stimulated significantly lower levels of IL-1 β secretion from infected BMDMs as compared to KIM5, suggesting a requirement for effector translocation in this response (Figure 2.2, panel B).

The expression of the T3SS is maximal at 37°C and is downregulated at ambient temperatures (i.e., 28°C). As the above results suggest that translocation channel formation is required for caspase-1 activation, we would expect the amount of IL-1 β

secreted from macrophages to be dependent upon the temperature at which KIM5 is grown prior to infection. This is because by growing KIM5 cultures at 37°C, the T3SS apparatus would be fully formed at the surface of the bacterium ready for translocation channel insertion into macrophages upon infection. Bacteria grown at 28°C would demonstrate a delayed insertion of translocation channels; the bacteria will only be exposed to 37°C when added to the macrophages for infection, so it will take slightly longer for them to assemble the T3SS and translocation channels. To determine whether bacterial growth temperature would have any effect on IL-1 β secretion, we infected BMM with KIM5 pre-grown at 37°C or 28°C. Pre-growth of KIM5 at 28°C significantly reduced IL-1 β secretion (Figure 2.3), further demonstrating the importance of the T3SS for activating caspase-1.

Macrophage activation with LPS decreases secretion of IL-1 β from macrophages infected with *Y. pestis* KIM5. Bergsbaken *et al.* (6) have shown that pre-activation of BMDMs with LPS is required for activation of caspase-1 following infection with wild-type *Y. pseudotuberculosis* strain YPIII. BMDMs were exposed to 50 ng/ml LPS for 4 hrs prior to infection to investigate the role of macrophage activation in secretion of IL-1 β from macrophages challenged with *Y. pestis* KIM5. Interestingly, activated BMDMs secreted significantly less (2-fold) IL-1 β after infection with KIM5 as compared to naïve macrophages (Figure 2.2, panel C). We also observed little difference in the levels of IL-1 β secreted from activated or naïve macrophages following infection with wild-type IP2666 (Figure 2.2, panel C). However, activated BMDMs infected with the *Y. pseudotuberculosis* *yopJEHMOK* mutant reproducibly secreted higher levels of IL-1 β as

compared to naïve macrophages infected with the same strain, but this difference was not statistically significant (Figure 2.2, panel C).

The role of bacterial protein synthesis and intracellular survival for IL-1 β secretion from KIM5-infected macrophages. To determine whether bacterial protein synthesis is necessary for IL-1 β secretion during infection, the bacteriostatic antibiotic and protein synthesis inhibitor chloramphenicol (Cm) was used. As shown in Figure 2.4, the addition of Cm concurrently with KIM5 during infection greatly reduced the ability of the bacteria to induce secretion of IL-1 β from infected macrophages, indicating that de novo production of proteins by *Y. pestis* during infection is important for stimulating IL-1 β secretion.

Next, we wanted to determine if intracellular survival was necessary for IL-1 β secretion. To test this, we used a previously constructed *phoP* mutant in the KIM5 background. Previously published data indicated that a *phoP* mutant of *Y. pestis* is defective for intracellular survival and replication within macrophages (47). The *phoP* mutant stimulated significantly less IL-1 β secretion from infected BMMs as compared to the parental KIM5 strain, suggesting the importance of intracellular survival of *Y. pestis* for activation of caspase-1 (Figure 2.4).

Time course analysis of cytokine secretion from KIM5-infected macrophages. To begin to characterize the mechanism of caspase-1 activation in macrophages infected with KIM5, a time course analysis was performed. In addition to analyzing IL-1 β , we also measured secretion of IL-18, which is dependent upon cleavage by active caspase-1

for its processing and release, and TNF- α , which is not. A previous study has shown that *Y. pestis* strain EV76 can partially suppress secretion of TNF- α from infected RAW264.7 macrophage-like cells, and that YopJ is required for this suppression (143). BMDMs were infected with KIM5 or KIM5*yopB*, supernatants were collected at various time points, and ELISA was used to measure levels of secreted IL-1 β , IL-18 and TNF- α . IL-18 was detected in the supernatants of KIM5-infected macrophages at 4 hr post infection and IL-1 β was detected at 8 hr post infection (Figure 2.5, panels A, B). Both cytokines continued to accumulate in the supernatants of KIM5-infected macrophages over time, and only background levels of these cytokines were secreted from BMDMs infected with KIM5*yopB*. Quite different results were observed for TNF- α , as compared to IL-1 β and IL-18, under the same infection conditions (Figure 2.5, panel C). Both KIM5 and KIM5*yopB* stimulated infected BMDMs to secrete TNF- α , which accumulated in the supernatants over time, although higher levels (~2-fold) of TNF- α were secreted from macrophages infected with the *yopB* mutant (Figure 2.5, panel C). These results suggested that the T3SS of *Y. pestis* KIM5 was differentially regulating production of cytokines in infected BMDMs. The T3SS was partially inhibiting a pathway that regulates TNF- α production, likely via YopJ, while simultaneously activating a pathway required for IL-1 β and IL-18 secretion.

KIM5 induces caspase-1-independent cell death in infected macrophages. Previous studies have indicated that pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium can induce macrophage cell death via a process mediated by caspase-1 and termed pyroptosis (37, 58, 71, 91). Since we obtained evidence that caspase-1 was

activated in KIM5-infected macrophages, we wanted to determine if these BMDMs were dying of pyroptosis. Supernatants from infected BMDMs were tested for LDH, a marker of cell lysis, to quantify cell death. A time course analysis revealed that BMDMs infected with KIM5, but not KIM5*yopB*, were dying as measured by LDH release (Figure 2.6, panel A). A marked increase in LDH in the supernatant of KIM5-infected macrophages was first detected at 8 hr post infection, and approximately 40% of macrophages underwent cell lysis by 24 hrs (Figure 2.6, panel A). To determine if caspase-1 activity was required for cell death, macrophages were pretreated with the caspase-1 inhibitor YVAD and tested for both IL-1 β secretion and LDH release. Surprisingly, under conditions in which pre-treatment with 100 μ M of YVAD significantly reduced secretion of IL-1 β (Figure 2.2, panel A), LDH release from KIM5-infected macrophages was not significantly reduced (Figure 2.6, panel B). Additionally, LDH release was significantly lower in macrophages infected with CO92 Δ *pgm* as compared to macrophages infected with either KIM5 or KIM D27 (Figure 2.6, panel C). Thus, although *Y. pestis* KIM strains induced YopB-dependent caspase-1 activation and cell death in infected BMDMs, caspase-1 activity was not required for cell death. Therefore, KIM-infected macrophages did not appear to be dying of pyroptosis (36).

Identification of a region of pCD1 that is required for caspase-1 activation, IL-1 β secretion and LDH release from KIM5-infected macrophages. The data shown so far have demonstrated a role for the T3SS in stimulating the secretion of high levels of IL-1 β from macrophages infected with KIM5; however, this same phenotype was not seen in macrophages infected with KIM-D27, CO92 (Δ *pgm*) or *Y. pseudotuberculosis*. A

derivative of KIM5 unable to produce the F1 capsule (*caf1A* mutant) was used to infect BMDMs, and ELISA results showed that this strain stimulated the same high levels of IL-1 β secretion as compared to KIM5 (Figure 2.7). This result indicates that the F1 capsule encoded on pMT1 is not required for activation of caspase-1 in BMDMs infected with KIM5. To determine whether stimulation of IL-1 β secretion is specific to pCD1 of KIM5, I utilized a derivative of this strain in which pCD1 was replaced with pIB102, a virulence plasmid from *Y. pseudotuberculosis* YPIII. As shown in Figure 2.7, substituting pIB102 for pCD1 in KIM5 significantly reduced secretion of IL-1 β from infected BMDMs, implicating a factor encoded on pCD1 of KIM5 as the stimulator of caspase-1 activation. To determine if one of the Yop effectors encoded on pCD1 was essential for inducing IL-1 β secretion and LDH release from infected BMDMs, we obtained KIM8-derived strains that contained various deletions comprising ~10 kb regions of pCD1, as listed in Table 2.1 and 2.2 (4). The deletions were designed to remove genes encoding the six effector Yops, some of the chaperones, and all uncharacterized reading frames and transposable elements (4). In general, the deletions did not remove genes required for expression or assembly of the T3SS or for effector translocation, except for the deletion termed Δ 34, in which *sycH* was deleted. *SycH* has been shown to play a role in T3SS expression (14). The KIM8-derived strains harboring the mutant pCD1 plasmids were tested along with KIM5 and KIM5*yopB* in BMDM infection assays and the results are shown in Figure 2.8. All mutants containing the region 2 deletion (Δ 2, Δ 12, Δ 123, and Δ 1234), as well as the mutant with the regions 3 and 4 deletion (Δ 34), stimulated significantly lower levels of IL-1 β secretion from infected BMDMs (Figure 2.8, panel A). Strains with deletions of regions 1 (Δ 1) or 4 (Δ 4) showed a partial, but not statistically significant, reduction (~2-

fold) in IL-1 β secretion-stimulating activity (Figure 2.8, panel A). Levels of LDH released from macrophages infected with the different strains were similar in trend as to those seen for IL-1 β secretion and pointed to a critical role for region 2 (Figure 2.8, panel B). Furthermore, a factor encoded in region 2 was required for activation of caspase-1 activity, as shown by staining with FAM-YVAD-FMK (Figure 2.9). BMDMs infected with KIM5 or the $\Delta 2$ mutant were incubated with FAM-YVAD-FMK, then washed, fixed and stained with anti-*Yersinia* antibody to detect macrophage associated bacteria. Microscopic analysis showed the FAM-YVAD-FMK fluorescent signal associated with a percentage of KIM5-infected macrophages at 8 hr (Figure 2.9). Macrophages infected with the KIM8 $\Delta 2$ mutant showed only background staining with FAM-YVAD-FMK at either time point (Figure 2.9). From these results we concluded that a factor(s) encoded in region 2 is essential for inducing activation of caspase-1 and cell death in KIM5-infected BMDMs. The apparent decrease in IL-1 β secretion stimulating activity observed for the $\Delta 34$ mutant (Figure 2.8, panel A) might be due to the absence of SycH in this strain, since deletion of *sycH* results in a reduction in the secretion of T3SS effectors (14).

Phagocytosis of KIM5 by macrophages is not required for IL-1 β secretion or LDH release. Two different assays were employed to investigate how loss of region 2 ($\Delta 2$) might impact bacterial internalization and intracellular survival, and thus alter the response of BMDMs to *Y. pestis* KIM infection. First, a CFU assay was utilized to determine the number of KIM5, KIM5yopB and KIM8 $\Delta 2$ mutant bacteria associated with, and surviving within, macrophages at various time points. The 0 hr time point

revealed the total number of viable bacteria associated with the BMDMs prior to application of gentamicin containing media. As shown in Figure 2.10, similar numbers of KIM5, KIM5*yopB* and the $\Delta 2$ mutant were associated with BMDMs at 0 hr. The later time points reflected the abilities of the different strains to survive within the BMDMs, and as shown in Figure 2.10, the numbers of viable intracellular bacteria remained constant over time for KIM5, the *yopB* mutant and the $\Delta 2$ mutant and overall decreased only slightly. These results suggest that differences in bacterial association with macrophages or intracellular survival of *Y. pestis* were unlikely to explain the difference between KIM5 and the $\Delta 2$ mutant with respect to their ability to activate caspase-1 or induce macrophage cell death.

BMDMs were treated with cytochalasin D (CD), which blocks phagocytosis by inhibiting actin polymerization, to determine if bacterial internalization was required for caspase-1 activation and cell death. Macrophages were left untreated or treated with CD as described in Material and Methods, then infected, and ELISA was performed on supernatants collected after 24 hr. Macrophages left untreated or treated with CD and infected with KIM5 showed no significant difference in IL-1 β secretion (Figure 2.11, panel A) or LDH release (Figure 2.11, panel B). In addition, treatment of macrophages with CD did not alter the levels of IL-1 β secretion or LDH released following infection with KIM5*yopB* or the $\Delta 2$ mutant. To verify that CD was reducing bacterial internalization, a microscopic assay that measures levels of bacterial internalization was employed. Macrophages pretreated or not with CD were infected with KIM5/GFP (Table 2.1). After 20 minutes, the macrophages were fixed, processed for immunofluorescence microscopy, and percentage of intracellular bacteria was determined. We observed

approximately 70% of KIM5/GFP were intracellular in samples of untreated macrophages as compared to less than 1% of bacteria that were intracellular in samples of CD-treated macrophages. These results suggested that the factor(s) required for stimulating caspase-1 activation and cell death could be translocated into BMDMs by extracellular bacteria. Additionally, live bacteria are required for IL-1 β secretion, since there is a decrease in IL-1 β secretion from macrophages infected with a *phoP* mutant, which are defective for intracellular survival.

The activity of YopJ is required for caspase-1 activation and cell death in KIM5-infected macrophages. To determine if any of the known effectors encoded in region 2 of pCD1 (*yopH*, *yopO* and *yopJ*) were required for caspase-1 activation and cell death, KIM5 strains with mutations in these genes were constructed (Table 2.1). There was no significant difference in levels of IL-1 β secreted or LDH released from BMDM infected with *yopH* or *ypkA* mutants as compared to KIM5 (Figure 2.12). To determine if YopJ was important for stimulating IL-1 β secretion and LDH release from infected macrophages, a plasmid (pYopJ-GSK) (43) that expresses the KIM *yopJ* ORF under control of an arabinose-inducible promoter was used to transform KIM8 Δ 2. When the resulting strain (Δ 2/YopJ) was used to infect BMDMs, a significant increase in IL-1 β secretion and LDH release from infected macrophages was observed in the presence of arabinose (Figure 2.13, panels A and B, respectively). Levels of IL-1 β secreted and LDH released from macrophages infected with Δ 2/YopJ in the presence of arabinose were lower than observed in KIM5-infected BMDMs (Figure 2.13, panels A and B), suggesting that only partial complementation of phenotypes was obtained.

To determine if the KIM YopJ protein is sufficient to stimulate IL-1 β secretion and LDH release from infected macrophages, the pYopJ-GSK plasmid was used to transform the *Y. pseudotuberculosis yopJ* mutant IP2666 J-. As shown in Figure 2.13, the resulting strain (IP2666 J-/YopJ) exhibited an increased ability to stimulate IL-1 β secretion and LDH release from infected macrophages when cultured in the presence of arabinose (panels A and B, respectively).

A requirement for YopJ catalytic activity in the elicitation of cell death and caspase-1 activation was examined by constructing a mutant of KIM5 that expresses enzymatically inactive YopJ (KIM5*yopJC172A*). KIM5*yopJC172A* was as defective as the *yopB* mutant or the $\Delta 2$ mutant for stimulating IL-1 β secretion and LDH release from infected macrophages (Figure 2.13, panels C and D, respectively). KIM5*yopJC172A* was also defective for inhibiting TNF- α secretion from infected BMDMs, as expected from previous results obtained with *Y. pestis* EV76 (Figure 2.13, panel E) (143). Taken together, our results demonstrate a novel role for the YopJ protein of *Y. pestis* KIM strains in activation of caspase-1 in macrophages.

Caspase-1-deficient macrophages have a reduced ability to secrete IL-1 β during infection with KIM5, but undergo cell death at the same rate as wild type macrophages. We have shown that pretreatment with YVAD significantly reduced the amount of IL-1 β secreted from macrophages infected with KIM5 (Figure 2.2, panel A), suggesting that caspase-1 is important for maximal secretion of this cytokine. To verify these results, we infected BMDMs deficient for caspase-1 (*Casp-1*^{-/-}) with KIM5 or KIM5 *yopJC172A* for 4, 8 or 24 hrs and measured IL-1 β secretion and LDH release.

There was a significant decrease (~2-fold) in IL-1 β secretion at 24 hr post infection in the *Casp-1*^{-/-} macrophages infected with KIM5 as compared to *Casp-1*^{+/+} C57BL/6 BMDMs (Figure 2.14, panel A). In contrast, there was no significant difference in the rate of cell death in *Casp-1*^{-/-} and *Casp-1*^{+/+} macrophages infected with KIM5 (Figure 2.14, panel B), confirming that although caspase-1 is important for maximal secretion of IL-1 β , it is not required for death of BMDMs infected with *Y. pestis* KIM.

2.5 Discussion

This study was undertaken to determine if the T3SS and Yops of *Y. pestis* and *Y. pseudotuberculosis* inhibit caspase-1 activation and IL-1 β secretion during infection of naïve macrophages. We unexpectedly found that *Y. pestis* KIM-derived strains stimulate infected murine bone marrow derived macrophages to secrete high concentrations of IL-1 β . This phenotype was not seen in *Y. pestis* CO92 Δ *pgm* or the *Y. pseudotuberculosis* strains IP2777 or IP2666 (Figure 2.1), suggesting that KIM isolates differ genetically from these other *Yersinia* strains. Caspase-1 was activated in macrophages infected with KIM5 as shown by microscopic analysis of infected cells incubated with the fluorescent caspase-1 inhibitor Fam-YVAD-fmk (Figure 2.9). IL-1 β secretion from macrophages infected with KIM5 was significantly reduced when caspase-1 was inhibited (Figure 2.2, panel A) or absent (Figure 2.14, panel A) in BMDMs. Pro-IL-1 β released from caspase-1-deficient macrophages (Figure 2.14, panel A) may account for the residual amounts of IL-1 β detected by ELISA. In addition, other pro-inflammatory caspases may play a role in processing and release of IL-1 β during infection with KIM5 when caspase-1 is absent (Discussed further in Chapter 5). KIM5 also stimulated a higher level of cell death in infected macrophages as compared to CO92 Δ *pgm* (Figure 2.6, panel C). However, cell death in KIM5-infected BMDMs did not require caspase-1 activity, since release of LDH was not significantly decreased in the presence of YVAD (Figure 2.6, panel B) or the absence of caspase-1 (Figure 2.14, panel B). These results argued that, although caspase-1 was being activated in KIM5-infected macrophages, the BMDMs were not dying of pyroptosis. Analysis of a KIM5*yopB* mutant suggested that translocation of a T3SS effector into the macrophage cell was required for caspase-1 activation and cell death

(Figure 2.2, panel B). Pre-growth of KIM5 at 28°C significantly reduced IL-1 β secretion (Figure 2.3), further demonstrating the importance of the T3SS for activating caspase-1. Inhibition of actin polymerization with CD can suppress the induction of caspase-1 activation and cell death in macrophages infected with *Salmonella* and *Francisella* species (37, 78, 91). However, we found that caspase-1 activation and cell death in macrophages infected with KIM5 was not inhibited by CD treatment (Figure 2.11). This result suggested that caspase-1 activation and cell death could result from the translocation of a T3SS effector by extracellular *Y. pestis*. Both intracellular survival and bacterial protein synthesis were important for the activation of caspase-1 and secretion of IL-1 β ; this was shown by infecting macrophages with KIM5 Δ *phoP* or in the presence of chloramphenicol, respectively. Finally, analysis of *Y. pestis* KIM-derived mutants defective for expression of functional Yop effectors indicated that cell death and caspase-1 activation required T3SS-mediated delivery of active YopJ into macrophages (Figure 2.8 and 2.13). All together, these results indicated that macrophages infected with *Y. pestis* KIM strains undergo a YopJ-dependent form of cell death that is coupled to activation of caspase-1. Although we had set out with the goal of learning how Yop effectors of *Yersinia* species inhibit activation of caspase-1, we instead discovered that the YopJ protein of *Y. pestis* KIM strains is required for activation of this pro-inflammatory caspase.

The mechanism of caspase-1 activation in BMDMs infected with *Y. pestis* KIM appears to be different from what has previously been observed by other groups studying *Yersinia*-infected macrophages (6, 122, 124). Bergsbaken and Cookson (6) found that caspase-1 was activated in BMDMs infected with wild-type *Y. pseudotuberculosis* YPIII,

but only when the macrophages were pre-treated with LPS, and YopJ was dispensable for activation of caspase-1 under their infection conditions. In contrast, YopJ activity was required for caspase-1 activation (Figure 2.13), and IL-1 β secretion decreased, when BMDMs were pre-treated with LPS (Figure 2.2, panel C) in KIM5-infected macrophages. In *Y. enterocolitica* E40-infected Mf4/4 cells, activation of caspase-1 was only observed in strains deficient for production of YopE and YopT (122, 124), because the activities of these effectors normally prevent the formation of pores (136) that can apparently stimulate the inflammasome to activate caspase-1 (124). However, we found that activation of caspase-1 in KIM-infected BMDMs did not require the absence of YopE and YopT, and in fact the absence of these effectors appeared to result in decreased IL-1 β secretion by macrophages (Figure 2.8, $\Delta 1$ mutant is missing *yopT* and $\Delta 4$ mutant is missing *yopE*). Genetic differences that exist between the strains used in the studies discussed above and our study may partially explain the different results that have been observed with respect to mechanisms of caspase-1 activation in *Yersinia*-infected macrophages. In addition, differences in experimental variables such as type of macrophage (primary, cell line, activated vs. non-activated) and MOI may result in different conclusions being reached as to the role of Yops in modulating caspase-1 activation. For example, activation of caspase-1 as a result of T3SS-dependent pore formation requires a high MOI (≥ 50) and extended time of contact (≥ 2 hours) between live extracellular bacteria and macrophages (124). We used a relatively low MOI (10) and a short period of contact with live extracellular bacteria (20 min) and as a result activation of caspase-1 via pore formation was minimal, as shown by the low levels of IL-1 β secreted from naïve or LPS-stimulated BMDMs infected with multi-effector

mutants of *Y. pseudotuberculosis* IP2666 (Figure 2.1, panel C) or *Y. pestis* KIM8 (Figure 2.8, panel A).

Previous studies have shown that *Y. pestis* strains are limited in their ability to induce YopJ-dependent apoptosis in macrophages, unless high MOIs are used and the bacteria are centrifuged onto the host cells to force contact (140, 143). Similar to what has been reported for EV76 and Kimberly53 (143), CO92 Δ *pgm* was limited in its ability to kill macrophages under our infection conditions (Figure 4D). In contrast, O:8 serogroup strains of *Y. enterocolitica*, such as WA-314, have been shown to induce high levels of YopP-dependent apoptosis in macrophages at low MOI (116, 143). The limited ability of some *Y. pestis* strains to induce macrophage apoptosis, as compared to WA-314, has been correlated with decreased translocation of *Y. pestis* YopJ as compared to YopP of WA-314 (143). As discussed further below, it is possible that the YopJ protein of KIM has unique features or activities that result in high levels of cell death in macrophages infected at low MOI. We hypothesize that *Y. pestis* KIM strains induce a novel form of YopJ-dependent cell death in macrophages, which is coupled to activation of caspase-1. A previous study reported that murine bone marrow derived dendritic cells infected with *Y. enterocolitica* strain WA-314 died of a necrotic form of cell death that required YopP activity (49). Although activation of caspase-1 or secretion of IL-1 β was not investigated in that study, dendritic cells infected with *Y. enterocolitica* WA-314 released HMGB1 (49), which is a potent proinflammatory molecule (145). Thus, there is precedence for the idea that *Yersinia* infection can stimulate YopJ/P-dependent pro-inflammatory host cell death, although our study is the first to demonstrate YopJ-dependent activation of caspase-1 in infected host cells.

The ability of different *Y. enterocolitica* strains to effectively induce apoptosis in infected macrophages has been linked to variations at position 143 of the YopP sequence (116). Serogroup O8 strains such as WA-314 that effectively induce apoptosis in infected macrophages contain an Arg at position 143 (116). *Y. enterocolitica* strains that express YopP proteins containing the Arg at position 143 also strongly inhibit activation of NF- κ B in macrophages (116). However, the YopJ proteins encoded by KIM, CO92 and *Y. pseudotuberculosis* all contain an Arg at position 143 (116), so variations at this residue are not responsible for the phenotypic differences seen in the present study. The sequence of the KIM YopJ protein (NP_857908) differs by two amino acids from the sequence of CO92 YopJ (NP_395205.1), corresponding to L177F and E206K substitutions. The sequence of the KIM YopJ protein differs by one amino acid from the sequence of *Y. pseudotuberculosis* IP32953 YopJ (NP_395205.1), corresponding to an L177F substitution. The amino acid differences that exist between the YopJ proteins of KIM, CO92 and *Y. pseudotuberculosis* are likely responsible for the phenotypic differences observed in this study, and in the future it will be important to determine how these substitutions alter YopJ protein function. Several possibilities exist including differences in translocation efficiency, protein stability, or substrate specificity. In addition, we note that the sequence of a YopJ protein from *Y. pestis* Mediaevalis strain K1973002 (ZP_02318615) is identical to the sequence of YopJ from KIM, suggesting that the phenotype we are observing is not an artifact resulting from a mutation acquired during laboratory passage, but rather is due to a unique *yopJ* genotype associated with Mediaevalis strains.

Another key issue that remains to be addressed is the connection between cell death and caspase-1 activation in macrophages infected with *Y. pestis* KIM. Caspase-1 activity is not required for cell death (Figure 2.6 panel C, 2.14 panel B), which indicates that caspase-1 activation is a downstream effect of cell death, or that separate pathways regulate cell death and caspase-1 activation. The kinetics of IL-1 β /IL-18 secretion and LDH release from KIM5-infected macrophages were similar (compare Figure 2.5 A and B with 2.6, panel A), which is suggestive of a mechanistic connection between the two processes. LPS activation of macrophages decreases YopP-dependent apoptosis in response to *Y. enterocolitica* infection (115), and we observed that LPS pre-treatment decreased secretion of IL-1 β from KIM5-infected BMDMs (Figure 2.2, panel C), which is an indication that caspase-1 activation occurs downstream of the cell death program. One possibility is that YopJ-mediated inhibition of NF- κ B activation in KIM5-infected macrophages triggers cell death and caspase-1 activation. Greten *et al.* (48) have shown that gene products under control of NF- κ B negatively regulate caspase-1 activation in macrophages, and that inhibition of NF- κ B activation before stimulating macrophages with LPS results in enhanced secretion of IL-1 β . It may seem counterintuitive that strong inhibition of NF- κ B activation in KIM5-infected macrophages could result in enhanced secretion of mature IL-1 β , since expression of the pro form of IL-1 β is positively regulated by NF- κ B. In fact, Schotte *et al.* (122) reported that YopP inhibits expression of the pro form of IL-1 β in Mf4/4 cells infected with *Y. enterocolitica* E40. We observed inhibition of TNF- α secretion from BMDMs infected with KIM5 (Figure 2.13, panel E), indicating that the YopJ protein of KIM is reducing expression of NF- κ B target genes. However, we note that macrophages infected with KIM5 secrete more TNF- α than

uninfected BMDMs (Figure 2.13, panel E), which suggests that activation of NF- κ B and expression of NF- κ B target genes was occurring at a low level in even in the presence of YopJ^{KIM}. Low levels of activated NF- κ B may be sufficient for small amounts of pro-IL-1 β to be made, but not sufficient to prevent activation of caspase-1, resulting in measurable secretion of the mature form of IL-1 β .

Greten *et al.* (48) proposed that activation of caspase-1 in response to inhibition of NF- κ B represents a mechanism of host defense against pathogens that target this transcription factor. It is possible that the differential ability of *Y. pestis* KIM and CO92 strains to activate caspase-1 in BMDMs is a consequence of a differential ability of these strains to inhibit, via YopJ, activation of NF- κ B. Caspase-1 is known to play a protective role against several pathogens, including *Salmonella* and *Francisella* species, in murine infection models (71, 76, 112). If KIM and CO92 strains differentially activate caspase-1 in vivo, it could manifest in a virulence difference between these strains. This will be an important issue to address in future studies.

Acknowledgements

I thank Ying Zheng for contributing the time course ELISA results and the CFU assay to these studies. I am grateful for all the assistance and contributions she has made towards this work. Thanks to Stephen Smiley from the Trudeau Institute for the KIM-D27 strain, Robert Perry from the University of Kentucky for pCD1::Amp, Gregory Plano from the University of Miami for the KIM8 strains harboring the various deletions in pCD1 and the pYopJ-GSK plasmid and Richard Flavell and Craig Roy from Yale University for the caspase-1 deficient mice. A number of Bliska lab members contributed to this study: Hana Fukuto for construction of KIM5, Kathryn Klein for construction of KIM5*yopB*, Selina Myrczek for constructing the KIM5 *ypkA* and *yopH* mutants, Yue Zhang and Shirou Wu for construction of the KIM5 *yopJC172A* mutant, Céline Pujol for construction of CO92 Δ *pgm*. Galina Romanov isolated and propagated the murine macrophages used in the majority of these experiments.

TABLE 2.1. *Yersinia* strains used in this chapter

Strain name	Relevant Characteristics	Reference or source
<i>Y. pestis</i>		
KIM6+	Biovar Mediaevalis, pCD1 ⁻ , pMT1 ⁺ , pPCP1 ⁺ , <i>pgm</i> ⁺	(20)
KIM6	Biovar Mediaevalis, pCD1 ⁻ , pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i>	This study
KIM5	Biovar Mediaevalis, KIM6/pCD1Ap, pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i> , Ap ^{r(1)}	This study
KIM5/GFP	KIM5/pMMB207gfp3.1, Ap ^r , Cm ^{r(2)}	(46)
KIM-D27	pCD1 ⁺ , pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i>	(101)
CO92 Δ <i>pgm</i>	Biovar Orientalis, pCD1 ^{CO92+} , pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i>	This study
KIM5 <i>yopB</i>	pCD1Ap <i>yopB18</i> (in-frame deletion of nucleotides 496-774), Ap ^r	This study
KIM8 Δ 1	pCD1- Δ 1, pPCP1 ⁻ , pMT1 ⁺ , Δ <i>pgm</i> , Km ^{r(3)}	(1)
KIM8 Δ 2	pCD1- Δ 2, pPCP1 ⁻ , pMT1 ⁺ , Δ <i>pgm</i> , Km ^r	(1)
KIM8 Δ 34	pCD- Δ 34, pPCP1 ⁻ , pMT1 ⁺ , Δ <i>pgm</i> , Km ^r	G. Plano, unpublished
KIM8 Δ 4	pCD1- Δ 4, pPCP1 ⁻ , pMT1 ⁺ , Δ <i>pgm</i> , Tm ^{r(4)}	(1)
KIM8 Δ 12	pCD1- Δ 12, pPCP1 ⁻ , pMT1 ⁺ , Δ <i>pgm</i> , Km ^r	(1)
KIM8 Δ 123	pCD1- Δ 123, pPCP1 ⁻ , pMT1 ⁺ , Δ <i>pgm</i> , Km ^r , Cm ^r	(1)
KIM8 Δ 1234	pCD1- Δ 1234, pPCP1 ⁻ , pMT1 ⁺ , Δ <i>pgm</i> , Tm ^r , Km ^r , Cm ^r	(1)
KIM5 <i>yopJC172A</i>	pCD1Ap <i>yopJC172A</i> (codon change of Cys172 to Ala172), Ap ^r	This study
KIM5 <i>yopH::kan</i>	pCD1Ap (Km ^r cassette inserted into <i>yopH</i>), Ap ^r , Km ^r	This study
KIM5 <i>ypkA::kan</i>	pCD1Ap (Km ^r cassette inserted into <i>ypkA</i>), Ap ^r , Km ^r	This study
KIM5 <i>caf1A::kan</i>	pCD1Ap (Km ^r cassette inserted into <i>caf1A</i>), Ap ^r , Km ^r	(117)
KIM-D27 Δ <i>phoP</i>	pCD1Ap (in-frame deletion of nucleotides 127-429)	(46, 47)
KIM5 pIB102	pIB102, pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i>	This study

Y. pseudotuberculosis

IP2777	Serogroup O1, pYV ⁺	(125)
IP2666	Serogroup O3, pYV ⁺ (naturally <i>DyopTsysT</i>)	(125)
IP2666 E ⁻	pYV <i>yopE::kan</i> , alternative name is IP6, Km ^r	(8)
IP2666 J ⁻	pYV <i>yopJD1-867</i> , alternative name is IP26	This study
IP2666 EJ ⁻	pYV <i>yopE::kan yopJD1-867</i> , alternative name is IP31, Km ^r	This study
IP2666 JEHMOK ⁻	pYV <i>yopJD1-867 yopE::kan yopH::cam yopM yopO yopK</i> , alternative name is IP37, Km ^r , Cm ^r	This study

(1) Ap^r: Ampicillin resistance; (2) Cm^r: Chloramphenicol resistance; (3) Km^r: Kanamycin resistance; (4) Tm^r: Trimethoprim resistance.

TABLE 2.2. KIM8 pCD1 deletion mutant strains used in this chapter

Strain	Deleted genes or ORFs
KIM8 Δ 1	ylpA, yopK, yopT, sycT, ORF61, ORF60, yopM, ORF54
KIM8 Δ 2	ORF5, ORF7, ypkA, yopJ, yopH
KIM8 Δ 34	yadA, yadA', ORF85, ORF84, sycH, sycE, yopE, ORF75, ORF74, ORF73
KIM8 Δ 4	sycE, yopE, ORF75, ORF74, ORF73
KIM8 Δ 12	ylpA, yopK, yopT, sycT, ORF61, ORF60, yopM, ORF54, ORF5, ORF7, ypkA, yopJ, yopH
KIM8 Δ 123	ylpA, yopK, yopT, sycT, ORF61, ORF60, yopM, ORF54, ORF5, ORF7, ypkA, yopJ, yopH, yadA, yadA', ORF85, ORF84
KIM8 Δ 1234	ylpA, yopK, yopT, sycT, ORF61, ORF60, yopM, ORF54, ORF5, ORF7, ypkA, yopJ, yopH, yadA, yadA', ORF85, ORF84, sycE, yopE, ORF75, ORF74, ORF73

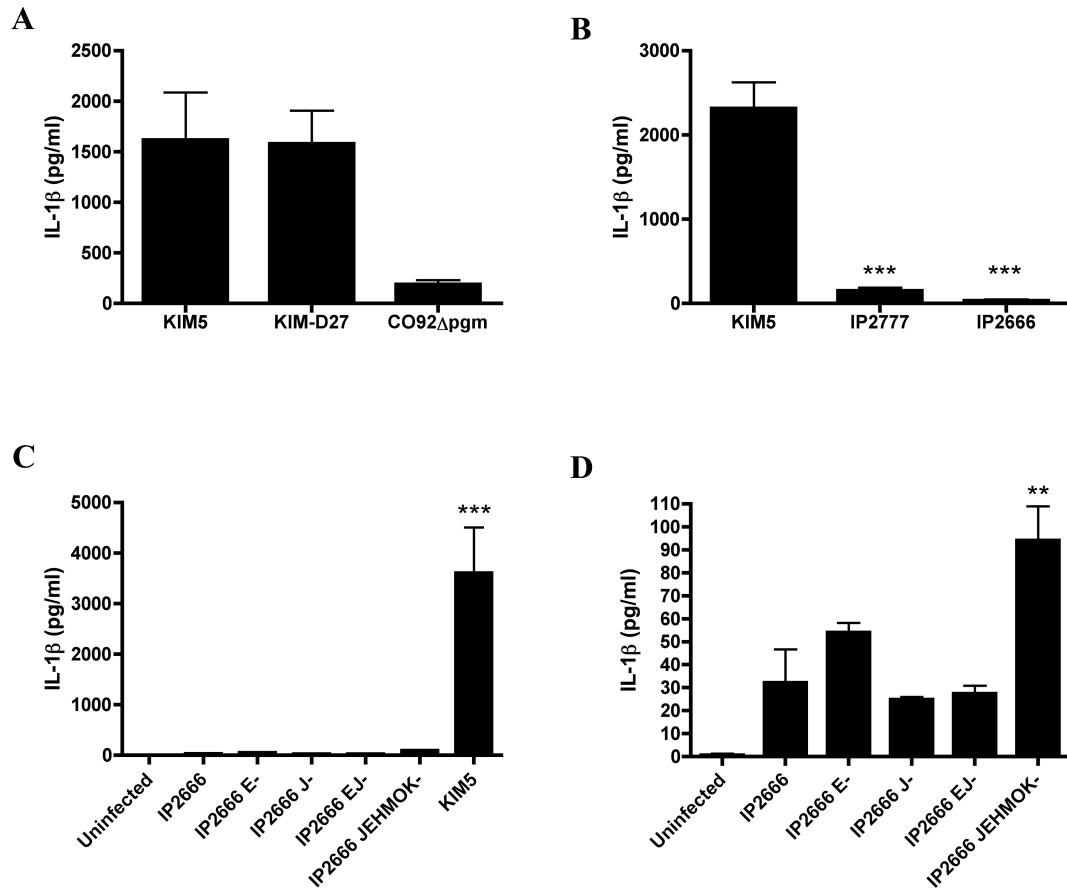
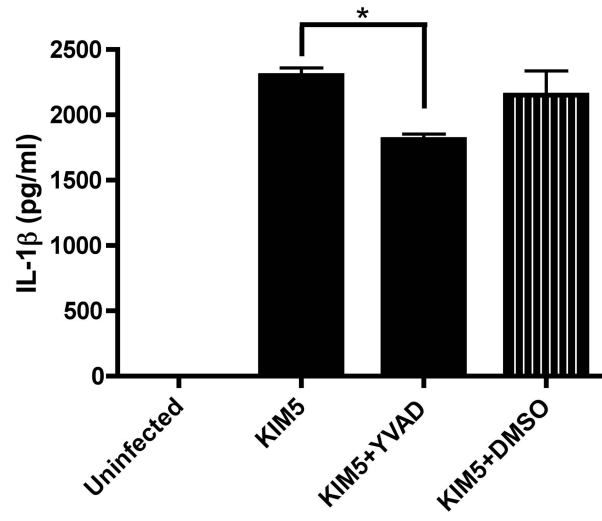


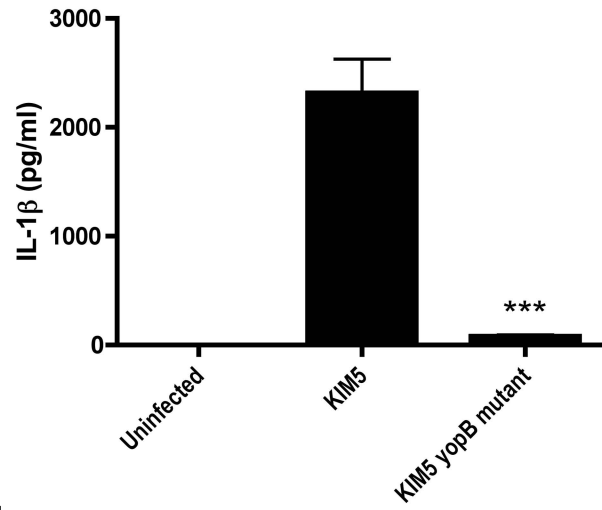
Figure 2.1. Determination of levels of IL-1 β secreted from macrophages infected with different *Y. pestis* or *Y. pseudotuberculosis* strains. BMDMs were left uninfected or infected with the indicated strains of *Y. pestis* or *Y. pseudotuberculosis* (Table 2.1). IL-1 β levels (pg/ml) from BMDM supernatants were measured by ELISA at 24 hr post infection. Results shown are the averages of three independent experiments. Error bars represent standard deviation. Statistical significance as compared to KIM5 (B) or IP2666 (C, D) was determined ($P < 0.01$, **; $P < 0.001$, ***).

Figure 2.2. Determination of factors required for IL-1 β secretion in macrophages infected with *Y. pestis* or *Y. pseudotuberculosis*. BMDMs were left uninfected or infected with the indicated strains of *Y. pestis* or *Y. pseudotuberculosis* (Table 2.1) and levels of IL-1 β secreted at 24 hr post infection were measured by ELISA. (A, B) Macrophages were left untreated or exposed to 100 μ M of the caspase-1 inhibitor Ac-YVAD-cmk (YVAD) or 140 mM DMSO as a solvent control for 1 hr prior to infection. (C) Macrophages were left untreated (white bars) or exposed to 50 ng/ml of LPS for 4 hrs prior to infection (black bars). Results shown are the averages of three independent experiments (B, C), or a single experiment with triplicate wells (A). Error bars represent standard deviation. Statistical significance ($P < 0.05$, *; $P < 0.001$, ***) as compared to KIM5 (A, B) or KIM5 untreated (C) was determined.

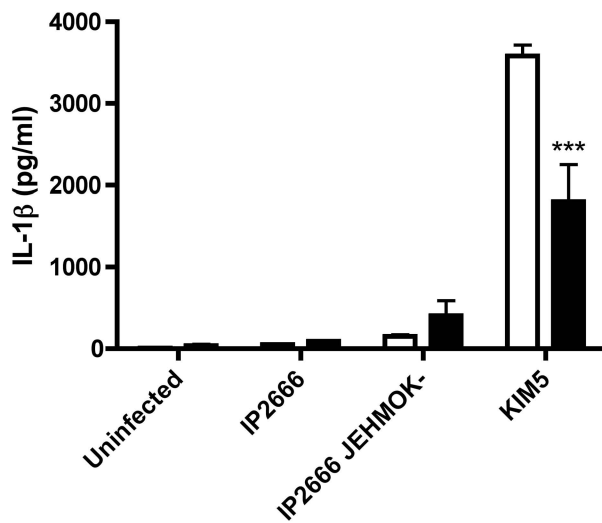
A



B



C



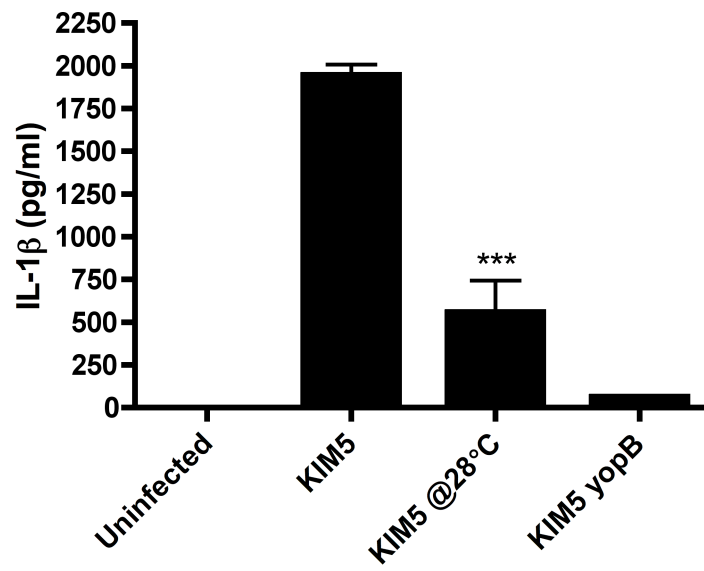


Figure 2.3. *Y. pestis* KIM5 grown at 37°C, but not at 28°C, induces maximal IL-1 β secretion during infection. KIM5 was grown at either 37°C or 28°C for 2 hr with shaking prior to infection of BMDM. Twenty-four hours later, supernatants were collected and analyzed by ELISA (R&D Systems). Results (pg/ml of IL-1 β) are calculated from 3 experiments with duplicate wells per experiment and analyzed by ANOVA using GraphPad Prism (**, $P < 0.01$; ***, $P < 0.001$).

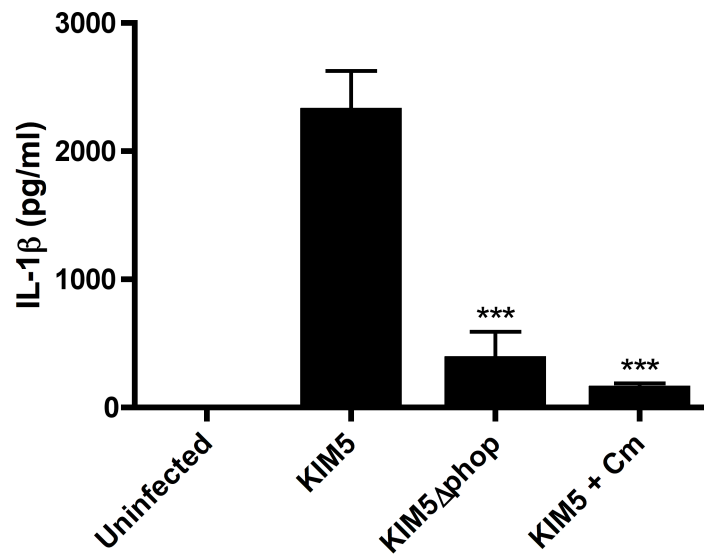


Figure 2.4. Bacterial protein synthesis and intracellular survival are important for IL-1 β secretion. BMDMs were infected with KIM5, a KIM5 *phoP* mutant or left uninfected. Where indicated, a concentration of 30 μ g/ml of chloramphenicol (Cm) was added to infection medium concurrently with bacteria. Twenty-four hours post-infection, BMDM supernatants were collected, and IL-1 β levels (pg/ml) were measured by ELISA. Results shown are the averages of three independent experiments and analyzed by ANOVA using GraphPad Prism. Error bars represent standard deviation (***, $P < 0.001$).

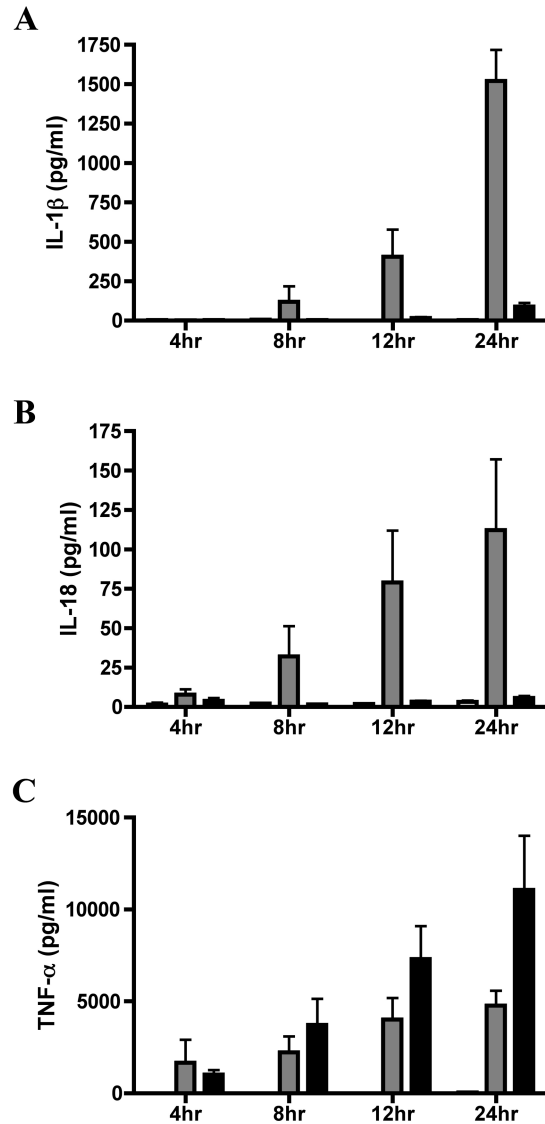
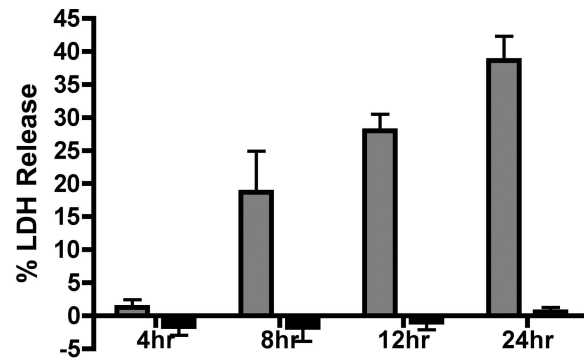


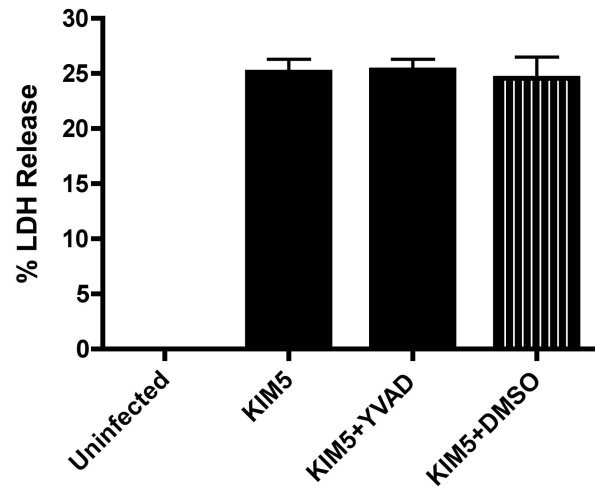
Figure 2.5. Time course analysis of cytokine secretion from macrophages infected with *Y. pestis*. BMDMs were left uninfected (white bars) or infected with either KIM5 (grey bars) or a KIM5yopB mutant (black bars). Supernatants were collected at the indicated timepoints, and levels of IL-1 β (A), IL-18 (B) and TNF- α (C) were quantified by ELISA. Results shown are the average of three independent experiments. Error bars represent standard deviation.

Figure 2.6. Determination of factors required for cell death in macrophages infected with *Y. pestis*. BMDMs were left uninfected or infected with *Y. pestis* KIM5, KIM5*yopB*, CO92Δ*pgm* or KIM-D27. Cell death was monitored by the percentage of lactate dehydrogenase (LDH) release. In (A) supernatants from macrophages infected with KIM5 (grey bars) or KIM5*yopB* (black bars) were collected at the time points indicated and percent LDH release was determined. In (B, C, D) macrophages were left untreated or exposed to 100 μM YVAD or DMSO as a solvent control for 1 hr prior to infection, and supernatants were collected at 24 hr post infection and analyzed for LDH release. Results shown are the averages of three independent experiments (A, C) or one experiment with triplicate wells (B). Error bars represent standard deviation. Statistical significance ($P < 0.01$, **) as compared to KIM5 (C) was determined.

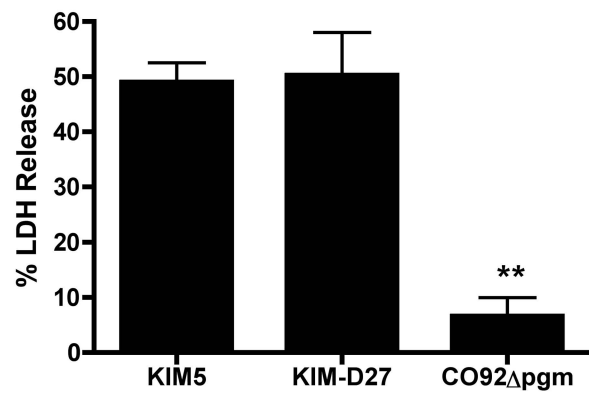
A



B



C



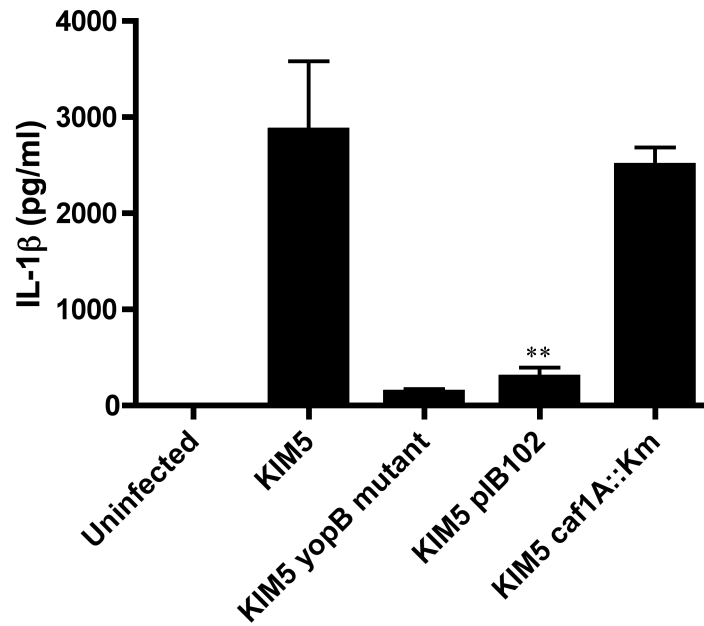


Figure 2.7. Substitution of pIB102 for pCD1 in KIM5 does not stimulate IL-1 β secretion in infected macrophages. BMDMs were infected with the indicated strains or left uninfected. Twenty-four hours post-infection, BMDM supernatants were collected and IL-1 β levels (pg/ml) were measured by ELISA. Results shown are the averages of three independent experiments and analyzed by ANOVA using GraphPad Prism. Error bars represent standard deviation (**, $P < 0.01$).

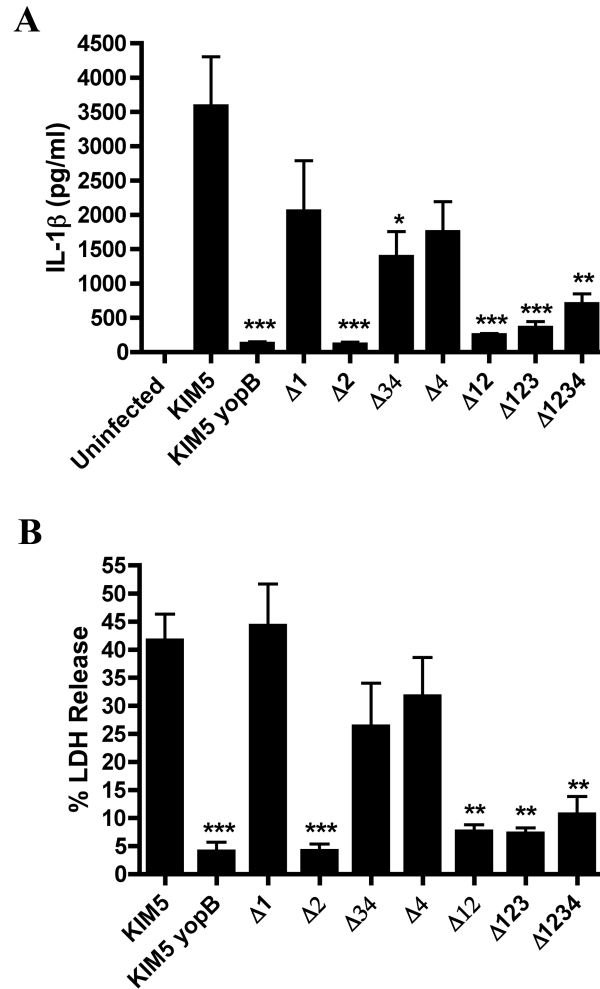


Figure 2.8. Identification of a region of pCD1 required for IL-1 β secretion and cell death in macrophages infected with *Y. pestis*. BMDMs were left uninfected or were infected with KIM5, KIM5yopB or the indicated KIM8-derived strains (Tables 2.1, 2.2). Supernatants were collected at 24 hr post infection and analyzed for secreted IL-1 β by ELISA (A) and cell death by LDH release (B). Results shown are the averages of three independent experiments. Error bars represent standard deviation. Statistical significance ($P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***) as compared to KIM5 was determined.

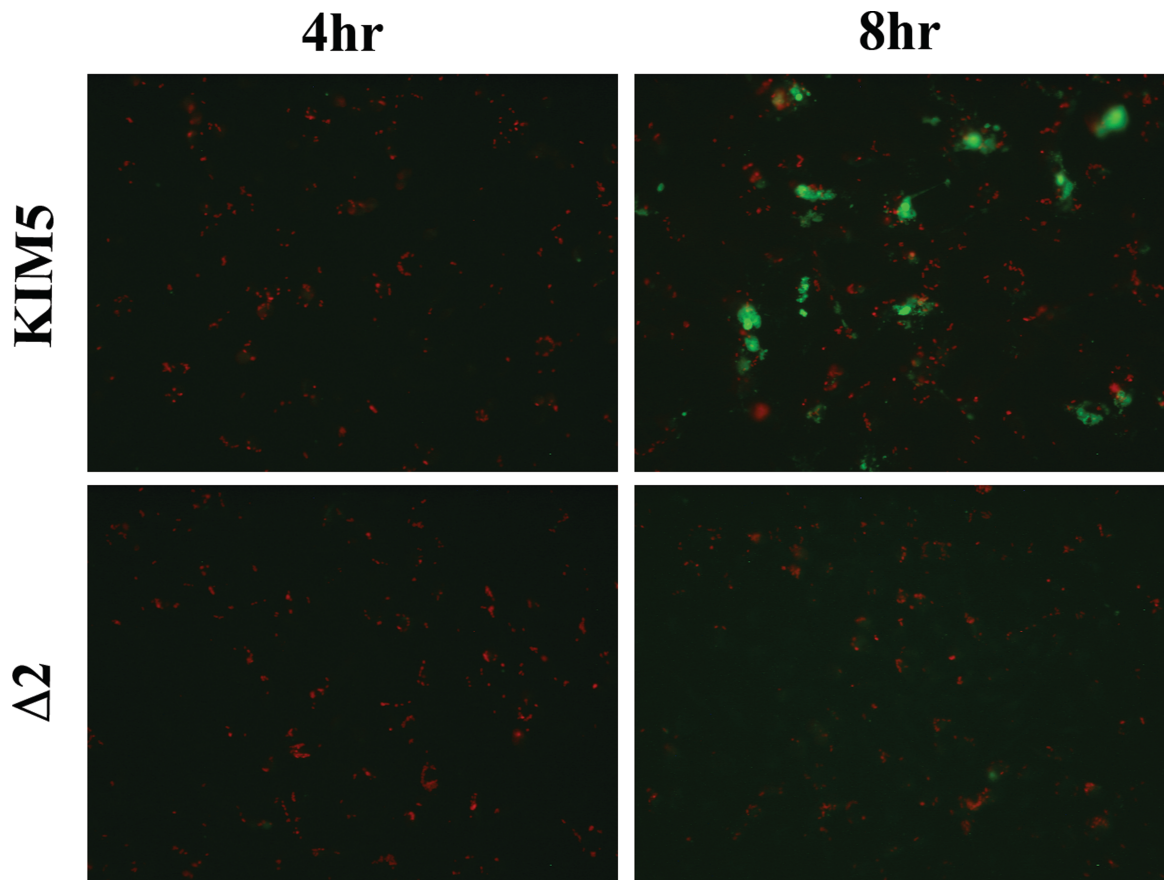


Figure 2.9. Detection of active caspase-1 in macrophages infected with *Y. pestis* by fluorescence microscopy. BMDMs attached to coverslips were infected with *Y. pestis* KIM5 (upper panels) or with the KIM8 $\Delta 2$ mutant (lower panels) for 4 hr (left panels) or 8 hr (right panels). Infected macrophages were incubated with FLICA reagent (FAM-YVAD-FMK) to stain for active caspase-1 (green). The samples were then fixed, and bacteria were immunolabeled with a rabbit anti-*Yersinia* antibody (red). Coverslips were mounted on slides, and fluorescence microscopy was used to detect red and green signals. Representative images were captured by digital photomicroscopy. Images shown are overlays of the red and green signals and are representative of three independent experiments.

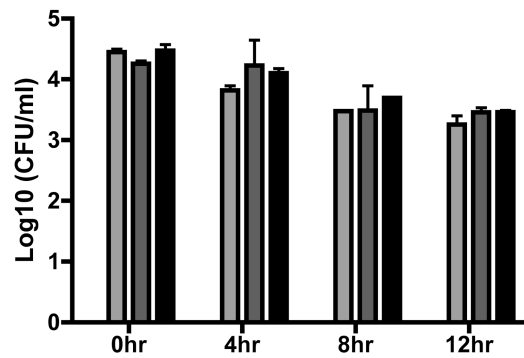


Figure 2.10. Analysis of bacterial association and intracellular survival in macrophages infected with *Y. pestis*. BMDMs were infected for 20 min with KIM5 (light grey bars), KIM5yopB (dark grey bars) or the KIM8 Δ2 mutant (black bars). At the end of the 20 min infection period, some of the infected macrophages were washed and lysed, and serial dilutions of the lysates were plated for CFU determination (0 hr time point). The infected macrophages remaining were exposed to gentamicin, and at the time points indicated the BMDMs were processed for CFU determination as above. Results shown are the averages of three independent experiments. Error bars represent standard deviation.

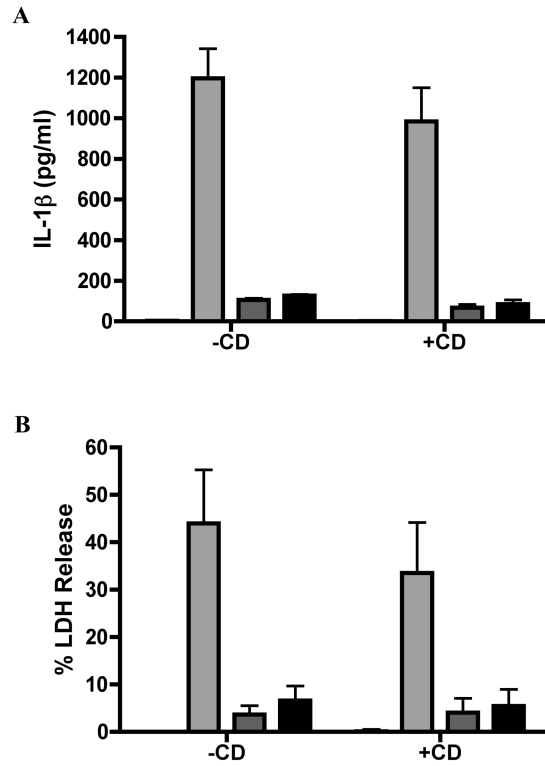


Figure 2.11. Determination of the role of bacterial phagocytosis for IL-1 β secretion and cell death in macrophages infected with *Y. pestis*. BMDMs were left untreated (-CD) or treated with cytochalasin D (+CD) and infected with KIM5 (light grey bars), KIM5yopB (dark grey bars) or the KIM8 Δ 2 mutant (black bars). Twenty-four hours post infection supernatants were collected and analyzed for secreted IL-1 β by ELISA (A) or released LDH (B). Treatment of macrophages with DMSO as a solvent control under identical conditions used for CD exposure did not affect IL-1 β secretion or LDH release (data not shown). Results shown are the averages of three independent experiments. Error bars represent standard deviation.

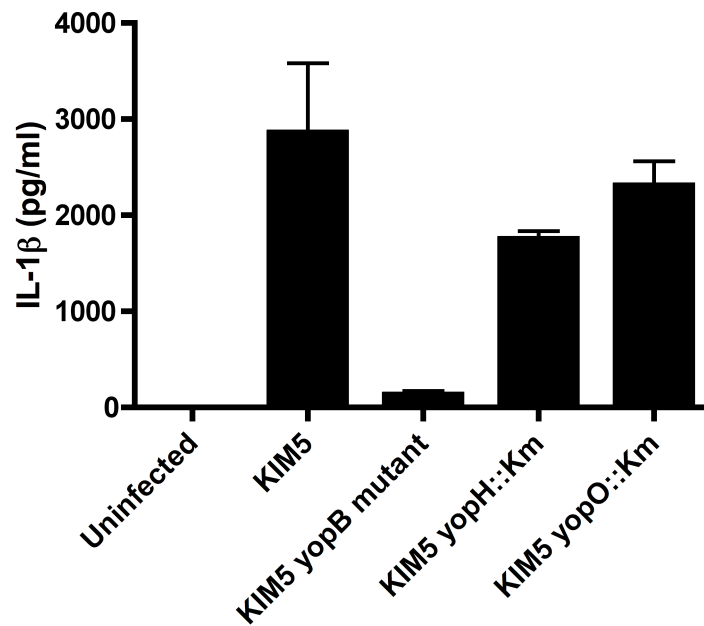


Figure 2.12. Determination of the role of YopH and YopO for IL-1 β secretion in macrophages infected with *Y. pestis* mutants. BMDMs were left uninfected or were infected with KIM5, KIM5yopB, KIM5yopH or KIM5yopO (Table 2.1). Supernatants were collected at 24 hr post infection and analyzed for secreted IL-1 β by ELISA. Results shown are the averages of three independent experiments. Error bars represent standard deviation.

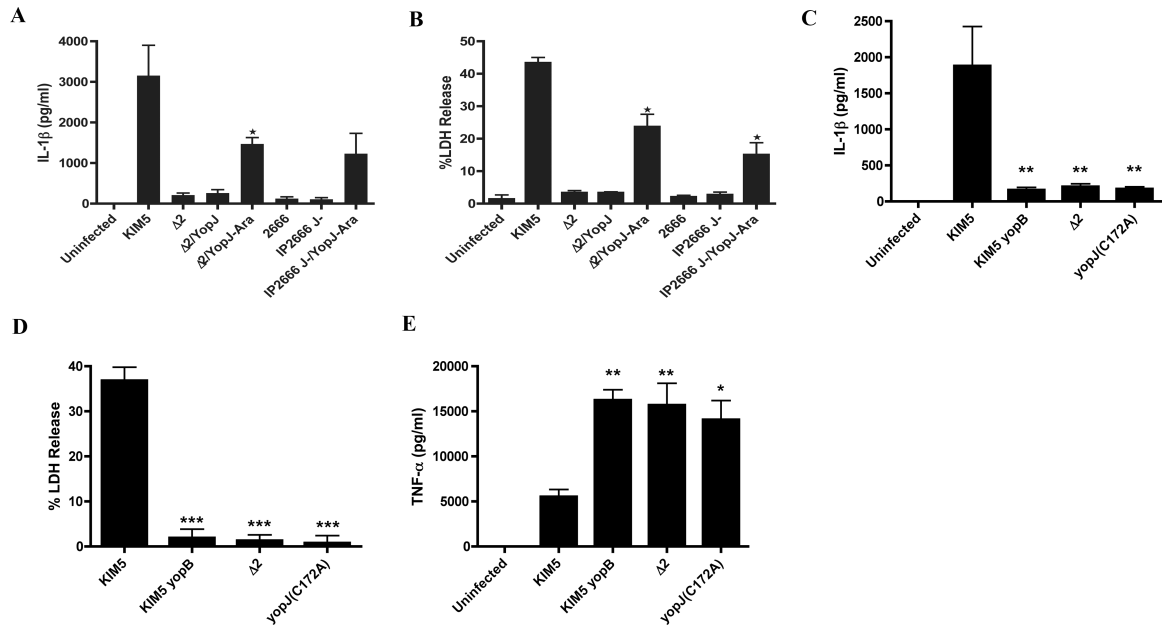


Figure 2.13. Determination of the role of YopJ for IL-1 β secretion, TNF- α secretion and cell death in macrophages infected with *Y. pestis* or *Y. pseudotuberculosis*. BMDMs were left uninfected or infected with KIM5, KIM5*yopB*, KIM5*yopJ*C172A, KIM8 Δ 2 IP2666, or IP2666 J-. Where indicated KIM8 Δ 2 and IP2666 J- carried the pYopJ-GSK plasmid (YopJ) and were cultured in the presence of arabinose (Ara). Supernatants were collected at 24 hr post infection and tested for IL-1 β by ELISA (A, C), cell death by LDH release (B, D), or TNF- α by ELISA (E). Results shown are the averages of three independent experiments. Error bars represent standard deviation. Statistical significance ($P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***) as compared to KIM5 was determined (C, D, E). In (A), $P < 0.05$, * comparing Δ 2 vs. Δ 2/YopJ-Ara. In (B) $P < 0.05$, * comparing Δ 2 vs. Δ 2/YopJ-Ara or IP2666 vs. IP2666 J-/YopJ-Ara.

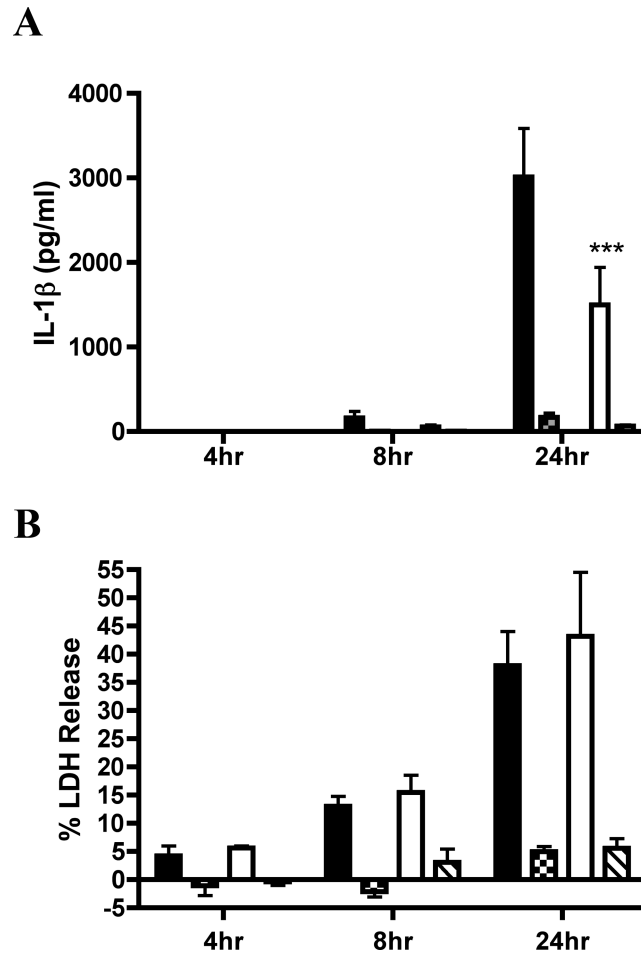


Figure 2.14. Determination of the importance of caspase-1 for IL-1 β secretion and cell death by infection of *Casp*^{-/-} macrophages with *Y. pestis*. BMDMs were left uninfected or infected with KIM5 (black bar *Casp*^{+/+}, white bar *Casp*^{-/-}) or KIM5yopJCI72A (checked bar *Casp*^{+/+}, striped bar *Casp*^{-/-}). Supernatants were collected at 4 hr, 8 hr and 24 hr post infection and monitored for IL-1 β secretion by ELISA (A) or cell death by LDH assay (B). Results shown are the averages of three independent experiments. Error bars represent standard deviation. Statistical significance ($P < 0.001$, ***) as compared to KIM5 was determined.

Chapter 3: The NALP3/ASC inflammasome complex and potassium efflux are important for caspase-1 activation and IL-1 β processing and release from KIM5-infected macrophages

3.1 Summary

Previous studies have demonstrated that the inflammasome complex is an important cytoplasmic component for the recognition of pathogenic bacteria and the subsequent recruitment and activation of caspase-1. Additionally, others have demonstrated that potassium efflux due to the activation of the P2X₇R by extracellular ATP can activate the NALP3 inflammasome complex (74, 102). Our studies aimed to elucidate the role of the inflammasome complex, potassium efflux and the P2X₇R in the detection of *Y. pestis* KIM5. To this end, macrophages deficient for the NLRs NALP3 and Ipaf, as well as ASC, were infected with *Y. pestis* KIM5 or a strain expressing a catalytically inactive form of YopJ (KIM5 *yopJ C172A*), and secretion of cytokines was measured by ELISA. Levels of IL-1 β and IL-18 secreted from NALP3- and ASC-deficient macrophages were decreased significantly as compared to wild-type macrophages after 24 hrs; Ipaf-deficient macrophages secreted similar levels of these cytokines as compared to wild-type. Additionally, levels of LDH release and TNF- α production from all macrophages tested were comparable to wild-type infected macrophages, indicating that these pathways are not dependent on inflammasome activation. Macrophages deficient for the P2X₇R showed no significant differences in IL-1 β , IL-18 or TNF- α production as well as cell

death after 24 hrs, as compared to wild-type macrophages, indicating that the P2X₇R does not play a role during KIM5 infection. Blocking the release of potassium from infected macrophages by the addition of 30 mM potassium chloride (KCl) inhibited the secretion of IL-1 β and IL-18, but not TNF- α , from infected macrophages at both 8 hrs and 24 hrs post-infection. These results suggest that the NALP3/ASC inflammasome complex, as well as potassium efflux, plays a significant role in the detection of KIM5 and activation of caspase-1, through recognition of YopJ.

3.2 Introduction

Caspase-1 activation and IL-1 β secretion from macrophages can occur through the recognition of various PAMPs by the inflammasome complex. The inflammasome is a multi-protein complex, composed of an NLR and the adaptor protein ASC, that activates caspase-1, resulting in the cleavage of the precursor forms of the pro-inflammatory cytokines IL-1 β and IL-18. The NALP3 inflammasome complex has been shown to be responsive to both microbial products and non-microbial “danger” signals (5, 60, 62, 63, 70, 82). Numerous studies have demonstrated that bacterial RNA, LPS, lipoprotein and PGN can activate NALP3, specifically in the presence of millimolar concentrations of adenosine triphosphate (ATP) (5, 60, 62, 70, 79, 128). ATP is released from damaged cells or cells undergoing death, and this extracellular ATP can activate the purinergic receptor P2X₇R, which rapidly induces the release of intracellular potassium (74, 102). The Ipaf inflammasome has been shown to recognize flagellin from *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Legionella pneumophila* (38, 40, 76, 87, 113, 114, 118, 127, 129). In this chapter, we aim to determine if the inflammasome, potassium

efflux and/or the P2X7R play an important role in the activation of caspase-1 and processing and release of IL-1 β and IL-18 in KIM5-infected macrophages.

3.3 Experimental Methods

Bacterial strains and growth conditions. The *Y. pestis* strains KIM5 and KIM5 containing a catalytically inactive form of YopJ (KIM5 *yopJCI72A*) were used in this study (see Table 2.1, Chapter 2). The construction of these strains is described in Chapter 2, Experimental Methods. *Y. pestis* strains were cultivated on heart infusion (HI) agar plates (Difco) for two days at 28°C. Cultures were grown overnight with aeration in HI broth at 28°C. The next day the cultures were diluted to an OD₆₀₀ of 0.1 in the same medium supplemented with 2.5 mM CaCl₂ and incubated for 2 hr at 37°C with aeration. Bacterial cultures were washed once with phosphate-buffered saline (PBS) then resuspended in PBS for OD₆₀₀ measurements. Bacterial growth medium was supplemented with ampicillin (25 μ g/ml) when appropriate.

Bone marrow macrophage isolation and culture conditions. Bone marrow derived macrophages (BMDM) and P2X₇ receptor-deficient macrophages were isolated as described in Chapter 2, Experimental Methods. Frozen stocks of macrophages deficient for the inflammasome components Ipaf, ASC or NALP3 and wild-type C57BL/6 macrophages were propagated in tissue culture medium supplemented with 10% fetal bovine serum, 30% L-cell-conditioned medium and penicillin-streptomycin (BMM-high). Briefly, macrophages were resuspended in 10 ml of DMEM containing 10% FBS and centrifuged for 5 minutes at 1200 rpm. A 20 ml suspension of each macrophage type was

plated in two 100 mm, non-tissue culture treated plates and incubated at 37°C with 5% CO₂ for 6 days, adding fresh BMM-high on day 4.

Macrophage infections. Infection assays were performed as described in Chapter 2, Experimental Methods. To inhibit potassium efflux from infected macrophages, potassium chloride (KCl) was added concurrently with 8 µg/ml gentamicin and 4.5 µg/ml gentamicin at concentrations of 30, 50, 70 and 90 mM above baseline for titration experiments and at a concentration of 30 mM above baseline for subsequent infection assays. Sodium chloride (NaCl) was used as a control and added at a concentration of 30 mM above baseline.

Cytokine measurements. Levels of IL-1β, IL-18 and TNF-α secreted into tissue culture media during infection assays were measured as described in Experimental Methods, Chapter 2.

Lactate dehydrogenase (LDH) release. Supernatants from infected macrophages were collected and analyzed for LDH release as described in Experimental Methods, Chapter 2.

IL-1β and caspase-1 immunoblots. At 8 hrs post-infection, infection medium was removed, and macrophage lysates from triplicate wells were collected in 100 µl of 1X lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 2 mM DTT and a protease inhibitor cocktail [Complete Mini, EDTA-Free, Roche]). Proteins were separated by SDS-PAGE (12% polyacrylamide) and transferred onto a nitrocellulose membrane overnight at 30 V using an electrophoresis apparatus. To detect IL-1β and caspase-1, membranes were blotted with goat anti-IL-1β (R&D Systems) or rabbit anti-caspase-1p10 (Santa Cruz Biotechnology, Inc.), respectively, in 2%

casein/PBS containing 0.1% Tween-20. Hamster anti-goat IRDye 700 or donkey anti-rabbit IRDye 800 secondary antibodies (Rockland) were used to detect samples, and blots were viewed on the Odyssey Infrared Imaging System (LI-COR). To control for loading, blots were probed with a rabbit anti-actin antibody (Sigma-Aldrich).

Statistical analysis. In general, experiments analyzed for significance were performed three independent times. Results presented in Figure 3.4 were performed once. Probability (P) values were calculated by one-way ANOVA and Tukey's multiple comparisons post test and were considered significant if less than 0.05 (GraphPad Prism 4.0).

3.4. Results

Previous studies have demonstrated that the inflammasome can be activated by a number of microbial products and host “danger” signals, which leads to the activation of caspase-1 and secretion of mature IL-1 β and IL-18 from macrophages (5, 60, 62, 63, 70, 82). We investigated the role of the NLRs NALP3 and Ipaf, as well as the adaptor protein ASC, in the caspase-1-dependent secretion of IL-1 β and IL-18 by using NALP3-, ASC- and Ipaf-deficient bone marrow derived macrophages. BMDMs were infected with KIM5 or KIM5 expressing a catalytically inactive form of YopJ (KIM5yop*JC172A*) (see Table 2.1). Tissue culture supernatants were collected and analyzed by ELISA to measure the levels of IL-1 β and IL-18 present after 24 hr of infection. As shown in Figure 3.1, NALP3- and ASC-deficient macrophages infected with KIM5 secreted significantly lower levels of IL-1 β and IL-18 as compared to wild-type macrophages infected with KIM5. Ipaf-deficient macrophages released similar levels of these cytokines as compared to wild-type, suggesting Ipaf does not play a significant role in caspase-1 activation and cytokine secretion during KIM5 infection. As observed previously, macrophages infected with KIM5yop*JC172A* secreted low levels of IL-1 β and IL-18 as compared to wild-type macrophages infected with KIM5 (73); this result was observed in the inflammasome-deficient macrophages as well (Figure 3.1). Different results were observed for TNF- α , as compared to IL-1 β and IL-18, under the same infection conditions (Figure 3.2, A and B). Both KIM5 and KIM5yop*JC172A* stimulated infected BMDMs to secrete TNF- α , although higher levels (~2 to 3 fold) of TNF- α were secreted from macrophages infected with KIM5yop*JC172A* regardless of macrophage type infected (Figure 3.2, A and B). These results suggest that NALP3, ASC and Ipaf are specifically required for the

caspase-1-dependent secretion of IL-1 β and IL-18, but not TNF- α production. Additionally, we have demonstrated that NALP3 and ASC, but not Ipaf, are involved in the secretion of IL-1 β and IL-18 from KIM5-infected macrophages.

A number of published reports have demonstrated a type of macrophage cell death mediated by caspase-1, termed pyroptosis (37, 58, 71, 91). We have previously demonstrated that the cell death observed during KIM5 infection is not caspase-1-dependent, and macrophages did not appear to be dying of pyroptosis (36, 73, 143). To determine whether NALP3, Ipaf and ASC play a role in cell death during KIM5 infection, macrophages deficient for these inflammasome components were infected with KIM5 and KIM5yop*JC172A*. Tissue culture supernatants were collected 24 hr post-infection and analyzed for lactate dehydrogenase (LDH) release, a marker of cell death. Similar levels of LDH release were observed from Ipaf-, ASC- and NALP3-deficient macrophages as compared to KIM5-infected wild-type macrophages (Figure 3.2, C and D). Low levels of LDH release occurred in all macrophages infected with KIM5yop*JC172A*, indicating that the induction of cell death by KIM5 requires the enzymatic activity of YopJ. These results demonstrate that cell death can occur in KIM5-infected macrophages in the absence of NALP3, Ipaf or ASC. Furthermore, these results support our previously published results that the cell death observed during KIM5 infection is not dependent on caspase-1 (73).

To assess the requirement for intracellular potassium loss in caspase-1 activation and IL-1 β release during infection with KIM5, increasing concentrations of potassium chloride (KCl) were added to tissue culture media (Experimental Methods). Cell culture supernatants were collected at 8 hr and analyzed for the presence of IL-1 β by ELISA and

cell death by LDH assay (Figure 3.3, A and B). IL-1 β secretion was reduced in a dose-dependent manner, while LDH release remained at similar levels compared to untreated infected macrophages. To determine if these observations could be related to inhibition of pro-IL-1 β expression, cell lysates from uninfected and infected wells were collected at 8 hr post-infection and analyzed by immunoblotting for IL-1 β . As shown in Figure 3.3C, pro-IL-1 β expression was inhibited at increasing concentrations of KCl, demonstrating that KCl can inhibit the expression of pro-IL-1 β at as low a concentration as 50 mM. This result is different from previous studies, which report concentrations as low as 70 mM or as high as 150 mM KCl as the minimal concentration to inhibit IL-1 β secretion (104, 138). The different results could be attributed to the different cell types used in each study (human monocytes (104, 138) versus primary mouse macrophages). For the purposes of our study, we utilized 30 mM KCl as the minimal concentration required to inhibit IL-1 β secretion, but not pro-IL-1 β expression, from macrophages infected with KIM5. To this end, BMDMs were infected with KIM5 or KIM5*yopJCI72A*, then treated with 30 mM KCl, 30 mM NaCl or unsupplemented infection medium (Experimental Methods). Cell culture supernatants were collected at 8 hr and 24 hr timepoints and analyzed for the presence of IL-1 β and TNF- α by ELISA (Figure 3.4). Significantly lower levels of IL-1 β (~5-fold) were secreted from macrophages infected with KIM5 in the presence of 30 mM KCl as compared to untreated macrophages at 8 hr post-infection (Figure 3.4, A). Macrophages infected with KIM5 in the presence of 30 mM NaCl secreted IL-1 β to slightly lower levels as compared to untreated infected macrophages at 8 hr post-infection (Figure 3.4, A). A similar trend of IL-1 β secretion was observed at 24 hr from macrophages infected with KIM5 as compared to the 8 hr timepoint (Figure 3.4,

C). Macrophages infected with *KIM5yopJC172A* secreted similar levels of IL-1 β regardless of treatment (Figure 3.4, A and C). KIM5- or *KIM5yopJC172A*-infected macrophages secreted TNF- α to similar levels in the presence or absence of 30 mM KCl or NaCl (Figure 3.4, B and D). Cell lysates from infected wells left untreated or treated with KCl or NaCl were collected at 8 hr post-infection and analyzed by immunoblotting for pro-IL-1 β . As shown in Figure 3.4, E, similar amounts of pro-IL-1 β were detected in the absence or presence of 30 mM KCl and 30 mM NaCl. In addition, slightly higher levels of pro-IL-1 β are expressed in macrophages infected with *KIM5yopJC172A* as compared to KIM5-infected macrophages in all conditions tested. This supports our previous results, which demonstrated that lower levels of mature IL-1 β are secreted from macrophages infected with *KIM5yopJC172A* as compared to KIM5 (73). Taken together, these results suggest that blocking potassium efflux from macrophages with 30 mM KCl can inhibit IL-1 β secretion from KIM5-infected macrophages, but has no significant effect on TNF- α production or expression of pro-IL-1 β . In addition, these results support the hypothesis that the NALP3 inflammasome plays an important role in the activation of caspase-1 and secretion of IL-1 β during infection with KIM5, since it has been demonstrated that the NALP3 inflammasome can sense potassium efflux and activate caspase-1 under different conditions (104).

The purinergic receptor, P2X₇, has been shown to play a role in NALP3 inflammasome activation by inducing the release of intracellular potassium. Activation of P2X₇ occurs by binding of extracellular ATP, which is released from damaged cells or cells undergoing cell death (74, 102). Upon activation by ATP, potassium efflux occurs, which is recognized by the NALP3 inflammasome and, subsequently, caspase-1

activation and secretion of IL-1 β occur. Since we demonstrated that inhibition of potassium efflux by the addition of 30 mM KCl to the infection medium could block IL-1 β secretion (Figure 3.4, A), we aimed to determine the role of the P2X₇ receptor (P2X₇R) during infection with KIM5. P2X₇R-deficient bone marrow derived macrophages were infected with KIM5 or KIM5yopJCI72A, and cell culture supernatants were collected and analyzed by ELISA to measure the levels of IL-1 β , IL-18, TNF- α and LDH release after 24 hr of infection (Figure 3.5). Similar levels of IL-1 β and IL-18 were secreted from P2X₇R-deficient macrophages as compared to wild-type macrophages infected with KIM5, indicating that this receptor does not play a significant role in inducing the secretion of these cytokines (Figure 3.5, A and B). Additionally, levels of both TNF- α and LDH were similar between the two cell types tested (Figure 3.5, C and D). Taken together, these results suggest that potassium efflux occurring during KIM5 infection is not dependent on the activation of the P2X₇ receptor, but possibly due to other factors, such as pore formation during cell death or by the T3SS of *Yersinia*.

3.5. Discussion

In this chapter, we aimed to determine if a specific inflammasome, potassium efflux and/or the P2X7R play an important role in the activation of caspase-1 and processing and release of IL-1 β in KIM5-infected macrophages. We have demonstrated that the NALP3 inflammasome, in conjunction with the adaptor protein ASC, play a significant role in the processing and release of IL-1 β and IL-18 during infection with KIM5 (Figure 3.1). The absence of Ipaf in macrophages did not significantly affect the production of these cytokines during infection with KIM5. Previous studies have demonstrated that cytosolic flagellin from *Salmonella* and *Legionella* can activate caspase-1 through the Ipaf inflammasome (38, 87, 89, 113). Our results were not surprising, since *Y. pestis* strains are non-motile and do not express flagella due to a mutation in *flhD* that results in the suppression of flagellin production (24). TNF- α secretion and cell death in macrophages infected with KIM5 were not significantly affected by the absence of the different inflammasome components as compared to wild-type macrophages (Figure 3.2). O'Connor *et al.* reported that NALP3 can suppress NF- κ B activation, thereby inhibiting the production of TNF- α and other cytokines (93). We did not observe significant differences between NALP3- or ASC-deficient macrophages and wild-type macrophages infected with KIM5 for the secretion of TNF- α , suggesting that in our system, NALP3 only affects caspase-1 and not NF- κ B activation. Additionally, these results indicate that the NALP3/ASC inflammasome does not play a significant role in inducing cell death in KIM5-infected macrophages; this is consistent with our observed results demonstrating that cell death occurred in a caspase-1-independent manner (73). Titration studies performed with KCl and NaCl of varying concentrations (Figure 3.3) demonstrated that a

concentration of 30 mM is optimal for use in our studies, since higher concentrations of KCl inhibited the expression of the pro-form of IL-1 β in infected macrophages (Figure 3.3C). Previous studies utilizing KCl as an inhibitor of potassium efflux used concentrations as low as 70 mM and as high as 150 mM (104, 138). We believe that the various cell types used (human monocytes/macrophage cell lines [THP-1] versus murine macrophages) could be affected by different concentrations of KCl (104, 138). Our studies reveal that the presence of 30 mM KCl in the infection medium inhibited the secretion of IL-1 β , but not TNF- α , from macrophages infected with KIM5 at either 8 hr or 24 hr post-infection (Figure 3.4). Furthermore, our results indicate that the release of potassium from macrophages infected with KIM5 was independent of the purinergic receptor, P2X₇R (Figure 3.5). Efflux of intracellular potassium mediated by the P2X₇R appears to be critical for caspase-1 activation and the secretion of IL-1 β from macrophages primed with LPS (59). Franchi *et al.* demonstrated that the intracellular bacteria *Salmonella* and *Listeria* do not require the activation of P2X₇R or potassium efflux for caspase-1 activation to occur (39); in contrast, our results showed that potassium efflux is important for the secretion of IL-1 β from KIM5-infected macrophages, but this efflux occurred independently of P2X₇R activation.

We propose the following model (Figure 3.6) for activation of caspase-1 in macrophages in response to KIM5 infection. First, KIM5 interacts with a macrophage and translocates effectors, including YopJ, into the host cell. YopJ has been shown to inhibit both the MAPK and NF- κ B pathways, blocking the production of pro-inflammatory cytokines and cell survival genes, leading to cell death. Second, the induction of cell death may lead to pore formation in the macrophage membrane, causing

the release of intracellular potassium into the extracellular space. The secretion of IL-1 β seems to be independent of P2X₇ receptor activation. Third, the NALP3/ASC inflammasome can recognize changes in intracellular potassium concentrations, leading to its activation, recruitment and activation of caspase-1 and subsequent cleavage of pro-IL-1 β to its mature form for secretion. Our findings have established a previously uncharacterized mechanism of caspase-1 activation by the NALP3/ASC inflammasome complex during infection with KIM5.

Acknowledgements

I would like to thank Gabriel Nuñez and Luigi Franchi from the University of Michigan for supplying the P2X₇ receptor-deficient mice for bone marrow macrophage isolation and Ruslan Medzhitov and Igor Brodsky from Yale University for the frozen stocks of inflammasome-deficient and C57BL/6 macrophages.

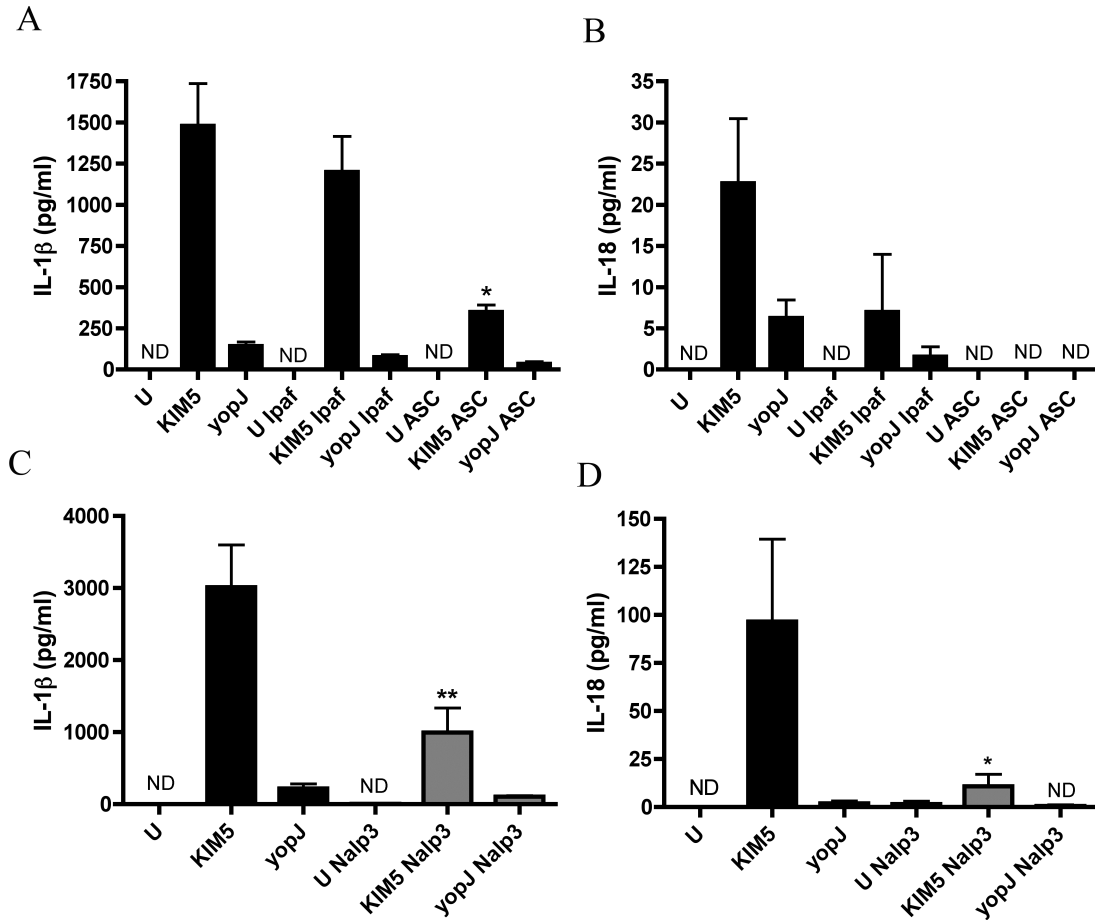


Figure 3.1. Determination of the importance of the inflammasome for IL-1 β and IL-18 secretion by infection of macrophages deficient for NALP3, ASC and Ipaf with KIM5. Wild-type or NALP3⁻, ASC⁻ or Ipaf⁻ deficient BMDMs were left uninfected (U) or infected with KIM5 or KIM*yopJC172A* (yopJ). Supernatants were collected at 24 hr post-infection and analyzed to determine the amount of IL-1 β (A and C) or IL-18 (B and D) by ELISA. Results shown are the average of three independent experiments. Error bars represent standard deviation. Statistical significance compared to KIM5 was determined (ND, below level of detection; P < 0.05, *; P < 0.01, **).

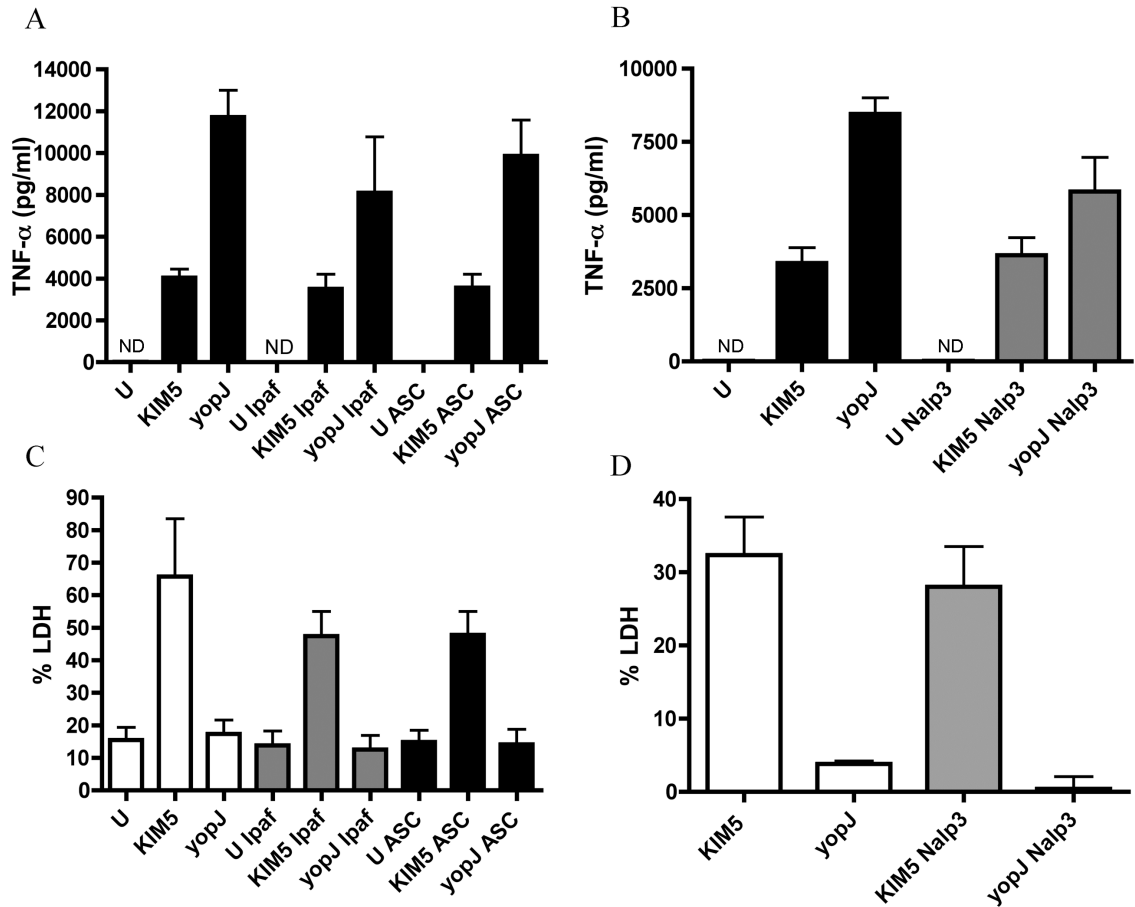


Figure 3.2. Determination of the importance of the inflammasome for TNF- α secretion and induction of cell death by infection of macrophages deficient for NALP3, ASC or Ipaf with KIM5. Macrophages were left uninfected (U) or infected as described in Figure 3.1. Supernatants were collected at 24 hr post-infection and analyzed for TNF- α production (A and B) and cell death (C and D) by ELISA and LDH release assay, respectively. Results shown are the average of three independent experiments. Error bars represent standard deviation. (ND, below level of detection).

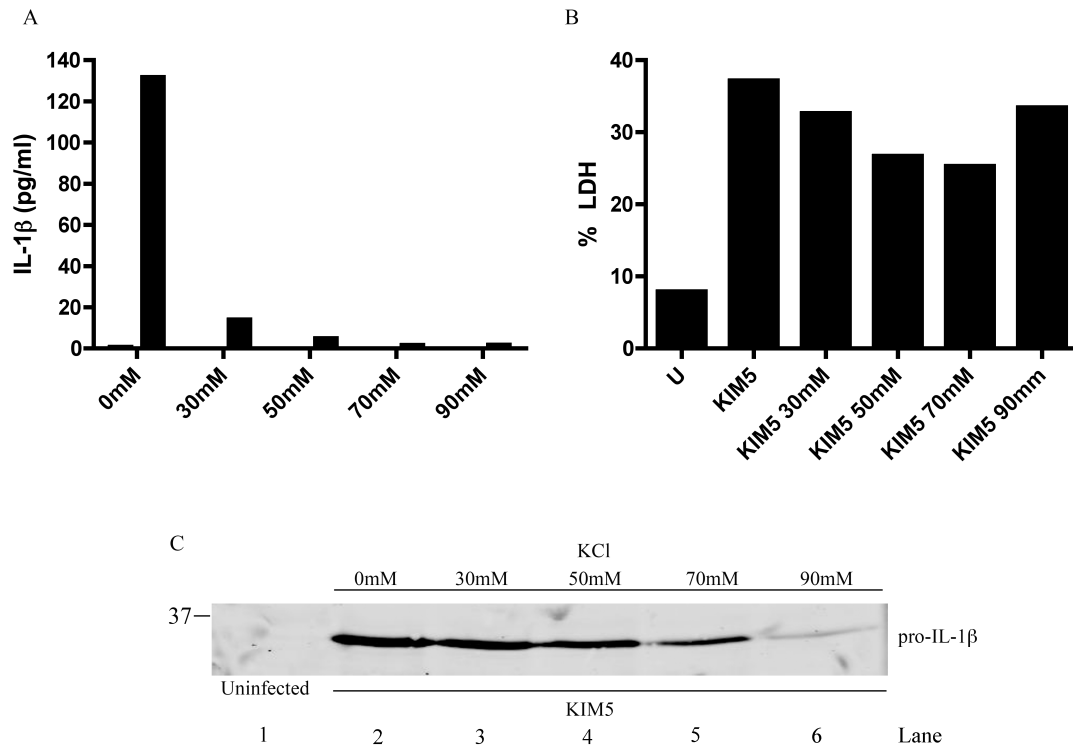
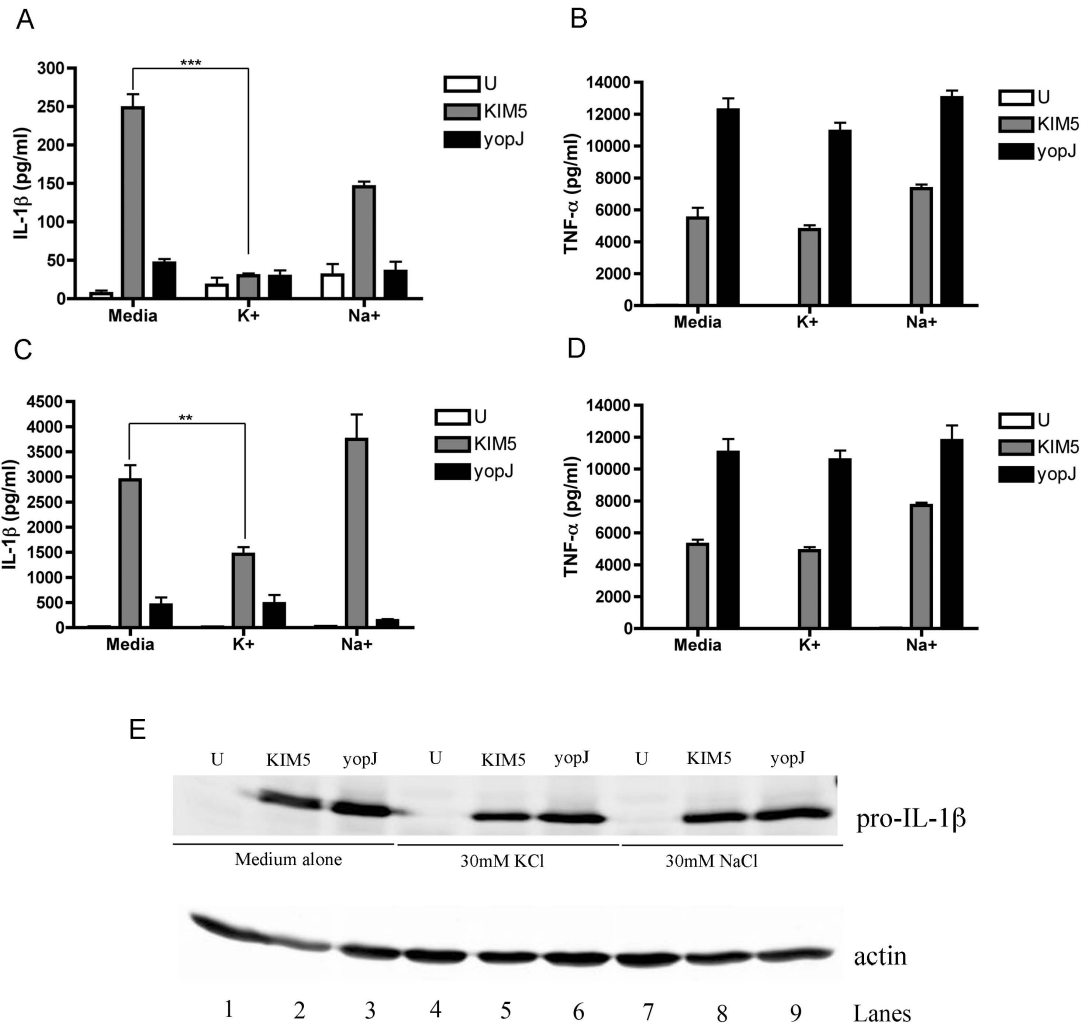


Figure 3.3. Determination of the effect of different concentrations of potassium chloride (KCl) on IL-1 β production and secretion in macrophages infected with KIM5. BMDMs were left uninfected or infected with KIM5 and treated with increasing concentrations of KCl or left untreated (U) as indicated. Supernatants were collected at 8 hr post-infection, and IL-1 β secretion (A) and cell death (B) were measured by ELISA and LDH release assay, respectively. (C) Precursor IL-1 β in cell lysates was detected by Western blotting with an anti-IL-1 β antibody. Results are from one independent experiment.

Figure 3.4. Secretion of IL-1 β , but not TNF- α , from KIM5-infected macrophages is significantly inhibited in the presence of 30 mM KCl at 8 hr and 24 hr post-infection. BMDMs were left uninfected or infected with KIM5 or KIM5*yopJC172A* and treated with 30 mM KCl (K⁺), 30 mM NaCl (Na⁺) or left untreated (medium) as indicated. Supernatants were collected at 8 hr (A and B) and 24 hr (C and D) post-infection, and IL-1 β secretion (A and C) and TNF- α production (B and D) were measured by ELISA. (E) Precursor IL-1 β in cell lysates prepared at 8 hr post-infection was detected by Western blotting with an anti-IL-1 β antibody. Actin in cell lysates was used as a loading control. Results shown are the average of three independent experiments. Error bars represent standard deviation. (P < 0.01, **; P < 0.001, ***).



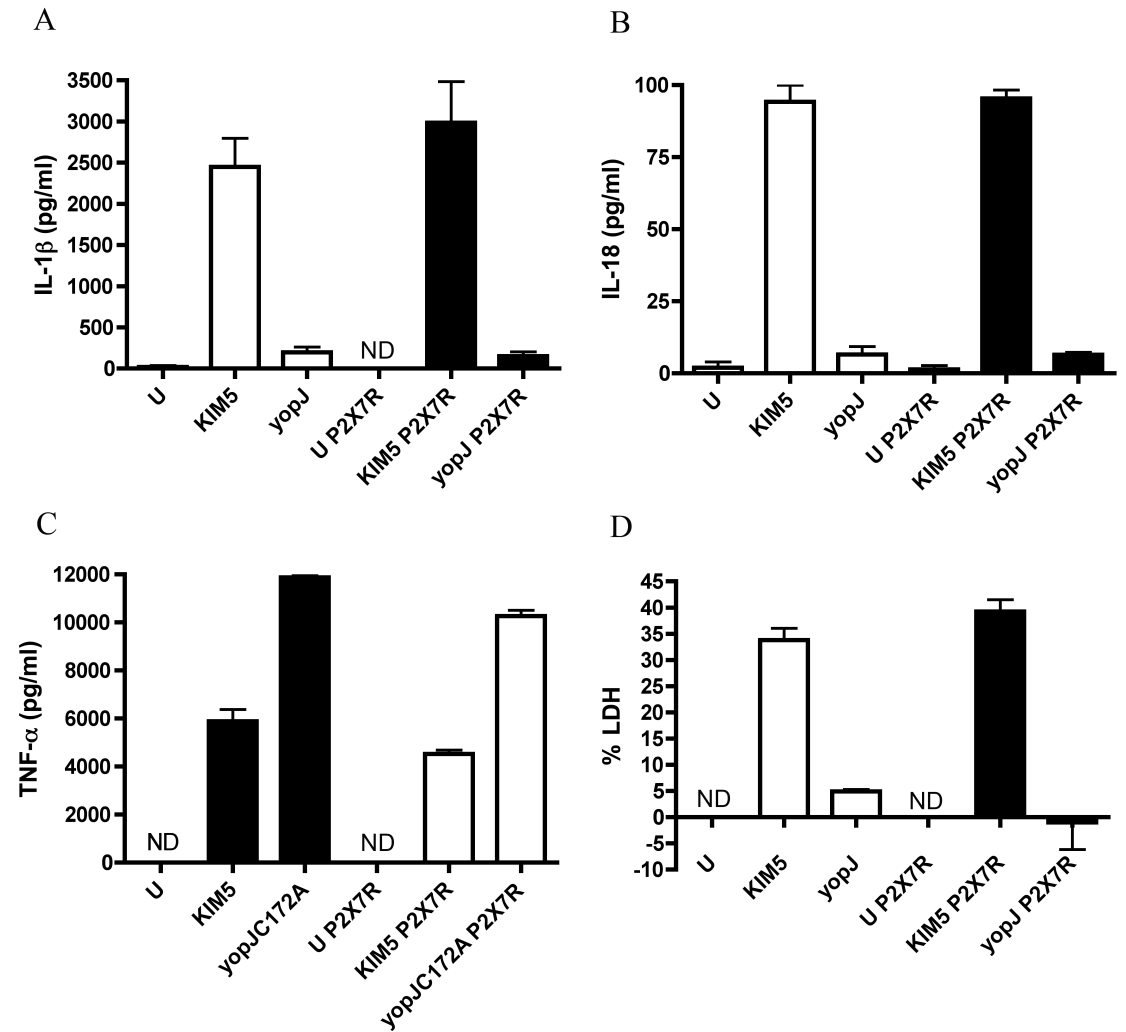


Figure 3.5. Determination of the importance of the P2X₇ receptor (P2X₇R) for cytokine production and induction of cell death by infection of P2X₇R-deficient macrophages with KIM5. Wild-type or P2X₇R-deficient BMDMs were left uninfected (U) or infected with KIM5 or KIM*yopJC172A*. Supernatants were collected at 24 hr post-infection and analyzed for IL-1 β (A), IL-18 (B) and TNF- α (C) secretion by ELISA. Cell death was measured by LDH release (D). Results shown are the average of three independent experiments (ND, below level of detection).

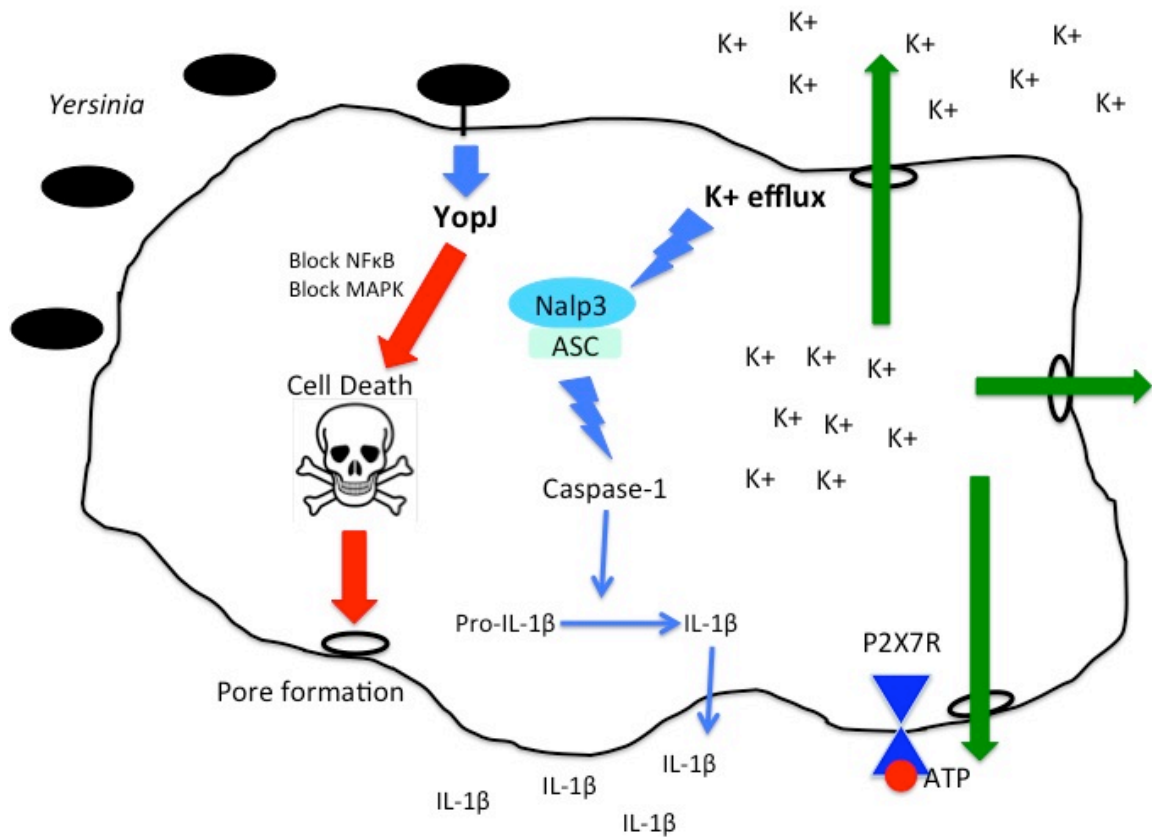


Figure 3.6. Proposed model for caspase-1 activation in macrophages in response to KIM5 infection. *Y. pestis* KIM5 (black ovals) translocates YopJ into the host cell during infection. YopJ inhibits MAPK and NF- κ B pathways, causing cell death by apoptosis. Potassium efflux (green arrow), possibly through pores formed during cell death, is recognized by the NALP3 inflammasome (blue lightning bolt), leading to caspase-1 activation and processing and release of mature IL-1 β . In our studies, potassium efflux is P2X₇R-independent.

Chapter 4: *Yersinia pestis* KIM5 activates caspase-1 in CD11b⁺ cells *in vivo*

4.1 Summary

Previous studies have demonstrated that caspase-1 plays a protective role against certain bacterial pathogens during infection of mice (58, 71, 112, 119). Alternatively, some have suggested that caspase-1 activation *in vivo* allows bacteria to disseminate into deeper tissues to establish infection (90, 91). In this chapter, we aim to elucidate the role active caspase-1 plays *in vivo* during infection of mice with KIM5. C57BL/6 mice and non-congenic mice deficient for caspase-1 were infected intravenously (I.V.) with KIM5. Surprisingly, caspase-1-deficient mice succumbed to infection at the same rate as C57BL/6 mice. These results suggest that caspase-1 does not play a protective role *in vivo* during I.V. KIM5 infection, although studies with congenic mice will need to be done to confirm this finding. We believe differences in IL-1 β secretion during KIM5 or CO92 Δ *pgm* infection of macrophages *in vitro* (73) may be due to amino acid differences present in YopJ. Sequence alignment of YopJ proteins from KIM5 and CO92 Δ *pgm* revealed amino acid differences at positions 177 and 206. The amino acid sequence of YopJ in KIM5 was altered to reflect the amino acids present in YopJ of CO92 Δ *pgm* and this strain (KIM5YopJ^{L177F E206K}, “177/206”) was used to infect mice I.V. to determine its ability to activate caspase-1 *in vivo* as compared to KIM5. No significant differences in survival between mice infected with KIM5 versus 177/206 were observed, but mice infected with 177/206 did succumb to infection more rapidly than mice infected with

KIM5. Flow cytometry analysis revealed that KIM5-infected mice had an increased percentage of caspase-1 positive CD11b⁺ macrophages as compared to 177/206-infected macrophages isolated from the spleen. Additionally, bacterial organ burden assays of lungs, spleen and blood revealed no significant differences in colonization between the two strains tested. Overall, our results suggest that the YopJ protein of KIM5 can activate caspase-1 *in vivo*, but that caspase-1 does not play a protective role during infection in the I.V. infection models we have used. In order to establish an infection model using a different route of challenge, intranasal (I.N.) infections of C57BL/6 mice were performed using KIM5, a strain expressing a catalytically inactive form of YopJ (KIM5yopJC172A) or CO92Δp_{gm}. Approximately 90% of mice infected with CO92Δp_{gm} succumbed to infection while ~60% of mice died during infection with KIM5 or KIM5yopJC172A. The I.N. challenge route may be useful for future studies to determine the role of caspase-1 in host protection against *Y. pestis* infection.

4.2 Introduction

In vivo studies using mice have demonstrated an important role for caspase-1 in controlling infections by *Salmonella*, *Francisella* and *Shigella* (78, 112, 119). It is suggested that by activating caspase-1 during infection, these bacteria induce inflammation and tissue destruction, leading to their dissemination to other organs and tissues of the host; eventually, however, the inflammatory response may eradicate the infection (71, 90, 91, 112, 119). Other studies have indicated that the activation of caspase-1 can help control the infection, as shown by the use of caspase-1-deficient mice, which are highly susceptible to infection and are unable to efficiently eradicate bacteria

(71, 78, 112, 119). These results suggest that caspase-1 plays an important role in the control and clearance of certain bacterial pathogens during *in vivo* infection. In this chapter, we aim to determine if KIM5 can activate caspase-1 *in vivo*, if YopJ of KIM is important for this response, and what the consequences of caspase-1 activation are for *Y. pestis* pathogenesis.

4.3 Experimental Methods

Bacteria strains and growth conditions. The construction of *Y. pestis* strain KIM5, KIM5*yopJ**Δ**172A* and CO92*Δ**pgm* were described previously in Chapter 2 and are listed in Table 4.1. To construct the KIM5*yopJ*^{L177F E206K} double mutant (Table 4.1), the *yopJ*^{L177F E206K} gene was moved from a pBAD-YopJ-GSK plasmid into the suicide vector pSB890. This plasmid was conjugated into KIM5 by use of *Escherichia coli* strain S17λ*pir*, and transconjugants were selected on *Yersinia* selection medium containing tetracycline. Transconjugants were grown as overnight cultures and plated on sucrose-containing plates to force recombination and pSB890 excision. The DNA segments encoding *yopJ* genes from mutant colonies were amplified by colony PCR. To verify the presence of the codon changes, the L177F mutation was detected by mismatch PCR. The forward mismatch primer was complementary to the sequence of the KIM5 *yopJ*, but not to *yopJ*^{L177F}, so the PCR was negative for *yopJ*^{L177F} and positive for KIM5 *yopJ*. The E206K codon change was detected by HphI digestion. A PCR product containing the KIM5 *yopJ* sequence was digested by this enzyme, but not if it contained the mutation E206K. The KIM5*yopJ*^{L177F E206K} double mutant was verified by DNA sequencing.

To prepare cultures for intravenous (I.V.) and intranasal (I.N.) infection, samples of bacteria from frozen stocks were plated on heart infusion (HI) plates containing ampicillin and allowed to form single colonies at 28°C for two days. Isolated colonies of the indicated bacteria were grown in HI broth plus antibiotics when appropriate and incubated at 28°C overnight with shaking. Twenty-four hours later, cultures were diluted in HI broth plus 25 μg/ml ampicillin to an OD₆₀₀ of ~0.02 and incubated overnight at

28°C with shaking. The morning of infection, cultures were centrifuged, and bacterial pellets were suspended and subsequently serially diluted in PBS.

Infection of mice. All experiments were carried out with the approval of the Stony Brook University IACUC. Female 6- to 8-week-old C57CL/6 mice were purchased from Jackson Laboratories. Caspase-1-deficient mice (backcrossed 7 generations to C57BL/6) were obtained from Craig Roy, Yale University School of Medicine. C57BL/6 and caspase-1-deficient mice were injected via the lateral tail vein with 100 µl of KIM5 suspended in PBS (~10 CFU or 50% lethal dose [LD₅₀]). For flow cytometry analysis and bacterial organ burden studies (see below), C57BL/6 mice were injected I.V. with 100 µl of KIM5 or KIM5yopJ^{L177F E206K} suspended in PBS (~100 CFU or 10 LD₅₀). For I.N. infections, mice were anesthetized by intraperitoneal (I.P.) injection of a ketamine-xylazine solution (0.1 ml/10 g body weight) followed by intranasal inoculation of 2 x 10⁵ CFU of KIM5, KIM5yopJ^{ΔC172A} or CO92Δpgm in 25 µl of PBS. Mice were monitored twice a day for signs of illness and lethality for 14 days for survival studies. Mice exhibiting severe disease and deemed incapable of survival were humanely euthanized by CO₂ narcosis.

Bacterial organ burden studies. C57BL/6 mice infected I.V. with KIM5 or KIM5yopJ^{L177F E206K} (see above) were euthanized on day 4 by CO₂ narcosis and spleen, lungs and blood were harvested. Lungs were homogenized in 5 ml of sterile Dulbecco's modified Eagle medium (DMEM) for 60 seconds using a stomacher (Seward Stomacher 80 Biomaster); spleens were manually disrupted with 18 gauge 1-1/2 inch needles in 5 ml of DMEM. Homogenates were plated on HI plates containing ampicillin and incubated for 2 days at 28°C prior to counting colonies to determine CFU/g tissue. The number of

viable bacteria in blood was determined by spreading dilutions onto HI plates containing ampicillin, incubating plates for 2 days at 28°C and counting colonies to determine CFU/ml of blood.

Flow cytometry. Spleens from KIM5- or KIM5yopJ^{L177F E206K}-infected mice were harvested on day 4 and manually disrupted as described above. Cell suspensions were subjected to centrifugation (300-400 x g) for 10 minutes at 4°C. Pellets were treated with a sterile cell lysis solution (1 M NH₄Cl, 1 M Tris pH 7.4 in H₂O) to lyse red blood cells. Cells were passed through a 70 µm filter to create a single-cell suspension and counted by hemacytometer. Approximately 10⁶ cells suspended in 300 µl of FLICA wash buffer was used for staining of active caspase-1 by the fluorescent inhibitor of apoptosis (FLICA) detection kit as per manufacturer's protocol (Immunochemistry Technologies, LLC). Briefly, 10⁶ cells were incubated for 1 hr at 37°C with 5% CO₂ with 1X FAM-YVAD in DMEM. Cell suspensions were washed twice to remove unbound FLICA reagent, suspended in 50 µl of FLICA wash buffer and incubated with anti-FcγR antibody (clone 2.4G2) (BD Pharmingen) for 5 minutes at room temperature to inhibit non-specific binding. Appropriate amounts of PerCP-Cy5.5 anti-mouse Mac-1 (CD11b) (clone M1/70) and isotype matched control antibodies (BD Pharmingen) were incubated with cells for 30 minutes at 4°C to stain for macrophages. Cells were washed twice, centrifuged for 5 minutes at 300-400 x g at 4°C and fixed in 500 µl of 1% formalin in PBS. At least 20,000 viable cells were acquired on the basis of forward and side light scattering, then quantified for CD11b and FAM-YVAD staining using a BD FACSCalibur instrument and analyzed with WinList software (Verity Software House).

Statistical analysis. Probability (P) values for survival curves were calculated by the Log Rank test; organ burden analyses were calculated by the Mann-Whitney test. Probability values for the flow cytometry results were calculated by the Kruskal-Wallis test with Dunn's multiple comparison post-test. All were considered significant if less than 0.05 (GraphPad Prism 4.0).

4.4 Results

Previous studies have indicated that activation of caspase-1 *in vivo* is important for the control and clearance of certain pathogenic bacteria in that caspase-1-deficient mice exhibited higher bacterial organ loads and mortality as compared to wild-type mice (78, 112, 119). To evaluate the *in vivo* role of caspase-1 in KIM5 infection, wild-type C57BL/6 mice and mice deficient for caspase-1 (backcrossed on the C57BL/6 line for 7 generations) were infected I.V. with 10 CFU (~LD₅₀) of KIM5 and monitored for survival over 14 days. We expected that caspase-1 deficient mice would succumb to infection more rapidly than wild-type mice. Surprisingly, we observed no significant difference in survival of wild-type or caspase-1-deficient mice over time (50-55% mortality) (Figure 4.1). This result suggested that caspase-1 does not play a protective role during KIM5 infection *in vivo*, although use of fully backcrossed caspase-1-deficient mice will be required to confirm this finding.

Our previous studies have demonstrated that *Y. pestis* strain CO92 Δ *pgm* and *Y. pseudotuberculosis* strain IP2666 cannot induce macrophages to secrete IL-1 β to high levels as compared to KIM5 (73). We hypothesize that differences in the YopJ sequence between these three strains could account for differences we observe *in vitro*. A sequence

alignment of YopJ from these strains was performed and revealed two amino acid differences (Figure 4.2). First, a leucine at position 177 (L177) was present in KIM5; CO92 Δ *pgm* and IP2666 contain a phenylalanine at this position (F177). Secondly, at position 206, CO92 Δ *pgm* contains a lysine (K206) as opposed to the other two strains, which contain a glutamate at this position (E206). To determine if these amino acids have an effect on caspase-1 activation *in vivo*, a strain of KIM5 was constructed to express a YopJ protein with the amino acids present in CO92 Δ *pgm* (Experimental Methods, KIM5 $yopJ^{L177FE206K}$, “177/206”). KIM5 and 177/206 were used to infect wild-type mice I.V. at a dose of 100 CFU (~10 LD₅₀). Mice were monitored for survival over 14 days (Figure 4.3). We observed no significant difference in survival among infected mice, but surprisingly, mice infected with the 177/206 strain succumbed to infection more rapidly (starting at day 3) as compared to KIM5 infected mice.

We have previously shown caspase-1 activation to occur in BMDMs infected with KIM5 utilizing the FLICA reagent, which stains for active caspase-1 and can be detected using fluorescence microscopy (73). To determine if KIM5 can activate caspase-1 *in vivo*, mice infected with KIM5 or 177/206 intravenously with a dose of 100 CFU (two mice per group per experiment), as well as one uninfected mouse, were euthanized on day 4 post-infection. Spleens, lungs and blood were collected from each mouse and processed for bacterial organ burden assay. Spleens were processed for flow cytometry (Experimental Methods). KIM5 and 177/206 colonization levels were not significantly different among the spleen (P = 0.0667), lungs (P = 1) or blood (P = 0.2897) (Figure 4.4 A-C). Upon analysis of flow cytometry results, we observed a significant decrease in caspase-1 activation in CD11b⁺ macrophages isolated from the spleens of 177/206

infected mice (~5%) as compared to those infected with KIM5 (~30-35%) (Figure 4.4 D). Our results demonstrate that KIM5, but not 177/206, can activate that caspase-1 in macrophages *in vivo*, suggesting that KIM YopJ is required for this response.

Next, we wanted to use an I.N. route of infection to determine if there are differences in survival among *Y. pestis* strains. Wild-type mice were infected I.N. with a dose (2×10^5 CFU) of KIM5, CO92 Δ *pgm* or KIM5*yopJC172A* and monitored for survival over 14 days. We observed no significant differences in survival among the strains tested, but mice infected with CO92 Δ *pgm* did succumb to infection earlier (day 4) and ~90% died (Figure 4.2). Approximately 70% of mice died during infection with either KIM5 or KIM5*yopJC172A*, but mice infected with the latter survived three days longer (~9 days) than KIM5 infected mice (~6 days), suggesting that enzymatically active YopJ may play a role during infection *in vivo*. The I.N. route of infection may be useful for future studies to determine the role of caspase-1 in host protection against *Y. pestis* infection.

4.5 Discussion

In this chapter, we aimed to determine whether *Y. pestis* KIM5 could induce caspase-1 activation *in vivo* and what consequences this process might have for *Y. pestis* pathogenesis. We have preliminary evidence that caspase-1 does not play a protective role during infection with KIM5, since both wild-type and non-congenic caspase-1-deficient mice survived to similar levels over a 14-day period (Figure 4.1). This was a surprising result, since previous studies have demonstrated that caspase-1-deficient mice have an increased susceptibility to infection with some other bacteria and have higher bacterial loads in infected organs (52, 71, 78, 112). Cheng *et al.* describe similar results with *Chlamydia*, where normal mice and mice deficient for caspase-1 displayed similar survival rates during a course of chlamydial infection, suggesting that caspase-1 does not play an important role in controlling infection (18). However, they do point out differences in inflammation in the genital tracts of mice infected with *Chlamydia*, citing decreased inflammation in mice deficient for caspase-1. Further studies are needed to determine whether there are differences in inflammation or organ burden in wild-type and caspase-1-deficient congenic mice infected with KIM5.

We believed that differences in the YopJ sequence of *Y. pestis* KIM5 and CO92 strains contribute to the differences in caspase-1 activation and IL-1 β secretion we observed in macrophages infected *in vitro*. We observed no significant differences in colonization levels or survival between mice infected with wild-type KIM5 and 177/206. Taken together, these results indicate that caspase-1 does not play a protective role *in vivo* during I.V. infection with KIM5; however, caspase-1 activation does occur and seems to be dependent on KIM5 YopJ. Utilizing a different route of infection, such as the

I.N. route, and repeating the experiments discussed in this chapter may help elucidate the role of caspase-1 activation during infection with KIM5. KIM5 administered by the subcutaneous route is highly attenuated (134). Additionally, it would be beneficial to infect mice with the fully virulent (*pgm*⁺) strain of *Y. pestis* to determine if this locus contributes to caspase-1 activation *in vivo*.

Intranasal infections of wild-type mice were performed using KIM5, KIM5*yopJCI72A* and CO92Δ*pgm* (Figure 4.5). We observed that both KIM5 and KIM5*yopJCI72A* infected mice succumbed to infection at similar rates, but the *yopJ* mutant infected mice survived a few days longer than KIM5 infected mice, suggesting that enzymatically active YopJ may be important for KIM5 to establish a systemic infection. Lemaître *et al.* observed similar levels of survival of Brown Norway rats infected intradermally with *Y. pestis* 195/P or a 195/P *yopJ* mutant at an LD₅₀ of 10 CFU (72). CO92Δ*pgm* infected mice succumbed to infection rapidly as compared to KIM5, but these results were not significant. The differences in the strains used (KIM5 biovar *Mediaevalis* and CO92Δ*pgm* biovar *Orientalis*) may play a role in the survival of infected mice.

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Table 4.1. *Yersinia* strains used in this chapter

Strain name	Relevant Characteristics	Reference or source
<i>Y. pestis</i>		
KIM6+	Biovar Mediaevalis, pCD1 ⁻ , pMT1 ⁺ , pPCP1 ⁺ , <i>pgm</i> ⁺	(20)
KIM6	Biovar Mediaevalis, pCD1 ⁻ , pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i>	(73)
KIM5	Biovar Mediaevalis, KIM6/pCD1Ap, pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i> , Ap ^{r(1)}	(73)
CO92 Δ <i>pgm</i>	Biovar Orientalis, pCD1 ^{CO92+} , pMT1 ⁺ , pPCP1 ⁺ , Δ <i>pgm</i>	(73)
KIM5yopJCI72A	pCD1Ap yopJCI72A (codon change of Cys172 to Ala172), Ap ^r	(73)
KIM5YopJ ^{L177FE206K}	pCD1Ap yopJL177F E206K (codon change of Leu177 to Phe177, Glu206 to Lys206), Ap ^r	This Study

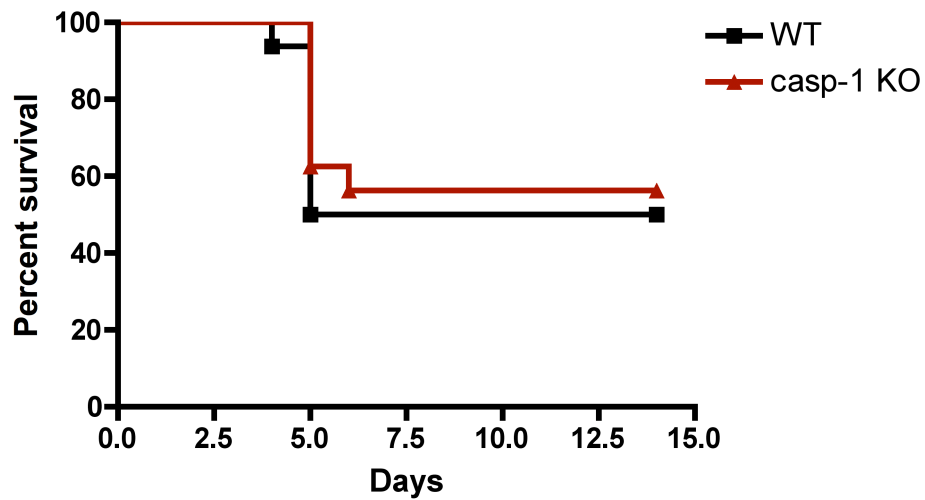


Figure 4.1. Susceptibility to KIM5 infection of mice deficient for caspase-1. C57BL/6 (black square) and caspase-1-deficient (red triangle) mice were infected intravenously with a dose of 10 CFU of KIM5 in 100 μ l PBS (LD_{50}). Mice were monitored twice a day for 14 days for survival. Approximately 55% of caspase-1-deficient mice survived infection; C57BL/6 mice survived to similar numbers (n = 16 per group).

IP2666	61	VIQANNKYPEMNLNLVTSPLDLSIEIKNVIENGVRSSRFIINMGEGGIHFSVIDYKHING	120
KIM5	61	VIQANNKYPEMNLNLVTSPLDLSIEIKNVIENGVRSSRFIINMGEGGIHFSVIDYKHING	120
CO92Δpgm	61	VIQANNKYPEMNLNLVTSPLDLSIEIKNVIENGVRSSRFIINMGEGGIHFSVIDYKHING	120
IP2666	121	KTSLILFEPANFNSMGPAMLAIRTKTAIERYQLPDCHFMSVEMDIQRSSECGIFSFALA	180
KIM5	121	KTSLILFEPANFNSMGPAMLAIRTKTAIERYQLPDCHFMSVEMDIQRSSECGIFSLALA	180
CO92Δpgm	121	KTSLILFEPANFNSMGPAMLAIRTKTAIERYQLPDCHFMSVEMDIQRSSECGIFSFALA	180
IP2666	181	KKLYIERDSSLKIHEDNIKGILSDGENPLPHDKLDPYLPVTFYKHTQGKKRLNEYLNTNP	240
KIM5	181	KKLYIERDSSLKIHEDNIKGILSDGENPLPHDKLDPYLPVTFYKHTQGKKRLNEYLNTNP	240
CO92Δpgm	181	KKLYIERDSSLKIHEDNIKGILSDGKNPLPHDKLDPYLPVTFYKHTQGKKRLNEYLNTNP	240

Figure 4.2. Alignment of the amino acid sequence of YopJ from different *Yersinia* strains. Protein sequence alignment of YopJ from *Y. pestis* strains KIM5 and CO92Δpgm and *Y. pseudotuberculosis* strain IP2666. Shown are amino acids 61-240 out of 288 total. Red letters display amino acid differences at positions 177 and 206.

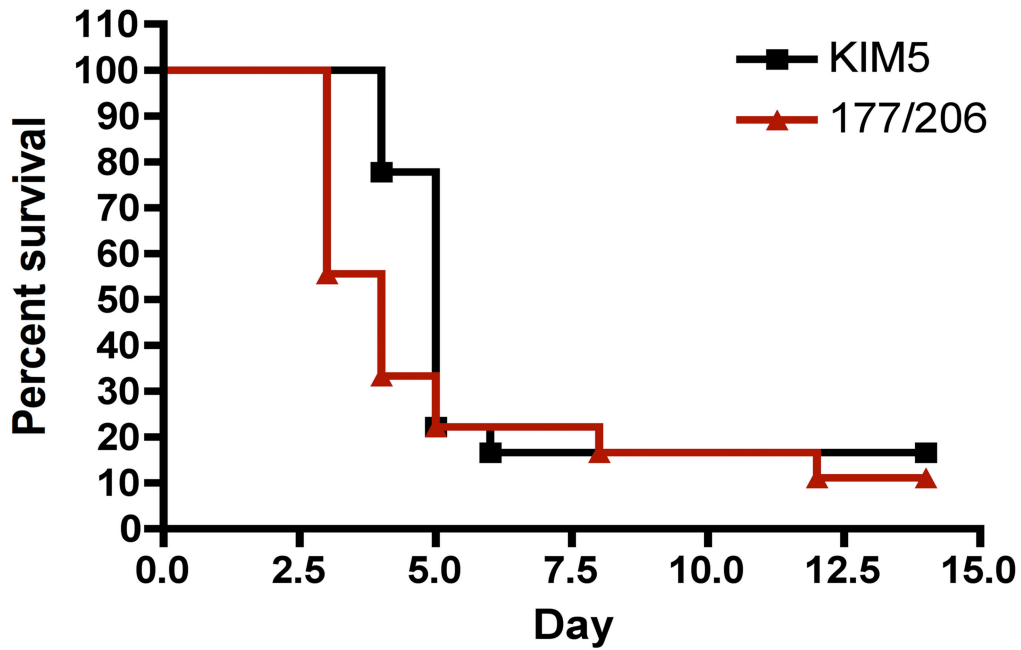


Figure 4.3. Susceptibility of mice to KIM5 or to the double mutant $KIM5yopJ^{L177F E206K}$. C57BL/6 mice were infected intravenously with KIM5 or $KIM5yopJ^{L177FE206K}$ (177/206) at a dose of 100 CFU per 100 μ l PBS (10 LD₅₀). Mice were monitored twice a day for 14 days for signs of disease (n = 18 mice per group).

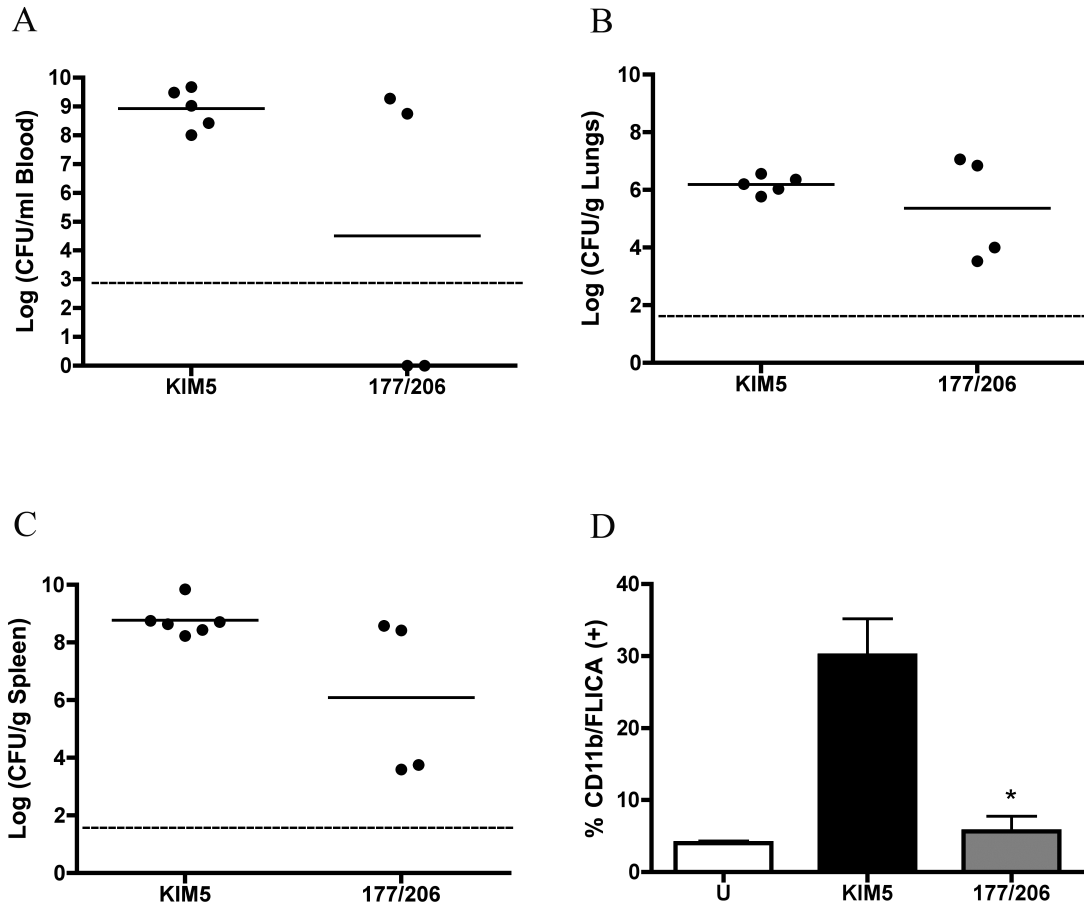


Figure 4.4. Analysis of bacterial organ burden and caspase-1 activation in macrophages isolated from *Y. pestis*-infected mouse spleens. C57BL/6 mice were infected intravenously with KIM5 or KIM5 $yopJ^{L177F E206K}$ (177/206) at a dose of 100 CFU/100 μ l per mouse. On day 4, two KIM5 infected, two 177/206 infected and one uninfected mice were euthanized and blood (A), lungs (B) and spleen (C) were harvested and plated for CFUs. Spleen isolates were processed for flow cytometry to identify macrophages (CD11b⁺) positive for active caspase-1 (FLICA). Results are of three independent experiments. Dashed lines represent the detection limit for CFUs. Error bars represent standard deviation (D) ($P < 0.05$, * as compared to KIM5).

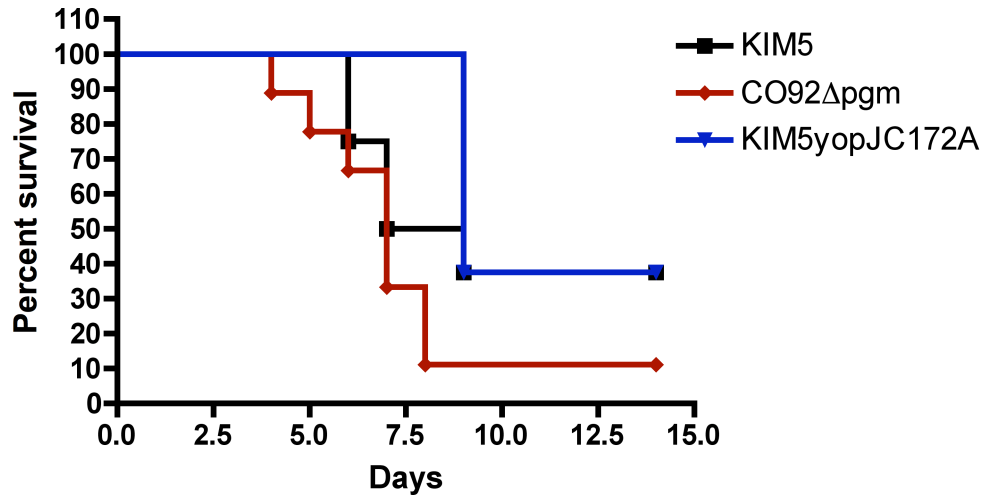


Figure 4.5. Survival of mice infected with *Y. pestis* strains. C57BL/6 mice were infected intranasally with KIM5, CO92Δpgm or KIM5yopJC172A at a dose of 2×10^5 CFU in 25 μ l PBS. Mice were monitored twice a day for 14 days for survival. (n = 8 per group [KIM5 and KIM5yopJC172A] and n = 9 [CO92Δpgm]).

Chapter 5. Concluding Remarks and Future Directions

The studies discussed in this dissertation were undertaken to determine the mechanism and importance of caspase-1 activation by *Yersinia pestis* KIM5 both *in vitro* and *in vivo*. In chapter 2, we aimed to determine if *Y. pestis* could inhibit caspase-1 activation and IL-1 β secretion, since previous studies with *Y. enterocolitica* had indicated that YopE, and to a certain extent YopT, could inhibit caspase-1 mediated IL-1 β maturation (122). Our results revealed that *Y. pestis* KIM5 could *induce* the production of IL-1 β from infected macrophages, and, furthermore, we demonstrated a novel role for the effector YopJ in this response. These were surprising results, since YopJ has been shown to inhibit the expression of pro-inflammatory cytokines, such as IL-1 β , by inhibiting the activation of the MAPK and NF- κ B pathways during infection with *Yersinia*. In chapter 3, we determined, through the use of macrophages deficient for the inflammasome components NALP3, ASC and Ipaf, that caspase-1 activation was in part regulated by the inflammasome complex composed of NALP3 and ASC. Previous studies have shown that potassium efflux induced by potassium depleting agents can activate the NALP3 inflammasome (79, 104, 128, 138). In our experiments, the addition of 30 mM potassium chloride to the infection medium inhibited the secretion of IL-1 β from KIM5-infected macrophages. These results are the first to demonstrate a role for the NALP3/ASC inflammasome complex and potassium efflux for the activation of caspase-1 during infection with a Gram-negative bacterium.

We observed a significant decrease in IL-1 β secretion in macrophages deficient for caspase-1, but not a complete loss of production of this cytokine (Chapter 2, Figure 2.14, A). This result indicated that other pro-inflammatory caspases might be involved. Wang *et al.* demonstrated that mouse caspase-11 in conjunction with caspase-1 plays a crucial role in the processing and release of IL-1 β and IL-18 (139). Mice deficient for caspase-11 exhibited a similar phenotype to caspase-1 deficient mice. Additionally, they showed that caspase-11 was upstream of caspase-1, since the absence of caspase-11 inhibited the activation of caspase-1, and caspase-11-null mice were resistant to septic shock after LPS administration. Martinon *et al.* initially identified the inflammasome as a multi-protein complex composed of NALP1, ASC and both caspase-1 and caspase-5 (80). Little is known about caspase-5; human caspase-5 and murine caspase-11 have 54% sequence homology, but it is suggested that they are functional homologs (80). It is possible that caspase-5 may play a role in inducing IL-1 β secretion through the inflammasome during KIM5 infection in humans. Lastly, a recent study revealed a novel role for caspase-7 in host defense against *Legionella* (2). The Ipaf inflammasome is activated upon recognition of flagellin from *Legionella*; this in turn activates caspase-1. Akhter *et al.* demonstrated that caspase-1 could activate caspase-7, leading to restriction of *Legionella* growth and mediating the fusion of *Legionella* containing phagosomes with lysosomes for degradation of the pathogen (2). It would be beneficial elucidate the role of these various caspases, as well as the possible interactions with the NALP3/ASC inflammasome, in macrophages infected with KIM5.

In chapter 4, we aimed to determine if caspase-1 activation occurs during infection with KIM5 *in vivo* and whether this process impacts pathogenesis. Caspase-1

was shown to not play a protective role during infection with KIM5, since there was no significant difference in survival between wild-type and caspase-1-deficient mice (Figure 4.1). The caspase-1-deficient mice obtained for these experiments were backcrossed 7 generations; we are currently in the process of backcrossing them out to 10 generations. Others have indicated that genetic differences among mouse strains may affect the activation of caspase-1 (112); certain mice may be more susceptible or resistant to infection. Studies with *Salmonella* have shown that mice containing the *nramp* allele (*nramp* +/+) are more resistant to infection as compared to mice (C57BL/6 and BALB/c) with a mutant *nramp* allele (*nramp* -/-) (71, 112). Additionally, intranasal (I.N.) infections utilizing KIM5, a strain of KIM5 expressing a catalytically inactive form of YopJ and another *Y. pestis* strain, CO92 Δ *pgm*, did not show significant differences in survival (Figure 4.2). For future studies, these infections should be repeated, and organs (spleen and lungs) should be assessed for differences in colonization levels as well as for inflammation by histopathological examination. The I.N. route of infection could also be used to determine if the route of infection affects the activation of caspase-1 and survival in mice.

We determined through the use of flow cytometry that caspase-1 is activated in macrophages isolated from KIM5-infected mice; a strain of KIM5 expressing YopJ containing two amino acid substitutions (L177F and E206K, “177/206”) was not able to induce the activation of caspase-1 (Figure 4.5). No significant differences in survival or colonization levels were observed in mice infected with KIM5 or 177/206 (Figure 4.4 and 4.5). Similar results were observed in mice infected with *Chlamydia*; it was noted that caspase-1 activation did not play a significant role in resolving the infection (18).

Examination of histopathology on genital tract tissue from *Chlamydia*-infected mice indicated a significant decrease in inflammation in caspase-1-deficient mice as compared to wild-type mice (18). It would be beneficial to look at histopathology of lung and spleen sections from KIM5- and 177/206-infected mice to determine the extent of inflammation in these organs.

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