Mechanisms of inflammasome activation and inhibition during *Yersinia* infection

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ABSTRACT

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The host inflammatory response is strikingly delayed during the initial stages of infection with *Yersinia pestis* and *Y. pseudotuberculosis*, pathogens that can suppress immune defenses. This work identifies the bacterial leucine-rich repeat protein YopM as an inhibitor of pyroptosis, an inflammatory programmed cell death pathway. Pyroptosis, which depends on the protease caspase-1, restricts *Yersinia* infection. Consequently, YopM subversion of caspase-1 is essential for bacterial virulence. Caspase-1 is activated after a multistep process of inflammasome formation, and YopM is bifunctional in preempting pyroptosis: it blocks caspase-1 recruitment to pre-inflammasome complexes and directly inhibits caspase-1 proteolysis. These data highlight the importance of caspase-1 in combating microbial infection and indicate that *Yersinia* are highly susceptible to pyroptosis, but normally prevent caspase-1 activation with YopM.

Though *Yersinia pestis* actively antagonizes inflammasome formation to delay inflammatory processes, *Yersinia* PAMPs are rapidly recognized by NLRs during infection. This work shows that the *Yersinia* insecticidal-like toxin complex (YTc) activates the inflammasome

and is a predominant PAMP of *Y. pestis*. YTc forms pores in the phagolysosome membrane that activate the NLRP3 inflammasome. These pores result in release of Cathepsin B, ROS, and protons, but only Cathepsin B is responsible for inflammasome formation. Thus it appears that YTc-mediated intracellular survival comes with the liability of stimulating an inflammatory anti-microbial response that is detrimental to *Yersinia* survival. Together, these results describe previously unknown functions of YopM and YTc contributing to the virulence of *Yersinia*.

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LIST OF ABBREVIATIONS

ASC	apoptosis-associated speck-like protein containing a CARD
CARD	caspase activation and recruitment domain
Casp1	caspase-1
Caspase	cysteinyl aspartate-specific proteinase
CthB	Cathepsin B
DAMP	danger-associated molecular pattern
EtBr	ethidium bromide
LAMP1	Lysosomal-associated membrane protein 1
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LT	lethal toxin
NLR	nucleotide-binding domain, LRR–containing receptor
NLRC4	NLR, CARD-containing 4
NLRP3	NLR, PYD-containing 3
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
PYD	pyrin domain
T3S	Type III secretion
TLR	Toll-like receptor
WT	wild-type
YCV	*Yersinia*-containing vacuole
Yop	*Yersinia* outer protein
Yp	*Yersinia pestis*
Ypstb	*Yersinia pseudotuberculosis*
YTc	*Yersinia* insecticidal-like toxin complex

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Chapter 1. Introduction

The natural history of *Yersinia* sp.

Yersinia pestis is the etiologic agent of plague, one of the most devastating epidemic bacterial diseases. Plague has killed over two hundred million people, primarily during the two major European pandemics of the 6th century and 14th centuries referred to as Justinian's plague and the Black Death. Throughout most of the world, *Y. pestis* is endemic in natural foci where it is spread by fleas as a zoonotic disease of mammals such as mice and ground squirrels (1, 2). Transmission from these foci results in sporadic individual infections and occasional epidemics in human populations that have been promoted by war, poverty, globalization, and climate change (2-4). *Y. pestis* has also been weaponized throughout history, and remains a significant potential threat to public health by acts of bioterrorism.

Y. pestis evolved from *Yersinia pseudotuberculosis* approximately two thousand years ago (5). *Y. pseudotuberculosis* is an enteropathogen that causes enteritis and mesenteric adenitis in humans that is normally self-limiting. With few genetic changes *Y. pestis* has gained a dramatically enhanced virulence toward mammalian hosts and the ability to colonize an additional host, the flea. Gene reduction has been important in plague evolution as several virulence factors of *Y. pseudotuberculosis* have been inactivated during the evolution of *Y. pestis* including flagella (6, 7), LPS O-antigen (8), urease (9), and multiple adhesins (10). Additional genes loss disrupts biosynthetic and intermediary metabolic pathways necessary for *Y. pseudotuberculosis* to compete for nutrients in enteric and environmental niches, but apparently dispensable for the obligate blood-born lifestyle of *Y. pestis* (10). Some of this gene loss enhances

Y. pestis transmission and virulence, suggesting it is due to positive selection, rather then neutral selection (11, 12). However, *Y. pestis* from the 14th century is highly similar to modern isolates (13), indicating the pathogen's genome has stabilized and the complement of virulence factors maintained is important for the pathogenesis of *Y. pestis*.

Virulence factors of *Yersinia*

When a flea bites an infected rodent, *Y. pestis* is ingested with the blood meal. The *Y. pestis* ancestor *Y. pseudotuberculosis* is unable to colonize or be transmitted by the flea, thus *Y. pestis* has gained factors enabling flea-borne transmission (14). One factor is the plasmid pMT1, encoding Ymt, a phosopholipase that detoxifies a bactericidal byproduct from flea digestion of blood (15). Additionally, a specialized biofilm encoded by the chromosomal *hms*-locus impairs function of the proventriculus, a valve closing off the flea's midgut. This allows backflow of infectious blood from the flea midgut into subsequently bitten mammals; *hms* mutants grow normally in the flea, but are inefficiently transmitted (16). A final factor important for transmission by the flea is Pla, a protease that hydrolyzes fibrin and collagen to break down tissue in the mammal and potentiate dissemination from the flea bite site (17, 18).

Following successful transmission to a mammal, *Y. pestis* is taken up by phagocytic cells. Though all *Yersinia sp.* are predominantly extracellular throughout infection of mammals, intracellular survival is thought to be important immediately post-transmission (19, 20). Within phagocytes, the *Yersinia*-containing vacuole (YCV) gains markers of the lysosomal compartment, suggesting normal maturation, but perplexingly the YCV does not significantly acidify (21-23). The YCV also gains markers of the autophagosome (21, 23), a compartment permissive for the

survival of *Listeria monocytogenes* and several other pathogens (24). However, autophagosomal proteins do not aide the intracellular survival of *Y. pestis* and so the contribution of the autophagosomal machinery to virulence is unknown (23). There are no gross disturbances in the membrane of the YCV of infected cells that observable by electron microscopy and *Y. pestis* cells appear to remain contained within a vacuolar membrane (19, 25). Thus it remains unclear how *Yersinia* is able to de-acidify the YCV to promote intracellular survival.

All *Yersinia sp.* have an additional virulence plasmid encoding for a type III secretion system (T3S). T3Ss are key virulence determinants in numerous Gram-negative pathogens including *Burkholderia pseudomallei*, *Chlamydia trachomatis*, *Escherichia coli*, *Salmonella enterica*, *Shigella flexneri*, and *Pseudomonas aeruginosa* (26). The T3S needle complex spans the bacterial membranes and protrudes from the cell. The proteins YopB and YopD are secreted through the T3S needle and form a channel in the eukaryotic cell membrane through which effector Yop proteins are injected (26). *Yersinia* have an accessory T3S component not present in other bacteria, YopK, which regulates pore size and translocation rate (26, 27). *In vivo* during murine infection, *Yersinia* specifically target macrophages, dendritic cells, and polymorphonuclear monocytes with this T3S to deliver Yop effector proteins that act to subvert host defenses (28).

There are five Yop effectors all *Yersinia* have in common: YopO, YopE, YopM, YopH, and YopJ (Table 1). The effectors YopO, YopE, and YopH, inhibit Rho GTPases (29-31), Rho-GAPs (32-34), and focal adhesion complexes (35-39), respectively. These eukaryotic factors are all involved in intracellular actin dynamics, and the synergistic activity of these effectors counteracts phagocytosis of the microbe. YopJ acetylates mitogen-activated protein kinases involved in cellular activation of pro-inflammatory signaling, thus contributing to the anti-

inflammatory activities of *Yersinia* (40-43). YopJ additionally can induce programmed cell death by apoptosis in naïve macrophages (44, 45). Activation of apoptosis specifically eliminates cells with anti-microbial or pro-inflammatory potential, preempting their priming of an immune response (46).

The final T3S effector, YopM is a poorly characterized leucine-rich repeat (LRR) protein that is associated with the anti-inflammatory activities of *Yersinia* (47-57), though by specific mechanisms that are unclear and will be discussed further (Chapter 3). Additional factors of *Y. pestis* that have probable virulence contributions but unclear mechanisms are a possible second T3S system (22), a type VI secretion system (58), LcrV (59, 60), catalase (61), and the *Yersinia* toxin complex (discussed in further detail in Chapter 4). Many additional virulence factors remain to be identified, accounting for *Yersinia*'s lympho-trophism (62), intracellular survival (22, 25), subversion of autophagy machinery (21), pneumonic transmission (63), catabolism of the host (64), and maintenance in the environment between epidemics (65).

Host detection of infection

Following successful transmission to a mammal, *Y. pestis* cells traffic to the lymph nodes where they replicate prodigiously; the engorged lymph nodes are a hallmark characteristic of bubonic plague termed a bubo (66). The enteric pathogen *Y. pseudotuberculosis* has a similar lympo-tropism and selectively colonizes the mesenteric lymph nodes after crossing the mucosal barrier (9). In both infections, bacterial replication spills into the blood stream, causing septicemia and colonization of additional organs such as the liver and spleen. Septicemia is essential for transmission of plague, so that that bacteria can be taken up during the feeding of

uninfected fleas to continue the transmission cycle. While the LD_{50} of *Y. pestis* toward mammals is less than 10 CFU, infection of *Xenopsylla cheopis* fleas is significantly less efficient and requires several thousand CFU (67). Since fleas only ingest approximately 0.1 μl of blood (67), *Y. pestis* therefore must grow to at least 10^7 CFU per milliliter in the blood for transmission to occur. Additionally, this high septicemic burden must not result in immediate death of the mammalian host, since fleas only bite living, warm-blood animals. *Y. pestis* delays host death during septicemia by multiple mechanisms for evading immune detection and actively repressing immune mechanisms of the mammal.

As previously discussed, *Yersinia* can escape phagocytosis, and also subvert many of the important inflammatory signals involved in immune activation. One such signal is IL-10, an anti-inflammatory cytokine important in homeostasis and normally induced during recovery from inflammation, but which is aberrantly induced during *Yersinia* infection by the virulence factor LcrV (59, 68). Tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) play several important roles in host resistance such as stimulating the microbicidal activity of macrophages. TNF-α or IFN-γ, either administered directly or when induced during concurrent latent herpesvirus infection, is protective during *Y. pestis* infection; the normally poor induction of these cytokines during *Y. pestis* infection suggesting their signaling is actively repressed by the bacterium (69, 70). IL-1β and IL-18 are key mediators in the pro-inflammatory immune response, but are also notably absent during infection with *Y. pestis* (71). The lack of IL-1β and IL-18 during infection with *Yersinia* suggests there is a defect in the activation of caspase-1, a central regulator of these cytokines that is also important in the defense against several other bacterial pathogens including *Francisella, Legionella, Pseudomonas,* and *Salmonella* (Table 1.2).

Caspase-1 activation

Caspase-1 is synthesized as a zymogen and remains in a quiescent form until pathogen recognition receptors (PRRs) detect intracellular pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), cluing the cell to pathogen invasion or major damage. Diverse stimuli can act as PAMPs or DAMPs (Table 2); flagellin (72, 73), T3 proteins (74), dsDNA (75-79), ATP (80, 81), the potassium ionophore nigericin (80), and toxins (82), but the specific PAMPs of *Yersinia* that activate caspase-1 have not previously been described.

PAMPs and DAMPs are detected by a family of cytosolic PRRs named nucleotide-binding domain, LRR–containing receptors (NLRs). The human genome encodes at least 20 NLR family members and mice encode at least 30 (83); however, most of these proteins do not activate caspase-1, and have emerging roles in antagonizing NLRs that activate caspase-1, as well as regulating NF-kB, MAPK, and MHC pathways (84-87). These proteins typically consist of a nucleotide-binding domain (also known as NACHT), a LRR domain, and a scaffolding domain (Figure 1.2). The LRR either directly interacts with a PAMP, or with a secondary signal induced by the PAMP, or with a coordinating protein that acts as the PAMP receptor (78, 79, 81, 88-93). The NACHT domain mediates oligomerization of NLR proteins, which is inhibited by the LRR (92, 94). The scaffolding domain, either a pyrin domain (PYD) or a caspase activation and recruitment domain (CARD), directs the signaling that activates caspase-1. Only the proteins NLRP3, NLRC4, NLRP1, and AIM2 have been shown to direct caspase-1 activation (Figure 1.3). Of these proteins, only NLRC4 has a CARD, which allows it to directly activate caspase-1

by hetero-oligomerization with a CARD present in the zymogen pro-caspase-1 (92). NLRP3, NLRP1, and AIM2 all have a PYD scaffolding domains and require the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) for signaling (95). ASC consists of a PYD and CARD, thus linking NLRs to pro-caspase-1, resulting in the formation of a large, macromolecular, cytosolic complex called an inflammasome (94).

In the inflammasome, tight hetero- and homo- oligomerization via CARD domains directs the ordered autoproteolysis of the pro-caspase-1 zymogen into a mature active form (96). Caspase-1 is a cysteinyl aspartate-specific proteinase that binds substrates via a four amino-acid motif fitting into a cleft containing the catalytic cysteine of the protease (Figure 1.4). The pro-inflammatory cytokines IL-1β and IL-18 are synthesized in biologically inactive forms, but contain sites (Figure 1.4) cleaved by active caspase-1, converting these proteins into bioactive cytokines (97, 98). Caspase-1 activation can direct the exocytosis of lysosomes (99), nuclear condensation (100), activation of NF-kB (101), and the formation of plasma membrane pores which causes osmotic swelling of the cell that is followed by lysis (102).

Caspase-1 repression

Consistent with its highly pro-inflammatory activities and ability to induce cell death, the activation of caspase-1 is tightly regulated in cells. The first level of regulation, previously discussed, is dependent on the detection of pathogen- or damage- associated stimuli. Further regulation comes from additional NLR proteins which antagonize, rather than induce, caspase-1 activation by acting as dominant-negative binders of activated NLRs: NLRP2, NLRP4, NLRP7, NLRP10, NLRC3, and NLRX1 (84, 85). An additional member of the NLR-family, NLRP12

(also known as Monarch-1), has recently been suggested to detect *Yersinia* (13). The contribution of NLRP12 to *Yersinia* detection remains complicated and unclear, as NLRP12 has previously been described not to detect pathogens, but instead antagonize caspase-1 activation (103, 104) and NF-kB signaling (105). Furthermore, previous reports with NLRP12-deficient mice found it contributed not to caspase-1 activation, but rather migration of dendritic cells and neutrophils (106). Since NLRP12 missense mutations result in greater caspase-1 activation and auto-inflammatory disorders in humans (107), it remains likely NLRP12 is a regulator of factors that activate caspase-1.

The mechanism by which NLRs can inhibit caspase-1 activation is likely similar to how PYD-only proteins (POPs) act. The PYD domain of this family of proteins is a dominant-negative inhibitor of PYD hetero-oligomerization (Table 1.3), thus preventing the signaling of activator NLRs to ASC (108). Similar to POPs, CARD-only proteins (COPs) also regulate caspase-1 activation (Table 1.3). These proteins are also dominant-negative inhibitors of hetero-oligomerization, but instead inhibit the CARD:CARD interactions of NLRC4 or ASC with pro-caspase-1 (108-113). COPs and POPs are encoded on the chromosome adjacent to NLRs or caspase-1 and appear to be derived by neofunctionalization, where duplicated NLR or caspase-1 genes have become truncated, resulting in proteins containing only PYD or CARD domains (114).

Lastly, inhibitors can interact directly with caspase-1 via a pseudosubstrate site (Figure 1.3) to act as a protease inhibitor (Table 1.3). While caspase-1 is a cysteine protease, it has partially overlapping substrate specificities and a similar mechanism of substrate hydrolysis as serine proteases. Consequently, several endogenous serine protease inhibitors also can inhibit

caspase-1 activity (115-117). Flightless-1, a regulator of the actin cytoskeleton, also contains caspase-1 pseudosubstrate sites that block caspase-1 activity (118). With the combined activities of inhibitory NLRs, COPs, POPs, and pseudosubstrates, caspase-1 activation requires sgnalling that surpasses a threshold of stimulation, reducing potentially harmful spontaneous caspase-1 activation and low-level inflammation.

Pathogen inhibition of caspase-1

Some viruses mask infection and prevent inflammation by inhibiting caspase-1, either by inhibiting activation with dominant-negative inhibitors of inflammasome scaffolding, or inhibiting caspase-1 activity though the action of protease inhibitors specific to caspase-1 (Table 1.3). The Kaposi's sarcoma-associated herpesvirus protein Orf63 binds the NACHT domains of NLRP1 and NLRP3, reducing caspase-1 activation and pro-inflammatory cytokine secretion in virus-infected monocytes (119). NLRs are targeted by several poxviruses, which encode POP-family inhibitors that act like and appear to be derived from host POPs (120, 121). Poxviruses also inhibit activity caspase-1 with pseudosubstrate inhibitors that also appear to be host-derived (122-128). One exception from this pattern of cooption of factors from the host is the baculovirus protein p35, a pseudosubstrate protease inhibitor with broad specificity likely only with incidental activity against caspase-1 since the virus only infects invertebrates, which lack caspase-1 (129).

No bacterial pathogens have previously been shown to act against the inflammasome by mechanisms so direct as viral pathogens; however, there are several factors that indirectly impact caspase-1 activation (Figure 1.5). *Mycobacterium tuberculosis* lacking Zmp1 induces greater

caspase-1 activation and inflammation, which leads to clearance of the bacterium (130). Zmp1 is a zinc metalloprotease required for intracellular survival that acts by altering phagosome maturation and neutralizing phagolysosomal Cathepsin G (131). While it is unclear how these activities are related to caspase-1 activation, disruption of the phagolysosome and the release of Cathepsin B, a protein related to Cathepsin G, are stimuli that activate caspase-1 (91, 132, 133). Thus it appears Zmp1 may block a pathogen-generated signal that activates caspase-1. The *Pseudomonas aeruginosa* T3S effector ExoU also contributes to diminished caspase-1 activation (134). ExoU is itself cytotoxic, and it appears to pre-empt pyroptosis activation by directing the cell to die by necrosis before caspase-1 can be activated (135).

Several other factors act by restricting the expression or presentation of PAMPs by the microbe. The regulator YdiV allows *Salmonella enterica* serovar Typhimurium to fully repress the expression of FliC, a very potent PAMP detected by NLRC4 (Table 1.2), during systemic infection (136). Two *Francisella tularensis* loci implicated in repression of caspase-1 activation were identified in a screen: an uncharacterized IclR-family transcriptional regulator and a CRISPR (clustered regularly interspace short palindromic repeats)-associated nuclease (137), both of which are not secreted and most likely alter PAMP availability indirectly (138). *F. tularensis* lipid II flippase MviN mutants have defective peptidoglycan, and consequently lyse easily (139) which results in greater caspase-1 activation (140); AIM2 detects DNA released by spontaneous bacteriolysis of cytosolic *F. tularensis* and *Listeria monocytogenes* (75-77). *Legionella pneumophilia* utilize the effector SdhA to alter membrane trafficking, maintaining the integrity of *Legionella*-containing vacuole and preventing the activation of caspase-1 that would occurs if this vacuole was disrupted (141, 142).

The NLRP3 inflammasome is only formed *in vitro* if cells are primed with TLR ligands or TNF-α to induce NF-kB-dependent expression of NLRP3 (143). Furthermore, expression of the precursor forms of IL-1β and IL-18 cytokines is induced by the TLR/IL-1R receptor family through NF-kB (144). Thus cell priming through TRIF, MyD88, and NF-kB -dependent signaling is important for pyroptosis, and may be impacted by pathogens that target these pathways. *F. tularensis* RipA reduces pyroptosis by restricting availability of an unknown factor that activates NF-kB and primes cells in this manner (145). Bacterial effectors can more directly inhibit the signaling of proteins involved in NF-kB activation, and are encoded *Bordetella pertussis, Brucella melitensis, Chlamydia trachomatis, Escherichia coli, Listeria monocytogenes, Salmonella enterica*, *Shigella flexneri*, and multiple *Yersinia* sp. (146), though it remains unknown whether any of these proteins can impact pyroptosis during infection.

Yersinia and caspase-1

While no direct inhibitors inflammasome formation or caspase-1 activity have previously been identified in *Yersinia*, several factors alter the ability of the macrophage to activate caspase-1 in response to the pathogen. *Y. pestis* has a reduced number of PAMPs available for detection; *Y. pestis* LPS is poorly recognized by TLR4 (147) and *Y. pestis* no longer expresses flagellin (10), precluding recognition by NLRC4 for caspase-1 activation or TLR5 for cell priming. YopK, a T3S factor unique to the *Yersinia sp.,* also impacts activation of caspase-1 during infection (148). The phenotype of a YopK mutant is complicated; at late time points it appears to hyper-translocate substrate (149) and also forms larger T3S translocon pores (27), which are stimuli

believed to contribute to caspase-1 activation during *Yersinia* infection (150). Thus, YopK appears to act by restricting the availability of PAMP for detection by host cells (148).

The T3S effector YopJ induces cell death by apoptosis (44, 151, 152), which pre-empts caspase-1 activation and cell death by pyroptosis (46). YopJ also an inhibitor of MAPKK and NF-kB pathways (41-43, 153) and as previously discussed, NF-kB signaling is required for inflammasome formation in response to numerous PAMPs and DAMPs (143). However, it appears in some circumstances YopJ activates caspase-1, rather than activating caspase-3 and inducing apoptosis (154), possibly by inducing NOD2 function by an unclear mechanism (155). This is not observed in several other infection models, possibly due to differing infection conditions, phenotypic heterogeneity of YopJ between *Yersinia* species, and interactions with additional factors (46, 154, 156, 157).

Perplexingly, the effector YopE may inhibit caspase-1 activation in a manner that coordinates with YopJ; macrophages infected with double mutant *ΔyopJΔyopE Y. enterocolitica* release IL-1β (dependent on caspase-1), but single mutants of *ΔyopJ* or *ΔyopE* do not induce release IL-1β (156). YopE is a RhoGAP mimic, and a member of the same effector family as ExoS of *P. aeruginosa*, which may also prevent caspase-1 activation (158). However, this effect on caspase-1 has not been seen by other groups (159) and may be a result of ExoS-induction of cell death by apoptosis (160). The *Salmonella* effector SopE also targets eukaryotic Rho family proteins, but contrastingly is instead an activator of caspase-1 (156), again suggesting this family of proteins may play an role in caspase-1 regulation, but by an unknown and complex mechanism.

Concluding remarks

The caspase-1 signaling pathway is very effective at combating microbial pathogens. However, previously described mechanisms of bacteria evasion of this response are based on avoidance rather than direct inhibition of this response. Chapter 3 of this work describes the mode of action of YopM, a direct inhibitor of caspase-1 that contributes to the remarkable ability of *Y. pestis* to evade immune defenses. The requirement to counteract caspase-1 activation comes as a consequence of detection of the *Y. pestis* toxin YTc as a PAMP, as described in Chapter 4. Together these findings describe major factors in the interactions of *Y. pestis* with its hosts. The mechanism YopM utilizes to inhibit caspase-1 provides novel insights into the regulation of caspase-1, and investigation of the cellular requirements for YTc detection clarifies how several other important pro-inflammatory stimuli are sensed. Chapter 5 explores how these findings relate to the field as a whole and the future directions of this work.

Table 1.1. Activities of major toxins of the *Yersinia sp.*

Factor	Target	Activity	Function	Ref
YopJ	MAP kinase kinases, IKKs; TRAF2, TRAF6, IkBa, RICK, TACK1	Ser/Thr acetyltransferase; deubiquitinase	Anti-inflammatory; pro-apoptotic; Pro-inflammatory?	(40-44, 46, 153, 155, 161)
YopO / YpkA	Rho GTPases	Ser/Thr kinase; RhoGDI mimicry	Anti-phagocytosis	(29-31)
YopE	Rho GTPases; additional unknown targets?	RhoGAP mimicry	Anti-phagocytosis; ROS inhibition	(32-34)
YopH	Focal adhesion complex proteins	Protein-Tyr-phosphatase	Anti-phagocytosis; Inhibition of T & B cell signaling	(35-39)
YopM	Rsk1, Prk2, thrombin, elastase, calpain, alpha-1-antitrypsin, Caspase-1	Scaffolding; Protease pseudosubstrate	Anti-inflammatory	(47-57)
YopT	Rho GTPases	Cys protease	(poor conservation) Anti-phagocytosis	(162, 163)
YopK	*Yersinia* YopBD	Unknown	Regulation of T3S rate; anti-pyroptosis	(27, 148, 149)
YTc	Unknown	Unknown	Anti-phagocytosis? Insecticidal?	(164-166)
Pla	Complement C3; Alpha 2-antiplasmin	Asp protease	(*Y. pestis* only) Fibrin & collagen breakdown; Tissue disruption	(17, 18)

Table 1.2. PAMP and DAMP stimuli activating caspase-1

Pathogen / Disease	Factor	Mechanism of recognition	NLR	Ref.
Bacillus anthracis	Lethal Toxin (LT)	Unknown	NLRP1	(89, 167)
Salmonella typhimurium *Legionella pneumophilia* *Pseudomonas aeruginosa* *Listeria monocytogenes* *Shigella flexneri*	Flagellin homologs	Direct interaction (via NAIP5)	NLRC4	(72, 73)
Salmonella typhimurium *Burkholderia pseudomallei* *Escherichia coli* *Pseudomonas aeruginosa*	T3S rod proteins	Direct interaction (via NAIP2)	NLRC4	(74)
Listeria monocytogenes *Francisella tularensis* Vaccinia virus Mouse cytomegalovirus	dsDNA	Lysis of cytosolic bacteria; dsDNA viruses	AIM2	(75-79)
Parkinsons disease Cancer Cell damage	Extracellular ATP	$P2X_7$ receptor	NLRP3	(80, 81)
Tissue injury	Biglycan	$P2X_7$ receptor	NLRP3	(168)
Tissue injury	Hyaluronan	Lysosome disruption	NLRP3	(169)
Type II diabetes	Islet amyloid polypeptide	Lysosome disruption	NLRP3	(170)
Hypertriglyceridemia	High glucose	ROS production	NLRP3	(171)
Gout	Monosodium urate	Lysosome disruption	NLRP3	(81, 172)
Chondrocalcinosis	Calcium pyrophosphate	Lysosome disruption	NLRP3	(172)
Alzheimer's disease	Amyloid-beta plaques	Lysosome disruption	NLRP3	(90)
Atherosclerosis	Cholesterol crystals	Lysosome disruption	NLRP3	(173)
Silicosis	Silica crystals	Lysosome disruption	NLRP3	(91)
Asbestosis	Asbestos	Lysosome disruption	NLRP3	(81)
Adjuvant	Alum, aluminum crystals	Lysosome disruption	NLRP3	(91, 174)
Gram-negative & Gram-positive bacteria	Muramyl dipeptide	$P2X_7$ receptor; Lysosome disruption	NLRP3	(175)
Malaria (*Plasmodium*) *Candida albicans*	Crystalline hemozoin Hyphae	Unknown	NLRP3	(176, 177)
Streptomyces sp.	Nigericin	$P2X_7$ receptor	NLRP3	(80)
Vibrio cholera, V. vulnificus *Bordetella pertussis*	RtxA CyaA	Pore formation (RTX toxins)	NLRP3	(178, 179)
Listeria monocytogenes *Streptococus pyogenes* *Clostridium tetani* *Streptococus pneumoniae*	Listeriolysin O Streptolysin O Tetanolysin Pneumolysin	Pore formation (Cholesterol-dependent cytolysins)	NLRP3	(133, 180-182)
Aeromonas hydrophila *Vibrio cholera* *Staphylococcus aureus* *Staphylococcus aureus*	Aerolysin VvhA Panton-Valentine leukocidin α-, β-, and γ- hemolysins	Pore formation (Beta-barrel toxins)	NLRP3	(178, 183-185)
Clostridium difficile	Toxin A, Toxin B	Pore formation	NLRP3	(186)
Mycobacterium tuberculosis	ESAT-6	Pore formation	NLRP3	(187)

Table 1.3. Mechanisms of inflammasome inhibition

Factor	Source	Target	Mechanism	Reference
orf63	Kaposi's sarcoma-associated herpesvirus	NLRs	NBD interference	(119)
POP1 POP2 Pyrin	endogenous	ASC	PYD interference	(108)
M013L gp013L SPV14L 18L DPV83gp024	Myxoma virus Shope fibroma virus Swinepox virus Yaba-like disease virus Mule deer pox virus	ASC	PYD interference	(120, 121)
ICEBERG, Caspase-12 INCA, CARD8, COP1, COP2	endogenous	Caspase-1	CARD interference	(108-113, 188)
Flightless-1, PI-9, PAI-2, Spi2a	endogenous	Caspase-1	Pseudosubstrate	(115-118, 189)
CrmA SERP2 B13R, B22R SPI-2 p35 p49	Cowpox virus Myxoma virus Vaccinia virus Ectromelia virus Baculovirus Baculovirus	Caspase-1	Pseudosubstrate	(122-128)

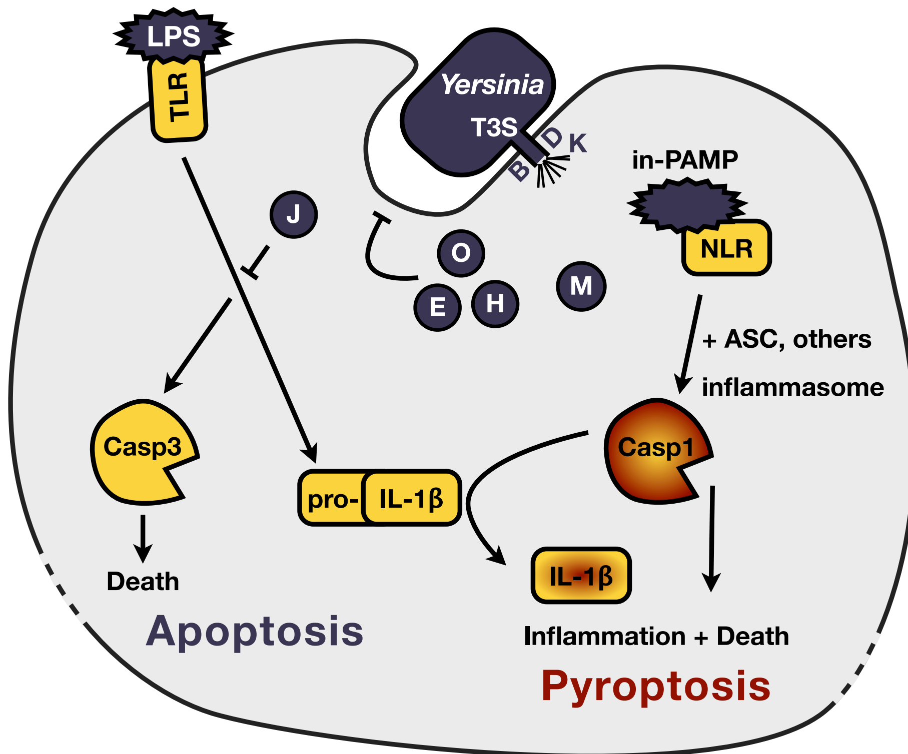

Figure 1.1: *Yersinia* remodeling of the macrophage
Yersinia deliver effector Yop proteins by T3S, through a channel formed by YopB and YopD and regulated by YopK. YopO, YopE, and YopH antagonize phagocytosis of the microbe. YopJ inhibits signaling involved in cellular activation and the priming of pro-inflammatory cytokines, and simultaneously, also induces the cell to die by non-inflammatory apoptosis. YopM has a previously poorly characterized function in virulence. A consequence of T3S is the delivery of PAMPs in the macrophage cytosol that are recognized by NLR, for the activation of caspase-1. With potentiation by extracellular PAMPs detected by TLRs, this leads to a rapid and pro-inflammatory program of cell death, pyroptosis.

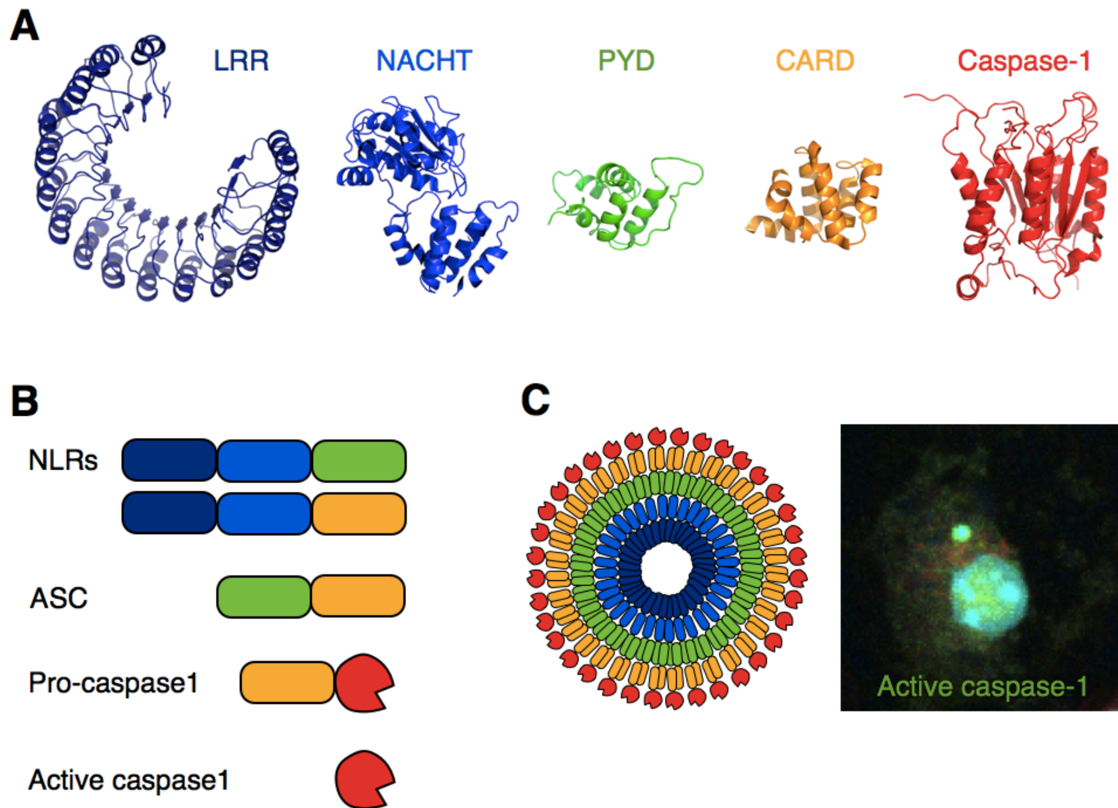

Figure 1.2: Mechanism of caspase-1 activation and inflammasome formation
(**A**) Inflammasome proteins are made up of several protein domain modules. Prototypical LRR (2BNH, (190)), NACHT, (3SFZ, (191)), PYD (1PN5, (192)), CARD (2B1W, (193)), and mature caspase-1 (1ICE, (96)). (**B**) NLRs contain a LRR, a NACHT, and either a PYD or CARD. The adapter ASC, containing a PYD and a CARD, is required for NLRs lacking a CARD to activate caspase-1. Pro-caspase-1 contains a CARD domain, which is cleaved off in the mature form of the protease. (**C**) A schematic of the inflammasome, a macromolecular complex of NLR, ASC, and caspase-1 formed *in vitro*, but of unknown and possibly undefined structure.

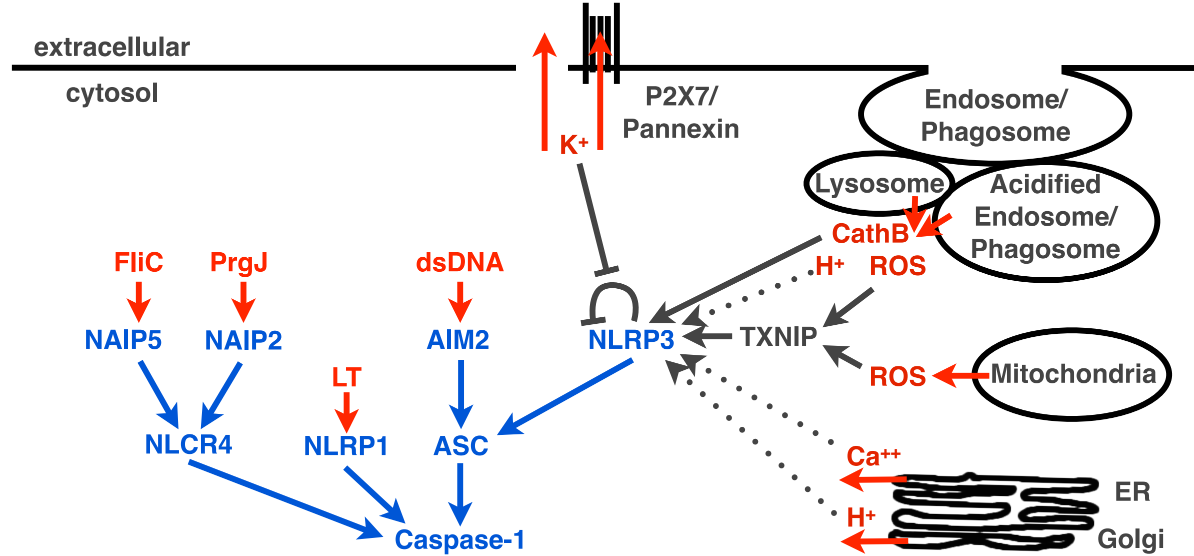

Figure 1.3: Mechanisms of PAMP detection by PRR for the activation of caspase-1
Distinct PAMPS activate the NLRC4-, NLRP1-, and AIM2- inflammasomes (Table 1.2). In contrast, NLRP3 responds to numerous stimuli related by the property of membrane disruption. Several different compartments have been implicated, and the leakage of various secondary signals has been proposed to integrate all of the stimuli: Cathepsin B (CathB), protons (H^+), reactive oxygen species (ROS), potassium (K^+) and calcium (Ca^{++}) cations. Indicated in blue are caspase-1 signaling events, red are PAMPs, DAMPs, or other caspase-1 activating signals; solid lines are direct interactions, while dotted lines may contain unknown intermediates.

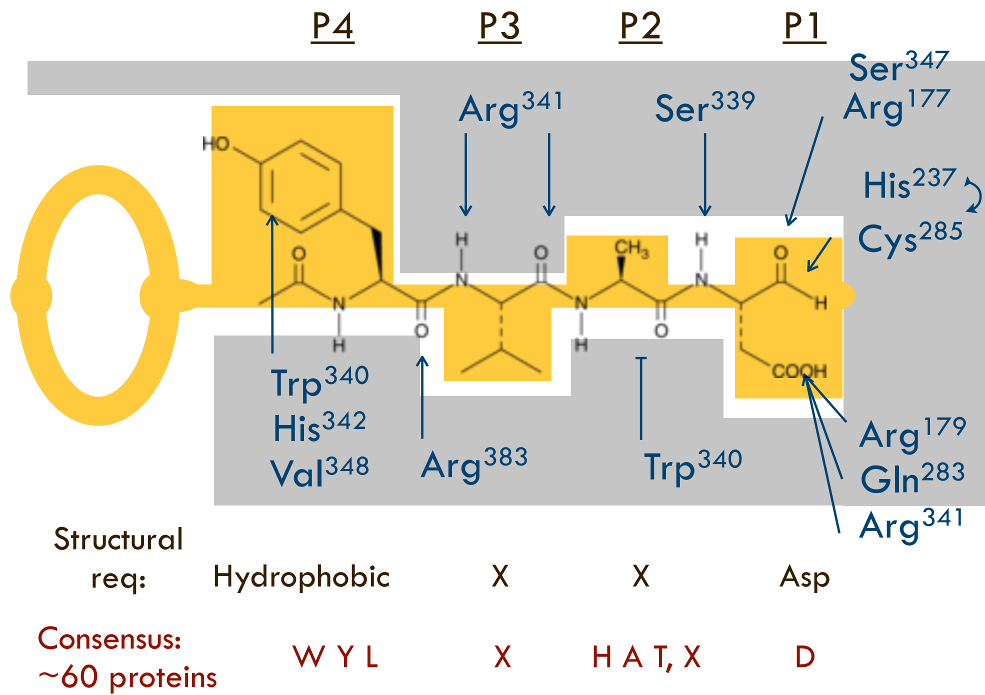

Figure 1.4: Mechanism of caspase-1 substrate and inhibitor recognition
Caspase-1 binds substrates and pseudosubstrate inhibitors by a "key-lock" mechanism. Residues of caspase-1 in close proximity to substrate that may mediate interactions as determined from previous data (96, 194) are indicated in blue. The consensus (195) includes a requirement for a bulky, hydrophobic residue in the P4 position and an aspartic acid in the P1 position.

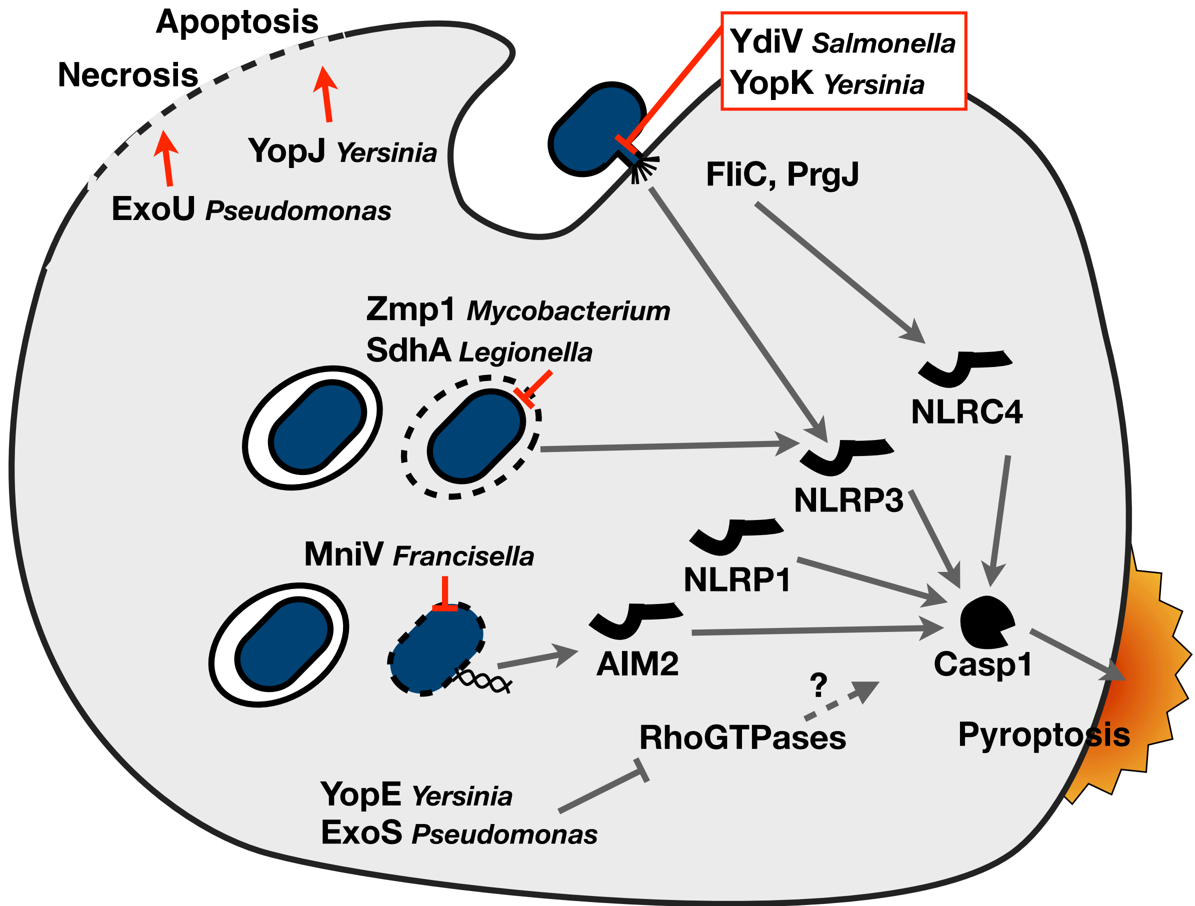

Figure 1.5: Pyroptosis avoidance mechanisms of bacterial pathogens
Inducing a macrophage to die by other mechanisms, such as ExoU-initiated necrosis or YopJ-initiated apoptosis, can preempt activation of pyroptosis. Additionally, restricting the availability of PAMPs are strategies utilized by *Yersinia, Salmonella, Mycobacterium, Legionella,* and *Francisella sp*. Some effectors that interact with host RhoGTPase proteins can also influence caspase-1 activation by an undiscovered mechanism.

Chapter 2. Material and Methods

Bacteria and plasmids

Bacterial strains are described in Table 2.1 and plasmids and the construction methods of strains described in Table 2.2. *Yersinia* were routinely grown in Luria Bertani broth or agar plates at 25°C unless otherwise noted.

Cell culture

Wild-type (Jackson Laboratories) or Caspase1$^{-/-}$11$^{-/-}$ (from C. Roy, Yale University) C57BL/6 mice were housed in specific-pathogen free conditions. Bone marrow-derived macrophages were generated from femur exudates of mice by culture for 7 days at 37°C in 5% CO2 in DMEM (Invitrogen) supplemented with 10% FCS, 5 mM HEPES, 0.2 mg/ml L-glutamine, 0.05 mM 2-ME, 50 mg/ml gentamicin sulfate, and 10,000 U/ml penicillin and streptomycin with 30% L cell-conditioned medium. Macrophages were then washed with PBS containing 1 mM EDTA, suspended in phenol red-free antibiotic-free DMEM supplemented with 5% FCS, activated with 100 ng/ml ultrapure LPS from *Salmonella* Minnesota (List Biologicals), and allowed to adhere for approximately 18 hr.

Retroviral vectors were constructed as in Table 2.1. Vectors were transfected into Ecotropic Phoenix cells (ATCC), The resulting recombinant viruses were transduced into macrophages and transformed cells selected with puromycin as described elsewhere (73).

Infection conditions

Yersinia grown overnight at 25°C in L broth were diluted 1:40 in L broth containing 20mM $MgCl_2$ and 20mM $Na_2C_2O_4$ and grown at 25°C for 1 hr followed by incubation at 37°C for 2 hrs. *Salmonella* were grown in L broth overnight at 37°C, diluted 1:40, and grown in L broth containing 0.3 M NaCl for 3 hrs. Bacteria with inducible expression plasmids were grown in media supplemented with 50 μg/ml carbenicillin and 0.02% arabinose. Bacteria, washed in cold PBS, were added to a multiplicity of infection of 10 and spun onto macrophages at 150 x g for 3 minutes. NLRP1 was induced by addition of 1 mg/ml anthrax lethal toxin subunits PA and LF (List Biologicals) and NLRP3 induced by addition of 20 mM nigericin (Sigma-Aldrich) as previously (167). Infections and toxin treatments of macrophages were for 90 minutes unless otherwise stated. Macrophages from lethal toxin-susceptible BALB/c mice (Jackson Laboratories) were used to examine caspase-1 activation in response to lethal toxin (Figures 3.5 and 3.11). LDH release was determined by Cytotox 96 kit (Promega) 2 hrs post infection. In all other experiments, infections were done in the presence of 5 mM glycine to protect against cell lysis (102).

Immunofluorescence microscopy

Caspase-1 activation was determined by Fam-YVAD-FMK (Immunochemistry Technologies) staining of macrophages infected on glass coverslips. After PBS washing, cells were fixed with Cytofix (BD). Lysosomal exocytosis was visualized by incubation of macrophages with anti-LAMP1 antibody (553792, BD Pharmingen) pre-fixation, as previously described (99).

Immunofluorescent staining of other proteins was performed post-fixation in Cytoperm (BD) with antibodies specific to the HA epitope tag (32-6700, Invitrogen), NLRP3 (sc-66846,

Santa Cruz), ASC (AL177, Enzo), Caspase-1 (sc-514, Santa Cruz), LAMP1 (553792, BD Pharmingen), YTc (antibodies against YitA, YitB, and YitC made by Syn Pep, previously described (164)), and *Yersinia* (KPL) with appropriate Alexa fluor-conjugated secondary antibodies (Invitrogen). DNA was stained with Hoescht (Immunochemistry Technologies) or To-Pro3 (Molecular Probes). Coverslips were mounted with ProLong (Molecular Probes) and examined by confocal microscope (Leica SL or Zeiss LSM 510) at the Wm. Keck Center for Advanced Studies in Neural Signaling. Caspase-1 activation or foci formation was enumerated by counting the fraction of positive cells in at least four separate fields.

As previously described, macrophages were treated 30 minutes pre-infection with 50 uM APDC (Enzo) (196), 30 mM KCl (167), 10 uM Ca-074-Me (Calbiochem) (181), and 250 nM bafilomycin A1 (Sigma) (197). Cathepsin B activity was visualized with MR-$(RR)_2$ added to infected cells 30 minutes pre-fixation, by the manufacturer's protocol (Immunochemistry Technologies). LysoTracker Red DND-99 (Invitrogen) was used as a reporter of vacuole acidification, added to 1 mM concurrent with infection.

Immunoblotting and cytokine analysis

Supernatants from infected macrophages were sterilized by passage through a 0.22-mm filter and IL-1β release was quantified by ELISA (R&D Systems) or concentrated using a 10,000 MWCO Centricon Plus-20 centrifugal filter device (Millipore), separated by SDS-PAGE, transferred to PVDF membrane, and immunobloted with anti–IL-1β antibody (AF-401-NA, R&D Systems). For immunoblots, cells were lysed in M-PER protein extraction reagent (Thermo) with sonication. After SDS-PAGE separation and transfer to PVDF membrane,

proteins were visualized with anti-HA (sc-805 or 32-6700, Invitrogen), anti-6xHis antibodies (37-2900, Invitrogen), an anti-YTc (164), or anti-caspase-1 p10 (sc-514, Santa Cruz) antibodies.

Protein interaction studies

Pyroptosis was induced in 10^7 macrophages by infection with *Yersinia*Δ at MOI 10 for 2 hrs. Lysates were added to 10 μg of recombinant 6xHisYopM or 6xHisYopM$_{271A}$ bound to HisPur Cobalt Resin (Thermo Fisher) and incubated at 4°C for 2 hr. After four washes with binding buffer, bound proteins were eluted with SDS-PAGE sample buffer containing imidazole and analyzed by immunoblotting.

Computational methods

The suitability of the YLTD motif of YopM as a caspase-1 pseudosubstrate was confirmed with the software PeptideCutter (ExPASy). The model interaction between YopM and caspase-1 was created in PyMOL by superimposing the YLTD sequence from the YopM (1G9U, (198)) with the YVAD inhibitor sequence of substrate bound to caspase-1 (1ICE, (199)).

Enzymatic studies

10U recombinant caspase-1 (Enzo) was pre-incubated with 6xHisYopM or 6xHisYopM$_{271A}$ for 5 min at 37°C in assay buffer (Enzo). Alternatively, lysates of 10^4 macrophages, treated as indicated, were centrifuged at 8,000 g for 5 min at 4°C, and the clarified lysate used for assays. Caspase-1 substrate Ac-YVAD-pNA (Enzo) was added to 200 uM and

cleavage detected by monitoring absorbance at 405 nm (Spectramax M3, Molecular Devices). Substrates WEHD-AMC, YVAD-AMC, LEVD-AMC, IETD-AMC, DEVD-AMC (Enzo) were similarly processed, with fluorescence measured at an excitation of 360 nm and emission 460 nm.

Circular dichroic (CD) measurements

Recombinant YopM and $YopM_{271A}$ were prepared in PBS and far-UV CD spectra collected (Avi Biomedical Model 420) in a 0.1 cm cuvette at 1 nm intervals at 25 °C. The PBS spectrum was subtracted from each sample and the mean molar ellipticity was calculated.

Toxin purification

Due to their high molecular weights, large toxin complexes can often be isolated without affinity tags by size exclusion chromatography (200, 201), so similar methods were used to isolate the *Yersinia* toxin complex. YTc was isolated from pCD-virulence plasmid-cured *Y. pestis* or from otherwise isogenic *ΔyitC Y. pestis*, both transformed with pYitR to promote YTc expression (164). Cultures were grown to OD_{600} 0.2 at 15°C, Arabinose added to 0.2%, and cultures further incubated at 15°C for 72 hrs. Cells were pelleted, lysed by three passes through a cell homogenizer (Avestin), and the cell debris removed by spinning 40,000 rpm at 4°C for 40 minutes in ultracentrifuge (Beckman). The supernatant was loaded onto an anion exchange column (HiTrap Q FF, GE Healthcare) and eluted across a 0-100% gradient of 1M NaCl in 25 mM Tris pH 8.0 on an Acta FPLC system (Amersham). Fractions containing YTc proteins were identified by immunoblot, concentrated using a 30 kDa molecular weight-cutoff Centricon

centrifugal filter device (Millipore). Complexes were purified by gel filtration with a superose 6 column (GE Healthcare) on an Acta FPLC (Amersham).

Toxin characterization techniques

Native-PAGE analysis of YTc isolated by size exclusion chromatography was performed on 3-12% Bis-Tris gels (Invitrogen) with proteins treated with 0.5% digitonin (Invitrogen). Ponceau S staining and immunoblotting were performed on these gels by standard procedures. Stoichiometry of the YTc was determined by subjecting the purified toxin to denaturing gel electrophoresis, 4-20% SDS-PAGE (Bio-Rad), followed by Coomassie blue staining and imaging. Densitometry with correction for relative molecular weights of the bands (NIH ImageJ) was used to determine the molar equivalents of each subunit. For caspase-1 activation experiments, LPS was removed from YTc samples with Detoxi-Gel Endotoxin Removing Columns (Pierce Biotechnology) and samples used in experiments had undetectable amounts of LPS remaining when measured by LAL assay (GenScript USA).

Intracellular replication assays

Caspase-1$^{-/-}$ bone marrow-derived macrophages were infected with the *Yersinia* strains indicated at a multiplicity of infection of two. Bacteria were grown as indicated then spun into contact with macrophages at 150 x g for 3 minutes. One hour post-infection, the cell culture media were replaced with media supplemented with 50 ug/ml gentamicin sulfate to kill extracellular bacteria. At the indicated time points, macrophages were washed once in PBS, lysed with 0.1% Triton X-100, and dilutions plated for colony-forming units on LB agar plates.

Animal experiments

Yersinia overnight cultures enumerated by Coulter counter (Multisizer 4, Beckman Coulter) were diluted in PBS for intraperitoneal delivery of 1000 CFU in 100 µl to 6–8 wk female mice. Mice were monitored for survival (5 mice each group) or sacrificed on day 4 and tissues homogenized and plated for CFU. Mice were housed in specific pathogen-free conditions and experiments performed according to University of Washington Institutional Animal Care and Use Committee guidelines.

Statistical analysis

Statistical significance was calculated by unpaired Student t test (*$P < 0.05$, **$P < 0.005$) using GraphPad Prism, unless otherwise indicated. Data are representative of at least three independent experiments.

Table 2.1. List of bacterial strains used in these studies

	Properties	**Reference**
Strains		
Y. pstb.	*Yersinia pseudotuberculosis* YPIII serogroup O:3, naturally deficient in YopT	(202)
*Yersinia*Δ	YPIII *yopJ-, yopO-, yopE-, yopH-, yopK-, yopM-*	(202)
Δ*yopJ*	(YP26)	(202)
Δ*yopO*		Cookson lab
Δ*yopE*	(YP9)	(202)
Δ*yopH*	(YP15)	(202)
Δ*yopK*	(YP36)	(202)
Δ*yopM*		Cookson lab
Salmonella enterica serovar Typhimurium LT2		Cookson lab
Yp pCD-	*Yersinia pestis* KIM10 (pgm-, pCD-)	Plano lab
Yp pCD- Δ*YTc*	*Yersinia pestis* KIM10 Δ*yitA-yipB* (YP1229)	(164)
Yp pCD- Δ*yitC*	*Yersinia pestis* KIM10 Δ*yitC* (YP1105)	(164)
Yp	*Yersinia pestis* KIM8 (pgm-, pCD+)	Plano lab
*Yp*Δ*YTc*	*Yersinia pestis* KIM8 Δ*yitA-yipB*	(164)
*Yp*Δ*yops*	*Yersinia pestis* KIM8 Δ1234 (pgm-, pCD T3S+, *yopJ-, yopO-, yopE-, yopH-, yopK-, yopM-*)	(203)
*Yp*Δ*yops*Δ*YTc*	*Yersinia pestis* KIM8 (pgm-, pCD+) Δ*yitA-yipB*	This study

Table 2.2. List of vectors and primers used in these studies

	Properties	**Reference**
Plasmids		
pBAD	pBAD30-HA	This study
pYopM	Bacterial expression of YopM in pBAD; primers 5'pM & 3'pM	This study
pYopM$_{CO92}$	YopM of Y. pestis CO92 in pBAD; primers 5'pM & 3'pM	This study
pYopM$_{271A}$	Site-directed mutagenesis of pYopM with primers 271A.F & 271A.R	This study
pMXsIP	Retroviral transfection	(73)
pMXS-YopM	For YopM macrophage transfection; primers 5'XsM & 3'XsM	This study
pMXS-YopM$_{271A}$	Amplified pYopM$_{271A}$ with primers 5'XsM & 3'XsM	This study
pET	pET30 Xa/LIC	Novagen
pET-6xHis-YopM	Protein expression of YopM; primers 5'XaM & 3'XaM	This study
pET-6xHis-YopM$_{271A}$	Amplified pYopM$_{271A}$ with primers 5'XaM & 3'XaM	This study
pYitR	Upregulation of YTc	(164)
Primers		
tataGAATTCcAccATGgTCATAAATCCAAGAAATGTATC	5'XsM	
tataCTCGAGCtaagcgtaatctggaacatcgtatgggtaCTCAAATACATCATCTTCA	3'XsM	
tttttGAGCTCgaaggagatatacatATGTTCATAAATCCAAGAAATG	5'pM	
ttttctagaCTCAAATACATCATCTTCA	3'pM	
ggtattgagggtcgcATGTTCATAAATCCAAGAAATG	5'XaM	
AGAGGAGAGTTAGAGCCctactcaaatacatcatc	3'XaM	
gtcagagataattatttaactgctctgccagaattaccgcag	271A.F	
Ctgcggtaattctggcagagcagttaaataattatctctgac	271A.R	

Chapter 3. *Yersinia* YopM arrests inflammasome assembly and processing

Summary

The host inflammatory response is strikingly delayed during the initial stages of infection with *Yersinia pestis*, a pathogen that suppresses immune defenses. We identify the bacterial leucine-rich repeat protein YopM as an inhibitor of pyroptosis, an inflammatory programmed cell death pathway. Pyroptosis, which depends on the protease caspase-1, restricts *Yersinia* infection. Consequently, YopM subversion of caspase-1 is essential for bacterial virulence. We demonstrate that caspase-1 is activated after a multistep process of inflammasome formation, and YopM is bifunctional in preempting pyroptosis: it blocks caspase-1 recruitment to pre-inflammasome complexes and directly inhibits caspase-1 proteolysis. Thus, a bacterium obstructs pyroptosis utilizing a mechanism distinct from viral or host inhibitors and therefore provides new insights into regulation of caspase-1 and inflammation.

Background

Pyroptosis is an inherently inflammatory program of cell death directed by the cysteine protease caspase-1. Activation of caspase-1 protects against numerous pathogens but aberrant activation or dysregulation is harmful to the host and is involved in several inflammatory disorders (204). Correspondingly, this system is controlled by Nod-like receptor (NLR) proteins that detect pathogen-associated molecular patterns (PAMPs) and initiate a response to an invading pathogen by activating caspase-1. The resulting cytokine release and cell lysis creates an

inflammatory environment directing immune responses to the site of infection. Accordingly, several pathogens antagonize this pathway to enhance their virulence (205).

The etiologic agent of plague, *Yersinia pestis* (*Yp*), and the closely related pathogen, *Y. pseudotuberculosis* (*Ypstb*), initially replicate in the host without inducing inflammation (46, 63, 206). *Yersinia* promote their survival by both evading detection and actively subverting immune signaling. *Yersinia* target several cell signaling pathways, through multiple type III secretion (T3S) system translocated effector proteins: YopO, YopE, YopM, YopH, and YopJ (207). One consequence of T3S by pathogenic bacteria is the cytosolic delivery of microbial factors that act as PAMPs for the activation of caspase-1 (208). While *Yersinia* can activate caspase-1, the specific factors detected as PAMPs have not yet been identified (46, 148).

One factor altering caspase-1 activation during *Yersinia* infection is YopK. This T3S regulator restricts effector translocation into host cells, likely limiting PAMP translocation, and consequently limiting caspase-1 activation as well (148, 149). We examined *Yersinia* effectors individually for repression of caspase-1 activation, and identified YopM as a potent antagonist of both caspase-1 activity and activation. We demonstrate that activation of caspase-1 is detrimental to *Yersinia* survival in the host; cell death and inflammation limits bacterial replication *in vivo* and promotes host survival. Consequently, YopM inhibition of caspase-1 is a requirement of *Yersinia* pathogenesis. YopM acts by directly binding caspase-1 to block caspase-1 activity and this binding also sequesters caspase-1 and aborts inflammasome formation. The resulting "pre-inflammasome" contains NLR and ASC, but not caspase-1, and appears to be an intermediate step of normal inflammasome development. Together these data indicate that repression of

inflammation by YopM is important for potentiating the virulence of the deadly pathogen *Yersinia pestis*.

Results

YopM prevents macrophage pyroptosis in response to *Yersinia* infection

During infection with *Y. pseudotuberculosis* or *Y. pestis*, caspase-1 activation occurs predominantly in activated cells and either TLR2 or TLR4 stimuli are sufficient signals for this priming (46). We observe caspase-1 activation is accelerated in macrophages infected with *Yersinia*Δ (Figure 3.1A), a *Y. pseudotuberculosis* mutant competent for type III secretion but lacking effector proteins. We therefore hypothesized that one or more effector blocks caspase-1 activation. YopJ induces death by caspase-1-independent apoptosis (46) and a YopJ allele unique to *Y. pestis* KIM activates caspase-1 (161), however, YopJ deletion in *Y. pseudotuberculosis* did not impact caspase-1 activation (Figure 3.1B). The effectors YopO, YopE, and YopH also did not alter caspase-1 activation (Figure 3.1B). YopK mutant bacteria activated greater caspase-1 (Figure 3.1B), likely due to its regulatory role in effector translocation (148, 149). Significantly enhanced caspase-1 activation also occurred upon deletion of YopM (Figure 3.1B), a protein of enigmatic function, but nonetheless linked with anti-inflammatory activity (48, 52, 56). Complementation with YopM from *Y. pseudotuberculosis* YPIII or *Y. pestis* CO92 (99.5% amino acid identity (Figure 3.2A)), depressed caspase-1 activation by *Yersinia*Δ (Figure 3.2B). Thus, YopM can act independently of other effectors and has conserved function between species, suggesting YopM is important for both pathogens.

Caspase-1 activation is typically accompanied by processing of caspase-1 into p20 and p10 subunits, and this cleavage is inhibited by YopM (Figure 3.3A). Active caspase-1 is required for activation of the pro-inflammatory cytokines IL-1β (204), and release of bioactive IL-1β is also prevented by YopM (Figure 3.3B). Additionally, lysosomal exocytosis, a caspase-1-dependent release of antimicrobial factors that can act on extracellular bacteria (99), is blocked by YopM (Figure 3.3C). The terminal cellular event directed by caspase-1 is lysis, where the release of inflammatory cellular contents amplifies local inflammatory responses (204, 209) and deprives intracellular pathogens a replicative niche (210). *Yersinia*Δ induces caspase-1-dependent lysis, prevented by YopM (Figure 3.3D). Infection with *Yersinia*Δ does not induce lysis of macrophages from *casp1*$^{-/-}$ mice (which are also *casp11*$^{-/-}$, an upstream activator of caspase-1 in some circumstances (211)) (Figure 1G). Together these data indicate YopM, delivered during *Yersinia* infection into relevant host target cells (28), blocks important antimicrobial responses directed by caspase-1.

YopM promotes virulence by inhibiting caspase-1 *in vivo*

Timely inflammation is important in the host's response to infection, suggesting that the ability of *Yersinia* to inhibit caspase-1 could impact bacterial virulence *in vivo*. YopM mutants are highly attenuated (47-49, 56), and our *in vitro* observations (Figures 3.1-3.3) suggested attenuation may result from the inability to inhibit caspase-1. Two orders of magnitude more *Y. pseudotuberculosis* were recovered from wild-type mice when the bacteria expressed YopM (Figure 3.4A), highlighting the requirement of YopM during infection. In *caspase-1*$^{-/-}$ mice, growth of Δ*yopM Y. pseudotuberculosis* was restored and comparable to WT *Y. pseudotuberculosis* (Figure

3.4A), indicating a major function of YopM *in vivo* is preventing caspase-1 activation. Wild-type and *caspase-1*$^{-/-}$ mice are both susceptible to WT (YopM^{+}) *Y. pseudotuberculosis.* (Figure 3.4B). Wild-type (*caspase-1*$^{+/+}$) mice survive infection with Δ*yopM Y. pseudotuberculosis* but there is attenuation reversal of for Δ*yopM Y. pseudotuberculosis* in *caspase-1*$^{-/-}$ mice (Figure 3.4C). These results demonstrate that *Y. pseudotuberculosis* is sensitive to the potent anti-microbial responses directed by caspase-1, thus YopM inhibition of caspase-1 *in vivo* is required for virulence.

YopM binds caspase-1 to inhibit activity

Caspase-1 is activated in the inflammasome, a macromolecular complex formed by NLR family proteins in response to PAMP stimuli (94, 143). The NLR protein NLRC4 detects bacterial flagellin; NLRP3 activates caspase-1 in response to numerous stimuli including inorganic irritants, pore-forming toxins, and nigericin; and NLRP1 responds to *Bacillus anthracis* lethal toxin (LT) (Table 1.2). Transduced YopM protects macrophages from caspase-1 activation in response to all of these stimuli (Figure 3.5), indicating YopM is sufficient to block caspase-1 activation triggered by multiple NLRs and diverse cognate PAMPs.

Avoiding caspase-1 activation is a beneficial strategy for many pathogenic bacteria (204, 210), although bacterial inhibitors specifically antagonizing caspase-1 have yet to be described. For viruses, which similarly benefit from avoiding or delaying inflammatory responses, several proteins structurally resembling endogenous host regulators have been shown to block caspase-1 activity (Table 1.3). We identified a four amino acid sequence in a surface-exposed loop of YopM similar to the idealized caspase-1 substrate (YVAD) and to endogenous and virus-encoded inhibitors of caspase-1 (Figure 3.6A). Immature and active caspase-1 both bind substrates with

equal affinity (96); pseudosubstrate inhibitors can block either the activation or activity of caspase-1 (118), suggesting YopM may similarly bind and inactivate caspase-1 (Figure 3.6B). This putative pseudosubstrate sequence is non-structural in nature, and can be mutated without disrupting protein folding (Figures 3.7A, 3.7B).

Binding analysis with recombinant YopM as bait for pull-down of proteins from lysates of macrophages induced for pyroptosis with *YersiniaΔ* infection, confirms YopM binds endogenous caspase-1 (Figure 3.8A). Caspase substrates and pseudosubstrate inhibitors contain an aspartic acid residue critical for the protein:protein interaction (Figure 1.3); altering this amino acid of YopM (Figure 3.6A, 3.7B) renders the protein ($YopM_{271A}$) incapable of binding caspase-1 (Figure 3.8A). Since YopM preferentially binds cleaved, active caspase-1 (Figure 3.8A), we determined whether substrate hydrolysis by caspase-1 is blocked by YopM binding. YopM, but not $YopM_{271A}$, directly blocks substrate cleavage by purified active caspase-1 (Figure 3.8B) without being cleaved itself (Figure 3.8C), indicating that YopM and caspase-1 directly interact, that YopM occupies the substrate site of caspase-1, and that a pseudosubstrate sequence of YopM is required for interaction.

YopM blocks formation of mature inflammasomes

During pyroptosis, NLRs complex with the adapter ASC to form a structure where pro-caspase-1 is cleaved into an active state (94, 95, 143). *In vivo*, these inflammasome structures are visualized as a macromolecular complex containing active caspase-1 (167). We hypothesized that YopM binding of caspase-1 might alter the formation or content of natural inflammasomes formed in response to infection. *Y. pseudotuberculosis* induces formation of NLRP3 foci, a

process unaltered by YopM (Figure 3.9A and 3.9B). NLRP3 requires the adapter protein ASC to bind and activate caspase-1; formation of ASC-containing pyroptosomes (212) were similarly unaltered by YopM (Figure 3.9C and 3.9D). Despite assembly of this NLR-ASC 'pre-inflammasome' scaffold during infection, caspase-1 is not recruited into foci in the presence of YopM delivered by *Y. pseudotuberculosis* (Figure 3.9E and 3.9F). Caspase-1 was recruited to the inflammasome in the presence of $YopM_{271A}$, the mutant that cannot bind caspase-1 (Figure 3.9F). Further, caspase-1 activation was blocked in cells where it could not be recruited to form inflammasomes (Figure 3.9G and 3.9H) and caspase-1 colocalized with YopM (Figure 3.10). Consistent with these observations, YopM, but not $YopM_{271A}$, blocks caspase-1 activation in response to PAMPs that activate NLRP1-, NLRP3- or NLRC4- inflammasomes (Figure 3.11). Together these results indicate naturally delivered YopM preempts caspase-1 activation by binding and sequestering caspase-1, resulting in the formation of developmentally arrested pre-inflammasomes lacking caspase-1 (Figure 3.9G, 3.9H). Thus, YopM blocks pyroptosis by a unique bifunctional mechanism: the penultimate step of inflammasome formation is interrupted and substrate processing is directly inhibited.

Discussion

Timely induction of inflammation is important for immunity to many pathogens. Inflammation during *Yersinia* infection is biphasic; an initial non-inflammatory phase of bacterial replication is followed by inflammation (46, 63, 206). We have demonstrated that *Y. pseudotuberculosis* not only evades innate immune detection, as previously presumed, but more specifically targets immune cells with an effector to bind and disable caspase-1. To our

knowledge this is the first report of a bacterial pathogen directly inhibiting this pathway, and these results provide insight into the exceptional ability of *Yersinia* to initially replicate in the host without eliciting inflammation.

Our results explain a virulence requirement for YopM that has previously been enigmatic. *Yersinia* primarily target macrophages for T3S translocation during infection (28). Using relevant host-derived cells and physiologic quantities of T3S-delivered YopM, we have identified a new target of YopM. Several reports have shown that Δ*yopM Yersinia* are hyper-inflammatory and attenuated *in vivo*; however, previously identified proteins that can bind YopM, α-thrombin, α1-antitrypsin, Rsk1, and Prk2, do not account for this phenotype (47, 52, 56). Thus, YopM appears to have multiple targets and functions *in vivo*, like many other toxins and effectors, and these activities may be interconnected. The caspase-1 binding site of YopM is in a distinct central region of the protein not required for Rsk1 binding (47, 48) or nuclear translocation (213, 214), but previously shown to be important for virulence (215). Mutation of the Rsk1 binding site of YopM also disrupts a nuclear localization signal, and may contribute to caspase-1 inhibition, as both caspase-1 and viral inhibitors of caspase-1 also translocate to the nucleus (125, 216). Our observations that YopM targets caspase-1 provides a mechanism for the anti-inflammatory properties of YopM.

Disruption of inflammasome formation and caspase-1 activation is a successful strategy of Poxviruses, which use multiple inhibitors that either block caspase-1 proteolysis or inhibit inflammasome formation (208). Remarkably, *Yersinia* has both activities in a single dual function virulence factor. The bacterial origin of YopM possibly explains the lack of structural homology to viral inhibitors, which appear to be coopted host regulators (118, 217). Based on

structural similarity, YopM is a potent, but only partial molecular mimic of Flightless-1, an endogenous pseudosubstrate inhibitor containing a gelsolin-like domain and leucine-rich repeats (118). Furthermore, like YopM, Flightless-1 inhibits both the activation and activity of caspase-1. However, YopM also inhibits inflammasome formation, like CARD-containing inhibitors of caspase-1 activation. Together these observations suggest *Yersinia* acquired caspase-1 inhibitory activity through convergent evolution, accounting for its unique, multipronged mechanism for inhibiting caspase-1 and regulating inflammation.

These data highlight the importance of caspase-1 in combating microbial infection and indicate that *Yersinia* are highly susceptible to pyroptosis, but normally prevent caspase-1 activation with YopM. Multiple *Yersinia* factors contribute to minimizing inflammation by restricting availability of PAMPs; directly targeting caspase-1 and inflammasome formation with YopM ensures *Yersinia* can limit host inflammatory responses to establish infection (Figure 3.12).

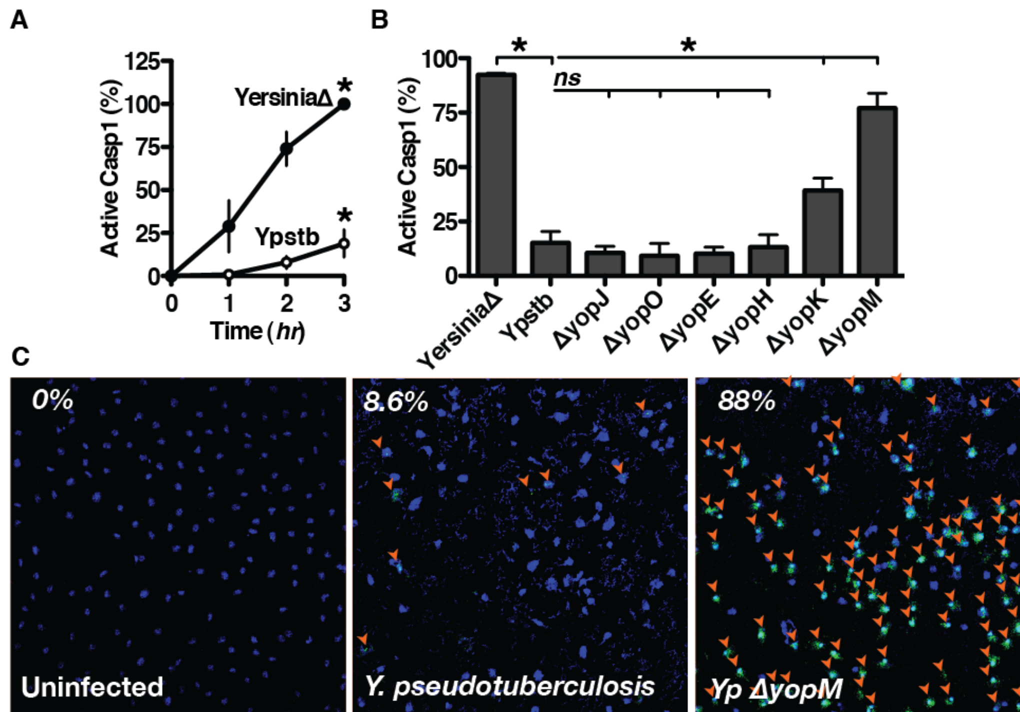

Figure 3.1. ΔYopM *Y. pseudotuberculosis* induce greater caspase-1 activation. Bone-marrow derived macrophages were infected with the *Y. pseudotuberculosis* (*Ypstb*) strains indicated. Cells with active caspase-1 were counted at the times indicated (**A**) or after 90 minutes of infection (**B** and **C**). (**C**) Representative micrographs *Ypstb*-infected macrophages and stained for active caspase-1. Arrows call out cells considered positive for caspase-1 activation, and the number indicates the percentage of total cells positive. *, $p<0.05$; "ns", $p>0.05$

A

```
pstbO3 YPIII    1 MFINPRNVSNTFLQEPLRHSSNLTEMPVEAGNVKSKTEYYNAWSEWERNAPPGNGEQREM
pestO CO92      1 MFINPRNVSNTFLQEPLRHSSNLTEMPVEAENVKSKTEYYNAWSEWERNAPPGNGEQREM

pstbO3 YPIII   61 AVSRLRDCLDRQAHELELNNLGLSSLPELPPHLESLVASCNSLTELPELPQSLKSLLVDN
pestO CO92     61 AVSRLRDCLDRQAHELELNNLGLSSLPELPPHLESLVASCNSLTELPELPQSLKSLLVDN

pstbO3 YPIII  121 NNLKALSDLPPLLEYLGVSNNQLEKLPELQNSSFLKIIDVDNNSLKKLPDLPPSLEFIAA
pestO CO92    121 NNLKALSDLPPLLEYLGVSNNQLEKLPELQNSSFLKIIDVDNNSLKKLPDLPPSLEFIAA

pstbO3 YPIII  181 GNNQLEELPELQNLPFLTAIYADNNSLKKLPDLPLSLESIVAGNNILEELPELQNLPFLT
pestO CO92    181 GNNQLEELPELQNLPFLTAIYADNNSLKKLPDLPLSLESIVAGNNILEELPELQNLPFLT

pstbO3 YPIII  241 TIYADNNLLKTLPDLPPSLEALNVRDNYLTDLPELPQSLTFLDVSENIFSGLSELPPNLY
pestO CO92    241 TIYADNNLLKTLPDLPPSLEALNVRDNYLTDLPELPQSLTFLDVSENIFSGLSELPPNLY

pstbO3 YPIII  301 YLNASSNEIRSLCDLPPSLEELNVSNNRLIELPALPPRLERLIASFNHLVEVPELPQNLK
pestO CO92    301 YLNASSNEIRSLCDLPPSLEELNVSNNKLIELPALPPRLERLIASFNHLAEVPELPQNLK

pstbO3 YPIII  361 QLHVEYNPLREFPDIPESVEDLRMNSERVVDPYEFAHETTDKLEDDVFE 409
pestO CO92    361 QLHVEYNPLREFPDIPESVEDLRMNSERVVDPYEFAHETTDKLEDDVFE 409
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B

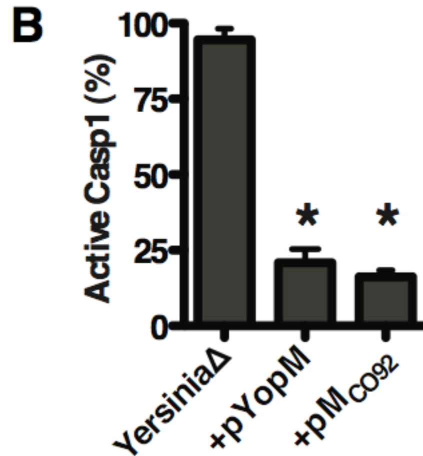

Figure 3.2. YopM function is conserved between *Y. pseudotuberculosis* and *Y. pestis*. (**A**) Identity of the Yop effectors (Table 1.1) between *Yersinia sp*. (**B**) Bone-marrow derived macrophages were infected with the strains indicated. Cells with active caspase-1 were counted at the times indicated after 90 minutes of infection. *, p<0.05; "ns", p>0.05; "nd", none detectable.

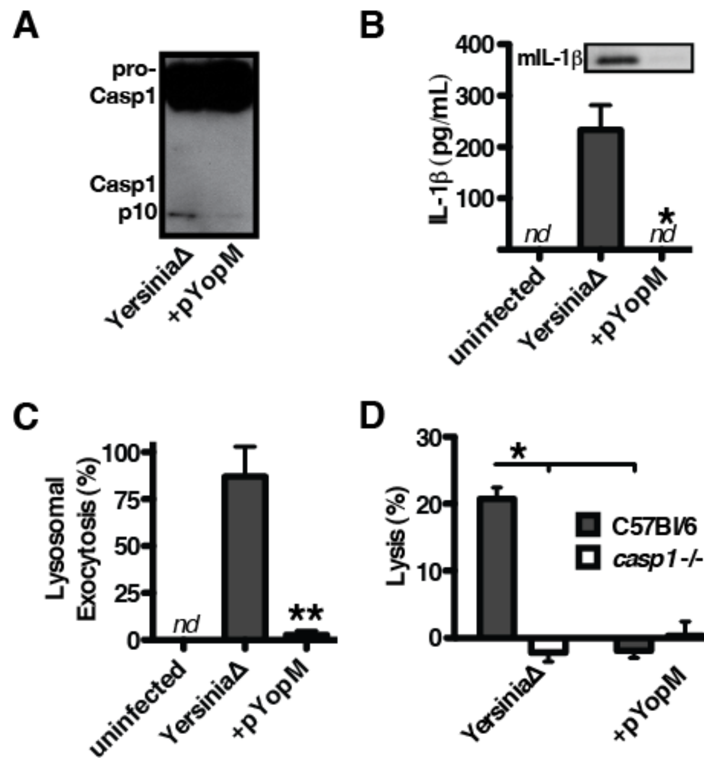

Figure 3.3. YopM prevents the pro-inflammatory processes directed by caspase-1. (**A**) Caspase-1 immunoblot of infected cells indicates YopM prevents caspase-1 maturation. (**B**) ELISA shows release of IL-1β, confirmed by western blot for mature IL-1β, during *Yersinia*Δ infection but not during infection with *Yersinia*Δ expressing YopM *in trans* (+pYopM). (**C**) Lysosomal exocytosis, determined by immunofluorescence staining of surface LAMP1 on intact cells, was induced by infection and blocked by YopM. (**D**) Cellular lysis was measured from macrophages infected 2 hrs with *Y. pseudotuberculosis;* lysis was caspase-1-dependent (indicating pyroptosis) and blocked by YopM. **, p<0.005; *, p<0.05; "ns", p>0.05; "nd", none detectable.

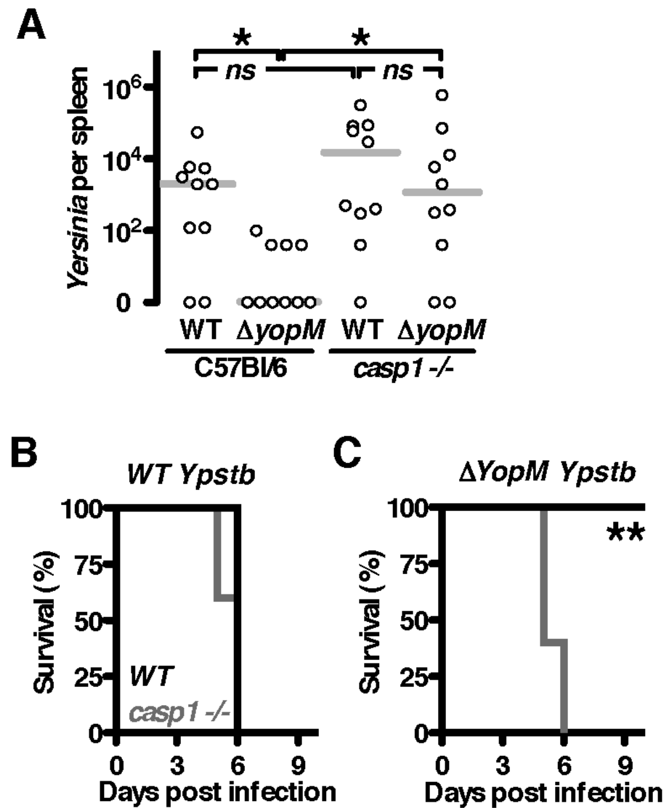

Figure 3.4. YopM is required *in vivo* for inhibition of caspase-1. (**A**) Four days post intra-peritoneal inoculation with 1000 CFU of *Y. pseudotuberculosis*, few bacteria were recovered from the spleens of C57BL/6 infected with Δ*yopM Y. pseudotuberculosis*, while this strain colonized *caspase-1*$^{-/-}$ mice similar to fully virulent *Y. pseudotuberculosis* (WT); each point represents the numbers recovered from a single mouse. (**B**) WT *Y. pseudotuberculosis* is lethal to both wild-type C57BL/6 mice (black) and *caspase-1*$^{-/-}$ mice (gray). (**C**) However, only *caspase-1*$^{-/-}$ mice are susceptible to *Y. pseudotuberculosis* lacking YopM (Δ*yopM Ypstb)*. Results are from combined experiments; asterisks indicate statistical differences by nonparametric Mann–Whitney U-test (titers, A) or Wilcoxon test (survival curves, B and C), **, p<0.005; *, p<0.05; "ns", p>0.05

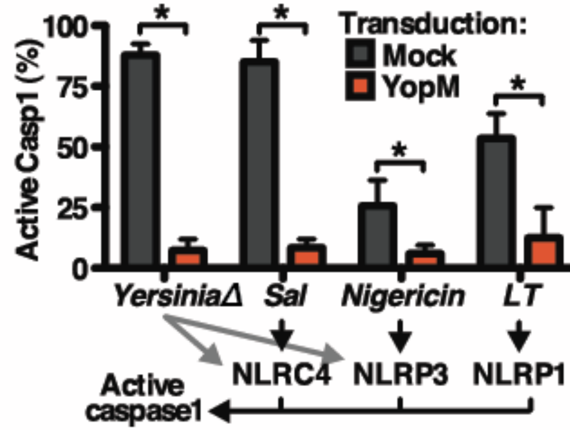

Figure 3.5. YopM transduction blocks multiple caspase-1 activation pathways. Macrophages were challenged or treated with *Yersinia*Δ, *Salmonella typhimurium* (Sal), nigericin, or anthrax lethal toxin (LT); retroviral transduction of YopM reduces caspase-1 activation in response to each of these stimuli relative to mock transduction. *, $p<0.05$

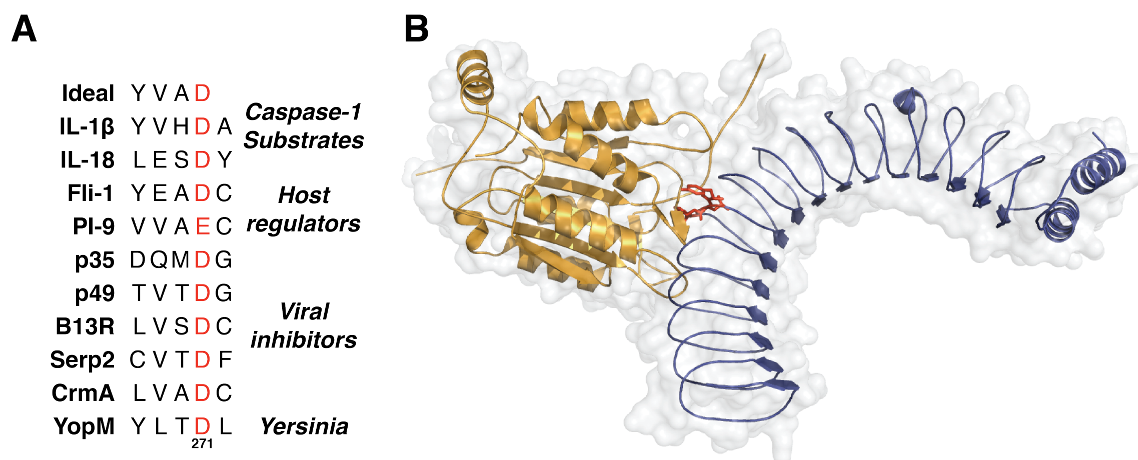

Figure 3.6. YopM contains a pseudosubstrate inhibitor domain against caspase-1.
(**A**) The conserved substrate recognition site bound by caspase-1 for selected known substrates and inhibitors, aligned to the similar sequence identified in YopM. The highly conserved aspartic acid is highlighted in red. (**B**) Model of YopM (blue, pdb: 1G9U) docking caspase-1 (yellow, pdb: 1BMQ) via pseudosubstrate site (red).

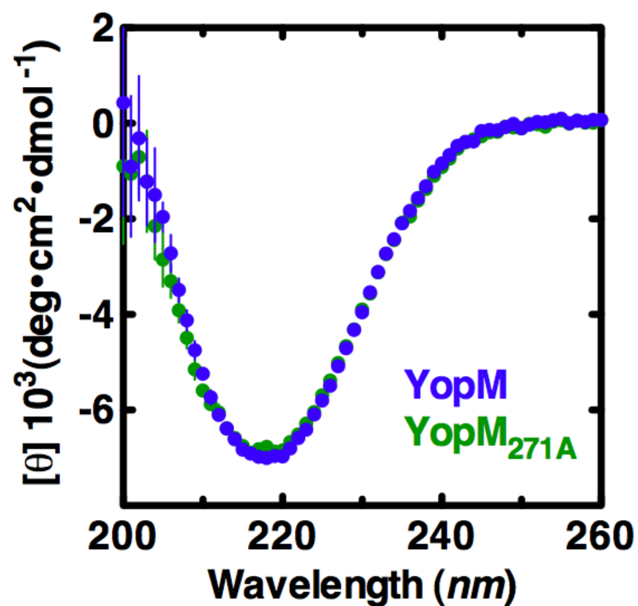

Figure 3.7. Alteration of the YLTD domain preserves YopM folding and structure.
Circular dichroism analysis of YopM and pseudosubstrate site mutant $\text{YopM}_{\text{D271A}}$ shows that these proteins are folded similarly, consistent with the folding previously observed of YopM (218, 219).

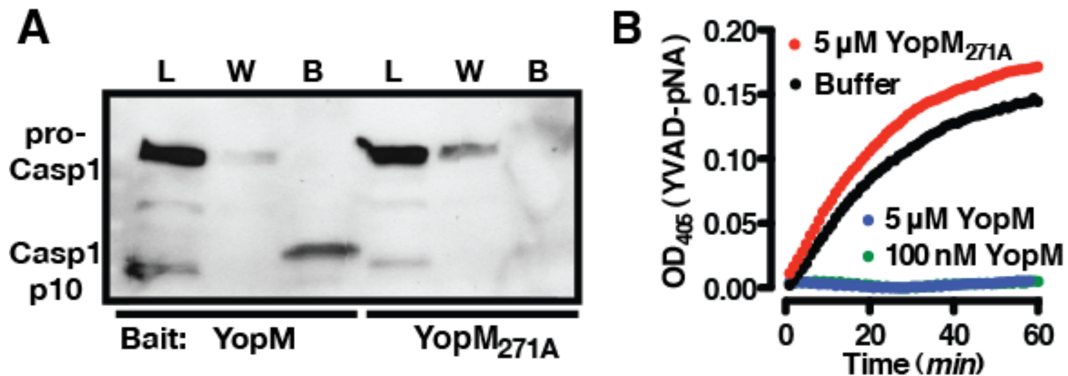

Figure 3.8. YopM binds directly to caspase-1, inhibiting substrate hydrolysis.
(**A**) Lysates from macrophages undergoing pyroptosis were incubated with 6xHis-YopM or 6xHis-YopM$_{271A}$ as bait for pull-down and immunoblot detection of caspase-1. The sample loaded (L) contains both the 45 kDa pro-caspase-1 as well as the cleaved p10 subunit indicative of mature caspase-1. Caspase-1 was detected in the wash volumes (W) with YopM$_{271A}$ but not YopM; the bound fraction (B) contained caspase-1 only when YopM was used as bait. Enrichment of p10 over pro-caspase-1 in the YopM-bound (B) fraction compared with the loading (L) indicates YopM may have a higher affinity for mature caspase-1. (**B**) YopM, but not YopM$_{271A}$, blocks proteolysis of caspase-1 substrate YVAD-pNA.

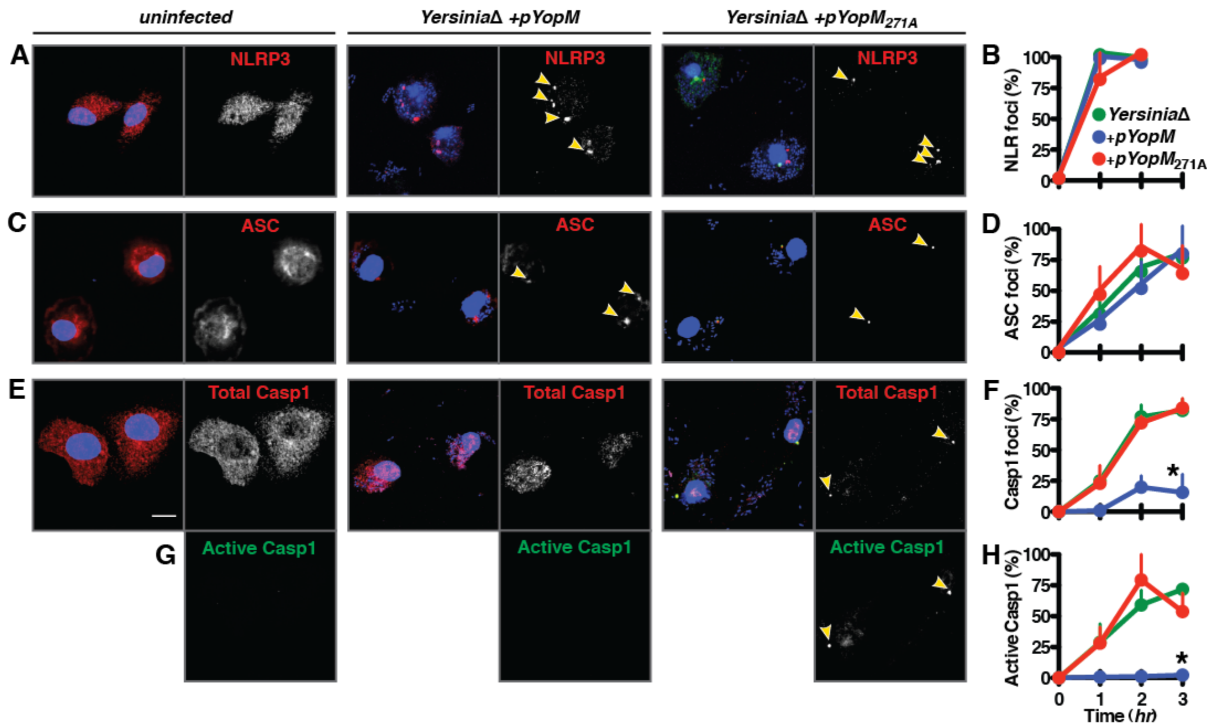

Figure 3.9. YopM inhibits inflammasome formation and caspase-1 activation. WT or *caspase-1*$^{-/-}$ macrophages infected with *Y. pseudotuberculosis* were fixed and protein localization visualized (A, C, E, G, I) and quantified (B, D, F, H) by immunofluorescence microscopy. Both NLRP3 (**A** and **B**) and the inflammasome adaptor protein ASC (**C** and **D**) rapidly form foci (arrowheads) in response to infection by *Y. pseudotuberculosis* whether expressing YopM (+pYopM), inactive YopM (+pYopM$_{271A}$), or no YopM at all (*Yersinia*Δ) (blue, red, and green lines, respectively, in B, D, F, H). (**E** and **F**) In contrast, recruitment of caspase-1 to foci is blocked by YopM but not YopM$_{271A}$. (**G** and **H**) Caspase-1 is not activated in cells in where can bind caspase-1. Representative confocal images are shown with macrophage nuclei visible by DNA staining (blue) and foci indicated with arrowheads; scale bars are 10 μm. *, $p<0.05$

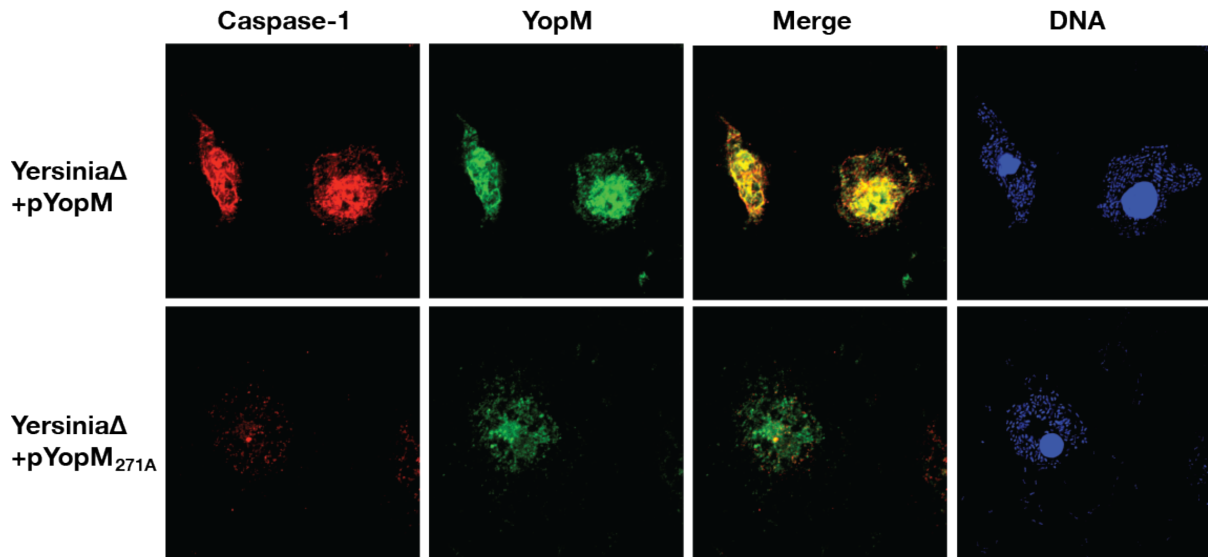

Figure 3.10. YopM colocalizes with caspase-1 during infection.
In YopM-transduced, *Yersinia*Δ-infected macrophages, immunostaining shows colocalization of YopM with caspase-1. Representative confocal images are shown with macrophage nuclei visible by DNA staining (blue) and foci indicated with arrowheads; scale bars are 10 μm.

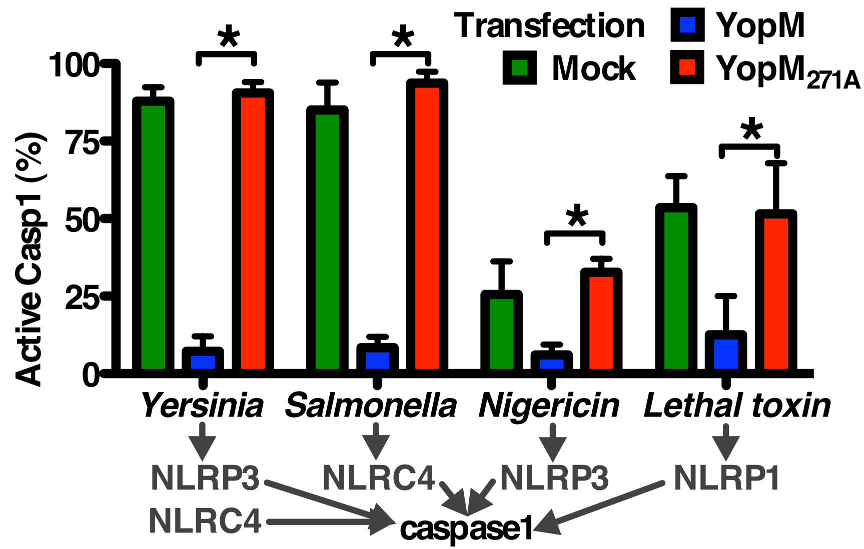

Figure 3.11. YopM$_{271A}$ cannot block caspase-1 activation by any pathway. Macrophages were challenged or treated with *Yersinia*Δ, *Salmonella typhimurium,* nigericin, or anthrax LT (*10*); retroviral transduction of YopM blocks caspase-1 activation in response to each of these stimuli. *, $p<0.05$

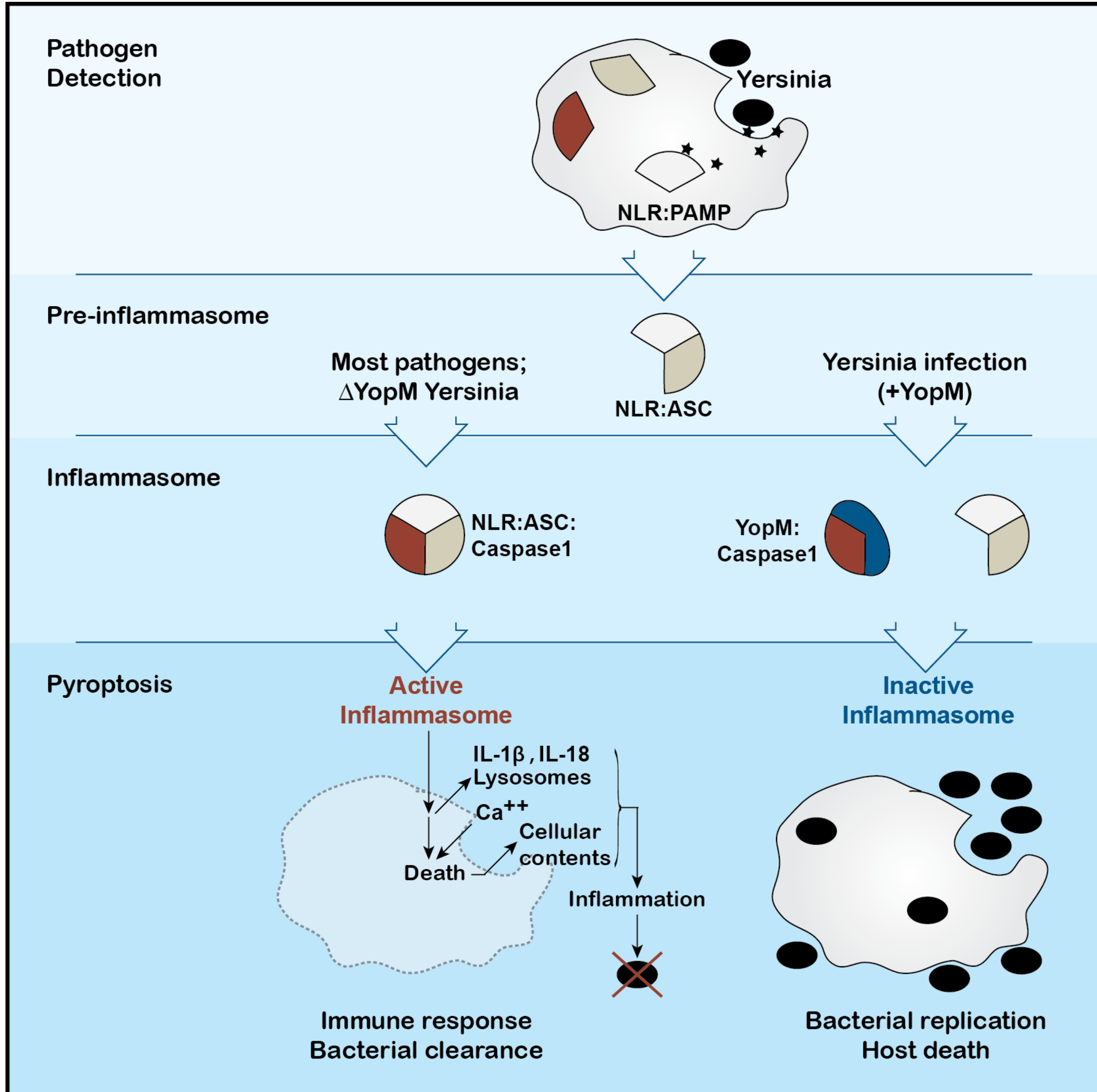

Figure 3.12. Model of YopM function in cells

In response to PAMP stimuli, NLRs form a caspase-1- and ASC- containing inflammasome, which leads to cytokine processing, release, and inflammatory cell death by pyroptosis. During infection with *Yersinia* expressing YopM, caspase-1 is not recruited to a pre-inflammasome complex to form an inflammasome. Consequently, the inflammatory pathway of pyroptosis is not initiated and *Yersinia* can continue to evade immune detection.

Chapter 4. The *Yersinia* toxin complex activates the NLRP3 inflammasome

Summary

Pyroptosis is a pro-inflammatory cell death program initiated by the inflammasome upon detection of pathogen-associated molecular patterns (PAMPs) by Nod-like receptor (NLR) proteins. NLR proteins recognize an unknown number of PAMPs of *Yersinia pestis*, a pathogen that actively antagonizes the inflammasome during infection. We demonstrate that the *Yersinia* toxin complex (YTc) activates the inflammasome and is a predominant PAMP of *Y. pestis*. YTc assembles a complex of over 1 mDa in size composed of 4:4:1:1 subunit stoichiometry. Purified YTc activates caspase-1, and the complex formed of the YitA and YitB subunits is sufficient for activation. Detection of YTc is dependent on endocytosis of the toxin, NLRP3, and cellular activation. During infection YTc is not exposed to extracellular antitoxin that can neutralize purified YTc and toxicity does not require endocytosis; it is instead dependent on the pCD-encoded type III secretion system. In infection, YTc forms lesions in the *Yersinia*-containing vacuole (YCV) that result in the release of Cathepsin B and efflux potassium from the cell, both of which are required to activate the NLRP3 inflammasome. YTc lesions in the YCV disperse the proton gradient across the vacuolar compartment. Interruption of YCV acidification, but not the release of Cathepsin B, protects *Y. pestis* residing intracellularly in the YCV. Thus, *Y. pestis* intracellular survival comes with the liability of detection by NLRP3 and the activation of pyroptosis.

Background

Plague, or the Black Death, is one of the most devastating epidemic bacterial diseases. Humans are incidental hosts of *Yersinia pestis,* the etiologic agent of plague, a zoonotic disease ordinarily spread between rodents by a flea vector. *Y. pestis* is predominantly extracellular throughout infection, however intracellular survival is important early during infection (19, 20). Upon initial transmission to a mammal, *Y. p*estis is endocytosed by phagocytes. Intracellular *Y. pestis* reside within a *Yersinia*-containing vacuole (YCV) that fuses with lysosomes but does not acidify (21-23). Within the YCV *Yersinia* begin to express virulence factors that antagonize host immune responses. The most well studied virulence factors are the Yop effectors (Yop E, H, O, M, and J) which are proteins delivered by type III secretion (T3S) into immune cells where they act to subvert phagocytosis, pro-inflammatory signaling, antigen presentation, and cell death processes (207).

Two distinct cell death programs occur during infection with *Yersinia*: apoptosis, dependent on caspase-3, and pyroptosis, dependent on caspase-1. Cell death by non-inflammatory apoptosis (44, 46) is thought to benefit *Yersinia* virulence by preempting pro-inflammatory pyroptosis, which can initiate an immune response protective against several pathogens including *Yersinia* (Chapter 3). *Yersinia* directly antagonizes pyroptosis with the T3S effector YopM (Chapter 3). YopJ antagonizes cellular activation in naïve cells which leads to activation of caspase-3 (44, 46), but unusually, YopJ of *Y. pestis* KIM instead activates caspase-1 in naïve cells (154). However, naïve cells often cannot undergo pyroptosis, as cellular activation can be a prerequisite for both caspase-1 activation and expression of the cytokines IL-1β and IL-18, key inflammatory components of pyroptosis (143, 144). Cellular activation of macrophages

occurs TLR2 priming during *Y. pestis* infection (60), and TLR2 and TLR4 can both prime macrophages for pyroptosis (46). Macrophages *in vivo* are found in a broad spectrum of activation states and infection with *Yersinia* leads to expansion of the number of activated cells (46). During *Yersinia* infection of mice, cellular activation and caspase-1 population coincide (46, 220), thus it is with activated macrophages that *in vitro* experiments are most relevant to pyroptosis during infection with *Y. pestis.*

While *Yersinia* have mechanisms inhibitory to pyroptosis *in vivo*, it is yet unclear how *Yersinia* is even detected to initiate pyroptosis. The key mediator of pyroptosis, caspase-1, remains in a quiescent form until activation by NLRs. NLRs are pathogen recognition receptors that detect the presence of intracellular PAMPs or damage-associated molecular patterns (DAMPs), cluing the cell to pathogen invasion or major damage. Diverse stimuli can act as PAMPs or DAMPs including flagellin, T3S proteins, dsDNA, ATP, potassium ionophores, and pore-forming toxins (204). *Y. pestis* does not express flagellin (10) and its T3S rod proteins do not activate caspase-1 (74), thus, these likely PAMPs *Yersinia* can be excluded and there remains new factors to be discovered.

The *Yersinia* toxin complex (YTc) has homology with the insecticidal toxin complex proteins of *Photorhabdus luminescens*. It is unclear whether YTc plays a role in mammalian pathogenicity of colonization of the flea vector, as it is expressed in both locations (165, 221). Further, at least one of the YTc components is translocated by the pCD1-encoded T3SS which also secretes the Yop effectors into mammalian cells (164). Some evidence suggests that genes regulated by the LysR-like response regulator, YitR, which includes the YTc genes, are important for increased resistance to phagocytosis of *Y. pestis* upon transmission from flea to mammal

(222). The mechanism of protection or whether this is attributed to the YTc proteins or other genes regulated by YitR is not known.

Here, we show that YTc acts as a PAMP detected by NLRP3 for induction of pyroptosis. YTc forms pores in the YCV that results in the release of Cathepsin B, a secondary signal detected by NLRP3. Concurrently, internalized *Y. pestis* are protected from acidification of the YCV by the YTc-dependent disruption of the vacuolar proton gradient, which promotes the intracellular survival of *Y. pestis*.

Results

***Yersinia* toxin complex activates caspase-1**

During infection, *Yersinia* PAMPs are rapidly detected by phagocytic cells, but the action of the T3S effector YopM greatly delays activation of caspase-1 (Chapter 3). Consistent with previous data using *Y. pseudotuberculosis*, macrophages detect infection of *Y. pestis* KIM and trigger the formation of ASC-containing foci indicating the formation of a pre-inflammasome (Figure 4.1A). As with *Y. pseudotuberculosis,* caspase-1 is not activated during infection with *Y. pestis* and this is dependent upon the Yop effectors (Figure 4.1A). A *Y. pestis* strain (*Yp*ΔYops) lacking effector proteins but otherwise competent for T3S readily activates caspase-1. Since the Yop effectors do not contribute to detection of the *Y. pestis* infection, we utilized the strain *Yp*ΔYops to examine which NLR sensors activate caspase-1 in response to infection.

Since *in vitro* cultivated cells are typically naïve and priming induces cytokine expression central to pyroptosis and provides a secondary signal often required for caspase-1 activation, we examined caspase-1 activation in LPS pre-treated bone marrow-derived macrophages. The major

NLR sensors implicated in inflammasome formation in response to bacterial infection are NLRP3, which responds to numerous stimuli, and NLRC4, which can detect flagellin and a rod-forming subunit of the T3S (72-74). Caspase-1 is poorly activated in NLRP3$^{-/-}$ macrophages but unaltered in NLRC4$^{-/-}$ macrophages when challenged with *Yp*ΔYops (Figure 4.1B), indicating that NLRP3 is critical for activating caspase-1 during *Y. pestis* infection.

The only bacterial PAMPs known to activate NLRP3 are pore-forming toxins, but no such toxins have been identified in *Yersinia*. However, the *Yersinia* toxin complex (YTc) has extensive homology with pore forming toxins and known PAMPs (Figure 4.2). The YTc subunit proteins YitA and YitB have homology with XptA2, the pore-forming subunit of *Xenorhabdus nematophilus* insecticidal toxin complex (201). YitC has similarity to SpvB, which may contribute to caspase-1 activation during *Salmonella* infection (223). YipA and YipB have homology with *Pseudomonas aeruginosa* RhsT, a pore-forming toxin that activates caspase-1 (224). The YTc is mostly closely related to insecticidal toxins of *Xenorhabdus* and *Photorhabdus*, which suggested it acts in the flea host of *Y. pestis* rather than the mammal. YTc is not toxic toward any insects or insect cell lines and it is toxic toward some mammalian cells (164-166). Together these observations suggest the YTc may be a PAMP that activates caspase-1.

Deletion of this toxin locus greatly reduces activation of caspase-1 during infection with *Yp*ΔYops (Figure 4.3A), indicating YTc is detected as a PAMP for the activation of caspase-1. Since this 20 kb locus is too large to complement *in trans*, we transformed *Y. pestis* with a vector allowing inducible expression of YitR, a transcriptional activator of YTc expression (164). YitR induction increased caspase-1 activation, but only when the YTc locus was intact (Figure 4.3B), indicating this effect is specifically due to upregulation of YTc. Together, these results indicate

that during infection with *Y. pestis*, the host detects YTc as a PAMP which activates caspase-1 and induces pyroptosis.

YTc forms a large multimeric complex

YitA and YitB are the predicted pore-forming subunits of the YTc and assemble independently of other subunits. YitC is essential for assembly of YipA or YipB in the complex (164). To further elucidate the mechanism of YTc activation of caspase-1, we purified the YTc from wild-type and Δ*yitC Y. pestis* using ion exchange followed by size exclusion chromatography (Figure 4.4A). Native gel analysis of the high molecular weight fractions isolated from size exclusion demonstrates the presence of a single, high molecular weight complex (Figure 4.4B) and immunoblot confirms that each complex contains YitA (Figure 4.4C). Denaturing gel electrophoresis shows that YTc is composed of YitA, YitB, YitC, and YipA and/or YipB, while the complex from Δ*yitC Y. pestis* contained only YitA and YitB (Figure 4.4D). Densitometry of these bands suggests the stoichiometry of YTc to be 4:4:1:1 of YitA:YitB:YitC:YipA/B (Figure 4.4E), similar to the 4:1:1 stoichiometry observed for the *X. nematophilus* toxin complex (201) and 5:5:1:1 stoichiometry of the *Yersinia entomophaga* toxin Yen-Tc (200). This stoichiometry adds up to a molecular weight of 1.15 mDa for the YTc, and 0.9 mDa for the YitAB complex, similar to gel filtration and native gel electrophoresis estimates and the sizes of heterologous toxin complexes.

The YTc subunits YitA and YitB are sufficient for NLRP3- and cell priming- dependent activation of caspase-1

To determine if YTc is sufficient to activate caspase-1, titrations of purified YTc were incubated with macrophages (Figure 4.5A). 1 μg/mL (~0.43 nM) of purified complex was sufficient to activate caspase-1, comparable to the sensitivity of caspase-1 activation to *Vibrio cholera* hemolysin (5 μg/mL) (178), *Streptococcus pyogenes* streptolysin O (10 μg/mL) (225) and *Staphylococcus aureus* α-toxin (5 μg/mL) (184). NLRP3 is required for caspase-1 activation in response to purified YTc (Figure 4.5B), in agreement with the dependence of NLRP3 for caspase-1 activation during *Y. pestis* infection (Figure 4.1B), and in response to other pore-forming toxins (Table 1.2).

Cellular activation is required for NLRP3-mediated detection of PAMPs (143). Naïve cells do not respond to purified, endotoxin-free YTc, but cells activated by LPS pre-stimulation can activate caspase-1 (Figure 4.5C). This requirement for LPS co-stimulation is similar to NLRP3 activation of caspase-1 observed during treatment with numerous PAMPs and DAMPs including *S. aureus* α-toxin (184), ATP (80), and monosodium urate crystals (172). Similarly, LPS-pretreatment greatly enhances caspase-1 activation by *Yp*ΔYops, in a manner also dependent upon the YTc (Figure 4.5D). Together these results support observations that cellular activation is a requirement of NLRP3 activation of caspase-1, and also for caspase-1 activation in response to *Y. pestis.*

With the notable exception of *Bacillus anthracis* Lethal Toxin, which is detected by NLRP1 rather than NLRP3 (89), bacterial toxins activate caspase-1 as a consequence of pore formation rather than translocating enzymatic subunits (178, 184, 225). YitA and YitB are heterologs of the pore-forming TcaA and TcaB subunits of the *P. luminescens* toxin complex, suggesting YitAB may be necessary and sufficient for pore formation, and consequently, caspase-

1 activation. Since Δ*yitC Y. pestis* forms a large toxin complex composed of YitAB (164), we tested caspase-1 activation by this toxin complex. Purified YitAB and native YTc similarly activated caspase-1 (Figure 4.6). This indicates that the putative enzymatic subunits of the complex, YipA and YipB are dispensable for caspase-1 activation.

Type III secretion potentiates YTc toxicity

Previous work demonstrated that the YTc proteins can be delivered into target cells by the pCD virulence plasmid-encoded T3SS (164). Furthermore, T3S is required for caspase-1 activation in the related pathogens *Y. pseudotuberculosis* and *Y. enterocolitica* (46, 150). Together, these results suggested that YTc may activate caspase-1 as a T3S-translocated toxin, rather than an exotoxin secreted during infection. The contribution of the T3S apparatus to activation of caspase-1 by YTc was examined with *Y. pestis* pCD^{-}, a strain lacking the T3S system. A fully functional T3S system is required for YTc activation of caspase-1 (Figure 4.7A), consistent with previous observations that YTc is T3S-translocated (164) and that activation of caspase-1 during *Yersinia* infection requires T3S (46).

Cellular uptake of many exotoxins can be pharmacologically blocked with colchincine, an inhibitor of endocytosis which also prevents caspase-1 activation during gout by blocking uptake of uric acid crystals (172). Colchine has no effect on T3S, therefore, colchicine can used to differentiate whether YTc acts as an exotoxin or a T3S substrate during infection. Colchicine blocks caspase-1 activation by purified YTc (Figure 4.7B), indicating that purified YTc requires endocytosis and acts intracellularly, rather than on the macrophage surface. Unlike with purified YTc, colchicine does not inhibit caspase-1 activation during *Y. pestis* or *Salmonella* (Figure 4.7B),

confirming colchicine does not directly affect caspase-1. Therefore, during infection the YTc does not require endocytosis to activate caspase-1, supporting the model that the YTc components are delivered by type III secretion into mammalian cells.

Antitoxins are antibodies that neutralize exotoxin uptake or activity. Antibody against YitA subunit of the YTc blocks the ability of purified YTc to activate caspase-1 (Figure 4.7C), demonstrating that YitA antibody can act as an antitoxin. However, YitA antibody is not protective during *Y. pestis* infection (Figure 4.7D), indicating that the extracellular antibody does not have access to YitA during infection. This suggests that caspase-1 is not activated by secreted YTc and that the toxin is instead delivered directly from bacterium to macrophage cytosol, a mechanism consistent with T3S. These independent observations, utilizing genetics, pharmacologic inhibitors, and specific antibodies, provide further support that the YTc is not an exotoxin during infection but is transferred by the T3S apparatus.

YTc induces lysosome disruption

Diverse PAMPs and DAMPs activate the NLRP3 inflammasome and these stimuli are thought to be integrated by several secondary signals: low cytoplasmic potassium concentrations, reactive oxygen species leaked from phagosomes or mitochondria, leakage of the lysosomal protease cathepsin B, and proton gradient disruption across organelles (Table 1.2). Identification of the stimuli that are involved in the activation of caspase-1 in response YTc provides information about the subcellular localization and activities of YTc. NLRP3 activation is typically proceeded by efflux of potassium from the cell and high levels of extracellular potassium can often block NLRP3 activation. Activation of caspase-1 by both purified YTc and YTc during

Y. pestis infection is blocked by supplementing the tissue culture media with 30mM KCl (Fig 4.9A). Thus, potassium efflux is necessary for NLRP3-dependent caspase-1 activation by YTc.

The contribution of ROS to NLRP3 activation is unclear; the ROS scavenger (2R,4R)-4-aminopyrrolidine-2,4,-dicarboxylate (APDC) prevents NLRP3 stimulation by several DAMPs, possibly by altering the activation state of the cell (81, 197). APDC did not reduce YTc-dependent caspase-1 activation, indicating ROS are dispensable for NLRP3 detection of YTc (Figure 4.9B). Lysosomal leakage of the protease Cathepsin B has also been implicated in NLRP3 inflammasome activation. The Cathepsin B-specific inhibitor, Ca-074-me, blocks NLRP3 activation by numerous stimuli including pore-forming toxins (91, 133). In agreement with these findings, Ca-074-me blocked NLRP3 activation by YTc (Figure 4.9C). Another signal that activates the NLRP3 inflammasome is proton leakage from lysosomes or other acidified compartments disrupted by internalized factors. Bafilomycin A is a vacuolar proton pump inhibitor that prevents vacuolar acidification and caspase-1 activation by these stimuli (197). Bafilomycin A did not decrease caspase-1 activation in response to *Y. pestis* infection or purified YTc, indicating proton efflux is not a requirement of YTc detection by NLRP3 (Figure 4.9D). These results indicate that the leakage of the lysosomal protease Cathepsin B, but not ROS or proton efflux, was detected by NLRP3 in response to YTc.

Since Cathepsin B activity is required for YTc activation of the NLRP3 inflammasome we performed microscopy to see if Cathepsin B is released from the phagolysosome into the cytosol. LAMP1, a marker of lysosomes and phagolysosomes, is recruited to the *Yersinia*-containing vacuole (YCV) (Figure 4.10). This indicates that *Y. pestis* does not inhibit fusion of lysosomes with phagosomes, consistent with previous findings (23, 226). The fluorescent

Cathepsin B substrate MR-$(RR)_2$ was confined to the $LAMP1^+$ YCV during infection with *Yp*Δ*Yops*Δ*YTc* (Figure 4.10). In contrast, there was significant MR-$(RR)_2$ staining outside of the $LAMP1^+$ YCV during infection with (YTc^+) *Yp*Δ*Yops* (Figure 4.10), indicating that YTc induces leakage of the lysosomal protease Cathepsin B, a stimulus that activates caspase-1.

Since YTc appeared to be disrupting the compartment containing intracellular *Yersinia*, we examined if additional factors leak out of this vacuole. If the YCV membrane is disrupted the efflux of protons prevents staining by the lysosomal marker, Lysotracker. The YCV contained Lysotracker during infection with *Yp*Δ*Yops*Δ*YTc while* little Lysotracker colocalized with (YTc^+) *Yp*Δ*Yops* bacteria, (Figure 4.11A). The significantly greater YCV acidification seen with Δ*YTc* bacteria (Figure 4.11A, 4.11B) indicates the lesion YTc forms in the YCV (Figure 4.10) also allows leakage of protons, although only Cathepsin B is detected by NLRP3 (Figure 4.9C).

YTc promotes the intracellular survival of *Yersinia pestis*

Intracellular survival of a pathogen requires a mechanism to resist the anti-microbial arsenal of the lysosome. Pathogens accomplish this in numerous ways: escaping the phagosome, disrupting fusion of the phagosome with the lysosome, or detoxifying the compartment through remodeling or with specific inhibitors. YCVs rapidly gain lysosomal markers; however, these compartments are not acidified and *Yersinia* are able to persist inside the vacuole (23, 226). Together, these observations suggest *Yersinia* has an uncharacterized mechanism to detoxify the phagolysosome. Disruption of YCV integrity and dilution of lysosomal contents by activity of the YTc (Figure 4.10, 4.11A) provides a possible mechanism for *Yersinia* resistance to lysosomal

factors. Thus, we hypothesized that YTc disruption of the YCV may contribute to the intracellular survival of *Y. pestis*.

Y. pestis were added to macrophages at an MOI of 2, and after a 30 minute incubation to allow adherence and internalization, extracellular bacteria were killed by the addition of gentamicin. Since induction of pyroptosis allows gentamicin entry into the macrophage, killing internalized bacteria, we utilized *caspase-1*$^{-/-}$ macrophages for these experiments. Two hours post infection, *Y. pestis* replicated to equal levels independently of YTc expression (Figure 4.11A). However, as the infection progresses *Y. pestis* lacking YTc decline in number while YTc^{+} *Y. pestis* persist (Figure 4.12A). Thus, YTc disruption of the YCV membrane appears to potentiate the intracellular survival of *Y. pestis*.

We next examined the contribution of individual lysosomal factors to the killing of intracellular *Yersinia*. Pharmacological inhibition of acidification with Bafilomycin A, but not neutralization of Cathepsin B with Ca-074Me or ROS with APDC, restored the intracellular growth of *Yp*ΔYopsΔYTc (Figure 4.12B). The combination of all three drugs was lethal to cells, explaining the lack replication under this treatment. Together, these data indicate that *Yersinia* are vulnerable to YCV acidification, which can be partially mitigated by YTc-mediated disruption of the YCV.

Discussion

Detection of pore formation by bacterial secretion systems or toxins is a very effective measure of pathogen invasion for the triggering of inflammatory immune defenses, evidenced by the protection conferred by NLRP3 against numerous pathogens. YTc pore formation is the

major trigger of pyroptosis during infection with *Y. pestis*, though this is delayed or negated *in vivo* through the combined actions of YopK and YopM (148). YTc only induces pyroptosis in activated macrophages, consistent with previous reports for NLRP3 agonists. As macrophages only become activated late during infection, macrophages likely cannot initially recognize YTc as a PAMP. However, *Y. pestis* has lost the ability to express flagellin and hexa-acylated LPS, two very potent PAMPs present in it's ancestor *Y. pseudotuberculosis*, suggesting there remains selection against PAMPs despite the ability of Yersinia to inhibit the anti-inflammation consequences of detection through the use of effectors. Thus, the possible liability from detection and activation of caspase-1 indicates YTc likely has an important contribution to the virulence of *Y. pestis*.

During infection, YTc disruption of YCV causes leakage of Cathepsin B which activates caspase-1, but is accompanied with efflux of antimicrobial compounds from the YCV, benefiting intracellular survival (Figure 4.13). This is consistent with prior observations that *Yersinia* are contained within phagosomes to which lysosomes fuse but there is no acidification (21-23). This mechanism is similar to that of toxins that form phagolysosomal pores, disrupting acidification of the vacuole or allow bacterial escape (227), at the expense of inducing caspase-1 activation (80).

Our data further indicate that YTc is not secreted extracellular during infection, but rather is delivered directly into the macrophage cytosol in a T3S-dependent manner. Treatment with toxin-neutralizing antibodies blocks caspase-1 activation by purified YTc added as an exotoxin, but not toxin produced during *Yersinia* infection. Furthermore, inhibition of toxin endocytosis is not protective during infection, and mutation of the T3S abrogates toxin

internalization and detection. Together, these data strongly support the previous report (164) that the pCD-encoded T3S is critical in secretion of the YTc.

Yersinia infection is characterized by biphasic infection where an inflammatory burst is preceded by an anti-inflammatory phase. Identification of YTc as a factor that activates caspase-1 builds on other recent work detailing how *Yersinia* greatly delays or negates caspase-1 activation *in vivo* through the combined actions of YopK and YopM (Chapter 3 and reference (148)). There appears to be a delicate balance to YTc expression; it promotes intracellular survival and likely has other pro-virulence activities dependent on it's enzymatic subunits, but it also stimulates an inflammatory, anti-microbial response detrimental to *Yersinia* survival.

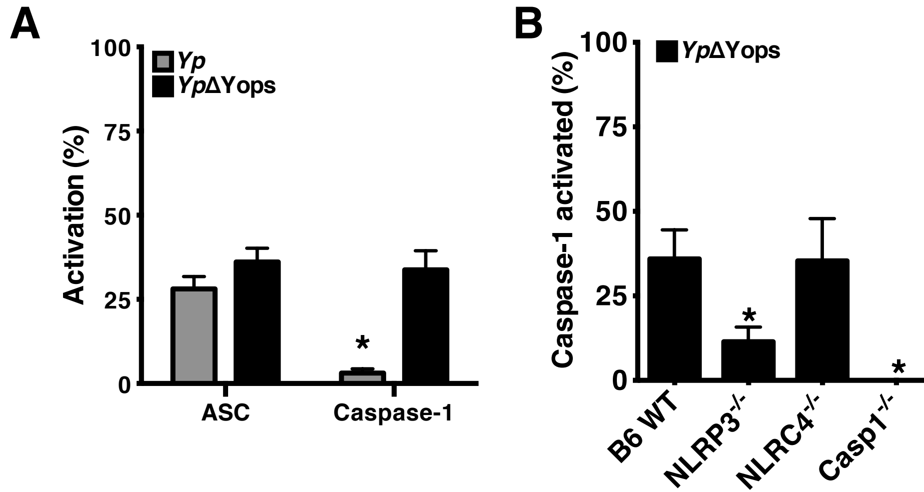

Figure 4.1. NLRP3 is important in macrophage detection of *Y. pestis*.
Macrophages derived from the bone marrow of C57Bl/6, NLRP3$^{-/-}$, NLRC4$^{-/-}$, or Casp1$^{-/-}$ mice were stimulated overnight with LPS then infected with the *Y. pestis* strains indicated at an MOI of 10. (**A**) Cells containing foci of caspase-1 or ASC, or (**B**) active caspase-1 were counted three hours post-infection. *, $p<0.05$

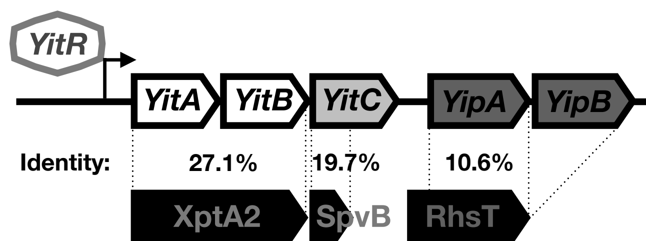

Figure 4.2. The Yersinia toxin complex has homology with toxins that activate caspase-1
Regions of homology of YTc constituent proteins with known caspase-1 activators (RhsT), known pore-forming toxins (XptA2), and virulence factors associated with inflammation and cell death (SpvB).

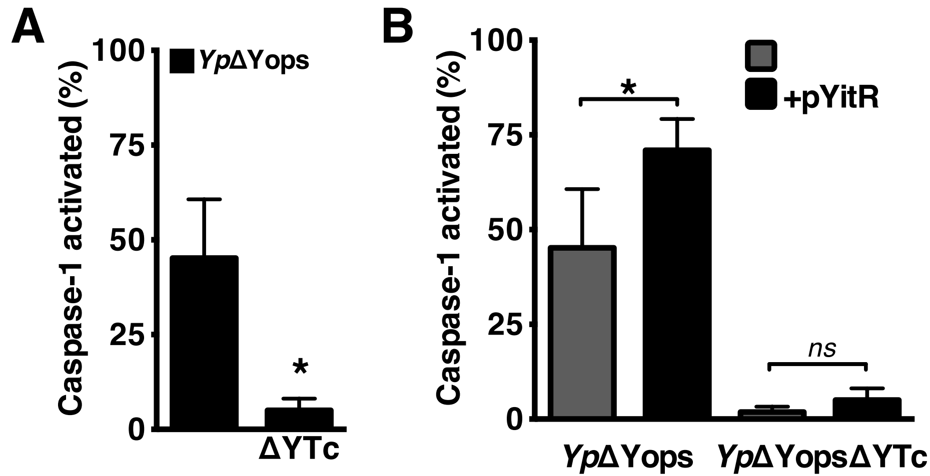

Figure 4.3. YTc is detected as a PAMP for the activation of caspase-1.
Macrophages from C57Bl/6 mice were stimulated overnight with LPS, then infected with the *Y. pestis* strains indicated for 3 hrs. (**A**) *Y. pestis* mutant for YTc induce less caspase-1, indicating YTc is detected as a PAMP. (**B**) *Y. pestis* hyper-expressing YitR, a transcriptional activator of the YTc operon, induce greater caspase-1 activation, while YitR has no effect in bacteria mutant for YTc. *, $p<0.05$

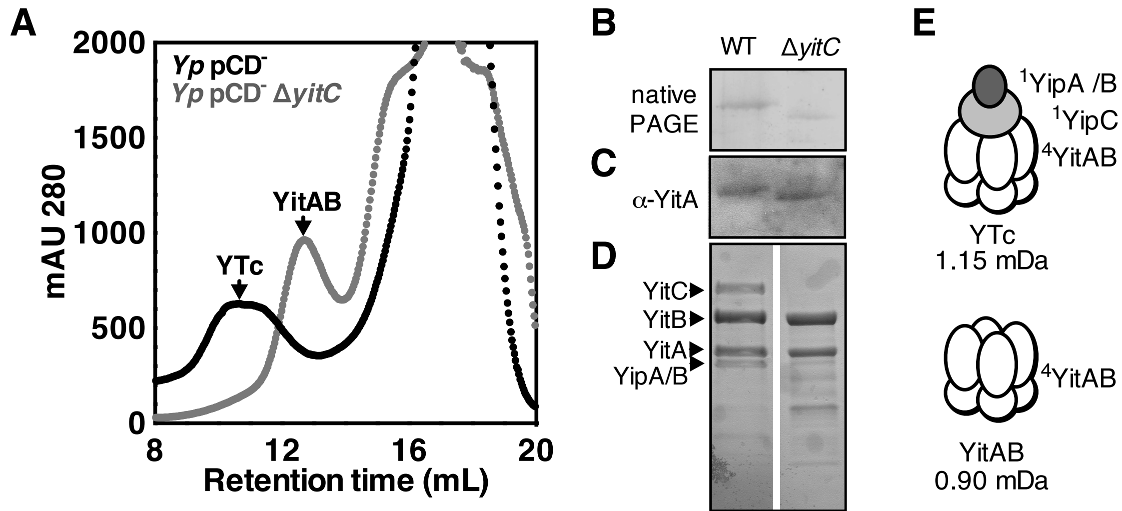

Figure 4.4. YTc forms a macromolecular complex composed of YitA, YitB, YitC and YipA or YipB.

(**A**) Resolution of high molecular weight complexes from WT or Δ*yitC Yersinia pestis* by gel filtration. The shift of the longer retention time in Δ*yitC* corresponds to a smaller complex sized YitAB complex relative to native YTc. (**B**) Native PAGE of the gel filtration elution peaks indicated in (A) confirms the presence of a stabile high molecular weight complex. (**C**) Immunoblot analysis of this native PAGE confirms the presence of Yit proteins in this complex. (**D**) Denaturing gel electrophoresis of the YTc and YitAB complexes shows protein sizes corresponding to known YTc complex components and an absence of additional proteins (**E**) Densitometry with correction for protein size indicates the YTc has a 4:4:1:1 subunit stoichiometry for YitA:YitB:YitC:YipA/B, for a total complex weight of approximately 1.15mDa.

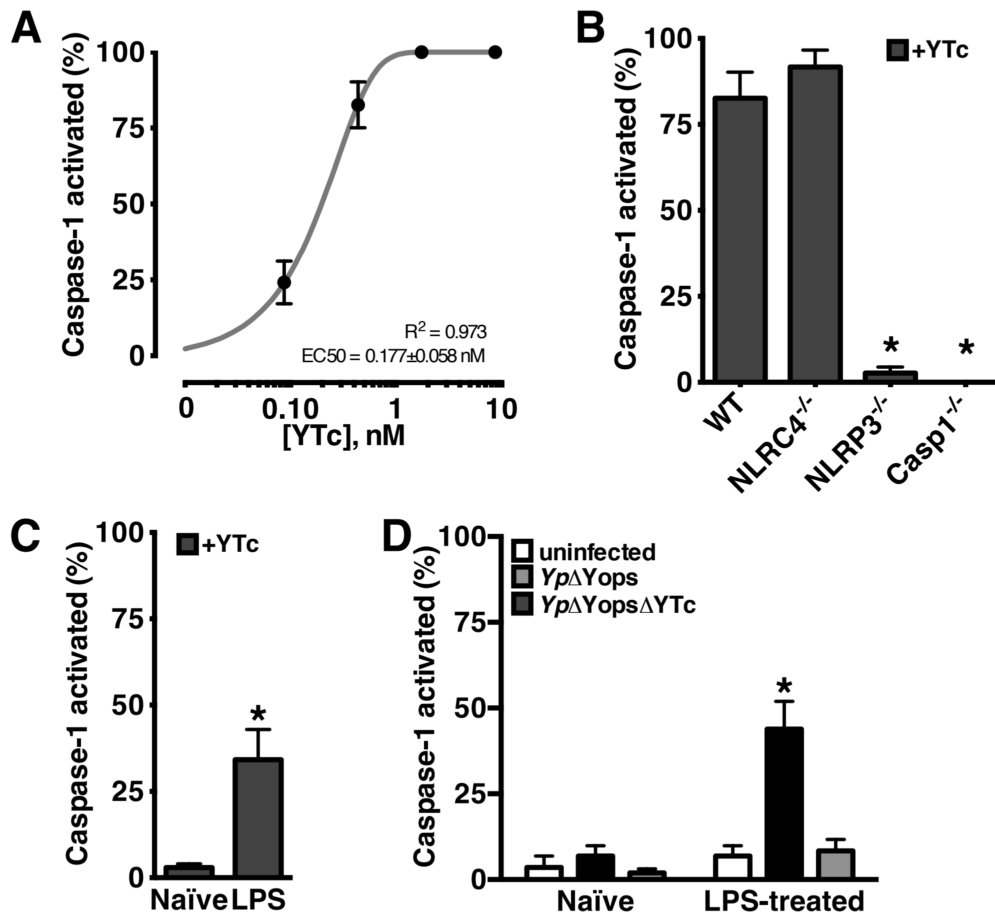

Figure 4.5. YTc activation of caspase-1 requires NLRP3 and a TLR co-signal.
(**A**) Purified YTc is sufficient to activate caspase-1 when added to LPS-primed macrophages. (**B**) NLRP3 is required for activation of caspase-1 in LPS-primed macrophages in response to treatment with 10 nM purified YTc for two hours. (**C**) Naïve or LPS-primed macrophages were treated with 10 nM endotoxin-free YTc for two hours and fraction of caspase-1 activated cells counted (**D**) LPS-pretreatment greatly increase caspase-1 activation in response to YTc^+ *Y. pestis* but not YTc-deficient *Y. pestis* (*YpΔYopsΔYTc*). *, $p<0.05$

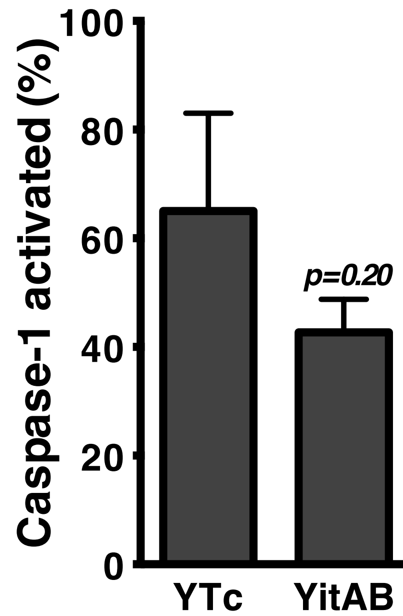

Figure 4.6. The pore forming YTc subunits YitAB are sufficient to activate caspase-1. LPS-primed macrophages activate similar levels of caspase-1 whether treated with 10 nM of the full YTc complex or to the YitAB complex lacking the components YitC, YipA, and YipB. The difference in activation between YTc and YitAB complexes was not statistically significant by T-test (p=0.20).

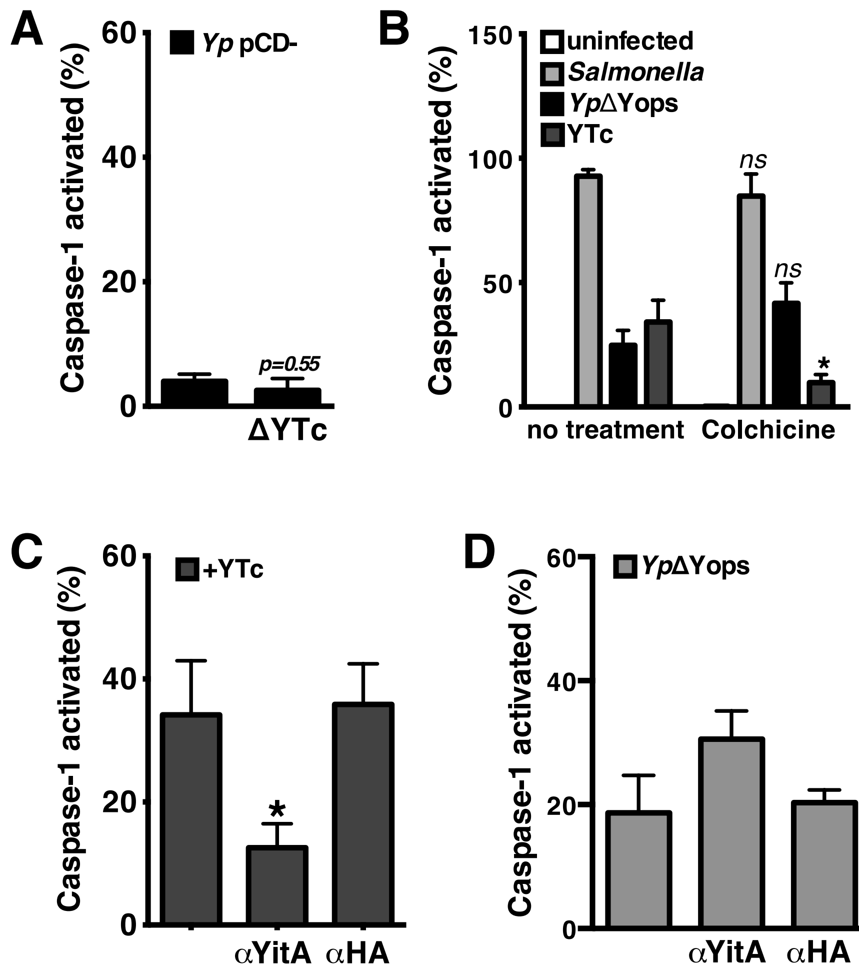

Figure 4.7. YTc detection requires type III secretion.
(**A**) LPS-primed macrophages infected with *Y. pestis* lacking the pCD virulence plasmid activate little caspase-1, regardless of YTc expression. (**B**) Colchicine inhibits caspase-1 activation in macrophages treated with purified YTc, indicating endocytosis is required for toxicity. Colchicine does not inhibit *Y. pestis* activation of caspase-1, indicating YTc is not normally trafficked by an endocytic pathway during infection. (**C**) Antibody against the YitA subunit of YTc blocks caspase-1 activation by YTc, suggesting the antibody acts as an antitoxin. (**D**) YitA antibody does not reduce caspase-1 activation during infection with YTc-expressing *Y. pestis* (*Yp*Δ*Yops*), indicating YitA is not accessible and unlikely to act as a exotoxin, but rather, a T3S substrate.

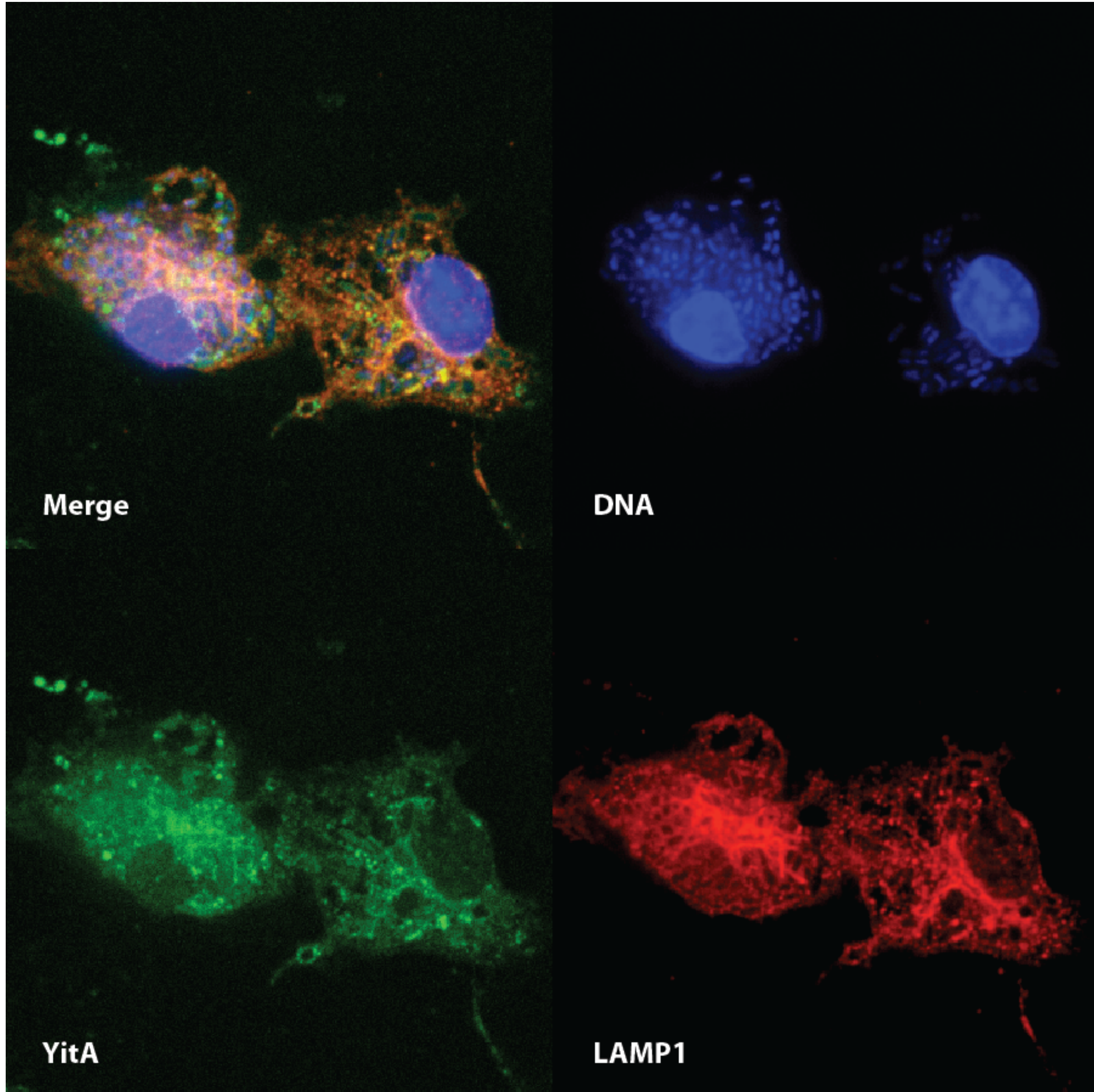

Figure 4.8. YTc colocalizes with the *Yersinia*-containing vacuole.
Macrophages were infected with *Yp*Δ*Yops* at an MOI of 20. 60 minutes post-infection, cells were washed, fixed, permeabilized, and stained for the YitA subunit of the YTc (green), for LAMP1 (red) as a marker of lysosomes, some endosomes, and the *Yersinia*-containing vacuole, and DNA with Hoescht dye (blue).

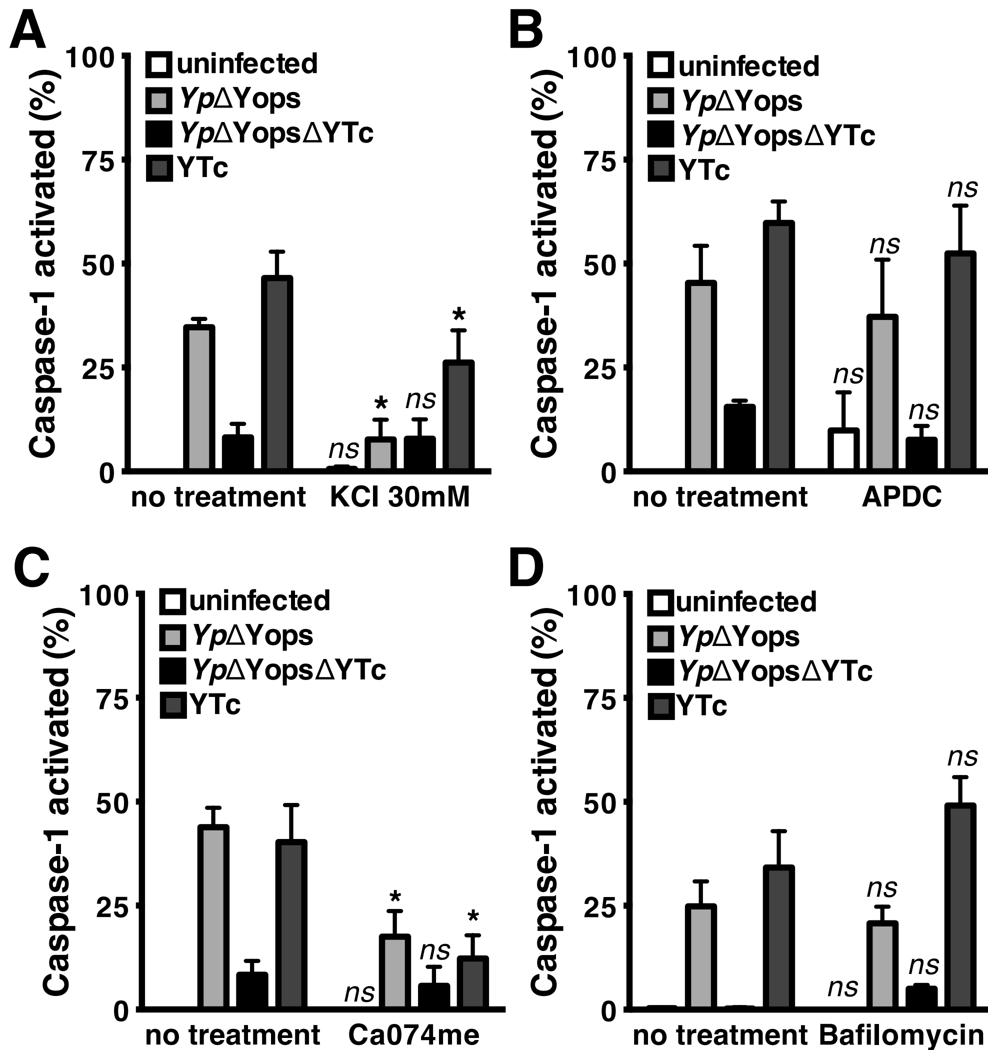

Figure 4.9. Potassium efflux and Cathepsin B are required for YTc activation of caspase-1. Macrophages were treated with the indicated inhibitors (KCl, APDC, Ca074me, or Bafilomycin) and infected or treated with purified YTc. (A) 30mM KCl inhibits caspase-1 activation in macrophages infected with *YpΔYops* or treated with purified YTc (B) APDC, an inhibitor of NADPH-oxidase dependent reactive oxygen species generation, does not significantly alter YTc-dependent capsase-1 activation. (C) Ca074-Me, a specific inhibitor of Cathepsin B, reduces caspase-1 activation by YTc during infection or upon treatment. (D) Bafilomycin, an inhibitor of the proton ATPase required for vacuole acidification, does not inhibit YTc activation of caspase-1.

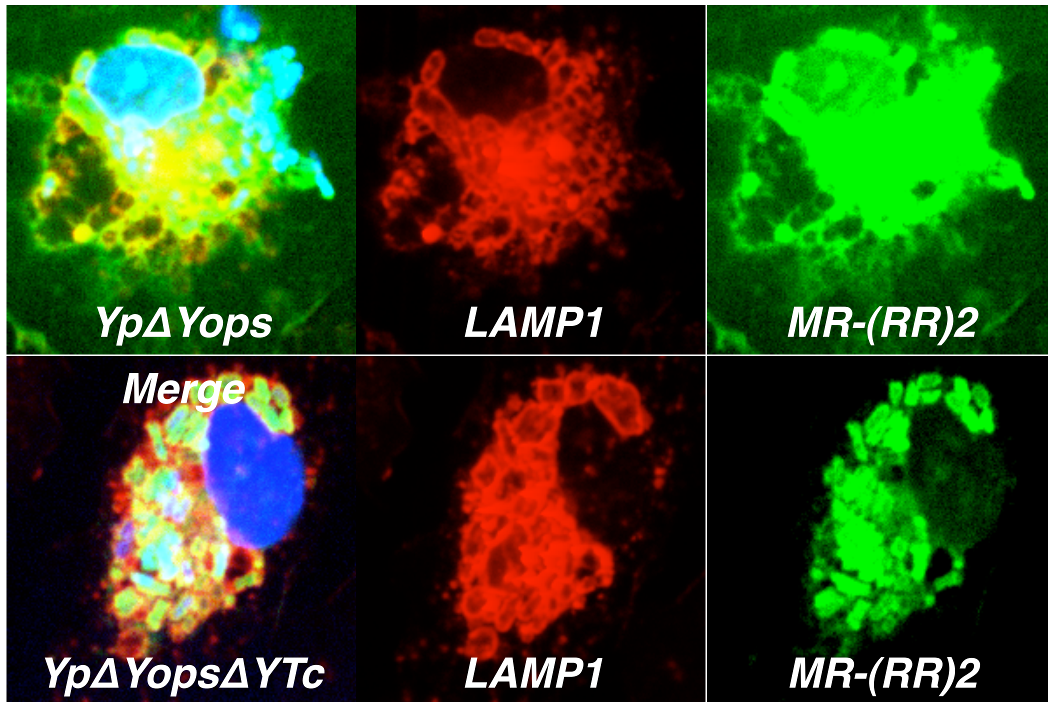

Figure 4.10. YTc induces leakage of the *Yersinia*-containing vacuole.
Macrophages were infected with *Yp*Δ*Yops* or *Yp*Δ*Yops*Δ*YTc* at an MOI of 10. 90 minutes post-infection, MR-$(RR)_2$, a substrate reporter of active Cathepsin B, was added. Two hours post-infection, cells were washed, fix, permeabilized, and stained for LAMP1, marking the *Yersinia*-containing vacuole, from which MR-$(RR)_2$ leaks in the presence of YTc.

Figure 4.11. YTc contributes to preventing acidification of the *Yersinia*-containing vacuole. (A) Macrophages were infected with *Yp*Δ*Yops* or *Yp*Δ*Yops*Δ*YTc* at an MOI of 10. 1 hour post-infection, lysotracker red was added to the cells. 2 hours post-infection, cell were washed, fixed and analyzed by immunofluorescence microscopy. (B) Enumeration of the number of *Yersinia* cells colocalized with lysotracker.

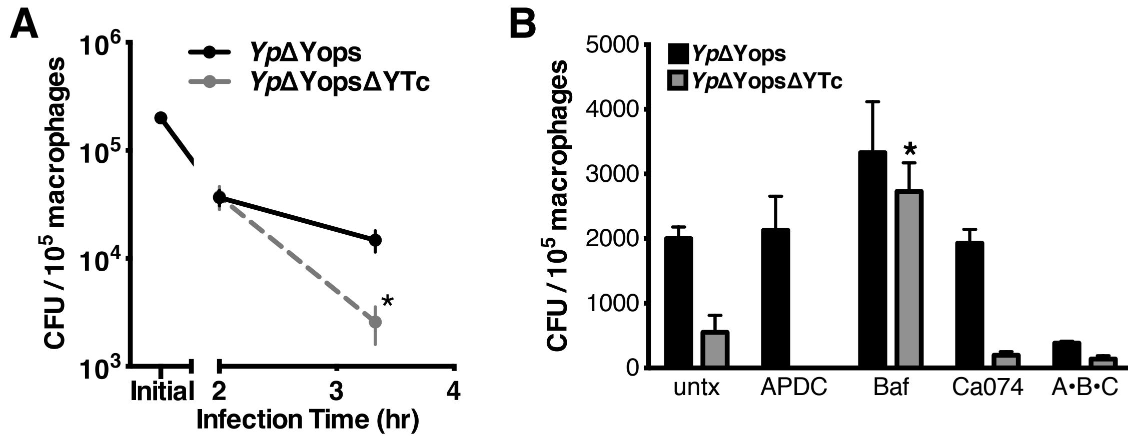

Figure 4.12. *Yersinia*-containing vacuole disruption by YTc promotes intracellular survival. (**A**) Caspase-1$^{-/-}$ macrophages were infected with the *Y. pestis* strains indicated at multiplicity of infection of two. One hour post-infection, the cell culture medium was replaced with media containing gentamicin to kill extracellular bacteria. At the indicated time points, macrophages were lysed and bacterial CFU enumerated. (**B**) Intracellular *Y. pestis* was enumerated four hours post-infection of macrophages pre-treated with APDC (ROS inhibitor), Bafilomycin (Baf; vacuolar acidification inhibitor), Ca074me (lysosomal protease inhibitor), or all three drugs (ABC).

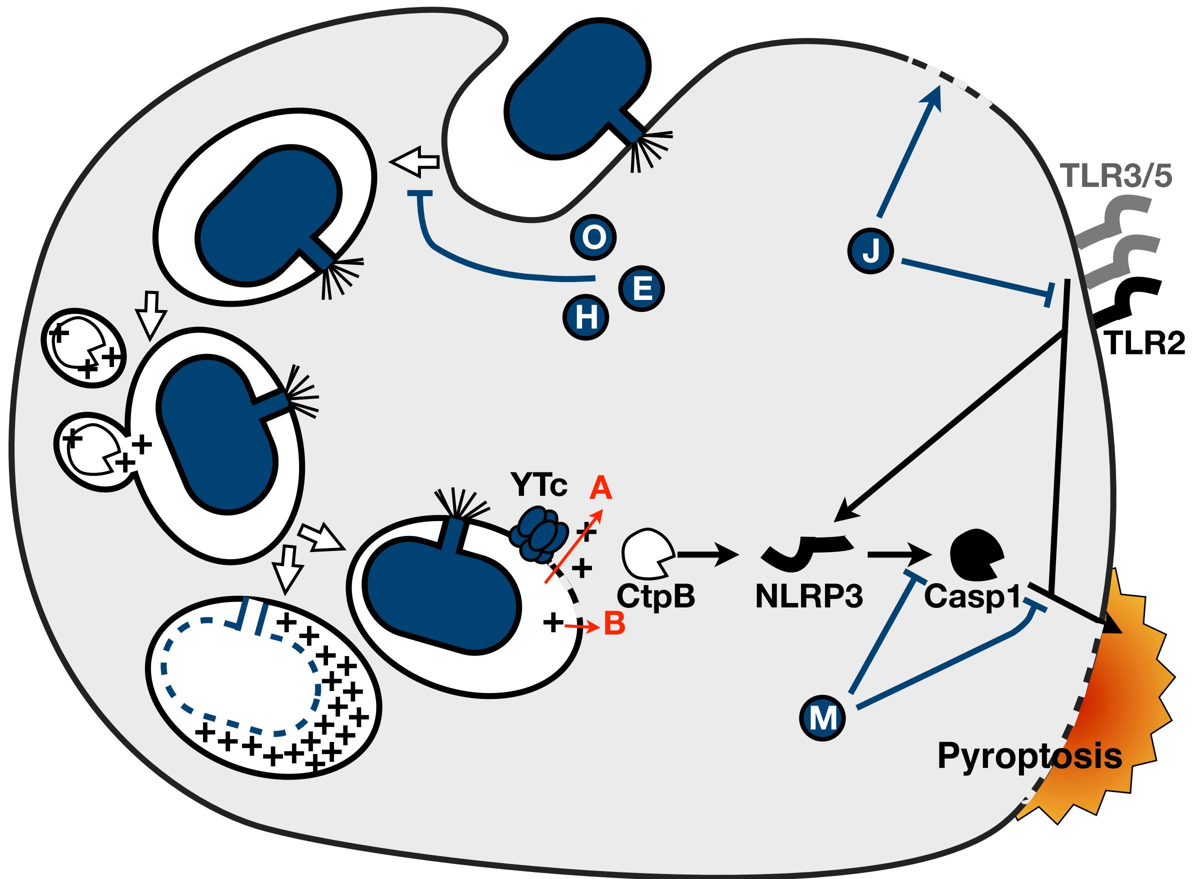

Figure 4.13. Model of YTc function in macrophages.
YTc disrupts the proton gradient (+) which protects *Y. pestis* from acidification of the YCV (A), but at the expense of Cathepsin B (CtpB) leakage (B), which is detected by NLRP3 to activate caspase-1 (Casp1). However, pyroptosis does not often occur as YopM (M) blocks the activation and activity of caspase-1 (Chapter 2) and YopJ can kill cells by apoptosis first (46).

Chapter 5: Perspectives and Future Directions

The goal of this study was to identify the factors involved in the activation of caspase-1 during *Yersinia* infection. This work identified both an inhibitor of caspase-1, YopM, and an activator, the *Yersinia* toxin complex. Direct inhibition of caspase-1 had not been previously described for a bacterium, and the unusual mechanism by which this occurs has intriguing parallels with viral inhibitors and broadens our understanding of caspase-1 regulation. Identification of this inhibitor allowed a follow-up study which identified one of the major PAMPs activating caspase-1: the *Yersinia* toxin complex. The mechanism by which the YTc is detected led to a clear demonstration that this toxin, with no previously known phenotype, promotes the intracellular survival of *Yersinia.* With the identification of new roles in virulence for these two enigmatic factors, several unresolved questions remain. This chapter will put these findings in a broader context with the goal of directing future studies.

Identification of caspase-1 inhibition by YopM (Chapter 3) brings to light a required function of the protein; however, YopM binds several other host proteins and may have multiple activities in cells. Though these binding partners are not required for YopM to bind and inhibit caspase-1 (Figure 3.8), they may coordinate or otherwise impact the binding and inhibition of caspase-1 *in vivo*. Caspase-11, an activator of caspase-1 that has similar substrate affinity (211), is bound by the endogenous pseudosubstrate inhibitor protein Flightless-1 (118). Since YopM and Flightless-1 have similar structures and pseudosubstrate sites (Figure 3.6a), YopM might also be able to bind caspase-11. Additionally, Flightless-1 binds actin and several other proteins (228), possibly explaining how it is able to spatially regulate caspase-11 in the cell (118). Thus one

possibility is that YopM binding of Rsk1 and Prk2 (47, 48, 52) helps coordinate the subcellular sequestration of caspase-1. Another possibility, since YopM activates and possibly alters the substrates of the kinases Rsk1 and Prk2 (52), is that YopM induces a modification of caspase-1. In an intriguing parallel, Rsk2 can phosphorylate caspase-8, leading to it's ubiquitination and degradation (229). Caspase-1 has not previously been observed to be modified, though several other inflammasome components can be regulated by phosphorylation, ubiquitination, or cleavage (230-233).

YopM may also coordinate with other bacterial factors to prevent caspase-1 activation (Figure 5.1). As described earlier (Figure 3.1B), YopK limits PAMP delivery, which delays bacterial detection by NLRs (148, 149). *Y. pestis* has also lost several likely PAMPs present in *Y. pseudotuberculosis*, including flagellin, which can both prime cells through TLR5 and activate caspase-1 through NLRC4 (10). Previous results have shown that flagellin is not necessary for *Y. pseudotuberculosis* to activate caspase-1 (148), likely because YTc is present to activate caspase-1 through NLRP3 (Chapter 4). Infection of NLRP3$^{-/-}$ macrophages, which are unable to activate caspase-1 in response to YTc (Figure 4.1B, 4.5B), shows that *Y. pseudotuberculosis* flagellin is sufficient to activate caspase-1 in an NLRC4-dependent manner (Figure 5.2). The lack of flagellin expression by *Y. pestis* is consistent with the small contribution of NLRC4 for caspase-1 activation in response to this pathogen (Figure 4.1B), and supportive of the model presented in Figure 5.1. There are likely additional, unknown PAMPs that activate the inflammasome. These factors might be identified screening for PAMPs in bacteria mutant for the PAMPs identified here, YTc and flagellin.

The ability of macrophages to be primed by *Yersinia* is likely another factor influencing caspase-1 activation; priming of NF-kB, such as with LPS, greatly potentiates activation of caspase-1 by NLRP3 (234) and AIM2 (77, 79), and macrophages positive for active caspase-1 *in vivo* are typically activated (46). Inhibition of MAPKs and NF-kB activation by YopJ (40-43) likely antagonizes these inflammasomes; thus formation of the NLRP3 inflammasome in response to YTc (Figure 4.5) could be impacted by YopJ as well as YopM (Figure 5.1). NF-kB stimulation might then be less necessary to activate caspase-1 in response to *Y. pseudotuberculosis*, which can occur independently of NLRP3 through activation of the NLRP4 inflammasome by flagellin (Figure 5.2), which does not require priming of the cell (72, 73).

Since *Y. pestis* primarily activates caspase-1 through NLRP3, the activation state of the cell is likely critical for induction of pyroptosis in response to this pathogen. The evolution of the LPS of *Y. pestis* might then also effect caspase-1 activation, since the LPS of *Y. pestis* grown at 37°C is poorly detected by human TLR4, though detected well by murine TLR4 (147, 235). However, macrophages *in vivo* are in a broad spectrum of activation states pre-infection and the requirement of a co-signal to activate the NLRP3 inflammasome appears to be relieved in many circumstances (46, 220). Additionally, signaling though numerous receptors other than TLR4 can prime for pyroptosis, including other TLRs, NOD receptors and Type I IFN, TNF, and IL-1 signals (46, 143, 236-238). Identification of which of these pathways is relevant to *Yersinia* infection remains an important question to be answered.

Identification of YTc as a PAMP (Chapter 4) also raises important questions as to its role in virulence. YTc is recognized in a manner analogous with other pore-forming toxin PAMPs, requiring NLRP3 (Figure 4.5c, 4.5d), cell priming (Figure 4.5c), K^+ efflux (Figure 4.9a), and

Cathepsin B (Figure 4.9c). *Y. pestis* has eliminated numerous PAMPs (LPS, flagellin) and has several mechanisms for antagonizing inflammation (YopJ, YopK, YopM), so it is striking that it would maintain this potent PAMP, YTc, if it were not important for virulence in some manner. Indeed, YTc promotes the intracellular survival of *Y. pestis* (Figure 4.11), like several other toxins that disturb the bacterial phagosome and consequently activate caspase-1 (80, 133, 180-182, 227). This observation may explain, at least in part, observations that the *Yersinia*-containing vacuole fails to acidify (21-23). However, the contribution of intracellular bacteria to the pathogenesis of *Yersinia* remains unclear. *In vivo*, intracellular *Yersinia* are rarely observed (19) and <5% are protected in a "whole-animal" gentamicin protection assay that eliminates extracellular bacteria (20). Nonetheless, the ability of *Yersinia* to replicate intracellularly suggests that this small population either contributes to virulence, or that current infection models have not captured all of the nuances that may be necessary for *Yersinia* virulence.

YTc likely has activities in addition to disruption of the YCV. Pore formation for many toxins is simply to create a channel for delivery of a toxin's enzymatic subunit across a cellular membrane, and the activities of the enzymatic subunits of the YTc, YipA and YipB, remain to be identified. The enzymatic subunits of the *Photorhabdus*, *Xenorhabdus,* and *Yersinia entomophaga* toxin complexes have glycoside hydrolase activity against chitin (200) or ADP-ribosylase activity targeting RhoGTPases to disrupt to actin cytoskeleton (239). Conversely, YipA and YipB appear to be protein tyrosine phosphatases, though this activity has not been demonstrated and the putative cellular targets remain to be identified (164). A high-throughput yeast two-hybrid screen (240) has identified interactions of the Yip proteins with the transcription factor NF-kB, SMAP2, a possible regulator of retrograde transport of endosomes, and TGS1, a

methyltransferase involved in transcriptional regulation. It remains unclear whether any of these proteins are relevant targets during *Yersinia* infection, if they are modified by the Yips, and if this activity contributes to bacterial virulence.

In summary, *Yersinia* is able to directly subvert the caspase-1 component of the inflammatory immune response, utilizing the effector YopM. The *Yersinia* toxin complex allows intracellular survival, at the expense of activating caspase-1. The involvement of these two factors in virulence has long been uncertain, and the future studies proposed will further build on their mechanisms. Additionally, the roles of intracellular survival and caspase-1 activation during *Yersinia* infection have been unclear, and identification of YopM and YTc as factors in these processes will allow the discovery of additional, cooperative factors.

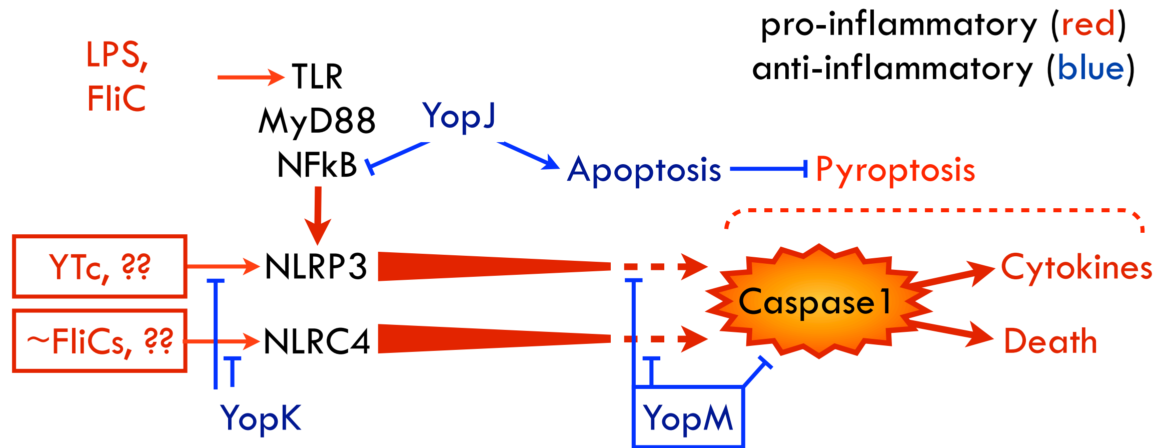

Figure 5.1. Factors involved in the activation of caspase-1 during *Yersinia* infection
Both NLRP3 and NLRC4 can activate caspase-1 during *Yersinia* infection, NLRP3 detecting YTc, NLRC4 detecting FliC (pro-inflammatory pathways in red). Either sensor, or possibly a third sensor, may detect additional PAMPs that remain to be identified. *Yersinia* antagonize this with several Yop proteins (blue): YopM, which directly inhibits caspase-1, YopK, which limits PAMP delivery, and YopJ, which inhibits cellular activation and induces death by apoptosis, preempting pyroptosis.

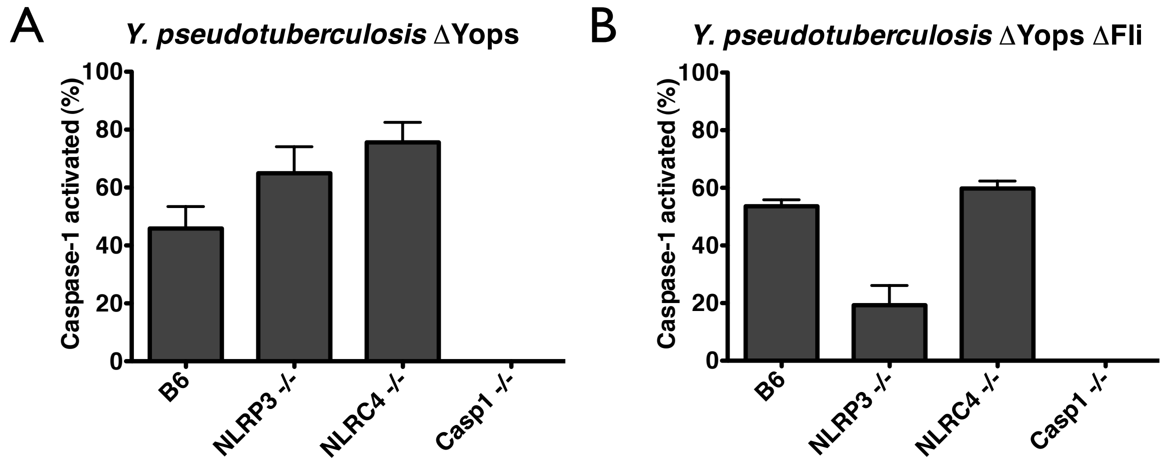

Figure 5.2. Flagellin can activate the NLRC4 inflammasome during *Y. pseudotuberculosis* infection

Macrophages from wildtype C57Bl6 (B6), NLRP3$^{-/-}$, NLRC4$^{-/-}$, or Caspase-1$^{-/-}$ mice were primed with LPS and infected with (**A**) *Y. pseudotuberculosis* Δ*Yops*, unable to inhibit caspase-1 activation, or (**B**) *Y. pseudotuberculosis* Δ*Yops* Δ*FliC*, unable to inhibit caspase-1 and further lacking flagellin. There is a reduction in NLRP3-dependent caspase-1 activation during infection with *Y. pseudotuberculosis* Δ*Yops* Δ*FliC*, indicating that FliC is detected to activate NLRC4, which is masked by and redundant with activation of NLRP3.

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EDUCATION

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PROFESSIONAL EXPERIENCE

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Research Associate — 2004-2006
University of Michigan Department of Microbiology & Immunology, Ann Arbor, MI
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Undergraduate Researcher — 2003-2004
Diagnostic Center for Population and Animal Health, East Lansing, MI
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Undergraduate Technician — 2002-2004
Michigan State University Genomics Technology Support Facility, East Lansing, MI

ACADEMIC AWARDS AND HONORS

Fellowship, UW Bacterial Pathogenesis Training Program (NRSA T32-AI055396) — 2009-2011

Associate Member, Sigma Xi Scientific Research Society — 2010

Poster Award, UW Department of Microbiology — 2010

PROFESSIONAL AFFILIATIONS & SERVICE

Expert Scientist, *www.askabiologist.org.uk* (K-12 web education program) — 2010-*present*

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Student President, UW Department of Microbiology — 2011-2012

Student Faculty Representative, UW Department of Microbiology — 2010-2011

Department Retreat Coordinator, UW Department of Microbiology — 2008-2009

PUBLICATIONS

LaRock CN, LaRock DL, Miller SI, Collins CM, Cookson BT. Pore formation by translocated *Yersinia* toxin complex activates the NLRP3 inflammasome. [*In preparation*]

LaRock CN*, Yu J*, Horswill AR, Parsek MR, Minion FC. *Yersinia pestis* uses AHL-based quorum sensing to regulate metabolic functions [*Submitted*] (*equal contribution)

LaRock CN, Cookson BT. (2012) A bacterial virulence protein arrests inflammasome assembly and processing. ***Cell Host and Microbe*** 12: (*In press*)

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Held KG, **LaRock CN**, D'Argenio DA, Berg CA, Collins CM. (2007) A metalloprotease secreted by the insect pathogen *Photorhabdus luminescens* induces melanization. ***Applied and Environmental Microbiology*** 73: 7622-8.

TEACHING EXPERIENCE

Guest Lecturer, Medical Bacteriology (Micro 442), University of Washington 2010

Guest Lecturer, Gene Action (Micro 411), University of Washington 2008

Teaching Assistant, Gene Action (Micro 411), University of Washington 2008

Teaching Assistant, Intro. Microbiology (Micro 302), University of Washington 2007

MENTORING EXPERIENCE

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Mentor, Rotation PhD Student Merika Treants, University of Washington 2011

Mentor, Undergraduate Allison Bray, University of Washington 2011

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Gordon Research Seminar on Microbial Toxins & Pathogenicity 2012
Waterville Valley Resort, NH. [*Speaker & Discussion Leader*]

Seattle Area Microbial Pathogenesis Work-in-Progress Meeting 2011
University of Washington, Seattle, WA.

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ABSTRACTS

Gordon Research Conference on Microbial Toxins & Pathogenicity 2012
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CSHL Symposium on Microbial Pathogenesis & Host Response 2011
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University of Washington Department of Microbiology Retreat 2011
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University of Washington Department of Microbiology Retreat 2010
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Michigan Branch of American Society for Microbiology 2004
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