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The N-Terminus Of Type III Secretion Needle Protein YscF From Yersinia Pestis Functions To Modulate Innate Immune Responses

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THE N-TERMINUS OF TYPE III SECRETION NEEDLE PROTEIN YSCF FROM *YERSINIA PESTIS* FUNCTIONS TO MODULATE INNATE IMMUNE RESPONSES.

By

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This dissertation, submitted by Patrick Osei-Owusu in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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ABSTRACT

The type III secretion system is employed by many pathogens, including the genera *Yersinia, Shigella, Pseudomonas*, and *Salmonella*, to deliver effector proteins into eukaryotic cells. The injectisome needle is formed by the polymerization of a single protein, e.g., YscF (*Yersinia pestis*), PscF (*Pseudomonas aeruginosa*), PrgI (*Salmonella enterica* SPI-1), SsaG (*Salmonella enterica* SPI-2), or MxiH (*Shigella flexneri*). In this study, we demonstrated that the N termini of some needle proteins, particularly the N terminus of YscF from *Yersinia pestis*, influences host immune responses. The N termini of several needle proteins were truncated and tested for the ability to induce inflammatory responses in a human monocytic cell line (THP-1 cells). Truncated needle proteins induced proinflammatory cytokines to different magnitudes than the corresponding wild-type proteins, except SsaG. Notably, N-terminally truncated YscF induced significantly higher activation of NF-κB and/or AP-1 and higher induction of proinflammatory cytokines, suggesting that a function of the N terminus of YscF is interference with host sensing of YscF, consistent with *Y. pestis* pathogenesis. To directly test the ability of the N terminus of YscF to suppress cytokine induction, a YscF-SsaG chimera with 15 N-terminal amino acids from YscF added to SsaG was constructed. The chimeric YscF-SsaG induced lower levels of cytokines than wild-type SsaG. However, the addition of 15 random amino acids to SsaG had no effect on NF-κB/AP-1 activation. These results suggest that the N terminus of YscF can function to decrease cytokine induction, perhaps contributing to a favorable immune environment leading to survival of *Y. pestis* within the eukaryotic host.

CHAPTER I

INTRODUCTION

History of Plague

The biblical account of an outbreak of a disease with buboes is possibly the oldest report of plague in human history (I Samuel V 9-12) (73). Other important accounts of possible plague epidemics are the plague of Athens in 430 BC and Antonine plague of 165-180 AD that prevailed at the beginning of the Roman Empire under the Antonine emperors (32, 73). There are doubts however in recent literature about these historical epidemics of plague as the term "plague" which was used to describe the diseases at that time referred to nonspecific diseases (107). Three confirmed major plague pandemics have been recorded worldwide (24, 95, 107).

The first pandemic, the "Plague of Justinian" that was named after the Roman Emperor during the time, occurred in the 6th century AD (24, 32, 42, 62, 73, 95, 101, 116). The Justinian Plague spread from either central Asia or Africa across the Mediterranean basin into Europe, from AD 532 until AD 595 and is said to have

caused the death of an estimated 100 million people contributing to the end of the Roman Empire (24, 63, 73, 95, 107). This pandemic was followed by several other epidemics in the following two centuries with an estimated mortality of 15-40% (24, 63, 73, 95, 107). Although the causative agent of this pandemic was long debated, sequencing of *Yersinia pestis*-specific genomic regions from skeletal material of individuals buried at that time has confirmed that the pandemic was caused by *Yersinia pestis* (107).

In contrast to the first pandemic, the second pandemic, called the Black Death, is well documented (62,101). This second pandemic, which is known to have introduced a lot of changes in society (economics, politics, and science), was introduced into Europe in the year AD 1347 and remained active until the year AD 1352 (24, 32, 45, 73, 95, 101, 107). As in the case of the Justinian Plague, a number of epidemics of different intensity followed the initial wave of the pandemic and continued into the early 18th century AD (73, 107). The Black Death encompassed the entire "known world" at that time and it is estimated to have killed 15 to 23.5 million Europeans, representing about one-fourth to one-third of the population at that time (24, 32, 62, 73, 95, 101).

The third and most recent pandemic started in AD 1885 in the Chinese province of Yunnan and quickly spread to the rest of the world reaching the United States by AD 1900 (24, 32, 62, 73, 95, 101, 107). Between 1898 and 1918, 12.5 million Indians alone were estimated to have died of plague (62, 73). This third pandemic led to the identification of the causative agent of plague by both

Shibasaburo Kitasato and Alexandre Yersin at about the same time (73). However, the bacterium was later named after Alexandre Yersin for his accurate and striking description of the bacterial characteristics (24, 73, 95, 101). Subsequently, through several investigations, the association between the plague of rats and human beings was established including the role of fleas in the transmission of the disease (24, 73, 107).

Epidemiology

Generally there has been a dramatic reduction in the number of plague cases recently compared to the past (24, 101). However, certain parts of Africa, India and China have reported a sharp rise in the number of human cases since the beginning of the 1990s (24, 45, 107). Although 90% of all reported human cases in recent years occur in Africa, specifically eastern Africa, central Africa and Madagascar, there have been reported epidemics in India and China as well (24, 45, 95,101,107). These reported human cases confirm the fact that despite the considerable reduction in the morbidity and mortality, plague has not been eradicated. With the exception of Australia, endemic foci continue to persist in all continents including Africa, North and South America, Europe and Asia (45, 62, 101). Human cases of plague cut across countries with different climates, altitudes and landscapes (24). For example, compared to Madagascar that is at a high altitude and has a temperate climate with sometimes high humidity and rich vegetation, the central Kazakhstan focus is largely a dry desert at sea level with very hot summers and extremely cold winters (24, 95). Climate variables such as temperature and seasonal precipitation

are known to be associated with frequency of human plague in the USA (11, 24). Similarly, it is reported that long-term human plague occurrence in China may be related to variation in sea surface temperature and oscillation (24).

From 1958 to 2008, the world health organization (WHO) reports that the number of confirmed and suspected human plague cases in all countries was 90,000 with 5,000 deaths. Madagascar reported 17,000 cases, 9,000 cases in Tanzania, 13,000 in Congo, 4,800 in India, 3,500 in Vietnam, 3,693 in Brazil, 4,001 in Peru and 438 in the United States. The current on-going epidemic in Madagascar is reported to have infected 224 people with 71 deaths. Human infections in the United States are rare, however, morbidity and death still occur. In 2006, 13 cases were reported in New Mexico, Colorado, California and Texas with 2 deaths. In spite of these relatively limited human cases, evidence of plague exposure in regions of the western USA in non-domestic rodents and carnivores is substantial (23, 62).

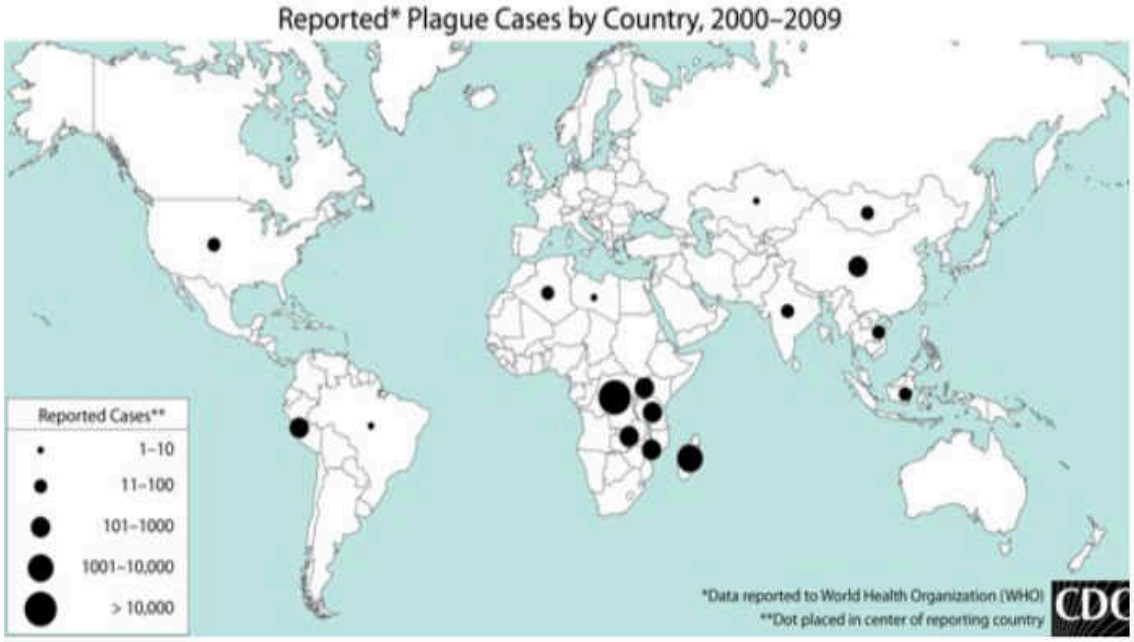

Figure1: Reported plague cases by country, 2000-2009

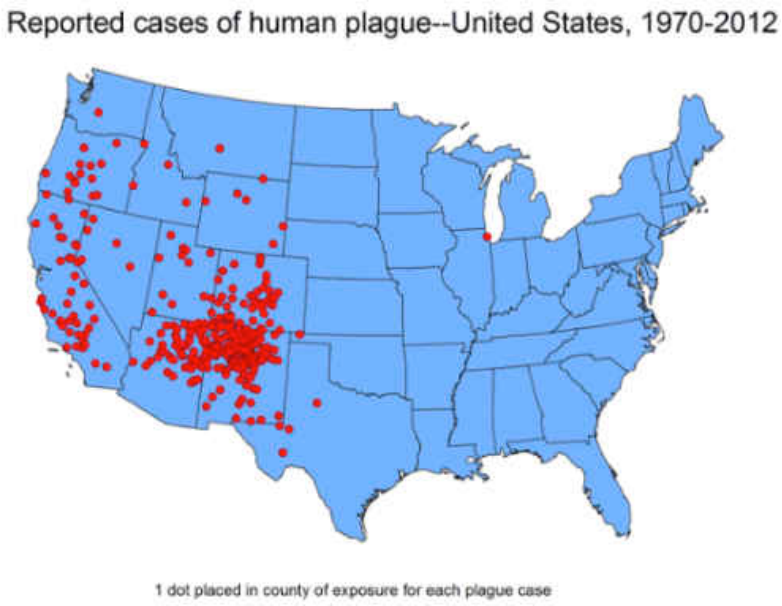

Figure 2: Reported cases of human plague – United States. 1970-2012.

Reservoir

Plague is a zoonosis maintained among rodents and their fleas (46, 45, 95, 107, 116). Humans and several other animal species including cats, rabbits and camels can however be infected (23, 24, 95, 116). While over 200 mammalian species in 73 genera have been reported to be naturally susceptible to *Y. pestis*, susceptibility to the disease varies considerably (11, 24, 95). Some animals are highly susceptible, but most are at least moderately resistant. Carnivores such as domestic dogs, raccoons, coyotes and black bears are reported to be highly resistant to plague (11, 24, 45, 116). Susceptibility and animal species that serve as natural

plague reservoirs vary greatly depending on geographical locations (11, 23, 45, 73, 116). For example while species of gerbils show various degree of susceptibility to plague and therefore serve as a primary carrier of the disease, rats serve as the common source of plague in Madagascar and Vietnam. Although some mammals such as the vole *Microtus californicus* have been cited as the source of plague cases in the United States, the main species involved are still unknown (11).

Vector

Plague is generally transmitted by fleas (23, 24, 62, 95). While over 1,500 species of fleas have been identified, only about 80 of them are implicated in maintaining the plague cycle (24, 95). Among these 80 different species of flea, the oriental rat flea *Xenopsylla cheopis* is the most common and efficient flea vector (23, 24, 95, 106). The proventriculus of the *X. cheopis* is more susceptible to blockage by a blood meal containing *Y. pestis* (23, 24, 95). The bacteria form biofilms on the spicules of the proventriculus, blocking blood from entering the stomach during feeding (24). This blockage leaves the flea hungry leading them to bite repeatedly and to regurgitate bacteria into the skin of its host (23, 24, 45, 95). Although blockage of the proventriculus by the bacterially formed biofilm has been the accepted paradigm for efficient transmission of *Y. pestis*, rapid and efficient transmission have also been demonstrated in *Oropsylla montana*, the flea commonly found on rock squirrels and the primary vector for plague transmission in North America (24, 45). This finding suggests that other fleas with poor blocking capacity could play key roles in the spread of *Y. pestis* (24).

Disease

Plague usually presents in one of three forms: bubonic, septicemic or pneumonic (24, 32, 62, 95,107). The majority of plague cases are either bubonic or septicemic (24). The clinical presentation of the disease depends on the mode of infection. Patients typically exhibit high fever, low blood pressure, chills, fatigue and in some cases cough and chest pain (24, 32, 62, 95).

Bubonic plague is the classical form of the disease. Within two days of infection, patients present with a nonspecific disease, usually developing symptoms of fever, chills, headaches and swollen tender lymph nodes (buboes) (32, 62, 95, 107). In addition, gastrointestinal complaints such as vomiting and diarrhea are common (62, 95). Cases without buboes are usually difficult to diagnose. Lymphadenitis due to plague is different from those due to other causes because plague related lymphadenitis is associated with systemic symptoms (hypotension and very high fever) and rapid clinical degradation (24, 32, 62). The bubo can be located in the groin, in the axilla, neck or head depending on the site of initial infection (24, 32). Bacteremia and secondary septicemia are frequently seen in patients with bubonic plague (24, 62).

Primary septicemia is generally defined as occurring in a patient with positive blood cultures but no palpable buboes (62, 95). Clinical septicemia from plague is not different from septicemia caused by other gram negative bacteria. The patients generally present with chills, headache, malaise and gastrointestinal

disturbances. The mortality rate for people with septicemic plague ranges from 30 to 50% (107).

Pneumonic plague may be primary or secondary following septicemia and can occur with or without buboes (24, 62, 95, 107). Primary pneumonic plague usually results from spread via aerosol from infected individuals through close contact or from infected animals. The pneumonic form of the disease is rare but is the most deadly form of the disease (32, 62, 95). The incubation period is 1 to 3 days (32, 62, 95), and it progresses rapidly from a febrile-like illness to an overwhelming pneumonia with coughing, chest pain and bloody sputum. Mortality for untreated pneumonic plague could be as high as 90% (45, 95, 101).

Diagnosis and Treatment

Clinical diagnosis is generally based on patients' exposure history and symptoms (95, 107). Thus people with painful swollen lymph nodes with fever and hypotension and have been exposed to fleas or rodents in a zone where plague exits are suspected of bubonic plague. Pneumonic plague is usually suspected in patients with bloody sputum containing gram-negative bacilli and who have been exposed to fleas and rodents (62, 95). Bacteriological, molecular and or serological examination of bubo aspirates, sputum and scrapings from skin lesions may provide presumptive or confirmatory evidence of plague infection (24, 62, 95, 107). These include but are not limited to polymerase chain reaction (PCR), antibody tests directed against purified F1 capsular antigen and isolation of the organism by culture (23, 24, 62, 107). Early diagnosis and specific treatment are critical to successful treatment.

Streptomycin has been and remains the standard treatment for plague (23, 24, 95). However, gentamycin and doxycycline are good alternatives (23, 24, 95).

Vaccines

Due to the rapid spread and high mortality associated with *Y. pestis*, epidemics of plague are thought to have killed more people than any other infectious disease in human history (24, 62, 107). *Y. pestis* has a broad range of reservoirs including rats, squirrels, mice and prairie dogs as well as several flea vectors for transmission (23, 24, 107). There is ubiquitous spread of zoonotic reservoirs with insect vectors and the possibility of using *Y. pestis* as a bioweapon is high, yet there are currently no approved vaccines to protect humans against this deadly disease (23, 24, 103). Killed whole cell vaccines have been explored extensively in the past (110, 132). The first FDA approved vaccine was the plague vaccine USP; a killed whole cell vaccine that was considered safe and effective at preventing bubonic plague (110). This vaccine was however associated with a number of adverse reactions as well as failure to provide protection against pneumonic plague (110). These reasons diminished the interest in using plague vaccine USP as a vaccine for plague. A live whole cell plague vaccine (Y. pestis EV76) was used extensively in Russia (23, 110, 132). Unlike the killed whole cell USP vaccine, the live whole cell EV76 vaccine has been reported to revert to wild type virulence (23, 110). Notwithstanding these shortcomings of plague vaccine development, there is ample evidence to suggest that plague vaccines can be developed. This evidence is based on the fact that clinical recovery from bubonic plague is associated with immunity to the disease (110). The ultimate goal of

vaccine development is therefore to develop a vaccine that is safe and protective against all forms of the disease especially pneumonic plague, which is likely to be the form a bioweapon would take (110, 132). This goal has shifted plague vaccine development towards subunit vaccines (110, 103, 132).

Two subunit antigens (F1 capsular antigen and LcrV) have shown to be protective against plague and are currently considered the only protective vaccine candidates for plague (23, 103, 131, 132). The use of these antigens either alone or in combination has been challenged (23, 11). Antibodies against the F1 antigen confer immunity against plague (121). However, *Y. pestis* variants lacking F1 capsule continue to cause lethal infections and therefore the F1 cannot be used as a sole antigen in plague vaccine (23, 110). This is further supported by the fact that the F1 capsule has only a contributory role and is not required for pathogenesis of plague. In contrast to the F1 capsule, LcrV is essential for pathogenesis of plague (18, 31, 34, 49). LcrV is deposited at the tip of the T3S system needle and enables the transport of effector proteins into host immune cells (58, 74, 84, 92). Mutants lacking LcrV have reduced virulence and antibodies against LcrV can confer immunity to plague (23, 93, 103, 110, 131). LcrV, however, interacts with TLR2/6 and CD14 to stimulate immune cells to release anti-inflammatory cytokines specifically IL-10 (10, 18). As a powerful anti-inflammatory cytokine, IL-10 prevents the release of pro-inflammatory cytokines, including TNF-α and IFN-γ, that are required for the clearance of *Y. pestis* (10, 71). This mechanism has the potential to impact host responses to *Y. pestis* and therefore makes LcrV unsuitable for use in a plague vaccine. To overcome this challenge, Quenne and colleagues have designed a

subunit vaccine that focused on an LcrV variant that lacks the immune-modulatory attributes of this peptide (103). Their LcrV variant (rV10) retained the ability to elicit immune response against plague and raised antibodies that block release of effector proteins into host cells without suppressing the release of pro-inflammatory cytokines (103). Although the rV10 vaccine has shown protection in different animal models, it is yet to complete toxicology or safety studies in rabbits (103). It is therefore necessary to develop other subunit vaccines that are safe and protective for humans.

The needle protein YscF, is a surface-expressed protein of the *Yersinia* T3S system. YscF forms the needle-like structure that enables the translocation of effector proteins into host cells and therefore essential for pathogenesis. Although antibodies against YscF do not show similar levels of protection compared to the F1 capsular antigen and LcrV (83, 133), Wang and his colleagues have reported enhanced protection when YscF is added to F1 antigen compared to F1 antigen DNA vaccines alone (133). These findings support the potential use of YscF as a vaccine at least in combination with other antigens.

The Bacteria

Yersinia species are gram-negative bacteria belonging to the family Enterobacteriaceae (11, 17, 59, 24, 95, 68, 107). The genus *Yersinia* contains 11 species of which three are human pathogens; *Y. pestis, Y. pseudotuberculosis and Y. enterocolitica* (23, 62, 95). *Y. pestis* causes the more lethal disease plague, while *Y. pseudotuberculosis and Y. enterocolitica* cause the self-limiting gastrointestinal

infection, yersiniosis (59, 62, 68). The three species of *Yersinia* share the T3S system encoding plasmid called pCD1 in *Y. pestis*, pYV in *Y. enterocolitica* and *Y. pseudotuberculosis* (23, 95). *Y. pestis* is thought to have evolved from the closely related *Y. pseudotuberculosis* as recently as 1,500 to 2,000 years ago with the loss of several genes while acquiring extra plasmids (pPCP1 and pMT1) (23, 24, 42, 45, 59, 68, 98, 101, 115). Compared to *Y. pseudotuberculosis, Y. pestis* has downsized its genome and has restricted itself to a strict pathogen depending on the infected host for essential nutrients (23, 45, 59, 109, 115).

Yersinia pestis is non-motile, non-spore forming coccobacillus with a size ranging from 0.5 to 0.8 μm in diameter and 1 to 3μm in length (62, 68, 95). The organism exhibits a bipolar staining with Giemsa, Wright's or Waysin stain (62). *Y. pestis* has an optimal growth temperature of 28^0C to 30^0C but can grow in a wide range of temperature from 4^0C to 42^0C (19, 92). Though the bacteria tolerate extremes of pH from 5 to 9, the optimal pH for growth is between 7.2 and 7.6 (95). Compared to *E. coli*, growth of *Y. pestis* is slow, requiring over 24 to 48 hours for colony formation on most rich media. The generation time in defined media can however be as short as 1.25 hours (62, 95).

Yersinia pestis has generally been grouped into four biovars based on their ability to convert nitrate to nitrite and fermentation of glycerol (41, 95). Biovar Antiqua is able to reduce nitrate to nitrite and ferment glycerol (41, 42, 95). Medievalis does not reduce nitrate but ferments glycerol while Orientalis ferments glycerol but does not reduce nitrate (41, 42, 95). Biovars Antiqua, Medievalis and Orientalis have been associated with the first, second and third plague pandemics

respectively (41, 95, 101). Recent analysis of teeth from plague victims has, however, linked all three pandemics with the biovar Orientalis (24, 41). The strains CO92 (Orientalis) and KIM (Medievalis) are the common lab strains used in research. Experiments performed in this manuscript were done with the KIM (Kurdistan Iran Man) strain of the biovar Medievalis.

Virulence Factors

Yersinia pestis has evolved efficient strategies to optimize resistance against host defense systems that enable the bacteria to colonize, invade and multiply in host tissues (23, 41, 59, 62, 109). It also acquired a mechanism to survive in the mid-gut of the flea as part of its evolution from *Y. pseudotuberculosis* (59, 109, 115). The majority of *Y. pestis* strains contain three plasmids of 9.5 kb, 70 kb and 110kb (23, 95). These plasmids are termed pPCP1 (pesticin, coagulase, plasminogen activator), pMT1 (murine toxin) and pCD1 (calcium dependent) in KIM strains (23, 41, 59, 95). The pMT1 plasmid encodes the 61 kDa murine toxin which is toxic to mice and rats but not guinea pigs, dogs or rabbits (95). The murine toxin is part of the phospholipase D family of proteins and functions to block the flea gut during the vector stage of the *Y. pestis* life cycle (41, 59). This has been proposed to play a role in the survival of *Y. pestis* in the flea. The pgm (pigmentation) locus contains the hms (hemin storage) locus, which is essential for *Y. pestis* survival in the flea. The hms locus is involved in blocking the flea mid-gut (95). This blockage has been established to aid frequent biting and regurgitation enabling efficient plague transmission from the flea to the mammalian host (59, 95). In addition to the hms

locus, the pgm locus contain the yersiniabactin (Ybt) system which functions in iron acquisition (59, 95). Iron is an essential micronutrient that is chelated by mammalian proteins in an attempt to make them less available to invading pathogens (95). *Y. pestis* uses its yersiniabactin to acquire iron to enable it survive in the iron restricted environment in the host (95, 96). Although Ybt has been reported to be dispensable in septicemic plague, it is essential in bubonic and pneumonic plagues (96).

Once in the host, *Y. pestis* should be able to resist host defenses and spread to the draining lymph nodes (17). This is achieved through a number of mechanisms. First is the expression of the plasminogen activator protein (Pla) that is encoded by the pPCP1 plasmid (59, 95). This surface protease is responsible for the dissemination of the bacteria from the site of the flea bite. In addition to the expression of Pla, *Y. pestis* also expresses a tetra-acylated LPS instead of the usual hexa-acylated LPS expressed in the flea (12, 17, 23). This tetra-acylated LPS is not only non-stimulatory for TLR4, it is actually antagonistic to TLR4 stimulatory LPS; the hexa-acylated LPS (12, 17, 23). This shift in LPS expression is a mechanism for the bacteria to evade host immune detection through reduced innate immune cell activation and expression of pro-inflammatory cytokines (118, 23). Another important mechanism used by *Y. pestis* to resist host defense is the expression of the fraction 1 (F1) capsule at 37^0C from the pMT1 plasmid (23, 59). The F1 capsule forms an envelope around the bacteria enabling it to resist phagocytosis (23, 59). In addition to all these survival mechanisms, *Y. pestis* possess a type III secretion (T3S) system to deliver anti-phagocytic effector proteins into host cells (27, 58, 80, 121).

Type III Secretion Systems

The type III secretion (T3S) system is a protein-secretion nano-machine used by many gram negative bacteria to deliver virulence factors into eukaryotic host cells (27, 58, 80, 121). T3S systems are found in both animal and plant pathogens (37, 134). Among these bacteria are the human pathogens *Salmonella spp., Pseudomonas spp.*, enteropathogenic *E. coli, Shigella spp.* and *Yesinia spp.* (37).

The T3S system is well conserved among these bacteria but the properties of the effectors and the resulting symptomatic effect on the host organism are species specific (27). However, the T3S system in all cases contributes to infection by allowing the bacteria to communicate with the eukaryotic cell (58, 80, 134). In *Y. pestis*, the effectors disturb the dynamics of the cytoskeleton and block phagocytosis by macrophages. They also suppress the production of pro-inflammatory cytokines, chemokines and adhesion molecules (30, 80). These allow the survival and replication of the bacteria in lymphoid tissues (30). The T3S apparatus is made of a basal body that spans both bacterial membranes and an extracellular exposed needle that protrudes from the bacterial surface (27,30,34,37,124,134). T3S systems are not constitutively active but they are activated to secrete by physical contact with host cells (27,34,36,37,124). Upon contact with the host cell, two distinct classes of proteins are exported; the translocators that form the pore in the target

membrane and the effectors that traffic through the pore to manipulate the host cell (30,34,36,37).

Structure

The T3S machinery, also called the injectisome, consists of two distinct parts: a basal body linked to the external hollow needle that protrudes from the bacterial surface (19, 25, 70, 89, 90). The basal body consists of two pairs of rings that span the two bacterial membranes and the periplasm joined by a rod protein (191, 25, 96, 90). Assembly of the needle complex requires around 25 different proteins that make up the basal body, the needle and translocon (30,50, 90). In *Yersinia* species, YscD and YscJ form the inner pair of rings while YscC forms the outer pair of rings that is associated with the outer membrane and peptidoglycan (90). The basal body has been reported to have elongation capacities that enable the bacterium to adjust to different environmental conditions (70). YscD, one of the proteins that form the inner ring, can stretch or contract by up to 50% of its original length while YscC can vary its length by 30-40% relative to that observed in intact bacterial membranes (70). In addition to the ring proteins, there are inner membrane and cytoplasmic proteins that are conserved throughout the T3S apparatus. These include the cytoplasmic protein ATPase (YscN in *Yersinia*) that provides energy to unfold the effectors for secretion (28, 61, 90).

Protruding from the bacterial surface is a hollow tube made by polymerization of a single protein that serves as a conduit for effector transport (28,

50, 60, 89, 90). The length of the needle is controlled by the molecular ruler YscP, and varies depending on the bacterial species (90). It could range in length from 45 to 85 nm with an internal channel of 2 to 3 nm (30, 130). Depending on the bacterial species, the number of needle complexes per bacteria ranges from 10 to 100. At the tip of the needle is the hydrophobic protein, LcrV (28, 74, 90, 106, 129, 130). The LcrV pentamer forms a structure that serves as a platform for the translocators (130). Translocation of effector proteins into the cytoplasm of host cells requires the formation of the translocon that forms a pore in the membrane of eukaryotic cells mediating the direct transfer of effectors into the cytosol of host cells (61, 74, 89, 90, 106). In *Yersinia* species, the translocon is formed by a hydrophobic pair of proteins, i.e. YopB and YopD. These two proteins have been shown to have the ability to insert and form pores in eukaryotic hosts (12, 28, 44, 61, 89, 90, 106).

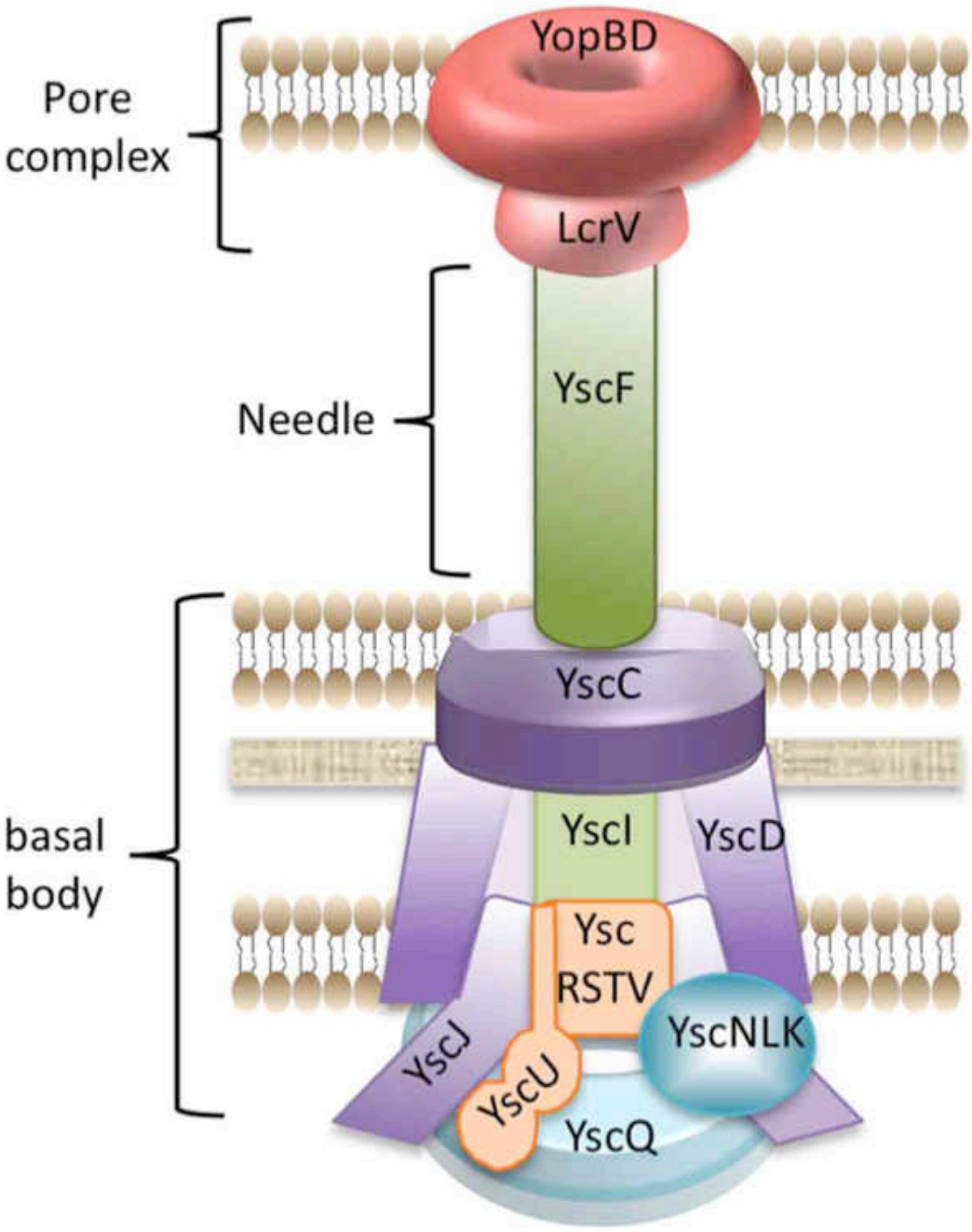

FIGURE 3: Model of the injectisome. (Used with permission)**. (38).** Shown is a cartoon depicting the structural components of the *Yersinia* injectisome. Purple, scaffold proteins: YscC, YscD, YscJ; Orange, export apparatus proteins: YscR, YscS, YscT, YscU, YscV; Blue, cytoplasmic components: YscQ (C-ring) and YscN, YscL, YscK (ATPase complex); Green, YscI (rod) and YscF (needle); Red, pore complex: LcrV (needle tip complex) and YopB/YopD (translocation pore).

Chaperones.

Type III secretion effector proteins mostly exert their action at specific sites in the eukaryotic host (19, 61, 90, 100, 106). Proper regulation of the T3S apparatus requires that the proteins are secreted in a specific order (19, 61, 90, 100, 106). To achieve this proper regulation, secretion of some T3S proteins requires chaperones for their secretion and presecretory stabilization (28, 30, 136). In *Yersinia pestis*, LcrH (also termed SycD) binds to YopB and YopD to prevent the premature interaction of these Yops with LcrV (28, 62, 90, 94, 107, 136). An LcrH mutant is reported to specifically lack YopB and YopD in culture supernatants. As with LcrH, SycE and SycH are chaperones for YopE and YopH respectively (28, 62, 90, 94, 107). Chaperones are generally predicted to play an antifolding role to maintain Yops in proper conformation suitable for secretion. By binding to YopE and YopH, SycE and SycH have been reported to maintain the stability and proper conformation and probably prevent premature association with other effector proteins (28, 62, 89, 90, 94, 107). Additionally, SycN and YscB are chaperones for YopN. No chaperones have been described for YopM, YopJ, YopK and LcrV (28, 62, 89, 90, 94, 107). In contrast YscF has two chaperones (YscE and YscG) and to date the only crystal structure of YscF was obtained in association with its chaperones (119, 136).

Effectors

A major function of the T3S system is to modulate host cell signaling to the benefit of the bacteria (28, 61, 129). This goal is achieved by the translocation of "toxic" effectors called Yops (Yersinia outer proteins) in the yersiniae into their

eukaryotic host (12, 28, 29, 30, 31, 61, 129). Six effector proteins are translocated into host cells. These effectors have different targets and effects in the host, however, they work together to hijack intracellular host signaling (61). Though they perform different functions in the host, all effector proteins require an N-terminal signal sequence for their translocation (28, 94, 100, 106). Their functions range from inhibition of the β-integrin signaling pathway to counteract phagocytosis to prevention of cytokine production by binding to and inhibiting activation of MAPK and NF-kβ signaling pathways (3, 12, 28, 59, 129). Specifically, YopH, which is a protein tyrosine phosphatase, counteracts phagocytosis by inhibiting the early steps in the β1-integrin signaling pathway (29, 90, 129). Similarly, YopE, YopO and YopT inhibit phagocytosis and cytokine production by inactivating Rho GTPases (3, 28, 29, 30, 90, 129). Additionally, YopJ prevents production of cytokines and cell survival factors by binding to and blocking activation of MKK and IKK in the MAPK and NF-kβ signaling pathways, respectively (3, 12, 28, 29, 90, 129). YopM has been implicated in the regulation of genes involved in cell cycle and cell growth (12, 28, 129).

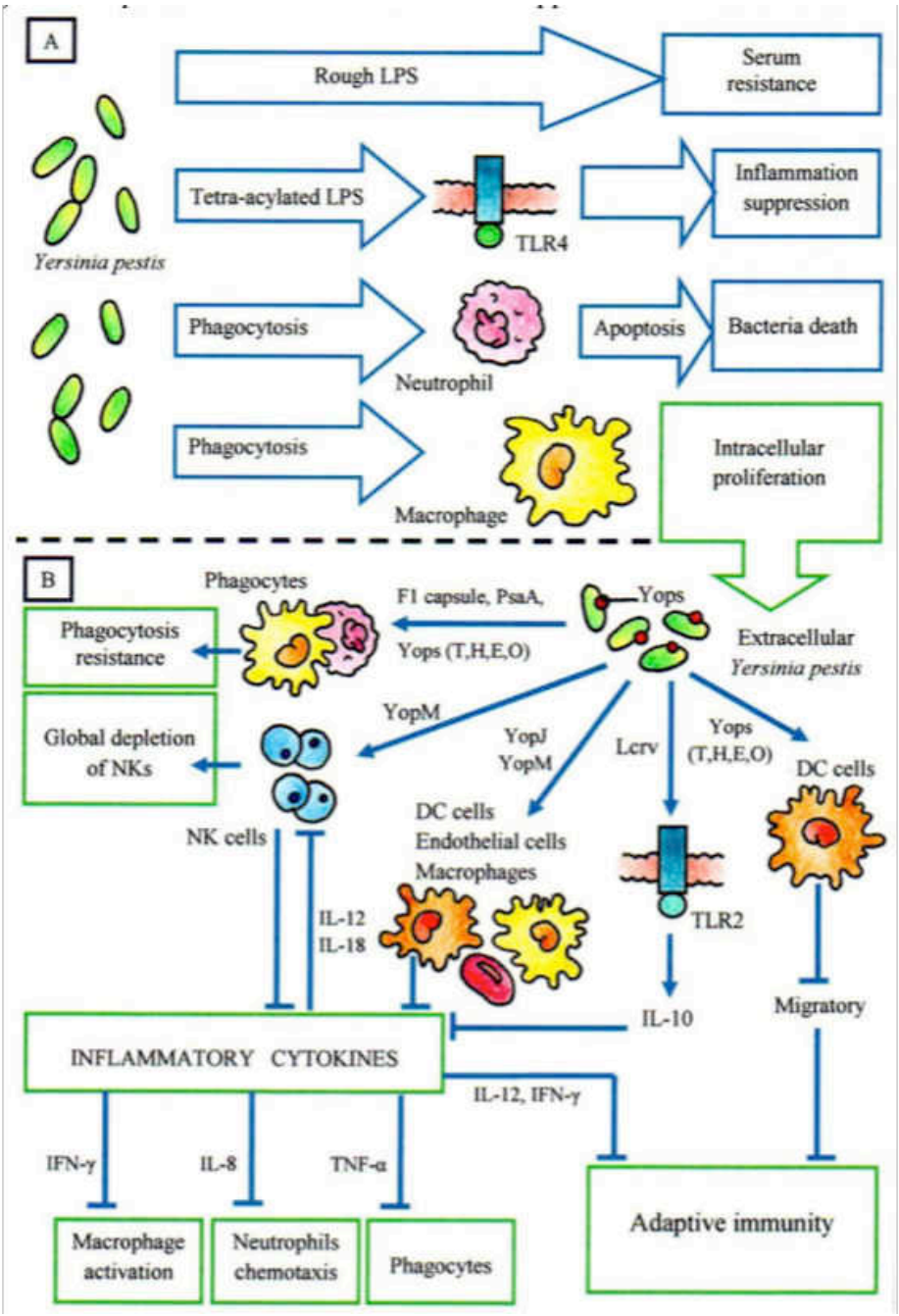

Figure 4*: Y. pestis* resistance mechanisms in opposition to host innate immunity. Used with permission. (3). (A) Resistance mechanisms at the early stage of infection. The LPS structure varieties of *Y. pestis* during transition between flea and host temperatures make the bacteria resistant to the serum-mediated lysis and repress the proinflammatory response. In the meantime, the bacteria phagocytosed by macrophages can grow and express different virulence determinants to act on host immune responses. (B) Resistance mechanisms after the release of *Y. pestis* from macrophages. The bacteria released from macrophages attain the capacity to resist phagocytosis and can inhibit the production of proinflammatory cytokines, which also attenuate the host's adaptive immunity.

Regulation of the Type III Secretion System

T3S systems are not constitutively active but they are activated by environmental conditions that usually correspond to conditions encountered by the bacteria in the host (90, 100). These environmental conditions include temperature, calcium levels and physical contact with host cells (90, 100). Regulation of the T3S system is achieved by the coordinated regulation of several proteins. LcrG blocks secretion in an inactive state by interacting with LcrV (46, 84). At 37^0C however, expression of LcrV increases which causes LcrV to bind and titrate away LcrG from the injectisome allowing for secretion of Yops (46,84). YopN also form a complex with TyeA in the bacterial cytosol also blocking the T3S apparatus in a non-triggering condition (28, 61). However, under triggering conditions, YopN secretion alleviates the blockage allowing for secretion (28, 61, 100 124).

Growth of *Y. pestis* is restricted at 37^0C in the absence of calcium. This growth cessation is associated with Yops secretion (29, 30, 84, 106). Addition of millimolar concentrations of calcium to the growth media rescues the growth restriction and represses Yop secretion (29). This phenomenon is called the low calcium response (LCR) (17, 28, 61, 106). Certain specific mutations in the regulatory genes of the T3S system results in phenotypes that do not require calcium for alleviation of growth restriction at 37^0C. This phenotype is described as calcium independent in contrast to the calcium dependent phenotype of wild type *Y. pestis* (17, 28, 61, 106). Another phenotype that is also as a result of altered regulatory genes is the calcium blind phenotype (29, 61). In contrast to the calcium independent phenotype, the calcium

blind phenotype constitutively expresses Yops even in the presence of calcium (29, 61).

Innate Immune Response and Type III Secretion System

The innate immune response is the first line of defense against invading pathogens and potentially harmful commensals (9, 15, 33, 104, 134). It consists of barrier structures, molecules (complement proteins, cytokines) and cells: macrophages, neutrophils, natural killer (NK) cells and dendritic cells (DCs) (3,10, 71). Innate immune responses are activated to provide protection once a bacterium enters the host. Pathogenic bacteria have, however, evolved mechanisms to overcome these host defenses. For example *Y. pestis* is able to resist serum-mediated killing and also prevent inflammation by inactivating TLR4 signaling (10). Upon entry into the host through the bite of an infected flea, *Y. pestis* encounters phagocytes including neutrophils and macrophages (3, 10, 111). Because neutrophils kill *Y. pestis*, the bacteria preferentially infect macrophages where they are able to survive and replicate (3, 10, 112). While in the macrophages, *Y. pestis* is protected from host serum mediated killing and is also transported to other sites within the host (3, 10, 112). *Yersinia* causes apoptosis in naïve macrophages to prevent inflammation but triggers pyroptosis in activated macrophages during the later stages of the infection (3, 10, 111).

Pro-inflammatory cytokines, especially TNF-α, are very important in limiting the severity of many bacterial infections (28, 69, 111, 129). They play significant roles in activating macrophages, neutrophils and NK cells (3, 10, 69, 112). The

suppression of pro-inflammatory cytokine through Yops secretion not only reduces activation of phagocytes and NK cells but also suppress the production of reactive oxygen species that are crucial for eliminating bacteria (3, 10, 111). Dendritic cells serve as the link between innate immune responses and the adaptive immune response (3, 10). Not only does *Y. pestis* suppress activation of innate immunity it also prevents the adaptive immune response by preventing maturation and movement of DCs (3, 10, 01, 111, 112).

Other roles of T3S apparatus; innate immune activation

Pathogens are recognized by the innate immune system via pattern-recognition receptors (PRRs) (9, 15, 33, 86, 108, 120, 140). The germ-line encoded PRRs recognize conserved microbial motifs termed pathogen-associated molecular patterns (PAMPs) (9, 15, 33, 86, 108, 120, 140). In addition to PAMPs, PRRs also respond to host damage-induced signals called danger-associated molecular patterns (DAMPs) or alarmins (15, 33, 71). Toll-like receptors (TLRs) were the first group of PRRs to be identified and characterized. Toll-like receptors detect either extracellular or endosomally located PAMPs (51, 71, 86). These PAMPs include LPS that is recognized by TLR4, lipoteichoic acid (TLR2 ligand) and flagella (TLR5 ligand) (51, 71, 86). When PRRs are engaged by their respect PAMPs, they induce gene expression leading to production of cytokines, chemokines and pro-inflammatory molecules through different adaptor molecules including MyD88 and TRIF (51, 53, 71).

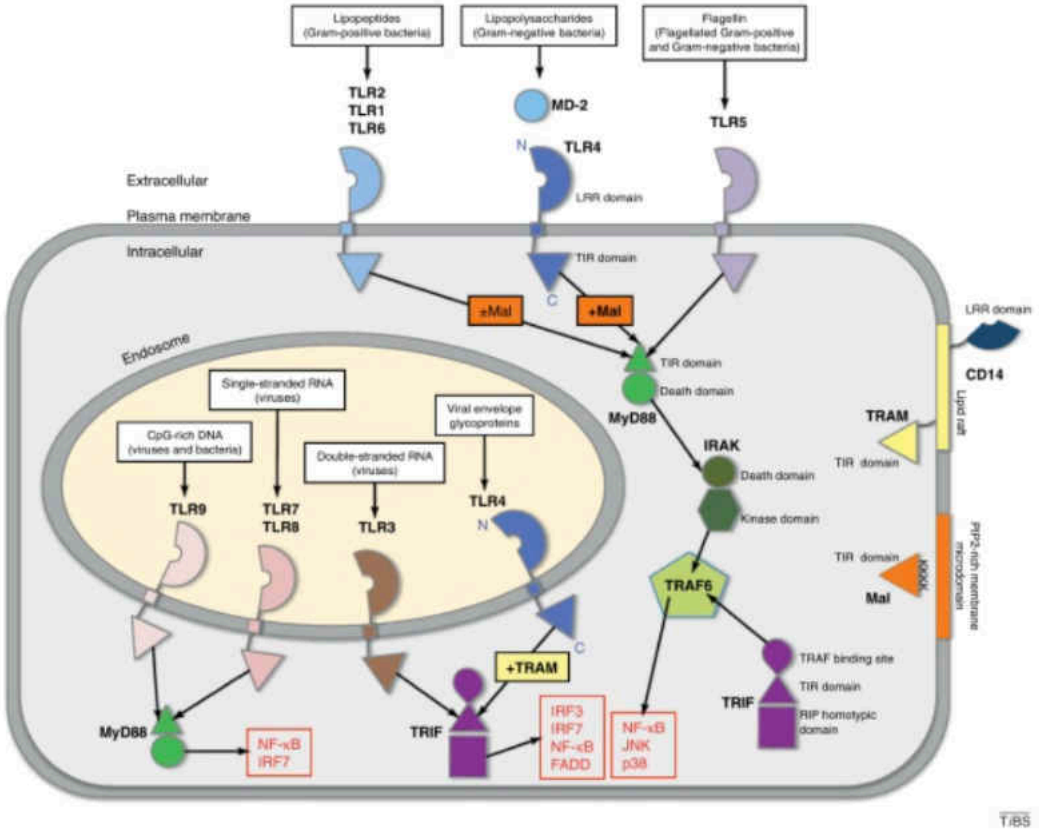

Figure 5. TLR signaling. Used with permission. (51). There are two types of TLRs, those located at the plasma membrane that sense microbial membrane components and the intracellular ones that sense microbial nucleic acids. TLR4 can signal both at the plasma membrane and at endosomes, where it can be activated by viral envelope glycoproteins. All TLRs signal via the adaptor MyD88, except TLR3 that can function only via TRIF. MyD88 leads to the activation of kinases such as IL-1 receptor-associated kinases (IRAKs). The resulting phosphorylation cascade activates members of TNF receptor associated factor 6 (TRAF6) and, ultimately, transcription factors NF-kB, JNK and p38 (as well as IRF7 for TLR7 and TLR9). The TRIF pathway stimulates TRAF6, Fas-associated protein with death domain (FADD) and the transcription factors IRF3, IRF7, NF-kB. TLR4 is the only receptor that can engage both MyD88-dependent and -independent pathways. It requires bridging adaptors TRAM and Mal to recruit MyD88 and TRIF, respectively. TLR2 depends only partially on Mal. Membrane signaling triggers an inflammatory response whereas intracellular TLR signaling leads to antiviral and adjuvant responses.

In addition to the TLRs, the cytosolic nod-like receptors (NLRs) serve in intracellular innate immune surveillance against invading pathogens (9, 15, 33, 49, 71, 108, 120).

Activation of these intracellular NLRs triggers a specific type of caspase-1 mediated cell death called pyroptosis (15, 71). NLRs initiate the assembly of multi-protein complexes called inflammasomes upon sensing pathogens or DAMPs. Inflammasomes serve as a platform for the activation of caspase-1 that processes pro IL-1β and IL-18 to their mature forms (15, 71). Assembly of this multi-protein complex usually require two signals: a priming signal via TLRs that is required for expression of certain inflammasome receptors and the substrates pro IL-1β and IL-18, before the second signal can activate the inflammasome (15, 33, 71). Activation of the inflammasome cleaves pro caspase-1 to its active form, caspase-1, that mediates the processing and release of IL-1β and IL-18 (15, 71). The majority of TLRs and NLRs play a pivotal role in sensing bacteria whereas RIG-like receptors generally sense viruses (71).

The T3S apparatus of many gram negative bacteria possesses conserved structural features (27, 58, 80, 121). Some components of these conserved structures are surface exposed and could be beneficial to the host by allowing direct detection of these components to mount immune responses to them (27, 58, 80, 121). The flagella, which is evolutionally and functionally related to the T3S system, has long been identified as a ligand of TLR5 that induces cytokine expression via NF-κB. Flagellin monomers from *Salmonella* are also known to activate NLRC4 in a NAIP5 dependent manner (53, 67, 127, 137, 139). Consistent with the wide distribution of flagella among bacterial species, innate immune recognition of bacteria mediated by the NLRC4 inflammasome have been identified (53, 86, 127, 137, 139). These bacteria include *Pseudomonas aeruginosa, Legionella. pneumophila*

and the gram positive bacterium *Listeria monocytogenes* (53, 137). Because flagella and the T3S system are related, different components of the T3S apparatus have also been examined for their ability to interact with PRRs. The SPI-1 T3S system rod protein PrgJ from *Salmonella enterica* Serovar. Typhimurium has been shown to activate caspase-1 through NLRC4 (67, 122, 72, 87). Similarly, several PrgJ homologs including BsaK (*Burkholderia pseudomellei*), EprJ (EHEC), EscI (EHEC and EPEC), MxiI (*Shigella flexneri*) and PscI (*P. aeruginosa*) have been determined to activate NLRC4 (67, 120, 122, 139).

The needle protein conserved among gram negative bacterial pathogens is a critical component for assembly and function of the T3S system (27, 58, 80, 121). The needle is the most exposed component of the T3S system and direct detection of the needle could be advantageous to the host to control infections. Notably, several needle proteins including CprI, BsaL, EprI and MxiH have been reported to trigger robust caspase-1 activation when introduced into C57BL/6 mouse derived bone marrow derived macrophages (BMDM) in an NLRC4 dependent manner (139). Not only does CprI trigger caspase-1 activation in BMDM, CprI has also been shown to activate the NLRC4 inflammasome in human U937 monocytes (139). Additionally, other bacterial needle proteins including PrgI from *S. typhimurium* and PscF from *P. aeruginosa* showed similar activity in inducing caspase-1 processing in THP-1 macrophages (133). Recently, YscF from *Y. pestis*, MxiH from *S. flexneri* and PrgI and SsaG from SPI-1 and SPI-2 of *Salmonella* have, respectively, been shown to be ligands of TLR2 and TLR4 that activate NF-κB in a MyD88 dependent manner (63).

Rationale

A key component of the T3S system is the needle–like structure that spans both bacterial membranes and through which effectors are delivered directly into the cytosol of host cells (31, 56, 108). The needle is formed by the polymerization of single proteins: YscF in *Yersinia spp.*, PrgI and SsaG (*Salmonella* Pathogenicity Island 1 (SPI-1) and *Salmonella* Pathogenicity Island 2 (SPI-2), respectively) in *Salmonella enterica* serovar Typhimurium, PscF in *Pseudomonas spp.* and MxiH in *Shigella flexneri* (60, 104, 105, 108, 119, 125). X-ray crystallography and NMR have been utilized to analyze structures of several needle proteins: MxiH from *Shigella* (35), BsaL from *Burkholderia pseudomallei* (138), and PrgI from *Salmonella* Typhimurium (134). The crystal structure of MxiH was used to generate a model of T3S needle structure (13, 26, 138). In this model the N-terminus of MxiH was predicted to line to the lumen of the T3S needle (26). Contrary to the previous model, recent reports by Loquet *et al.* and Demers *et al.* have revealed that the variable N-termini of needle proteins in *Salmonella* and *Shigella* respectively, are in fact, on the outside surfaces of the needles, exposing them to host elements, while the conserved carboxy ends face the lumen reflecting a bacterial strategy to evade host response. (37, 79).

The needle proteins share high sequence identity and their predicted structures have two α-helical segments separated by a loop at a P-(S/D)-(D/N)-P motif (66, 125). The C-termini of needle proteins are highly conserved between different bacterial species. As expected, the conserved residues of the C-termini mediate important intra- and intermolecular interactions of the complex. Deletion of 5

residues from the C-terminus of PrgI, MxiH and BsaL prevents needle polymerization (66, 134). The N-terminus of needle proteins in all these bacteria are suggested to be highly mobile and disordered (35) with no defined structure found for this portion of the proteins. YscF has only been crystalized in complex with its chaperones YscE and YscG (6, 119). Sun *et al*. reported the N-terminus in this crystal structure to be largely unorganized and not representative of YscF in its needle conformation (119). The N-termini of needle proteins are variable not only in amino acid composition but also in the number of amino acids.

Torruellas *et al* (124) has reported that YscF is a multifunctional structure that is involved in virulence protein secretion, translocation of virulence proteins as well as cell contact- and calcium-dependent regulation of T3S. However, mutations in the N-terminus of the needle protein have no significant impact on these essential processes (2, 51). A study by Allaoui et al (2) reports that truncation of 12-N-terminal amino acids from YscF reduced secretion of YopB and YopD but not the other Yops (2). Additionally, other studies have also reported that the hyper-variable N-termini do not mediate important needle sub-unit interactions (66, 134).

Needle proteins were recently identified as TLR2 and TLR4 ligands that activate expression and secretion of cytokines via a MyD88-dependent pathway (63). Purified needle proteins from different bacteria induced cytokine expression to different magnitudes (63). YscF comes from a T3S system from pathogens that have an anti-inflammatory infection objective (3, 56) and induces lower levels of pro-inflammatory cytokines. MxiH, from a bacterium with a pro-inflammatory infection objective (16,

98, 108), induces high levels of pro-inflammatory cytokines as did SsaG. SsaG is expressed when *Salmonella* is intracellular (47, 57, 135), and therefore is not exposed to surface expressed TLRs. Interestingly SsaG has fewer amino acids when compared to other needle proteins, notably lacking residues that correspond to the N-terminus of other needle proteins. The reported variation in the magnitude of cell activation by various needle proteins (63) coupled with the dispensability of the N-terminus for needle assembly led to the development of a hypothesis that the N-terminus of needle proteins could be involved in modulating TLR interactions.

In this study the role of the N-terminus of needle proteins in modulating host responses was investigated. Further, the region of the N-terminus that contributes to the host inflammatory activity was examined in YscF. A role for the N-terminus of YscF in modulation of host immune responses was demonstrated by constructing truncated, deleted and chimeric recombinant and purified needles.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains culture conditions and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. All strains were stored at -80°C in 25% (v/v) glycerol. *Escherichia coli* strains were grown at 37°C in LB broth (BD Difco, Sparks, MD) or on tryptose blood agar (BD Difco; TBA) plates with antibiotics added as needed. Kanamycin and carbenicillin were used at 50 μg/ml.

Plasmids used in this study to overexpress needle proteins were constructed in pET200 TOPO® using Champion^{TM} TOPO expression kits (Invitrogen, Carlsbad, CA). Primers for gene amplification were synthesized by Eurofins Genomics (Huntsville, AL). PCR primers designed to clone fragments of *yscF* missing regions of N-terminus of YscF into an expression vector, pET200 (Invitrogen) are listed in Table 2. SsaG was also N-terminally truncated to correspond to the 66 bp truncation of YscF using the forward primer 5'- CAC CCT CTC CCA CAT GGC GCA C-3'. The MxiH, truncated MxiH, and truncated PrgI encoding plasmids are a kind gift from Dr. Wendy Picking, Oklahoma State University. PCR fragments were cloned into pET200 TOPO® (Invitrogen). Template DNA for amplification was generated using the DNeasy kit (Qiagen; Valencia, CA); the manufacturer's instructions were followed. PCR was

performed using PFU Ultra II HS DNA polymerase (Agilent Technologies, Santa Clara, CA).

In-frame deletions in *yscF* that removed DNA sequences encoding residues S2-S5, S2-G10, S2-D15 and S2-A20 of YscF were constructed by PCR. Amplified DNA fragments were cloned into pET200 generating plasmids pDO1-pDO4 respectively. Site-directed mutagenesis of *yscF* in plasmid pDO3 was performed using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) according the manufacturer's instructions. Complementary oligonucleotides were designed to change one, two or three *yscF* codons, generating plasmids pDO5-pDO11. Plasmid pDO12 encoding the YscF-SsaG chimera was constructed by amplifying the sequence encoding the 15 N-terminal residues of YscF from pCD1 and the entire SsaG sequence from SPI-2 of *Salmonella enterica* serovar Typhimurium by PCR, using primers listed in Table 2. The 2 PCR products were hybridized to each other by overlap extension PCR (20), purified and cloned into pET200 for protein expression. Plasmid pDO13 containing the RN-SsaG chimera was constructed by making a +1 frameshift insertion in the region coding for N-terminal YscF of the YscF-SsaG chimera to change the amino acid sequence and following the *yscF* sequence the reading frame was corrected with a -1 deletion. Plasmids pDO16-pDO18 were constructed by cloning a EcoR1 and Sac1 cleaved SsaG, YscF-SsaG or RN-SsaG into pBAD-18 Kan (54). Primers used in this study are listed in Table 2. All of the gene constructs were verified by sequencing by Eurofins Genomics.

All plasmid constructs were transformed into commercially obtained TOP10 *E. coli* (Invitrogen) by chemical transformation. Plasmids for protein expression were purified from TOP10 *E. coli* by Qiaprep Miniprep kit (Qiagen). Purified plasmid DNA was then transformed into the expression host, BL21 (DE3) Star™ (Invitrogen).

Table 1. Bacterial strains and plasmids used in this study

Strains		
E. coli		
BL21 (DE3) Star	F$^-$ *ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3)	Invitrogen
Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL* (Str^r) *endA1* Δ$^-$	Invitrogen
Novablue	*recA1 endA1 hsdR17* (rK$^-$ mK$^-$) *supE44 thi-1 gyrA96 relA1 lac* (F' *proA*$^+$*B*$^+$) *lacI*^{q}*Z*ΔM15::Tn*10*	Novagen
Yersinia pestis		
KIM8-3002	KIM8 Sm^r	(124)
KIM8-3002.P61 (Δ*yscF*)	Sm^r pCD1 (Δ*yscF*), pPCP1$^-$, pMT1	(124)
Salmonella typhimurium		
I4028	*Salmonella enterica* serovaTyphimurium	American Type Culture Collection
BLS101	14028 ΔssaG	Brian Ahmer
Plasmids		
PET 200	T7 expression vector, N-terminal peptide containing the X-press ™ epitope and the 6XHis tag. Kan^r	Invitrogen
pBAD18-Kan	*araBADp* cloning vector, Km^r	(92)
pDO1	PET200 with YscF Δ(S2-S5) ΔN5	This study
pDO2	PET200 with YscF Δ(S2-G10) ΔN10	This study
pDO3	PET200 with YscF Δ(S2-D15) ΔN15	This study
pDO4	PET200 with YscF Δ(S2-A20) ΔN20	This study
pDO5	pDO3 L67A	This study

Table 1 cont.

pDO6	pDO3 D17A	This study
pDO7	pDO3 V19A	This study
pDO8	pDO3 L16A/D17A	This study
pDO9	pDO3 L16A/V19A	This study
pDO10	pDO3 D17A/V19A	This study
pDO11	pDO3 L16A/D17A/V19A	This study
pDO12	PET200 with YscF-SsaG	This study
pDO13	PET200 with RN-SsaG	This study
pDO14	pBAD18-kan with YscFΔ(S2-D15)ΔN15	This study
pDO15	pBAD18-kan with YscFΔ(S2-A20)ΔN20	This study
pDO16	pBAD18-kan with YscF-SsaG	This study
pDO17	pBAD18-kan with RN-SsaG	This study
pDO18	pBAD18-kan with SsaG	This study
pET15B	PET215b-MxiH	W. Pinking
pET200 PrgI	PET200-PrgI Km^r	(63)
pEt200 SsaG	PET200-SsaG Km^r	(63)
pJM119	PET24b-YscF	(83)

His-tagged protein purification

Protein purification was performed as reported previously (63). Briefly, *E. coli* BL21 (DE3) Star™ (Invitrogen) carrying plasmids for a given protein were grown overnight in non-inducing media (50xM, 1M $MgSO_4$, 40% glucose, 5% aspartic acid (118)) supplemented with antibiotic. Bacteria were then inoculated into auto-inducing media (50xM, 1M $MgSO_4$, 50x5052, NZ-amine, Yeast Extract, distilled water (118)) with antibiotic and grown to an $O.D_{620}$ of 0.6-0.8. Cells were harvested by centrifugation at 4,000 x *g* for 10 min at 4°C and re-suspended on ice in wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% (w/v) glycerol). The resulting

cellular suspension was then French pressed at 20,000 psi twice to lyse cells. The lysate was clarified by centrifugation at 12,000 x *g* for 20 min. The clarified supernatant was collected and diluted with 1,000 mL of wash buffer before application to a pre-equilibrated TALON metal affinity resin (Clontech, Mountain View, CA) column. The lysates were applied to the columns twice before washing with new wash buffer. Bound protein was eluted in buffer containing 50 mM sodium phosphate, 200 mM NaCl, 150 mM imidazole, and 20% glycerol (w/v). Purified protein was concentrated with Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA) and dialyzed against PBS + 10% glycerol (w/v) in Slide-A-Lyzer dialysis cassettes (Thermo Scientific, Rockford, IL). Protein concentrations were determined with the Bradford Protein Assay Kit (Thermo Scientific) and purified proteins were stored at -20°C for future use. Purified proteins were visualized by Coomassie blue staining of 15% SDS-PAGE gels and 10% native gels (GelCode Blue stain, Thermo Scientific, Rockford, IL) followed by immunobloting with anti-YscF.

Preparation of purified needle samples

Bacterial cultures were grown at 37^0C for 8 h in 60 ml heart infusion broth (HIB, BD Difco) subcultured 1:100 into fresh HIB and incubated overnight at 37^0C. Samples were prepared as reported previously (63). Briefly, overnight cultures were harvested by centrifugation (10 min at 10,000 x *g*), washed twice in 20 mM Tris-HCl (pH 7.5). The cell suspension was transferred to a 40 ml Dounce glass-glass tissue grinder (Wheaton, Millville, NJ) that shaves needles from the surface of cells through the exertion of sheer force for 60 cycles. Unbroken cells and debris were

removed (10 min at 10,000 x *g*) and the supernatant was passed through a 0.45 µm cellulose acetate membrane filter (Whatman, GE Healthcare Life Sciences, Pittsburgh, PA) to remove all bacteria. The supernatant was then centrifuged at 60,000 x *g* for 30 min using a Beckman Coulter JA 25.15 rotor. The sediment was suspended in 50 µl of 20 mM Tris-Hcl (pH 7.5) + 5% sucrose and subsequently loaded onto a 6 ml step-gradient of 70%, 20% and 10% sucrose (2 ml each). 1 ml fractions were collected and analyzed by Coomassie blue staining of 15% SDS-PAGE gels and 10% native gels (GelCode Blue stain, Thermo scientific, Rockford, IL) and immunobloting for YscF.

Electrophoresis and protein detection.

Purified needle proteins were used to load 15% SDS-polyacrylamide gels electrophoresis (SDS-PAGE) or 10% native gels. Samples were boiled in 2X sample buffer for 10 minutes before loading on gels. For silver staining, SDS-PAGE and native gels were ethanol fixed and stained according to the manufacturer's instructions. Gels were also fixed with methanol and stained with Coomassie blue (GelCode Blue stain, Thermo Scientific, Rockford, IL) over night and subsequently destained with deionized water to enhance staining sensitivity and clear background. SDS-PAGE and native gels separated proteins were transferred to Immobilon-P membrane for immunoblot analysis of YscF using specific anti-YscF antibody. Primary antibodies (rabbit polyclonal antibody) were used at 1:20,000 dilution followed by detection with alkaline phosphatase conjugated secondary antibodies (goat anti-rabbit immunoglobulin G: Pierce) at a dilution of 1:20,000.

Standard colorimetric detection method using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP, Thermo Fisher Scientific, Chicago, IL) was used for color development.

Enzyme digestion of purified needle protein

Needle proteins and flagellin (standard flagellin from *Salmonella* Typhimurium; Invivogen) were incubated with 40μg/ml proteinase K (Thermo-Fisher) at 37°C for 16 h. Proteinase K was then inactivated with 1.6 mg/ml phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO). Needle proteins and Pam3CSK4 (Invivogen) were incubated with 50 μg/ml of bacterial lipoprotein lipase (LPL) (lipoprotein lipase from *Pseudomonas* sp.; Sigma-Aldrich) in 1× PBS at 37°C for 12 h. THP-1 cells were stimulated with 20 μg/ml of polymyxin B (Invivogen) and needle proteins or lipopolysaccharide (LPS) as a control for 24 h. PBS treated with proteinase K, lipoprotein lipase, or polymyxin B was used as a negative control.

Stimulation of cell line with needle proteins

THP1-XBlue cells were seeded at 2 × 10^6 cells/ml. Cells were suspended in infection medium (RPMI or DMEM alone) as described by the manufacturer. Proteins were added to a final concentration of 1 μg/ml or as indicated. As designated, 20 μg/ml of anti-YscF antibodies (control polyclonal antibody) was added to cell cultures prior to addition of needle proteins. Cells were stimulated at 37°C and 5% CO_2 for 24 h.

Cell culture

The human monocyte cell line THP-1 (ATCC TIB-202) and THP1-XBlue Cells (Invivogen, San Diego, CA) were maintained in RPMI 1640 (Corning Cellgro, Manassas, VA) containing 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), 25 mM HEPES (Fisher Scientific, Pittsburgh, PA), 2 mM L-glutamine (Corning Cellgro), 1 mM sodium pyruvate (Corning Cellgro) and 50 µg/ml pen-strep (Corning Cellgro) at 37°C with 5% CO_2. The THP1-XBlue cells contain the secreted embryonic alkaline phosphatase (SEAP) reporter gene under control of NF-κB and AP-1. Stimulation of THP1 and THP1-XBlue cells were performed as reported previously (63). Briefly, THP1-XBlue cells were seeded at 3 x 10^6 cells/ml into 96 well plates and THP1 cells seeded at 8x10^5 cells/ml into 24 well plates. Cells were suspended in infection medium as described by the manufacturer. Proteins were added at a final concentration of 1 µg/mL. Cells were stimulated at 37°C with 5% CO_2 for 5 h or 24 h, as indicated.

SEAP reporter assays

Quantification of secreted embryonic alkaline phosphatase (SEAP) from the supernatant was detected using Quanti-Blue reagent (Invivogen) according to manufacturer's protocol. A microplate reader (Synergy HT, BioTek, Winooski, VT) was used to quantify SEAP activity by measuring absorbance at 630 nm, data was collected using KC4 v3.3 software (BioTek).

Cytokine analysis

THP1 cells were stimulated with PBS, 1 μg/ml heat killed *Listeria monocytogenes* (HKLM, Invivogen), 1 μg/ml LPS (LPS-EK Ultrapure, Invivogen) 1 μg/ml flagellin (FLA-ST, Invivogen) or 1 μg/ml of needle proteins for 5-24 h and cellular supernatants were collected and stored at -20^{0}C before analysis with Quantikine Elisa kits from R&D systems (Minneapolis, MN). Human TNF-α, IL-6 and IL-8 kits were used as instructed by the manufacturer.

Transcomplementation

Plasmids were isolated using the Qiaprep Miniprep Kit (Qiagen). DNA fragments encoding for the N-terminally deleted YscF constructs or the SsaG chimeras were amplified by PCR and purified using the QiaQuick PCR purification kit (Qiagen). The primers used to amplify the DNA fragments are listed in Table 2. Gene amplification was performed with PFU Ultra II HS DNA polymerase (Agilent Technologies, Santa Clara, CA) in a Mastercycler gradient thermal cycler (Eppendorf, Hauppauge, NY). The amplified sequences were digested with EcoR1 and ligated into EcoR1 and Sma1 cleaved pBAD18-kan (54). Mutations were confirmed by sequencing and the resulting plasmids transformed into a Δ*yscF Y. pestis* or ΔSsaG *Salmonella entrica* serovar Typhimurium. Transformants were selected on a TBA plates with kanamycin, streak purified and subsequently used for growth curves, cytotoxicity assay or gentamicin protection assay.

Growth curves

Growth curves were performed as described by Straley *et al* (117). Briefly, bacteria were grown in TMH medium (57) at 26^0C overnight, subcultured into fresh TMH medium at an A_{620} 0.1 (plus 2.5 mM $CaCl_2$ and 0.1% L-arabinose (BD Difco, Sparks, MD)) and grown until the A_{620} reached 0.2. The temperature was shifted to 37^0C and incubated for 6 h while taking A_{620} readings at 1 h intervals. 1 mL of sample was aliquoted into 1.5mL tubes, centrifuged at 20,000 x *g* for 5 min at 4^0C and pellets separated from the supernatant, which contains secreted proteins. The pellet and supernatants were precipitated with 10% (w/v) trichloro acetic acid (TCA) followed by SDS-PAGE and silver staining.

HeLa cell infection assay

Yop translocation was monitored visually by cytotoxicity (cells rounding up) as described previously (92, 124). Briefly, HeLa cells were grown in Dulbecco's Modified Eagle Minimum essential medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen), and 50 µg/ml pen-strep (Corning Cellgro) at 37°C with 5% CO_2. HeLa cells were seeded into 24-well tissue culture plates and after the HeLa cells reached near confluency the growth medium was removed and the cells washed twice with L15 medium and placed in fresh L15 medium containing 0.1% (w/v) L-arabinose (BD Difco, Sparks, MD). Bacteria were added to cell monolayer at a multiplicity of infection (moi) of 10:1. The plates were centrifuged at 800 x *g* for 5 min to allow cell contact. The plates were incubated at 37°C for 2–6 hours to check

for cytotoxicity and photographed at 4 h. Micrographs were captured on a Nikon D70 digital camera.

Gentamicin-Protection survival assay.

For macrophage infections, THP-1 cells were seeded at $5x10^5$ cells per well in 24-well tissue-culture dishes and were differentiated into macrophages by addition of 50 mM phorbol 12-myristate 13-acetate (PMA) for 48 h. Macrophage infections were performed as described by Forest *et al.,* (48) with minor modifications. Briefly, bacteria were grown overnight without shaking in LB broth. The overnight culture was sub-cultured to an A_{600} of 0.1 and grown to an A_{600} of 0.6. Bacteria were added to cell monolayer at a multiplicity of infection (moi). of 10:1 and centrifuged at 800 x *g* for 5 min to synchronize bacterial uptake. After incubation at 37^0C for 2 h, extracellular bacteria were removed by washing cells with PBS and incubated again with medium containing 100 μg/ml of gentamicin to kill extracellular bacteria for 2 h, then the cells were washed and the medium replaced with fresh medium containing 10 μg/ml gentamicin and 0.1% (w/v) L-arabinose (78) for another 20 h. Gentamicin resistant intracellular bacterial counts was determined by lysis of cells for 30 min at 37^0C with 1 ml 0.1% (v/v) Triton X-100 in H_2O and bacteria were enumerated by serial dilution in PBS and plating on LB agar containing kanamycin.

Image acquisition and production

All immunoblots, Coomassie and Silver stained gels were scanned on Epson 4490 Perfection scanner at 4800 dpi using VueScan Software (v.8.4.40; Hamrick

Software, (http://www.hamrick.com)). Scanned images were exported into Adobe photoshop (CS5.1, Adobe Software, San Jose, CA) and converted to grayscale. Images were assembled in Adobe illustrator (CS5.1) and downscaled to 600 dpi upon export to the TIFF image file format.

Data analysis and statistics

Data were assembled into graphs using GraphPad Prism, version 5.0d (GraphPad Software). Statistical analysis was completed using one-way analysis of variance with Bonferroni or Dunnett's Multiple Comparisons post-test as indicated.

CHAPTER III

RESULTS

Alignment of Needle proteins

Type III secretion systems found in animal pathogens can be divided into three main families: Ysc type injectisomes (e.g. *Yersinia* and *Pseudomonas*), *Shigella* and *Salmonella* SPI-1 type injectisomes, and *E. coli* and *Salmonella* SPI-2 type injectisomes (31). Our assessment of needle proteins includes at least one needle protein homolog from each T3S system family and represents both *Salmonella* injectisomes: needle proteins from *Yersinia pestis* (YscF), *Salmonella enterica* serovar Typhimurium SPI-1 (PrgI), SPI-2 (SsaG), and *Shigella flexneri* (MxiH). Needle proteins of T3S system are highly conserved, except for the N-termini (Figure 6). Representative needle protein sequences were aligned and this information was used to guide the construction and expression of truncated proteins for a select set of needle proteins. Truncated needle proteins were made by deletion of residues from the N-termini, the truncations correspond to the twenty-second amino acid of *Y. pestis* YscF. This resulted in truncation of amino acids A2-K15 from PrgI, S2-T18 from MxiH, and D2-M9 from SsaG.

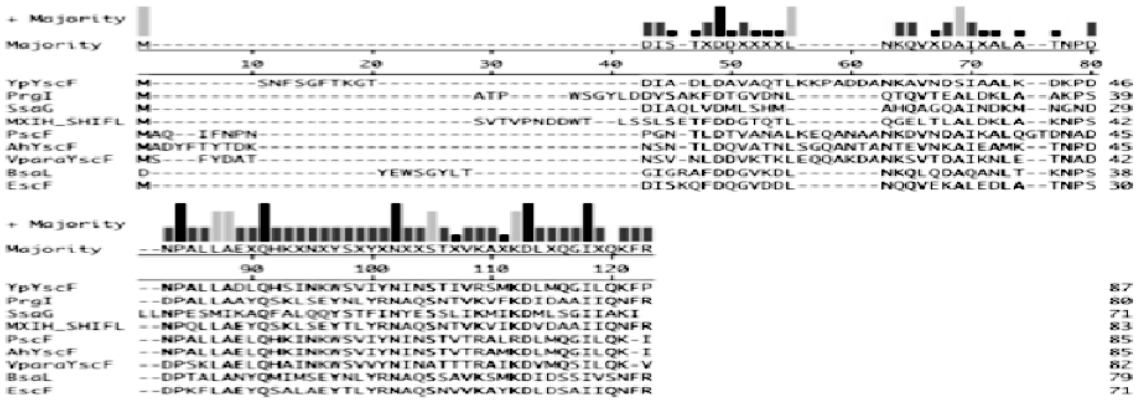

Figure 6. Multiple-sequence alignment of needle proteins demonstrates that the N termini of T3S needle proteins are not conserved.

Needle protein sequences from several species of bacteria were aligned with Megalign from the DNAStar Lasergene package (v. 10.1), using the Jotun Hein algorithm with the PAM250 matrix. Identical residues are shown in shaded boxes. Aligned needle proteins are from *Y. pestis* (YpYscF), *S. enterica* (PrgI and SsaG), *S. flexneri* (MXIH_SHIFL), *P. aeruginosa* (PscF), *Aeromonas hydrophila* (AhYscF), *Vibrio parahaemolyticus* (VparaYscF), *Burkholderia pseudomallei* (BsaL), and *E. coli* (EscF).

N-terminally Truncated Needle Proteins induce NF-κB/AP-1 activation

Recently T3S needle proteins were demonstrated to function as PAMPs for TLR2 and TLR4 (63). Published work suggests that the N-terminus of needle proteins is not necessary for needle assembly or function (66, 134). These observations led to a hypothesis that the non-conserved N-terminus may function in the recognition of needle proteins by the host. To examine this possibility, activation of NF-κB/AP-1 (using a reporter system) was used to evaluate the effect of removing the N-terminus of needle proteins on immune stimulation. Recombinant whole needle proteins and truncated forms of needle proteins were used to treat THP1-XBlue cells and NF-κB/AP-1 activation was assessed by measuring secreted embryonic alkaline phosphastase (SEAP) production. SEAP expression in THP1-XBlue cells is under control of the transcriptional activators NF-

κB and AP-1 that activate cytokine and chemokine expression critical for innate immune responses. Therefore an increase in SEAP expression equated to an increase in NF-κB and/or AP-1 activity. All proteins were applied at 1 μg/ml to cells and, as expected, all of the full-length proteins induced NF-κB/AP-1 activation. Truncated forms of YscF and PrgI significantly activated NF-κB/AP-1 more than their full-length counterparts (Figure 7A). However the truncated form of MxiH had lower levels of NF-κB/AP-1 activation compared to the full length MxiH (Figure 7A). These results showed that the truncated forms of YscF and PrgI increase NF-κB/AP-1 activation to higher levels in comparison to the full-length proteins. SsaG, which is only expressed within eukaryotic cells, activated cells only slightly more than the truncated form. MxiH activated NF-κB/AP-1 more than the truncated form of MxiH. The results suggested that the N-terminus (notably residues corresponding to the first 15 amino acids of YscF) of needle proteins act to influence host recognition of the proteins. The responses from the N-terminal truncations of YscF and PrgI suggested that the presence of the non-conserved N-termini may inhibit NF-κB/AP-1 activation in some cases, whereas, the presence of the N-terminus of MxiH may increase NF-κB/AP-1 activation by MxiH in THP1-XBlue cells.

When needle proteins (both truncated and full length) were incubated with THP1-XBlue cells deficient in MyD88, an adaptor protein required for most TLR-mediated responses (1, 21), the activation of NF-κB/AP-1 was abolished (Figure 7B). The lack of NF-κB/AP-1 activation in cells lacking MyD88 indicated that the NF-κB/AP-1 activation by the truncated needle proteins was most likely occurring

through TLR2 and TLR4 recognition of the needle proteins as previously demonstrated (63) for the full length proteins.

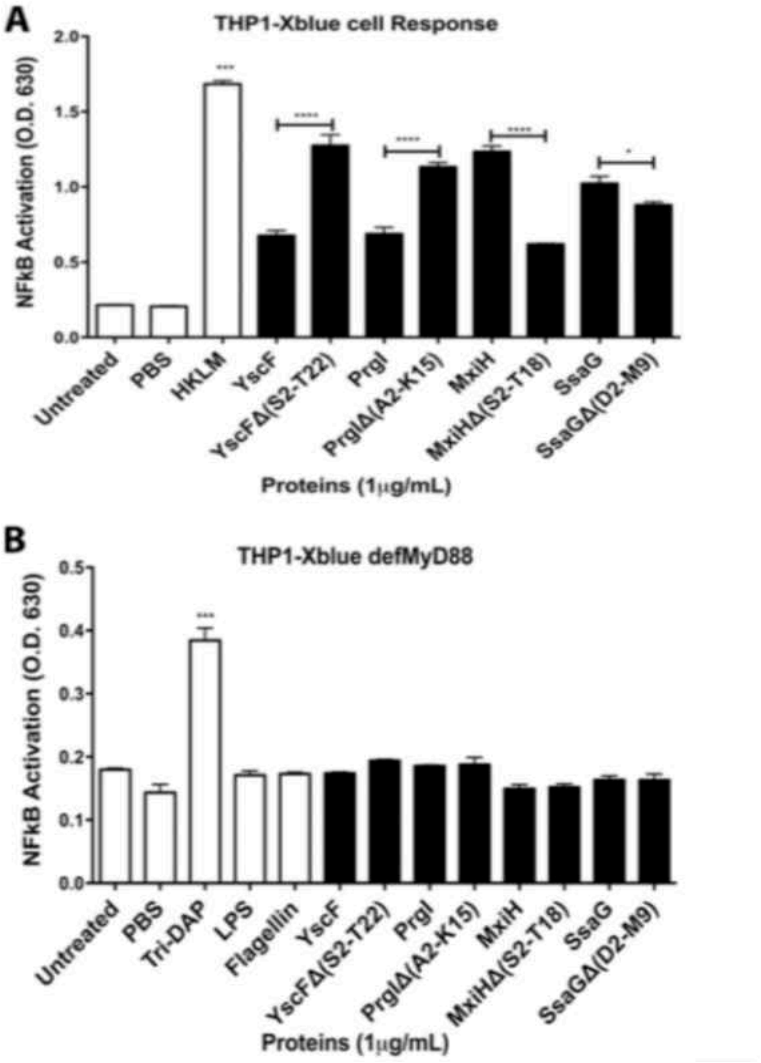

Figure 7. Full length and truncated needle proteins activate NF-ϰB/AP-1 in THP1-XBlue cells in a MyD88 dependent manner.

THP-1 (A) and THP-1 defMyd88 (B) cells were seeded in wells and treated with PBS, 1 μg/ml HKLM (Heat Killed *Listeria monocytogenes*), 1 μg/ml LPS, 1 μg/ml Flagellin, 1 μg/ml L-Ala- γ-D-Glu-meso-diaminopimelic acid (tri-DAP) or 1 μg/ml of needle protein dissolved in PBS. SEAP levels were measured as representation of NF-κB/AP-1 activation. Error bars represent SEM. n=3. Data shown are representative of at least three experiments. *, *P* is between 0.05 and 0.01; ***, *P* is between 0.001 and 0.0001, ****, *P* <0.000

Protein in purified needle protein preparations is responsible for induction of SEAP in THP1-XBlue cells.

Needle proteins and flagellin digested with proteinase K were added to THP1-XBlue cells to confirm that proteins were activating NF-κB/AP-1 (Fig. 8A). Proteinase K is a serine protease that cleaves after hydrophobic amino acids. Proteinase K in PBS was used as a negative control. As expected, flagellin (a TLR5 agonist; positive control) treated with proteinase K abrogated the NF-κB/AP-1 response, and proteinase K alone did not activate NF-κB/AP-1, demonstrating that the proteinase K used did not contain a TLR agonist and that, as expected, proteinase K treatment eliminated the ability of flagellin to activate NF-κB/AP-1 (Fig. 8A). The proteinase K (Fig. 8A)-treated needle proteins also failed to elicit NF-κB/AP-1 activation, confirming that proteins caused the cellular activation of NF-κB/AP-1.

To further demonstrate that the needle proteins, not contaminants, were responsible for induction of cellular responses, the purified needle proteins and His6-LcrG (purified in the same manner as the needle proteins) were incubated with bacterial lipoprotein lipase (LPL) to inactivate bacterial lipoproteins (Fig. 8B) or treated with polymyxin B (40 μg/ml) to neutralize LPS (Fig. 8C). Treatment of YscF with LPL slightly decreased SEAP induction. Treatment of all the other needle

proteins with LPL did not affect the ability of needle proteins to induce SEAP expression in THP1-XBlue cells (Fig. 8), showing that the induction seen was not due to contaminating lipoproteins. Treatment with polymyxin B resulted in no significant change in the ability of all tested needle proteins to induce SEAP expression, demonstrating that induction of SEAP was not due to LPS. Treatment of the LPS and Pam3CSK4 positive controls with either polymyxin B or lipoprotein lipase abolished their ability to induce SEAP activity by THP1-XBlue cells (Fig. 8). His_6-LcrG failed to induce SEAP expression in THP1-XBlue cells, indicating that the increase in SEAP expression by THP1-XBlue cells in response to the needle proteins does not occur with all proteins purified by our method (Fig. 8A and B).

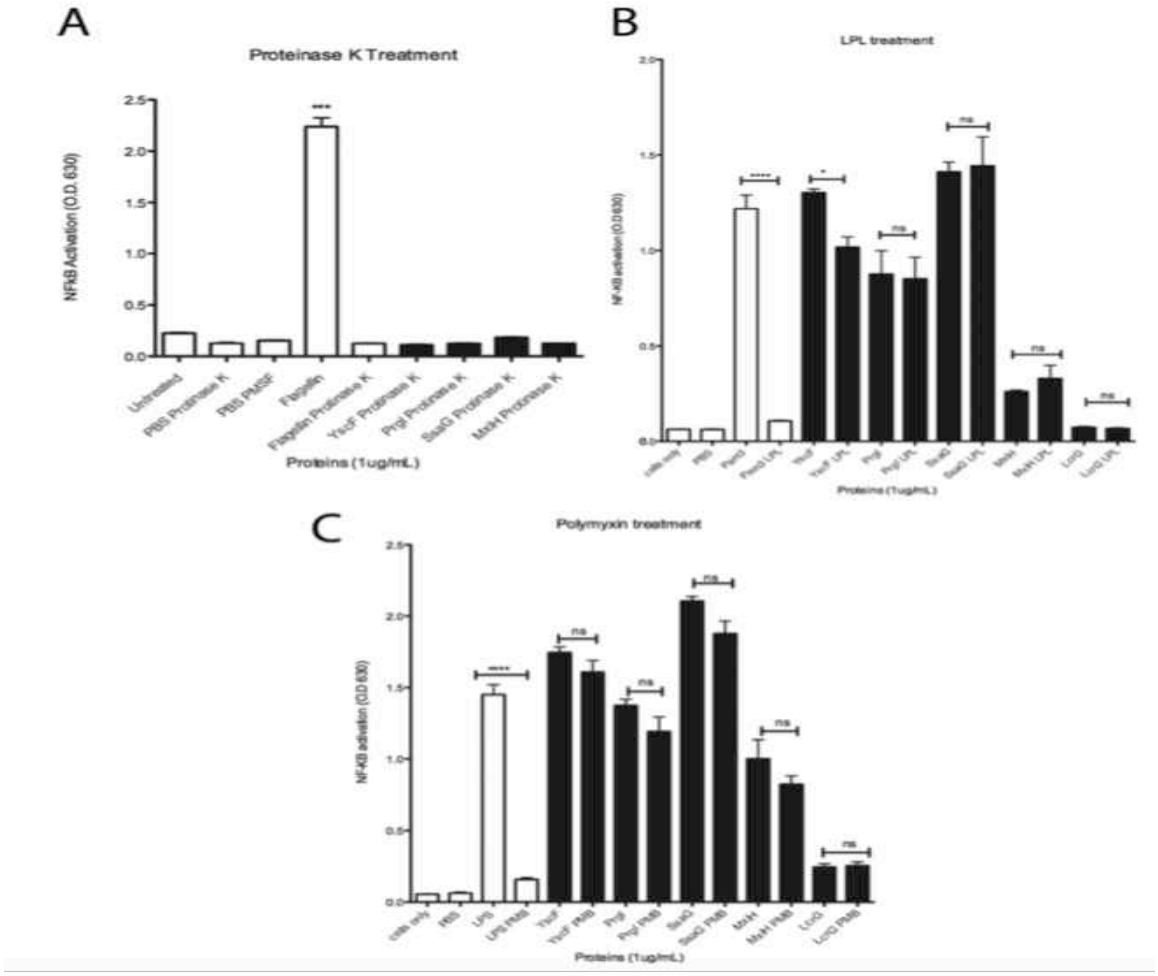

Figure 8. Treatment of needle proteins with proteinase K, lipoprotein lipase, and polymyxin B retains NF-κB/AP-1 activation.

Needle proteins (1 μg/ml) (A, B, and C) and LcrG (1 μg/ml) (B and C), PBS, flagellin (1μg/ml) (A), Pam3 (1 μg/ml) (B), or LPS (μg/ml) (C) were incubated with proteinase K (A), lipoprotein lipase (LPL; 50 μg/ml) (B), or polymyxin B (PMB; 20 μg/ml) (C) before addition to THP1-XBlue cells. *, *P* is between 0.05 and 0.01; ***, *P* is between 0.001 and 0.0001; ****, *P* < 0.0001; ns, not significant.

Cytokine expression in response to needle proteins.

Because the needle protein-induced NF-κB/AP-1 responses in THP1- XBlue cells were detected using a reporter gene, we wanted to examine if those responses translated to cytokine secretion from nontransfected THP-1 cells. Therefore, THP-1 cells were treated with PBS, HKLM, or needle proteins. After 5 h, supernatants were collected and assessed via ELISA for TNF-α and after 24 h, supernatants were collected and analyzed for IL-6 and IL-8 production. As expected, PBS-treated cells produced no TNF-α, IL-6, or IL-8. HKLM and all needle protein-treated cells led to production of all 3 cytokines (Fig 9). These results indicated that the NF-κB/AP-1 activation triggered by needle proteins led to increased expression of TNF-α, IL-6, and IL-8 that resulted in secretion of the cytokines into cell culture supernatants.

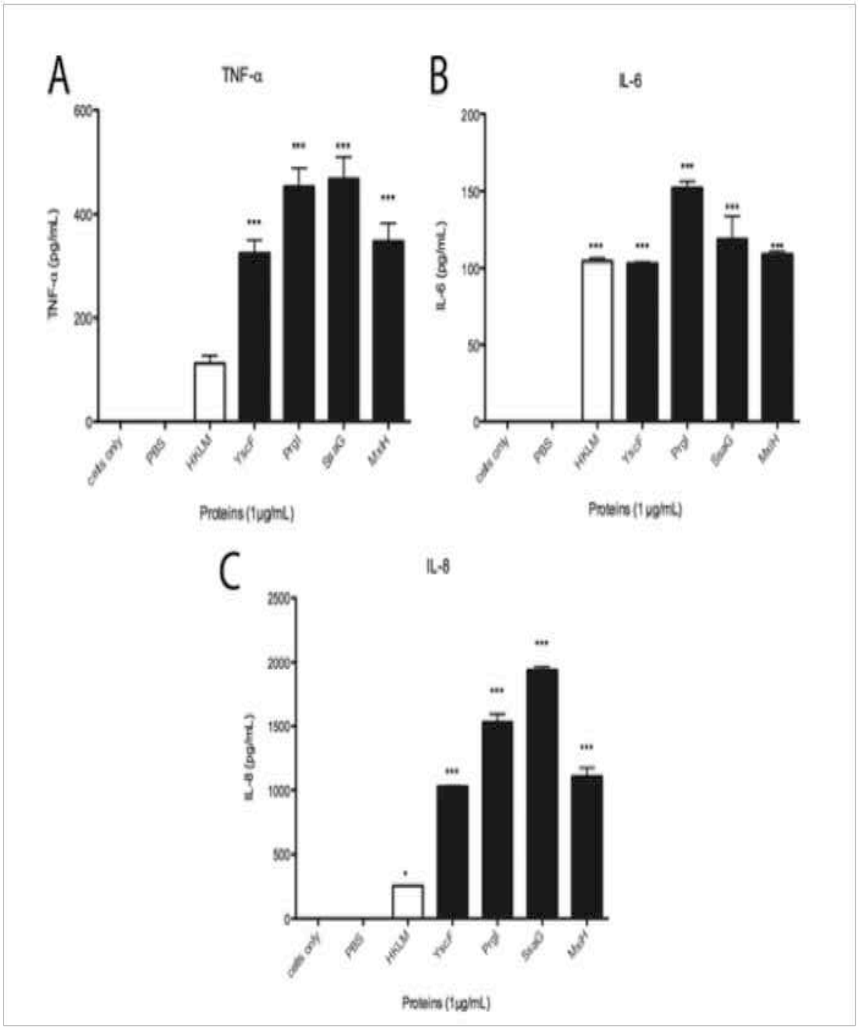

Figure 9. Activation of THP-1 cells by needle proteins results in cytokine secretion.

THP-1 cells were treated with PBS, 1 μg/ml of HKLM, or needle proteins (1 μg/ml). After 5h (TNF-α) and 24 h (IL-6 and IL-8), supernatants were collected and tested by ELISA for production of TNF-α (A), IL-6 (B), and IL-8 (C). Error bars represent SEM (n =3). Data shown are representative of at least three experiments. *, P is between 0.05 and 0.01**, ***, P is between 0.001 and 0.0001.

THP1-XBlue cells respond to sheared Ysc needles.

Our results demonstrated that recombinant needle proteins purified from *E. coli* activated NF- NF-κB/AP-1, leaving the question of whether native needle proteins could activate NF-κB /AP-1. Accordingly, purified needles from *Y. pestis* were tested for the ability to activate NF-κB /AP-1. Needles from *Yersinia pestis* were sheared from the bacterial surface to obtain YscF. The presence of purified YscF was confirmed by Coomassie blue staining and immunoblotting with YscF primary antibody (Fig. 10A). Addition of purified YscF to THP1-XBlue cells resulted in SEAP expression, while addition of a mock purified fraction from Δ*yscF Y. pestis* had no effect, demonstrating that native YscF could also induce NF-κB/ AP-1 (Fig. 10B). Treatment of needle fractions with lipoprotein lipase and THP1-XBlue cell treatment with polymyxin B indicated no contamination of lipoproteins. Activation of NF-kB/AP-1 in THP1-XBlue cells was seen in response to the YscF-enriched fraction (Fig. 10B), suggesting not only that recombinant needle proteins activate host cells but also that native YscF induces NF-κB/AP-1. This was further confirmed by obtaining a fraction from a strain of *Y. pestis* lacking *yscF* that did not induce NF-kB/AP-1. To confirm that YscF was activating NF-κB/AP-1, the THP1-XBlue cells were treated with anti-YscF alone or anti-YscF with native YscF (Fig. 10C). Antibody to YscF significantly inhibited activation of NF-κB/AP-1. This result demonstrates that neutralizing YscF can abrogate the effect of YscF to activate NF-κB/AP-1.

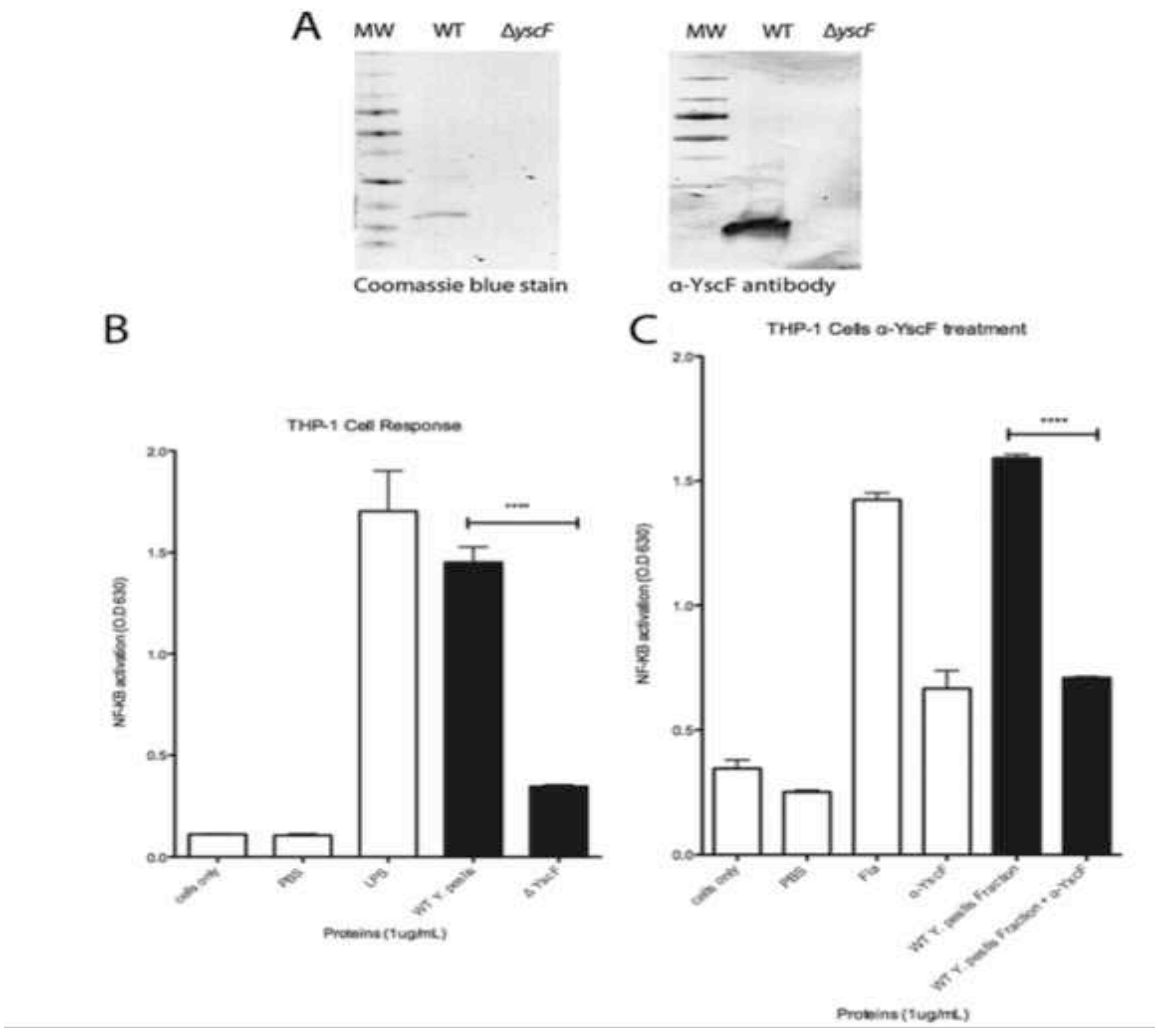

Figure 10 THP-1 X-Blue cells respond to YscF from *Y. pestis*.

(A) Coomassie blue-stained gel and immunoblot of YscF purified from *Y. pestis*, detected with rabbit anti-YscF primary antibody. (B) THP1-XBlue cells were seeded in wells and treated with PBS, 1 μg/ml of LPS, purified YscF needle fractions (1 μg/ml) from *Y. pestis*, or a mock purified preparation from a *Y. pestis* Δ*yscF* strain. (C) THP1-XBlue cells were treated with flagellin (1 μg/ml), anti-YscF alone, YscF (1 μg/ml), or YscF incubated with anti-YscF. SEAP activity was measured as a representation of NF-κB AP-1 activation. Error bars represent SEM (*n* =3). Data shown are representative of at least three experiments. ****, *P* value of 0.001 to 0.01.

Definition of amino acids within the N-terminus involved in TLR activation.

Having demonstrated an effect by the N-terminus of the needle proteins in modulating NF-κB/AP-1 activation, the specific amino acids in the N-terminus of YscF involved in NF-κB/AP-1 activation were sought. A panel of *yscF* truncations was constructed that resulted in deleting amino acids S2-S5, S2-G10, S2-D15 or S2-A20 from the N-terminus of YscF. Truncated YscF proteins were expressed and subsequently purified: their purity was assessed by Coomassie blue staining following SDS-PAGE (Figure 11). The ability of truncated YscF and full-length YscF to induce cytokines from THP-1 cells and NF-κB/AP-1 activation from THP1-XBlue cells was analyzed (Figure 12A). The truncated YscF proteins induced significantly higher cytokines levels (TNF-α, IL-6 and IL-8) (Figure 3B-D) and NF-κB/AP-1 activation (Figure 12A) than full-length YscF. Truncation of amino acids S2-D15 from the N-terminus of YscF resulted in the highest levels of cytokine and NF-κB/AP-1 activation, similar to levels observed with SsaG (Figure 7).

To confirm that proteins were activating NF-κB/AP-1 (20), needle proteins were digested with proteinase K and added to THP1-XBlue cells. As expected, the proteinase K treated needle proteins failed to activate NF-κB/AP-1 activation (Fig 12), suggesting that proteins caused the activation of NF-κB/AP-1. To further

demonstrate that needle proteins, not LPS or lipoprotein contaminants were responsible for the cellular response, needle proteins were incubated with lipoprotein lipase to inactivate lipoproteins (LPL) or treated with polymyxin B (PMB) to neutralize LPS. Treatment of needle proteins with either LPL or PMB did not affect the ability of the proteins to induce NF-κB/AP-1 activation (12 B&C), showing that the cytokine expression seen was not due to contaminating lipoproteins or LPS.

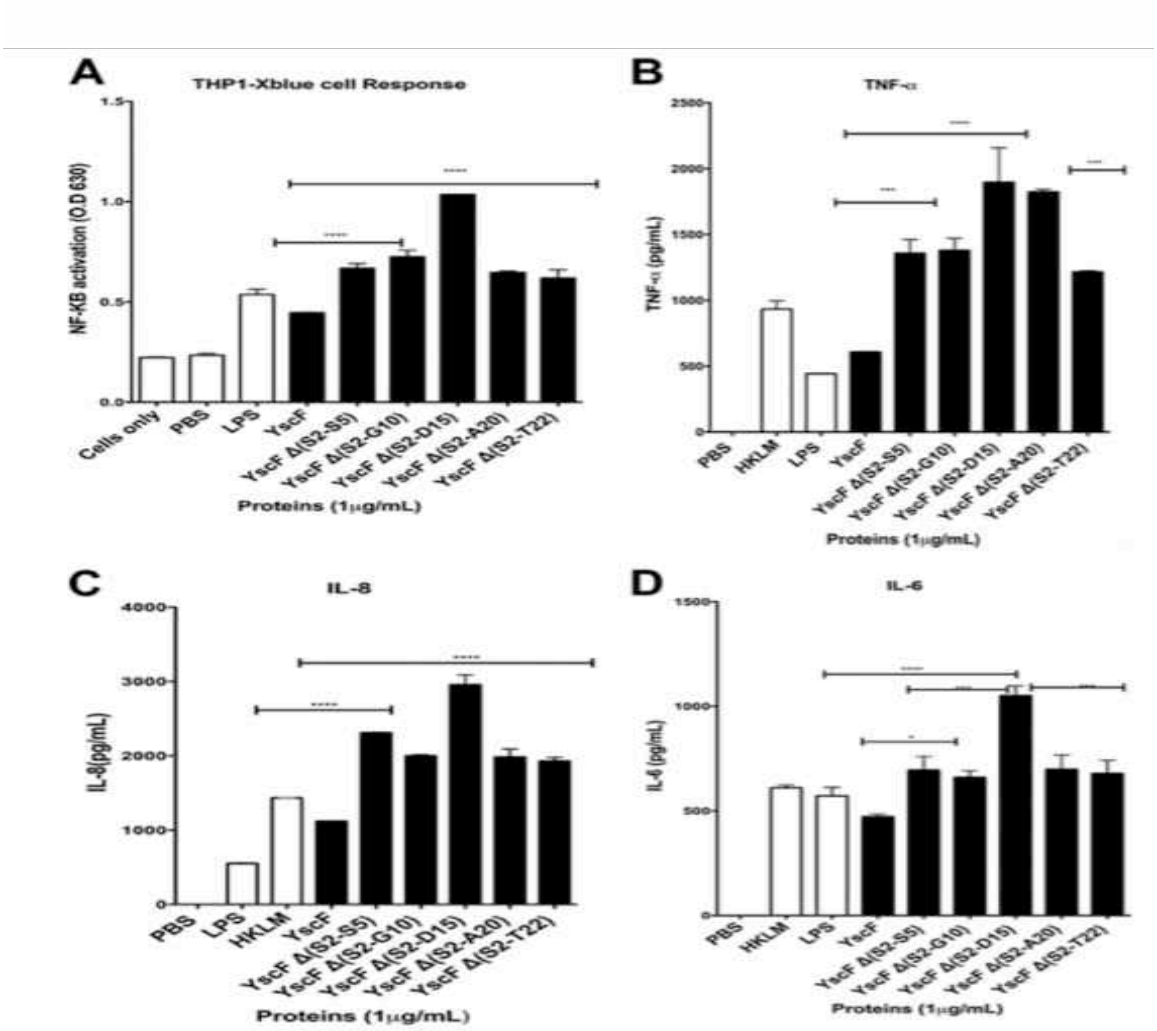

Figure 11. YscF truncated at 15 amino acids has the highest NF-ϰB/AP-1 and cytokine activation. (A) THP1-XBlue cells were seeded in wells and treated with PBS, 1 μg/ml LPS, or 1 μg/ml of recombinant needle protein dissolved in PBS. SEAP levels were measured as representation of NF-ϰB/AP-1 activation. (B-D) THP-1 cells were treated with PBS, 1 μg/ml of HKLM, or recombinant needle proteins (1 μg/ml). After 5 h (TNF-α) and 24 h (IL-6 and IL-8), supernatants were collected and tested by ELISA for production of (B) TNF-α, (C) IL-8 and (D) IL-6. Error bars represent SEM (n=3). Data shown are representative of at least three experiments. *, *P* is between 0.05 and 0.01; ***, *P* is between 0.001 and 0.0001; ****, P<0.000; ns, not significant.

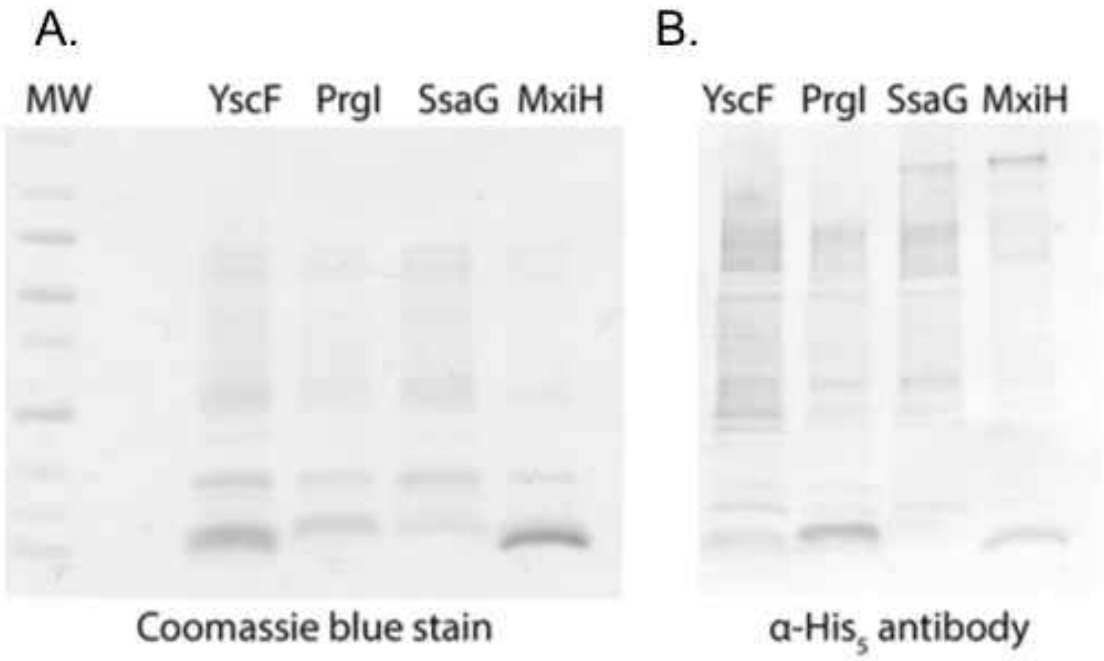

Figure 12: (A) Coomassie Blue stained SDS-PAGE gel of needle-protein purifications and (B) Immunoblot of needle proteins probed with Anti-His5 antibody.

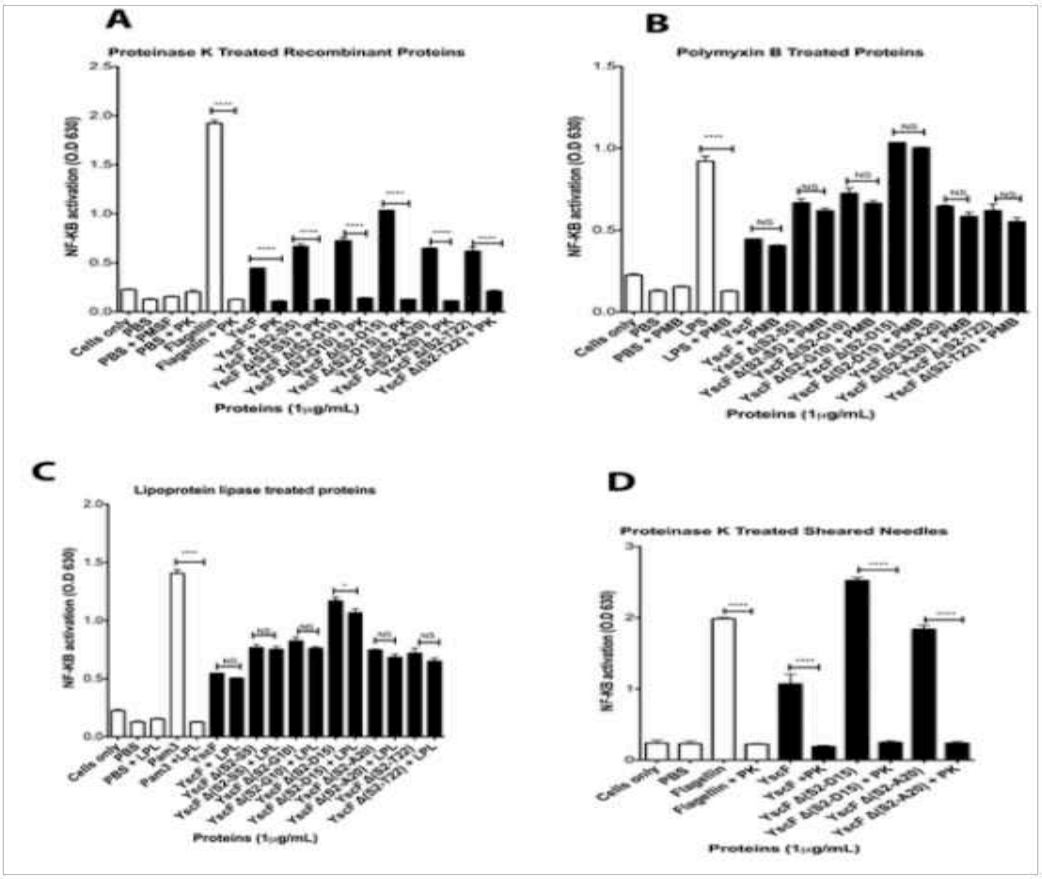

Figure 13. Purified Needles treated with Proteinase K, lipoprotein lipase, polymyxin B and anti-YcsF indicate needles cause NF-kB/AP-1 activation.

Purified recombinant needle proteins (1µg/ml), PBS, LPS (1 µg/ml) or Pam3 (1 µg/ml) were incubated with (A) Proteinase K (B) polymyxin B (PMB; 20 µg/ml) and (C) lipoprotein lipase (LPL; 50 µg/ml), before addition to THP1-XBlue cells. (D) Purified sheared needle proteins (1µg/ml) were incubated with Proteinase K, before addition to THP1-XBlue cells. SEAP activity was measured as a representation of NF-kB/AP-1 activation. Error bars represent SEM. n=3. Shown experiments are representative of at least three experiments. *, *P* is between 0.05 and 0.01; ***, *P* is between 0.001 and 0.0001; ****, P<0.000; ns, not significant.

Truncated sheared YscF behave similar to truncated recombinant proteins

Wild type sheared YscF needles are able to trigger NF-κB/AP-1 activation and cytokine (TNF-α, IL-6 and IL-8) expression (63). To verify whether truncated sheared YscF needles could also activate cytokine (TNF-α, IL-6 and IL-8) expression, a Δ*yscF Y. pestis* strain was transcomplemented with plasmids expressing truncated forms of YscF: YscF Δ(S2-D15) or YscF Δ(S2-A20). When these sheared needles composed of truncated YscF were used to treat THP-1 cells, activation was significantly higher with truncated YscF needles than wildtype needles (Figure 14A). The higher NF-κB/AP-1 activation translated to higher cytokine (TNF-α, IL-6 and IL-8) expression similar to what was observed with the recombinant proteins (Figure 14 B-D). The needle formed by N-terminal truncation of amino acids S2-D15 had the highest activation of NF-κB/AP-1 and cytokine expression. This result with sheared needles paralleled results seen in Figure 7 and Figure 11 with recombinant YscF needle proteins. Similar to the recombinant proteins, treatment of sheared needles with proteinase K abrogated NF-κB/AP-1 activation of THP1-XBlue cells (Fig 14D). To further confirm that YscF was activating NF-κB/AP-1, the sheared needles were treated with anti-YscF. Antibody to YscF significantly reduced NF-κB/AP-1 (Fig 14E), demonstrating that neutralizing YscF can abrogate the YscF-activation of NF-κB/AP-1. These results demonstrated that needles composed of the

truncated forms of YscF could activate NF-κB/AP-1 and cytokine expression in a manner analogous to wild type needles.

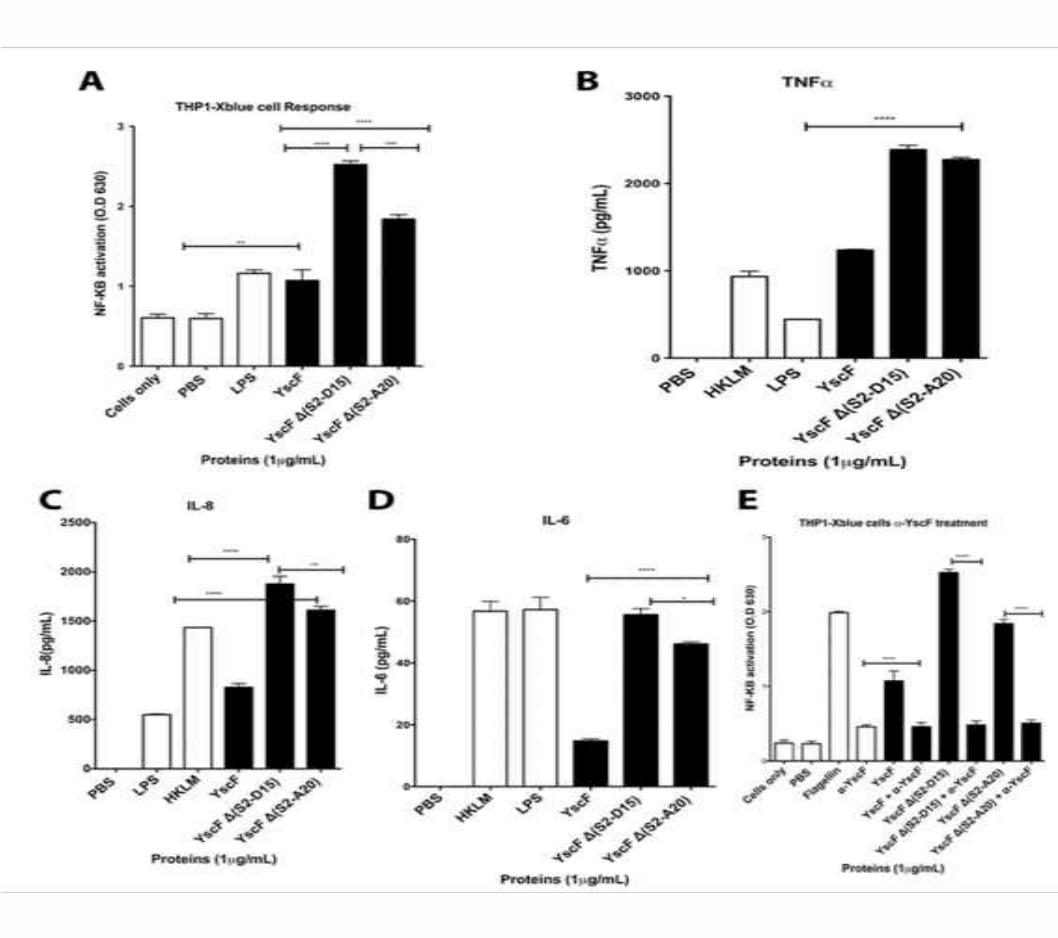

Figure 14. Ysc Needles lacking the N-terminus of YscF increases NF-κB/AP-1 and cytokine activation. (A) THP1-XBlue cells were seeded in wells and treated with PBS, μg/ml LPS, or 1 μg/ml of sheared needle dissolved in PBS. SEAP levels were measured as representation of NF-NF-κB AP-1 activation. (B-D) THP-1 cells were treated with PBS, 1 μg/ml of HKLM, or purified needles (1 μg/ml). After 5 h (TNF-α) and 24 h (IL-6 and IL-8), supernatants were collected and tested by ELISA for production of (B) TNF-α, (C) IL-8 and (D) IL-6. (E) THP1-Xblue cells were treated with anti-YscF alone, YscF (μg/ml), or YscF incubated with anti-YscF. Error bars represent SEM (n=3). Data shown are representative of at least three experiments. *, *P* is between 0.05 and 0.01**, *P* is between 0.01 and 0.001; ***, *P* is between 0.001 and 0.0001; ****, *P* <0.0001

N-terminal truncations of YscF are responsible for the differences in NF-κB activation of THP1-Xblue cells.

Full-length forms of monomeric needle proteins are known to have a strong tendency to oligomerize and self-associate (5). This needle polymerization can however be eliminated in some cases by truncating 5 or more amino acids from the C-terminus of PrgI, MxiH and BsaL (5). This modification does not have the same effect on YscF and PscF as they rapidly self-associate upon expression and purification (7). Consequently, truncation of 5 or 10 amino aids from the C-terminus did not eliminate needle polymerization of YscF (data not shown), suggesting that polymers could be the dominant protein species in our protein preparation samples.

To show whether the observed differences between the various mutants were due to differences in proportions of the protein species, purified recombinant and sheared needle proteins were visualized by Coomassie blue staining of SDS-PAGE gels and native gels, followed by immunoblotting with anti-YscF. There were no observed differences in band sizes between full length and truncated YscF. Additionally, all bands on the gel reacted with an anti-YscF antibody, demonstrating that the protein preparation samples were composed of YscF needle proteins (Figure 15). These results demonstrate that the differences in NF-κB and AP-1 activation were dependent on the number of amino acids truncated from the N-terminus of YscF and not differences in the proportions of the protein species. To further confirm that deleting amino acids from the N-terminus was responsible for

the observed differences in cytokine expression, THP1-XBlue cells were stimulated with C-terminally truncated YscF needle proteins. These C-terminally truncated YscF mutants activated NF-κB and AP-1 similar to full length YscF when added to THP1-XBlue cells (Fig 16). Taken together, our results argue that N-terminal truncations of YscF are responsible for the observed differences in NF-κB and AP-1 activation and not differences in proportion of protein species in our protein samples.

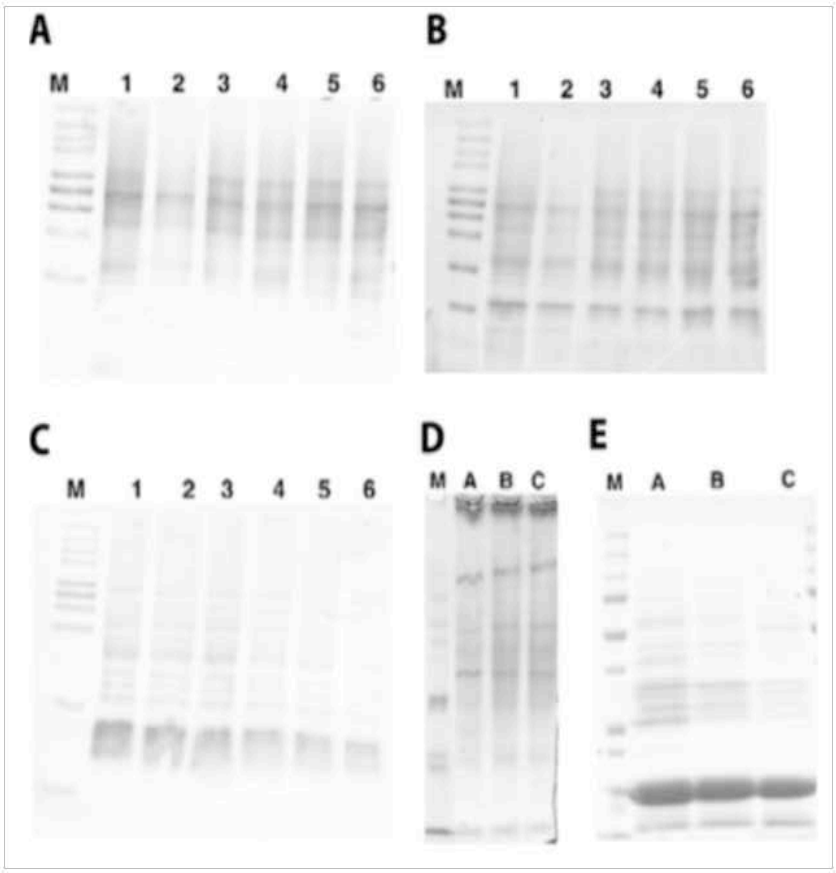

Figure 15 Coomassie Blue-stained SDS-PAGE and Native gels of purified needle proteins and immunoblot of YscF purified from *Y. pestis*, detected with anti-YscF primary antibody. (A&B) Coomassie blue stained Native gels (A) and SDS-PAGE gels (B) of recombinant needle proteins purifications. Lanes; YscF: 1 or YscFΔ((S2-S5)):2, (S2-G10):3, (S2-D15):4 (S2-A20):5 or (S2-T22):6). (C) Immunoblot of recombinant needle proteins probed with anti-YscF antibody. (D&E) Coomassie blue stained Native gels (D) and SDS-PAGE gels (E) of sheared needle proteins from *Y. pestis* KIM8-3002; (lanes A), *Y. pestis* Δ*yscF* KIM-3002.p61 containing plasmids pBAD18-Kan expressing YscF Δ(S2-D15) (lanes B) or YscF Δ(S2-A20), (lanes C)

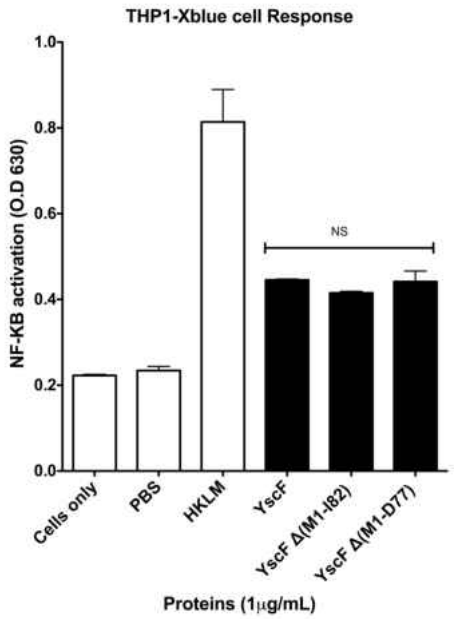

Figure 16. C-terminal truncation of YscF does not affect NF-kB activation in THP-1 Cells. THP1-XBlue cells were seeded in wells and treated with PBS, 1 μg/ml HKLM, or 1 μg/ml of recombinant needle dissolved in PBS. SEAP levels were measured as representation of κB/AP-1 activation. Error bars represent SEMs (n=3). Data are representative of at least three experiments. NS, not significant.

Truncated YscF forms functional needles.

The structure of these truncated proteins is not known and therefore the effect of these deletions on the function of YscF is uncertain. To address this issue a Δ*yscF Y. pestis* strain was transcomplemented with expression plasmids encoding YscF proteins (under control of the $araBAD_{pr}$) lacking amino acids S2-D15 (YscF Δ (S2-D15)) or S2-A20 (YscF Δ (S2-A20)). By comparing growth curves of Δ*yscF Y. pestis* transcomplemented with wildtype YscF to growth curves of Δ*yscF Y. pestis*

transcomplemented with mutant YscFs, no differences were seen between YscF Δ (S2-D15), YscF Δ (S2-A20) or full-length YscF (data not shown). Similar results were observed when Yops secretion and translocation profiles were compared (Figure 17 A, B); demonstrating that these truncated YscF proteins formed functional needles.

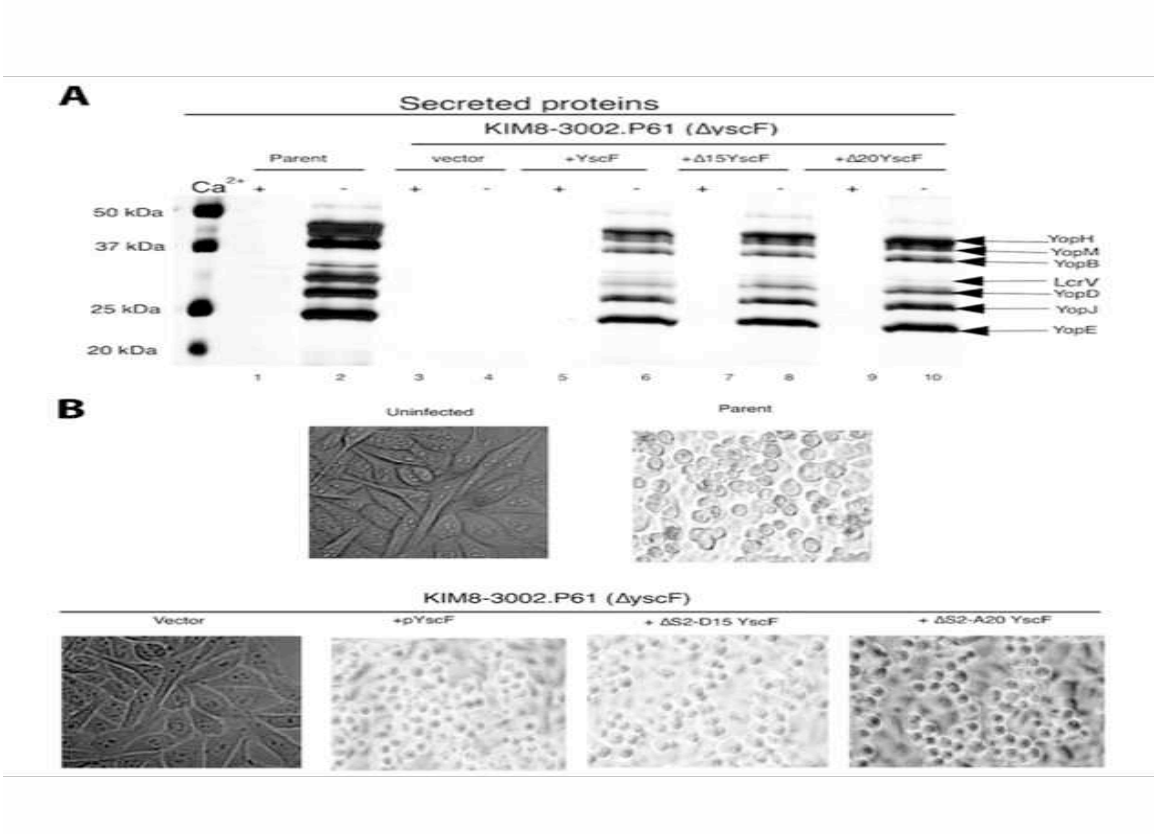

FIG. 17 Truncated forms of YscF form functional Needles. (A) Silver stain analysis of culture supernatant fractions from *Y. pestis* KIM8-3002; (Parent), *Y. pestis* Δ*yscF* KIM-3002.p61 containing plasmids pBAD18-Kan (+Vector), pBAD18-Kan expressing YscF, (+YscF), pBAD18-Kan expressing YscF Δ(S2-D15), (+S2-D15), pBAD18-Kan expressing YscF Δ(S2-A20), (+S2-A20) grown for 7 h at 37^0C in the presence (+) or absence (-) of 2.5 mM $CaCl_2$ and 0.1% (w/v) of L-arabinose.. Proteins were separated on a 12.5% SDS-polyacrylamide gel and detected by silver staining. (B) Hela cells were infected at MOI of 10 with same *Y. pestis* strains as in 5A. Images were captured 3 hours post infection on an Olympus IX50 inverted microscope fitted with a Nikon D70 digital camera (magnification 400×) to document cell cytotoxicity.

Alanine-scanning mutants of YscF reveal a region involved in cytokine expression

The above results showed that needles formed by N-terminal truncation of amino acids S2-D15 from YscF induced the highest activation of NF-κB/AP-1 and stimulation of cytokine (TNF-α, IL-6 and IL-8) production when used to treat THP-1 cells. This high activation was reduced by truncation of N-terminal amino acids S2-A20 similar to what was observed with the recombinant proteins. By comparing the aligned sequences (Figure 6), we identified 3 conserved amino acid residues in this region between amino acids D15 and Q21. This raised the possibility that certain amino acids within this region could be involved in NF-κB/AP-1 activation. The non-alanine codons (encoding residues L16, D17 and V19) in this region were selected for mutagenesis studies. Using site-directed mutagenesis the codons for these conserved residues (L16, D17 and V19) were substituted with alanine encoding codons. The individual alanine mutants were tested for NF-κB/AP-1 activation. The results demonstrated that 2 alanine mutants (YscF L16A and YscF V19A) had drastically reduced NF-κB/AP-1 activation while YscF D17A had no effect on NF-κB/AP-1 activation (Figure 18). Double mutant (YscF L16A V19A) and triple mutant (YscF L16A D17A V19A) YscF substitutions further reduced NF-κB/AP-1 activation. However, none of these YscF mutants reduced activation to wildtype levels. These

results suggested that the region between amino acids D15 and A20 were involved in the higher NF-κB/AP-1 activation of the YscF Δ (S2-D15) mutant, compared to the YscF Δ (S2-A20) mutant. Suggesting that the region of YscF between amino acid 15 and amino acid 20 plays a role in NF-κB/AP-1 activation. When these point mutants were examined in the TLR2 and TLR4 HEK 293 reporter cells, all point mutants showed similar cellular activation patterns to what was observed with the THP1-XBlue cells (Figure 18 B &C).

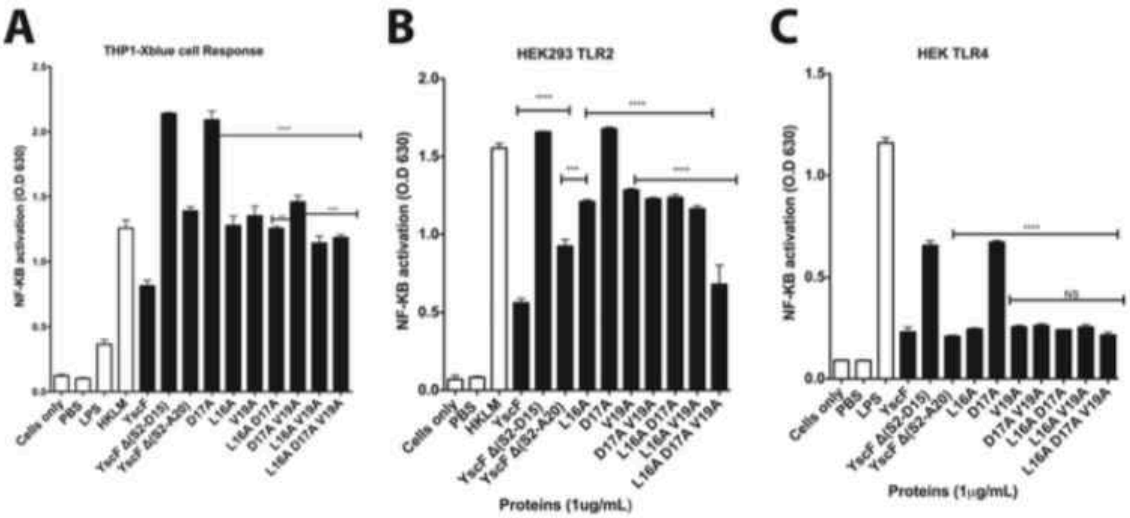

FIG 18: Alanine-scanning mutants of YscF Δ (S2-D15). (A) THP1-XBlue (B) HEK 293 TLR2 and (C) HEK 293 TLR 2 cells were seeded in wells and treated with PBS, 1 μg/ml LPS, 1 μg/ml HKLM or 1 μg/ml of recombinant needle proteins from the various YscF Δ(S2-D15) point mutants dissolved in PBS. SEAP levels were measured as representation of NF-ϰB/AP-1 activation. Error bars represent SEM (n=3). Data shown are representative of at least three experiments. **, *P* is between 0.01 and 0.001; ***, *P* is between 0.001 and 0.0001; ****, *P* <0.0001

The N-terminus of YscF reduces SsaG activation of NF-κB/AP-1 and cytokine expression

The results shown above demonstrated that the variable N-terminus of YscF modulates the interaction of the needle protein with host cells to influence NF-κB/AP-1 activation likely via TLR2 and TLR4. In order to confirm that the N-terminus of YscF interfered with NF-κB/AP-1 activation, the N-terminus of YscF was moved onto SsaG, a protein that highly activates NF-κB/AP-1. The YscF-SsaG chimeric protein was constructed by adding the 15 N-terminal amino acids of YscF to SsaG. As shown in Figure 19A, SsaG significantly activated NF-κB/AP-1 more than YscF. When the N-terminus of YscF was added to SsaG, the activation of NF-κB/AP-1 was reduced back down to the level seen with wild type YscF (Figure 19A-D). This showed that the N-terminus of YscF modulated the NF-κB/AP-1 activation ability of SsaG considerably, confirming that the N-terminus of YscF was capable of decreasing NF-κB/AP-1 activation. To determine whether the reduction in NF-κB/AP-1 activation was achieved by just adding an N-terminus to SsaG or if the reduction was due specifically to YscF residues, a frameshift mutation in the plasmid encoding the YscF-SsaG chimera was introduced after the start codon and then the frameshift was fixed to restore the original reading frame of SsaG. These mutations in the plasmid encoding chimeric YscF-SsaG changed the N-terminal amino acids of the new RN-SsaG chimera from MSNFSGFTKGTDIAD to MRYLLWIYERNRYRR. The RN-SsaG chimera had the same level of NF-κB/AP-1 activation as SsaG (Figure 19A) suggesting that the N-terminus of YscF was responsible for the observed decrease in NF-κB/AP-1 activation. As shown above, treatment of chimeric needle proteins with

proteinase K, but not LPL or PMB abrogated NF-κB/AP-1 activation of THP1-Xblue cells (Fig 20).

An alternative explanation for the ability of the YscF-SsaG chimera to reduce NF-κB/AP-1 activation is that the addition of the YscF N-terminus to SsaG resulted in a mis-folded or unstable protein. Therefore, a genetic analysis was devised to test whether these chimeric proteins could form functional needles. First a Δ*ssaG* *Salmonella enterica* serovar Typhimurium mutant was constructed and subsequently transcomplemented with YscF-SsaG, RN-SsaG or SsaG. Because SsaG is required for the intracellular survival of *Salmonella* (47, 57, 135), we tested the functionality of these chimeric proteins by the ability of these mutant *Salmonella* to survive in macrophages using a gentamicin protection assay (75). Our results showed that the Δ*ssaG* *Salmonella* transcomplemented with the YscF-SsaG or the RN-SsaG chimeras were able to survive in macrophages similar to wildtype *Salmonella* in contrast to the Δ*ssaG* *Salmonella* (Figure 21). This suggests that the chimeric proteins were able to form functional needles and that the addition of YscF's N-terminus onto SsaG had no effect on SsaG's function.

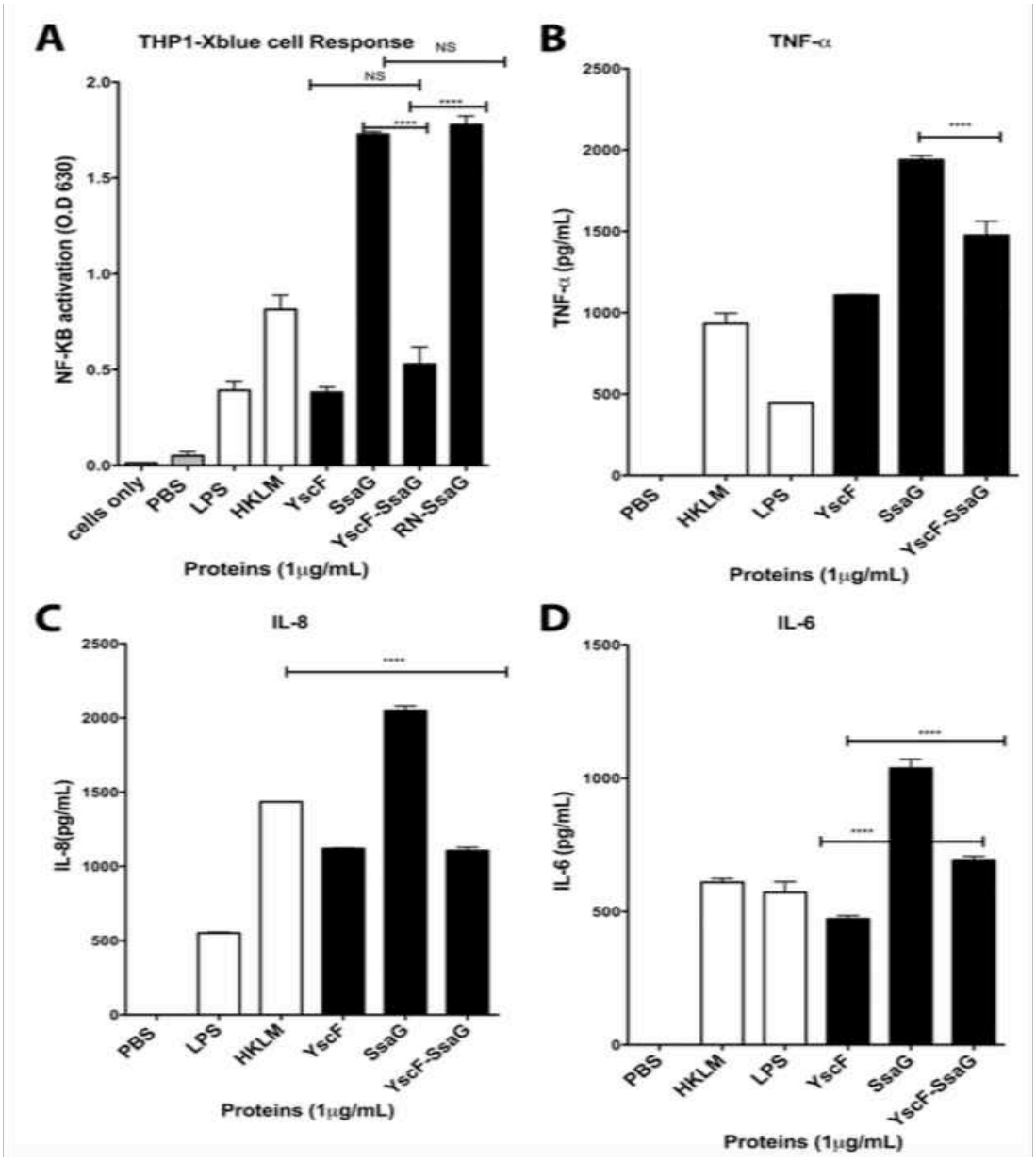

FIG 19: The N-terminus of YscF reduces NF-κB/AP-1 and cytokine activation by SsaG. THP1-XBlue cells were seeded in wells and treated with PBS, 1 μg/ml LPS, or 1 μg/ml of needle proteins (A) YscF, SsaG and Chimeric YscF-SsaG (E) YscF, SsaG and Chimeric YscF-SsaG and RN-SsaG dissolved in PBS. SEAP levels were measured as representation of NF-κB/AP-1 activation. (B-D) THP-1 cells were treated with PBS, 1 μg/ml of HKLM, or 1 μg/ml of needle proteins (YscF, SsaG and chimeric YscF-SsaG). After 5 h (TNF-α) and 24 h (IL-6 and IL-8), supernatants were collected and tested by ELISA for production of (B) TNF-α, (C) IL-8 and (D) IL-6. Error bars represent SEM (n=3). Data shown are representative of at least three experiments. **, *P* is between 0.01 and 0.001; ****, *P* <0.0001; ns, not significant

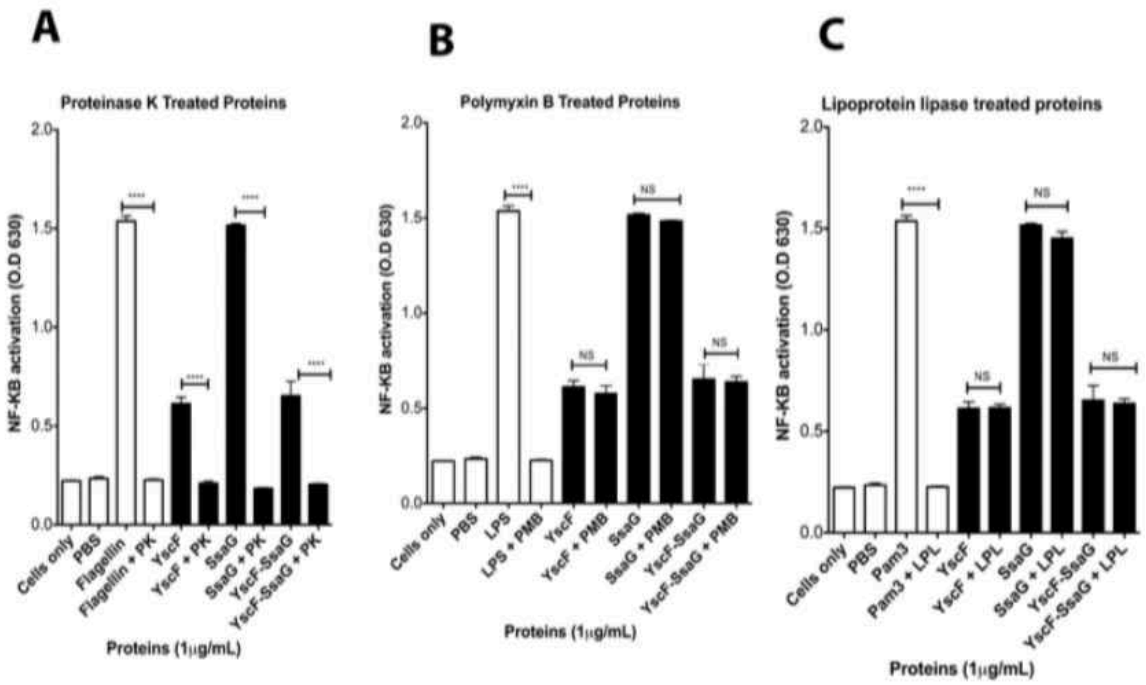

Figure 20. Purified Needles treated with proteinase K, lipoprotein lipase and polymyxin B indicate chimeric needle proteins cause NF-kB/AP-1 activation.
Purified Needles (1μg/ml), PBS, LPS (1 μg/ml) or Pam3 (1 μg/ml) were incubated with (A) Proteinase K (B) polymyxin B (PMB; 20 μg/ml) and (C) lipoprotein lipase (LPL; 50 μg/ml), before addition to THP1-XBlue cells. SEAP activity was measured as a representation of NF-kB/AP-1a ctivation. Error bars represent SEM. n=3. Shown experiments are representative of at least three experiments. ****, P <0.0001; NS, not significant**.**

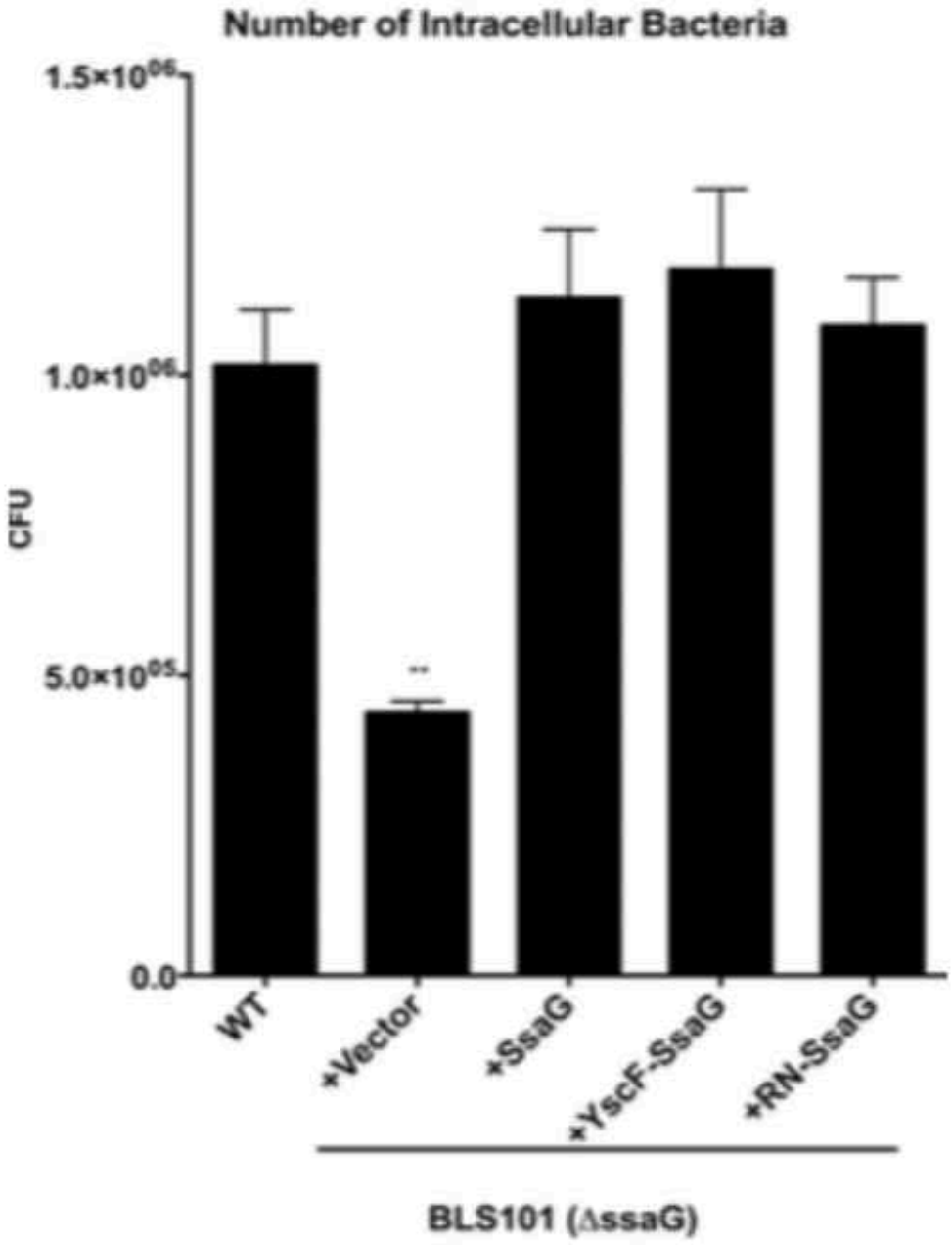

FIG 21. Addition of YscF's N-terminus to SsaG had no effect on SsaG's function.

THP-1 macrophages were infected with *S*. Typhimurium 14028 (WT), ΔssaG 14028 (BLS101) transcomplemented with plasmids pBAD18-Kan (Vector), pBAD18-Kan expressing SsaG (+SsaG), pBAD18-Kan expressing YscF-SsaG (+YscF-SsaG) or pBAD18-Kan expressing RN-SsaG (+RN-SsaG) in the presence of 0.1% L-arabinose. Intracellular survival was determined by comparing the number of bacteria 24 h post-infection. Error bars represent SEM (n=3). Data shown are representative of at least three experiments. **, *P* is between 0.01.

CHAPTER IV

DISCUSSION

Many pathogens induce inflammatory responses within few hours of infection (102). *Yersinia pestis* infection in contrast is characterized by 36 hours of rapid replication without an appreciable inflammatory response (30,80,102). This pre-inflammatory phase allows the bacteria to survive and multiply before the inflammatory response is fully activated (30,37,102). The disease is thus subdivided into two phases: an initial pre-inflammatory phase followed by pro-inflammatory phase that is typically too late to control the infection (30,102). The absence of an early pro-inflammatory response could result from the bacteria either avoiding detection by host responses or because the organism suppresses early innate immune responses (30,80,102).

Regardless of their pathogenic nature bacteria possess motifs called pathogen-associated molecular patterns (PAMPs), such as flagellin, LPS, and lipopeptide (9, 15, 33, 71, 108). Recognition of these PAMPs by the germ-line encoded pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs) and NOD-like receptors (NLRs), leads to induction of proinflammatory cytokines that facilitate direct elimination of the invading pathogen (9, 33, 49, 70, 71, 108). Consequently, successful bacterial colonization and multiplication within their hosts

relies not only on the virulence factors that modulates host responses but also in their ability to evade innate immunity and host responses (33, 108). Obviously, macrophages, dendritic cells and neutrophils that *Y. pestis* interacts with express a number of these PRRs that could be activated to induce proinflammatory cytokines to alert the immune system and directly eliminate *Y. pestis*. Yet, *Y. pestis* is able to stay under the radar and avoid activation of these diverse PRRs early in infection even before the translocation of Yops to manipulate host immune responses (43). The avoidance of early detection is important for the pathogenesis of *Yersinia pestis* because early pro-inflammatory responses to *Y. pestis* has been demonstrated to be detrimental to the bacterium (43). *Yersinia pestis* exhibits decreased replication in mice with heightened or altered states of immune activation, suggesting that pre-existing or early inflammatory responses are able to partially control or mitigate disease progression (43).

Like many other gram-negative bacterial pathogens, *Yersinia* employs the T3S system to interact with host cells (27, 58, 80, 121). The T3S systems play a significant role in the pathogenesis of *Y. pestis* by serving as conduits for the transfer of effector molecules that manipulate the host immune response (30, 37, 58). An important component of the T3S system is the hollow needle–like structure formed by polymerization of a single protein (YscF) that share sequence identity with needle proteins from other bacteria (37,79,124, 134). The location of YscF on the outside of the bacteria appears to be a prime location to interact with host innate immune receptors. Interestingly, YscF has shown to be a modest protective antigen in vaccinated mice (83, 133). Although antibodies against YscF do not show similar

levels of protection compared to the F1 capsular antigen and LcrV (83, 133), it has the potential to be a good antigen at least in combination with other antigens (83, 133). These findings support the potential role of needle proteins as an adjuvant.

A recent report demonstrated that T3S system needle proteins are pathogen associated molecular patterns (PAMPs) (63). These needle proteins function as ligands of TLR2 and TLR4 to induce cell activation via a MyD88-dependent pathway leading to expression of pro-inflammatory cytokines (63). Needle proteins from different species of gram-negative bacteria however induce cell activation to different magnitudes (63 and Figure 7). In the current study, the hyper-variable N-termini of these needle proteins were demonstrated to affect the levels of pro-inflammatory cytokines induced from a human monocyte cell line. These findings may give new insight into the pathogenesis of *Y. pestis* and other gram-negative bacteria that use T3S systems to inject bacterial effector proteins to subvert host defenses and promote infection.

T3S needle protein sequences from *Y. pestis* and other gram-negative bacteria have been compared and reported to be highly similar (66, 125 and Figure 6). T3S needle proteins show higher sequence conservation past the first ~20-25 amino acids. Short deletions of residues at the C-terminus of PrgI, MxiH and BsaL prevents needle polymerization (66, 134) suggesting that the conserved C-termini are involved in needle formation. However, the same observations cannot be made for the N-termini of needle proteins, which vary among bacterial species not only in amino acid composition but also in the number of amino acids (Figure 1). Because

the N-terminus of needle proteins does not appear to be involved in needle assembly (66, 134) and since the N-terminus of needle proteins may be exposed on the needle surface (37, 79), we hypothesized that the differences in cytokine expression (63) could be due to the variations in the N-termini of these proteins (40-42).

Consistent with this hypothesis, recombinant needle proteins lacking the variable N-termini showed different activation of NF-κB/AP-1 and cytokine expression (TNF-α, IL-6 and IL-8) when compared to their wild type counterparts (figure 7). These differences in cytokine expression indicate that the variable N-terminus could confer a selective advantage for the survival of these pathogenic bacteria. Accordingly, the removal of the N-terminus of YscF increased induction of NF-κB/AP-1 activation and cytokine expression compared to the full-length protein. Owing to the initial pre-inflammatory phase required for *Y. pestis* to survive and replicate (102), our results imply that the N-terminus of YscF in *Y. pestis* may function to interfere with host sensing in an attempt to either evade or suppress early host innate immune responses to prevent inflammation and bacterial clearance. Similar to YscF, the N-terminus of PrgI may play a role to dampen the host immune response by blocking inflammation resulting in a mechanism by which *Salmonella* control their population density during the initial stages of infection (99). In contrast to *Yersinia* and *Salmonella,* induction of immune responses leading to inflammation and subsequent neutrophil recruitment is known to promote invasion and dissemination of *Shigella* (16, 98, 108). Consequently, removal of the N-terminus of MxiH attenuated the high inflammatory response associated with the

full-length protein. This suggests that in accordance with the infection goal of *Shigella*, the N-terminus of MxiH may positively add to the pro-inflammatory environment possibly by enhancing activation of TLR2 and TLR4. These observations are in agreement with results obtained with flagellin by Smith et al. (114), who showed that the N-terminus of flagellin is involved in immune manipulation by some bacteria (*Helicobacter, Campylobacter* and *Bartonella*) to the advantage of the pathogens. Similar to flagella, our data shows that the N-terminus of needle proteins could be involved in manipulating host response to the advantage of the bacteria.

An exception to the observed differences in cytokine expression between full-length and truncated proteins was the *Salmonella* SPI-2 needle protein, SsaG. Truncated SsaG showed similar levels of NF-κB/AP-1 activation and cytokine expression when compared to the full-length protein (Figure 7). This result was not surprising because multiple sequence alignment of T3S system needle proteins (Figure 6) showed that SsaG lacked residues corresponding to the 15 N-terminal amino acids of YscF and comparable residues in other needle proteins. As a result N-terminal deletion SsaG was not expected to have a significant effect on SsaG's NF-κB/AP-1 activation and cytokine expression. Under a hypothesis that the N-terminus could modulate TLR2 and/or TLR4 interaction, SsaG would not need an extended N-terminus: because SsaG is only expressed when *Salmonella* is enclosed in the *Salmonella* containing vacuole (33, 47, 57, 135) and therefore is probably not exposed to TLRs 2 and 4 on the outside of the host cell. The observed variation between needle proteins of different bacteria may indicate that each needle protein

uniquely activates eukaryotic cells. Induction of innate immune responses by His_6-YscF was concentration dependent and saturable, demonstrating specificity: significant induction began at approximately 100 ng/ml of His_6-YscF and peaked at 5 μg/ml of His_6-YscF. These findings give strength to the notion that the variable N-terminus of T3S system needle proteins does appear to have an immune modulatory function.

Having shown that N-terminally truncated YscF induced higher pro-inflammatory cytokines than the full-length protein; it would be important to determine the region of YscF that is responsible for this induction of inflammation. To begin to address this issue, a mutational analysis of recombinant and sheared needle proteins was utilized. Deletion of amino acids S2-S5, S2-G10, S2-D15 or S2-A20 from the N-terminus of YscF increased pro-inflammatory activity (Figures 11 and 14). The highest activity was observed when amino acids S2-D15 were deleted from the N-terminus of YscF. The level of NF-κB/AP-1 activation and cytokine expression for the S2-D15 N-terminally truncated YscF (YscF Δ(S2-D15)) was similar to that of full-length SsaG. The observed similarity in cytokine expression was striking as the deletion of S2-D15 amino acids from the N-terminus of YscF corresponded to the beginning of SsaG according to our multiple sequence alignment (Figure 6). This strengthened the notion that the variable N-terminus of T3S system needle proteins may not play a role in maintaining a functional needle but may function in manipulating host immune responses. A second possibility is that the differences in NF-κB/AP-1 activation between the full-length and truncated proteins may be due to differences in proportions of protein polymers. However,

this seems unlikely given that NF-κB/AP-1 activation did not differ significantly between full-length and truncated SsaG (Figure 7A) neither did deleting residues from the C-terminus of YscF increase NF-κB/AP-1 activation compared to full-length YscF. Additionally, gels and blots of purified needle proteins confirm that they are all stable and demonstrate qualitatively similar patterns of polymer distribution. Furthermore, while it is possible to prepare soluble monomers that maintain their native secondary structures when five residues are deleted from the C-termini in MxiH, PrgI and BsaL, YscF and PscF rapidly self-associate upon expression and purification (Figure 15 and 5, 7), corroborating our results that deleting residues from YscF does not shift the balance between monomers and polymers.

Interestingly, truncation of amino acids S2-A20 from the N-terminus of YscF (YscF Δ(S2-A20)) had reduced cytokine expression compared to YscF Δ(S2-D15). These results open the possibility of a unique TLR binding signature between amino acids D15 and A20 in YscF that is blocked by the exposed N-terminus. The region (D15-A20) contains three non-alanine residues that are highly conserved among different bacterial species. As demonstrated in Figure 18, site-directed mutagenesis of two amino acids (L16A and V19A) showed significantly reduced NF-κB/AP-1 activation in THP1-XBlue cells. Substitution of all 3 amino acids further reduced NF-κB/AP-1 activation to almost wild type levels, however mutation of D17A alone had no effect on NF-κB/AP-1 activation. This indicated that amino acids L16 and V19 were important in NF-κB/AP-1 activation by YscF. Similar findings are reported in studies focusing on the PAMP activity of flagellin (4, 114). Those studies

demonstrated that mutant flagellin with substitutions of L88A, L94A, I411A, D412A and L425A either in single or in combination significantly reduced TLR5 recognition by *S.* Typhimurium FliC (4, 114). Recent studies with the Neisserial porin protein PorB provide insight into binding signatures for TLR2 in PorB. PorB is highly conserved between *Neisseria* species, except for the surface exposed loops (64, 77, 82, 126). Much like T3S needle proteins, PorB (specifically the exposed loops) was found to interact with TLR2. The interaction with TLR2 is dependent on specific amino acids and the variation between PorB of different *Neisseria* species creates unique "TLR2 binding signatures" similar to what our findings suggest. Specific binding signatures were found to be more or less inflammatory through interaction with TLR2 (64, 77, 82, 126), which corroborates our earlier report (63). Collectively our data suggest that some of the pro-inflammatory activity of YscF resides in the conserved region between amino acids D15-A20.

Transcomplementation studies were used to analyze the ability of the N-terminal YscF deletions to function in *Y. pestis.* N-terminal YscF deletions of S2-D15 or S2-A20 amino acids did not affect needle function (Figure 17). This observation is consistent with earlier reports that showed that deletion of 12 (2) or 19 (81) amino acids from the N-terminus of YscF from *Y. enterocolitica* and *Y. pseudotuberculosis* respectively did not affect needle assembly and function. Similar to Lwande and Wedemeyer's findings (81), the YscF deletions did not have a constitutive secretion phenotype in contrast to results reported in *Y. enterocolitica* by Allaoui *et al* (19). The discrepancy between these findings regarding the secretion phenotype could be

due to species-specific differences in *yscF* from *Y. pestis* and *Y. enterocolitica*. Another possible explanation for this discrepancy could be the differences in copy number of *yscF* used in the separate studies. In the current study the YscF mutants were expressed from a high-copy number plasmid (pBAD18-Kan), Allaoui *et al* made their truncation on the pYV plasmid. In spite of these explanations, the effect of various mutations in the N-terminus of YscF on pro-inflammatory activation, Yops secretion and translocation is no doubt a complex area and further work will be required to fully elucidate the role of the N-terminus of YscF in secretion and translocation of Yops. Additionally, changes in YscF structure due to the N-terminal deletions cannot be excluded, although the ability of the YscF truncations to transcomplement a *yscF* strain suggests that native structure is maintained. Furthermore, removal of the N-terminus was required to obtain crystals for structural data for several needle proteins and the N-terminus is reported to be unstructured and flexible (66, 119, 134).

In this study the N-terminus of YscF was found to modulate NF-κB/AP-1 activation by YscF. To further confirm the modulatory effect of YscF on host immune responses recombinant chimeric constructs were made to add the 15 N-terminal residues of YscF to the N-terminus of SsaG. The chimeric YscF-SsaG construct reduced NF-κB/AP-1 activation and cytokine expression compared to SsaG (Figure 19A-D). In contrast, the addition of random amino acids to the N-terminus of SsaG did not reduce NF-κB/AP-1 activation by SsaG (Figure 19A). This observation suggested that the modulatory effect of YscF on NF-κB/AP-1 activation was

sequence specific. This result is in agreement with Murthy et al (91) who showed that changes in amino acid sequences of the motif-N (91) of flagellin abolishes its pro-inflammatory activity. Moreover, genetic analysis showed that the addition of YscF's N-terminus to SsaG had no effect on SsaG's function, evidenced by the ability of a Δ*ssaG Salmonella* transcomplemented with these chimeric needle proteins to survive in macrophages (Figure 21). Together these results confirmed that the N-terminus of YscF can modulate host immune responses and further confirmed that sequence alterations at the N-termini of needle proteins have little or no effect on needle function (75).

This study independently provided a structural functional correlation that reflected a role for the N-terminus of needle proteins in manipulating host immune responses. Currently, the role that the N-terminus of YscF plays during *Y. pestis* infection is being investigated. These studies will allow us to begin to address the question of the role of N-terminus of YscF in the pathogenesis of *Yersinia* infections as well as other gram-negative bacteria that utilize T3SS to subvert host immune response. Detailed understanding of PRR-needle protein interaction will provide a clearer picture of host-pathogen interactions and permit design of needle proteins as adjuvants or immunomodulatory drugs.

REFERENCES

1. **Aderem A, Ulevitch J R.** 2000. Toll-like receptors in the induction of the innate immune response. Nature 406, 782-787

2. **Allaoui A, Schulte R, Cornelis GR.** 1995. Mutational analysis of the Yersinia enterocolitica virC operon: characterization of yscE, F, G, I, J, K required for Yop secretion and yscH encoding YopR. Mol Microbiol. 18(2):343-55.

3. **Amedei A, Niccolai E, Marino L, D'Elios MM.** 2011. Role of immune response in Yersinia pestis infection. J Infect Dev Ctries 5: 628-639.

4. **Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, Logan SM, Aderem A.** 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci U S A. 28;102(26):9247-52

5. **Barrett BS, Markham AP, Esfandiary R, Picking WL, Picking WD, Joshi SB, Middaugh CR.** 2010. Formulation and immunogenicity studies of type III secretion system needle antigens as vaccine candidates. J Pharm Sci. 99(11):4488-96.

6. **Barrett BS, Picking WL, Picking WD, Middaugh CR.** 2008. The response of type three secretion system needle proteins MxiHDelta5, BsaLDelta5, and PrgIDelta5 to temperature and pH. Proteins. 15;73(3):632-43.

7. **Bartra SS, Styer KL, O'Bryant DM, Nilles ML, Hinnebusch BJ, Aballay A, Plano GV.** 2008. Resistance of Yersinia pestis to complement-dependent

killing is mediated by the Ail outer membrane protein. Infect Immun. 76(2):612-22.

8. **Behnsen J, Perez-Lopez A, Nuccio SP, Raffatellu M.** 2015. Exploiting host immunity: the Salmonella paradigm. Trends Immunol. 36(2):112-120.

9. **Bergsbaken T, Cookson BT.** 2009. Innate immune response during Yersinia infection: critical modulation of cell death mechanisms through phagocyte activation. J Leukoc Biol. 86(5):1153-8.

10. **Bevins SN, Baroch JA, Nolte DL, Zhang M, He H.** 2012. Yersinia pestis: examining wildlife plague surveillance in China and the USA. Integr Zool. 7(1):99-109.

11. **Bliska JB, Wang X, Viboud GI, Brodsky IE.** 2013. Modulation of innate immune responses by Yersinia type III secretion system translocators and effectors. Cell Microbiol. 15(10):1622-31.

12. **Blocker AJ, Deane JE, Veenendaal AK, Roversi P, Hodgkinson JL, Johnson S, Lea SM.** 2008. What's the point of the type III secretion system needle? Proc Natl Acad Sci U S A 105: 6507-6513.

13. **Brodsky IE, Palm NW, Sadanand S, Ryndak MB, Sutterwala FS, Flavell RA, Bliska JB, Medzhitov R.** 2010. A Yersinia effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. Cell Host Microbe. 20;7(5):376-87

14. **Broz P, Monack DM.** 2011. Molecular mechanisms of inflammasome activation during microbial infections. Immunol Rev. 243(1):174-90.

15. **Broz P, Ohlson MB, Monack DM.** 2012. Innate immune response to Salmonella typhimurium, a model enteric pathogen. Gut Microbes 3: 62-70

16. **Brubaker RR.** 1991. Factors promoting acute and chronic diseases caused by Yersiniae. Clin Microbiol Rev. 4(3):309-24.

17. **Brubaker RR.** 2003. Interleukin-10 and inhibition of innate immunity to Yersiniae: roles of Yops and LcrV (V antigen). Infect Immun. 71(7):3673-81. 52

18. **Brutinel ED, Yahr TL.** 2008. Control of gene expression by type III secretory activity. Curr Opin Microbiol. 11(2):128-33.

19. **Bryksin AV, Matsumura I.** 2010. Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. Biotechniques. 48(6): 463-465

20. **Buckner MMC, Finlay BB.** 2011. Host–microbe interaction: Innate immunity cues virulence. Nature 472, 179–180

21. **Burkinshaw BJ, Deng W, Lameignère E, Wasney GA, Zhu H, Worrall LJ, Finlay BB, Strynadka NC.** 2015. Structural analysis of a specialized type III secretion system peptidoglycan-cleaving enzyme. J Biol Chem. 2015 Feb 12. pii: jbc.M115.639013

22. **Butler T.** 2009. Plague into the 21st century. Clin Infect Dis. 1;49(5):736-42.

23. **Carniel E.** 2008. Plague today. Med Hist Suppl. 27:115-22.

24. **Coburn B, Sekirov I, Finlay BB.** 2007. Type III secretion systems and disease. Clin Microbiol Rev. 20(4):535-49.

25. **Cordes FS, Daniell S, Kenjale R, Saurya S, Picking WL, Picking WD, Booy F, Lea SM, Blocker A.** 2005. Helical packing of needles from functionally altered Shigella type III secretion systems. J Mol Biol. 354(2):206-11.

26. **Cordes FS, Komoriya K, Larquet E, Yang S, Egelman EH, Blocker A, Lea SM.** 2003. Helical structure of the needle of the type III secretion system of Shigella flexneri. J Biol Chem. 278:17103-17107

27. **Cornelis GR, Boland A, Boyd AP, Geuijen C, Iriarte M, Neyt C, Sory MP, Stainier I.** 1998. The virulence plasmid of *Yersinia,* an antihost genome. Microbiol Mol Biol Rev. 62(4):1315-52.

28. **Cornelis GR.** 1998. The Yersinia deadly kiss. J Bacteriol;180(21):5495-504.

29. **Cornelis GR.** 2002. Yersinia type III secretion: send in the effectors. J Cell Biol. 5;158(3):401-8.

30. **Cornelis GR.** 2006. The type III secretion injectisome. Nat Rev Microbiol 4: 811- 825.

31. **Cunha** CB, Cunha BA. 2006. Impact of plague on human history. Infect Dis Clin North Am. 20(2):253-72

32. **Cunha LD, Zamboni DS.** 2013. Subversion of inflammasome activation and pyroptosis by pathogenic bacteria. Front Cell Infect Microbiol. 3:76.

33. **Deane J E, Abrusci P, Johnson S and Lea S M.** 2010. Timing is everything: the regulation of type III secretion. Cell. Mol. Life Sci 67: 1065-1075

34. **Deane JE, Cordes FS, Roversi P, Johnson S, Kenjale R, Picking WD, Picking WL, Lea SM, Blocker A.** 2006. Expression, purification, crystallization and preliminary crystallographic analysis of MxiH, a subunit of the Shigella flexneri type III secretion system needle. Acta Crystallogr Sect F Struct Biol Cryst Commun 62: 302-305.

35. **Deane JE, Roversi P, Cordes FS, Johnson S, Kenjale R, Daniell S, Booy F, Picking WD, Picking WL, Blocker AJ, Lea SM.** 2006. Molecular model of a type III secretion system needle: Implications for host-cell sensing. Proc Natl Acad Sci U S A. 103:12529-12533.

36. **Demers J-P, Sgourakis NG, Gupta R, Loquet A, Giller K, Riedel D, Laube B, Kolbe M, Baker D, Becker S, Lange A.** 2013. The Common Structural Architecture of *Shigella flexneri* and *Salmonella* typhimurium Type Three Secretion Needles. PLoS Pathog 9: e1003245.

37. **Deng W, Burland V, Plunkett G 3rd, Boutin A, Mayhew GF, Liss P, Perna NT, Rose DJ, Mau B, Zhou S, Schwartz DC, Fetherston JD, Lindler LE, Brubaker RR, Plano GV, Straley SC, McDonough KA, Nilles ML, Matson JS, Blattner FR, Perry RD.** 2002. Genome sequence of *Yersinia pestis* KIM. J Bacteriol. 184(16):4601-11.

38. **Dewoody RS, Merritt PM, and Marketon MM.** 2013. Regulation of the *Yersinia* type III secretion system: traffic control. Front. Cell. Infect. Microbiol.

39. **Donnelly MA, Steiner TS.** 2002. Two nonadjacent regions in enteroaggregative *Escherichia coli* flagellin are required for activation of toll-like receptor 5. J Biol Chem. 2002 Oct 25;277(43):40456-61.

40. **Drancourt M.** 2012. Plague in the genomic area. Clin Microbiol Infect; 18: 224-230

41. **Dykhuizen DE.** 2000. *Yersinia pestis*: an instant species? Trends Microbiol. 8(7):296-8.

42. **Eaves-Pyles TD, Wong HR, Odoms K, Pyles RB.** 2001. *Salmonella* flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. J Immunol. 2001 Dec 15;167(12):7009-16.

43. **Edqvist PJ, Aili M, Liu J, Francis MS.** 2007. Minimal YopB and YopD translocator secretion by *Yersinia* is sufficient for Yop-effector delivery into target cells. Microbes Infect. 9(2):224-33.

44. **Eisen RJ, Gage KL.** 2009. Adaptive strategies of *Yersinia pestis* to persist during inter-epizootic and epizootic periods. Vet Res. 40(2):1.

45. **Fields KA, Nilles ML, Cowan C, Straley SC.** 1999. Virulence role of V antigen of *Yersinia pestis* at the bacterial surface. Infect Immun. 67(10):5395-408.

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

47. **Forest CG, Ferraro E, Sabbagh SC, Daigle F.** 2010. Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of *Salmonella* pathogenicity island (SPI)-2. Microbiology. 156(Pt 12):3689-98.

48. **Franchi L.** 2011. Role of inflammasomes in *Salmonella* infection. Front Microbiol. 31;2:8.

49. **Fujii T, Cheung M, Blanco A, Kato T, Blocker AJ, Namba K.** 2012. Structure of a type III secretion needle at 7-Å resolution provides insights into its assembly and signaling mechanisms. Proc Natl Acad Sci U S A. 20;109(12):4461-6.

50. **Galán JE, Wolf-Watz H.** 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature 444(7119): 567-573.

51. **Gangloff M.** 2012. Different dimerisation mode for TLR4 upon endosomal acidification? Trends Biochem Sci. 37(3):92-8.

52. **Gong YN, Shao F.** 2012. Sensing bacterial infections by NAIP receptors in NLRC4 inflammasome activation. Protein Cell. 3(2):98-105

53. **Guzman LM, Belin D, Carson MJ, Beckwith J.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol. 1995 Jul;177(14):4121-30.

54. **Hacker J, Kaper JB.** 2000. Pathogenicity islands and the evolution of microbes. Annu Rev Microbiol. 54:641-79.

55. **Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA.** 2013. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science. 13;341(6151):1250-3.

56. **Hansen-Wester I, Chakravortty D, Hensel M.** 2004. Functional Transfer of *Salmonella* Pathogenicity Island 2 to Salmonella bongori and Escherichia coli. Infect Immun, 72(5), 2879–2888.

57. **Harmon DE, Murphy JL, Davis AJ, Mecsas J.** 2013. A Mutant with Aberrant Extracellular LcrV-YscF Interactions Fails To Form Pores and Translocate Yop Effector Proteins but Retains the Ability To Trigger Yop Secretion in Response to Host Cell Contact. J Bacteriol. 195:2244-2254

58. **Heroven AK, Dersch P.** 2014. Coregulation of host-adapted metabolism and virulence by pathogenic yersiniae. Front Cell Infect Microbiol. 20;4:146.

59. **Hoiczyk E, Blobel G.** 2001. Polymerization of a single protein of the pathogen *Yersinia enterocolitica* into needles punctures eukaryotic cells. Proc Natl Acad Sci U S A.10;98(8):4669-74.

60. **Hueck CJ.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev. 62(2):379-433.

61. **Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Koerner JF, Layton M, McDade J, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Schoch-Spana M, Tonat K.** 2000. Plague as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. JAMA. 3;283(17):2281-90.

62. **Jessen DL, Osei-Owusu P, Toosky M, Roughead W, Bradley DS, Nilles ML.** 2014. Type III Secretion Needle Proteins Induce Cell Signaling and Cytokine Secretion via Toll-Like Receptors. Infect Immun. 82(6):2300-9. doi: 10.1128/IAI.01705-14

63. **Kattner C, Toussi DN, Zaucha J, Wetzler LM, Rüppel N, Zachariae U, Massari P, Tanabe M.** 2014. Crystallographic analysis of *Neisseria*

meningitidis PorB extracellular loops potentially implicated in TLR2 recognition. J Struct Biol. ;185(3):440-7.

64. Kawai T, Akira S. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol. 21(4):317-37.

65. Kenjale R, Wilson J, Zenk SF, Saurya S, Picking WL, Picking WD, Blocker AJ. 2005. The needle component of the type III secreton of *Shigella* regulates the activity of the secretion apparatus. Biol Chem. 280(52):42929-37.

66. Kofoed EM, Vance RE. 2011. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature. 28;477(7366):592-5.

67. Kolodziejek AM, Hovde CJ, Minnich SA. 2012. *Yersinia pestis* Ail: multiple roles of a single protein. Front Cell Infect Microbiol. 6;2:103.

68. Kopp E, Medzhitov R. 2002. A plague on host defense. J Exp Med. 21;196(8):1009-12.

69. Kudryashev M, Stenta M, Schmelz S, Amstutz M, Wiesand U, Castaño-Díez D, Degiacomi MT, Münnich S, Bleck CK, Kowal J, Diepold A, Heinz DW, Dal Peraro M, Cornelis GR, Stahlberg H. 2013. In situ structural analysis of the *Yersinia enterocolitica* injectisome. Elife. 30;2:e00792.

70. Lightfield KL, Persson J, Trinidad NJ, Brubaker SW, Kofoed EM, Sauer JD, Dunipace EA, Warren SE, Miao EA, Vance RE. 2011. Differential requirements for NAIP5 in activation of the NLRC4 inflammasome. Infect Immun. 79(4):1606-14.

71. Ligon BL. 2006. Plague: a review of its history and potential as a biological weapon. Semin Pediatr Infect Dis. 17(3):161-70.

72. Ligtenberg KG, Miller NC, Mitchell A, Plano GV, Schneewind O. 2013. LcrV mutants that abolish *Yersinia* type III injectisome function. J Bacteriol. 195(4):777-87.

73. **Lim S, Kim B, Choi HS, Lee Y, Ryu S.** 2006. Fis is required for proper regulation of ssaG expression in *Salmonella enterica* serovar Typhimurium Microb Pathog. 41(1):33-42.

74. **Lippi D, Conti AA.** 2002. Plague, policy, saints and terrorists: a historical survey. J Infect. 44(4):226-8.

75. **Liu X, Wetzler LM, Nascimento LO, Massari P.** 2010. Human airway epithelial cell responses to *Neisseria lactamica* and purified porin via Toll-like receptor 2-dependent signaling. Infect Immun. 78(12):5314-23.

76. **Loessner H, Endmann A, Leschner S, Westphal K, Rhode M, Miloud T, Hämmerling G, Neuhaus K, Weiss S.** 2007. Remote control of tumour-targeted *Salmonella enterica* serovar Typhimurium by the use of L-arabinose as inducer of bacterial gene expression in vivo. Cell Microbiol. 9(6):1529-37

77. **Loquet A, Sgourakis NG, Gupta R, Giller K, Riedel D, Goosmann C, Kolbe M, Baker D, Becker S, Lange A.** 2012. Atomic model of the type III secretion system needle. Nature 486: 276-279.

78. **Luo D, Lin JS, Parent MA, Mullarky-Kanevsky I, Szaba FM, Kummer LW, Duso DK, Tighe M, Hill J, Gruber A, Mackman N, Gailani D, Smiley ST.** 2013. Fibrin Facilitates Both Innate and T Cell-Mediated Defense against *Yersinia pestis.* J Immunol. 190:4149-4161

79. **Lwande JS, Wedemeyer W.** 2010. Understanding the structure of YSCF, a Type III Secretion protein from *Yersinia* and how it forms pili . DAI/B 72-08 119 3458525

80. **Massari P, Visintin A, Gunawardana J, Halmen KA, King CA, Golenbock DT, Wetzler LM.** 2006. Meningococcal porin PorB binds to TLR2 and requires TLR1 for signaling. J Immunol. 2006 Feb 15;176(4):2373-80.

81. **Matson JS, Durick KA, Bradley DS, Nilles ML.** 2005. Immunization of mice with YscF provides protection from *Yersinia pestis* infections. BMC Microbiol 5: 38.

82. **Matson JS, Nilles ML.** 2001. LcrG-LcrV interaction is required for the control of yop secretion in yersinia pestis. J Bacteriol 183; (17), 5082-5091.

83. **Mejía E, Bliska JB, Viboud GI.** 2008. *Yersinia* controls type III effector delivery into host cells by modulating Rho activity. PLoS Pathog. 4(1):e3.

84. **Miao EA, Ernst RK, Dors M, Mao DP, Aderem A.** 2008. Pseudomonas aeruginosa activates caspase 1 through Ipaf. Proc Natl Acad Sci U S A.19;105(7):2562-7.

85. **Miao EA, Mao DP, Yudkovsky N, Bonneau R, Lorang CG, Warren SE, Leaf IA, Aderem A.** 2010. Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. Proc Natl Acad Sci U S A. 16;107(7):3076-80.

86. **Moore SM, Monaghan A, Borchert JN, Mpanga JT, Atiku LA, Boegler KA, Montenieri J, MacMillan K, Gage KL, Eisen RJ.** 2015. Seasonal fluctuations of small mammal and flea communities in a Ugandan plague focus: evidence to implicate Arvicanthis niloticus and Crocidura spp. as key hosts in *Yersinia pestis* transmission. Parasit Vectors. 8;8(1):11.

87. **Moraes TF, Spreter T, Strynadka NC.** 2008. Piecing together the type III injectisome of bacterial pathogens. Curr Opin Struct Biol. 18(2):258-66.

88. **Mota LJ, Sorg I, Cornelis GR.** 2005. Type III secretion: the bacteria-eukaryotic cell express. FEMS Microbiol Lett.1;252(1):1-10.

89. **Murthy KG, Deb A, Goonesekera S, Szabó C, Salzman AL.** 2004. Identification of conserved domains in *Salmonella muenchen* flagellin that are

essential for its ability to activate TLR5 and to induce an inflammatory response in vitro. J Biol Chem. 2004 Feb 13;279(7):5667-75.

90. Nilles ML, Fields KA, Straley SC. 1998. The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. J Bacteriol. 180(13): 3410-20

91. Overheim KA, Depaolo RW, Debord KL, Morrin EM, Anderson DM, Green NM, Brubaker RR, Jabri B, Schneewind O. 2005. LcrV plague vaccine with altered immunomodulatory properties. Infect Immun. 73(8):5152-9.

92. Page AL, Parsot C. 2002. Chaperones of the type III secretion pathway: jacks of all trades. Mol Microbiol. 46(1):1-11.

93. Perry RD, Fetherston JD. 1997. *Yersinia pestis*-etiologic agent of plague. Clin Microbiol Rev. 10(1):35-66.

94. Perry RD, Fetherston JD. 2011. Yersiniabactin iron uptake: mechanisms and role in *Yersinia pestis* pathogenesis. Microbes Infect. 2011 Sep;13(10):808-17.

95. Perry RD, Pendrak ML, Schuetze P. 1990. Identification and cloning of a hemin storage locus involved in the pigmentation phenotype of *Yersinia pestis.* J Bacteriol. 172(10):5929-37.

96. Phalipon A, Sansonetti PJ. 2007. Shigella's ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival? Immunol Cell Biol 85: 119-129.

97. Pilar AV, Reid-Yu SA, Cooper CA, Mulder DT, Coombes BK. 2013. Active modification of host inflammation by *Salmonella.* Gut Microbes 2:140-5.

98. Plano GV, Day JB, Ferracci F. 2001. Type III export: new uses for an old pathway. Mol Microbiol. 40(2):284-93.

99. Prentice MB, Rahalison L. 2007.Plague. Lancet. 7;369(9568):1196-207.

100. Price PA, Jin J, and Goldman EW. 2012. Pulmonary infection by *Yersinia pestis* rapidly establishes a permissive environment for microbial proliferation. Proc. Natl. Acad. Sci. 109:3083-3088

101. Quenee LE, Ciletti NA, Elli D, Hermanas TM, Schneewind O. 2011. Prevention of pneumonic plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines. Vaccine. 2;29(38):6572-83.

102. Quinaud M, Chabert J, Faudry E, Neumann E, Lemaire D, Pastor A, Elsen S, Dessen A, Attree I. 2005. The PscE-PscF-PscG complex controls type III secretion needle biogenesis in *Pseudomonas aeruginosa*. J Biol Chem. 280(43):36293-300.

103. Quinaud M, Plé S, Job V, Contreras-Martel C, Simorre JP, Attree I, Dessen A. 2007. Structure of the heterotrimeric complex that regulates type III secretion needle formation. Proc Natl Acad Sci U S A. 104(19):7803-8.

104. Ramamurthi KS, Schneewind O. 2002. Type III protein secretion in *Yersinia* species. Annu Rev Cell Dev Biol. 18:107-33.

105. Raoult D, Mouffok N, Bitam I, Piarroux R, Drancourt M. 2013. Plague: history and contemporary analysis. J Infect. 66(1):18-26.

106. Raymond B, Young JC, Pallett M, Endres RG, Clements A, Frankel G. 2013. Subversion of trafficking, apoptosis, and innate immunity by type III secretion system effectors. Trends Microbiol. 8:430-41.

107. Reuter S, Connor TR, Barquist L, Walker D, Feltwell T, Harris SR, Fookes M, Hall ME, Petty NK, Fuchs TM, Corander J, Dufour M, Ringwood T, Savin C, Bouchier C, Martin L, Miettinen M, Shubin M, Riehm JM,

Laukkanen-Ninios R, Sihvonen LM, Siitonen A, Skurnik M, Falcão JP, Fukushima H, Scholz HC, Prentice MB, Wren BW, Parkhill J, Carniel E, Achtman M, McNally A, Thomson NR. 2014. Parallel independent evolution of pathogenicity within the genus *Yersinia*. Proc Natl Acad Sci U S A. 6;111(18):6768-73.

108. Rosenzweig JA, Jejelowo O, Sha J, Erova TE, Brackman SM, Kirtley ML, van Lier CJ, Chopra AK. 2011. Progress on plague vaccine development. Appl Microbiol Biotechnol;91(2):265-86.

109. Sebbane F, Gardner D, Long D, Gowen BB, Hinnebusch BJ. 2005. Kinetics of disease progression and host response in a rat model of bubonic plague. Am J Pathol. 166(5):1427-39.

110. Shannon JG, Hasenkrug AM, Dorward DW, Nair V, Carmody AB, Hinnebusch BJ. 2013. *Yersinia pestis* subverts the dermal neutrophil response in a mouse model of bubonic plague. MBio. 27;4(5):e00170-13.

111. Sheahan KL, Isberg RR. 2015. Identification of mammalian proteins that collaborate with type III secretion system function: involvement of a chemokine receptor in supporting translocon activity. MBio. 17;6(1):e02023-14.

112. Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, Cookson BT, Aderem A. 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol. 4(12):1247-53.

113. Sokurenko EV, Hasty DL, Dykhuizen DE. 1999. Pathoadaptive mutations: gene loss and variation in bacterial pathogens. Trends Microbiol. 7(5):191-5.

114. Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, Carniel E, Gage KL, Leirs H, Rahalison L. 2008. Plague: past, present, and future. PLoS Med. 15;5(1)

115. Straley SC, Bowmer WS. 1986. Virulence genes regulated at the transcriptional level by $Ca^{2}+$ in *Yersinia pestis* include structural genes for outer membrane proteins. Infect Immun. 51(2):445-54.

116. Studier FW. 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr Purif 41: 207-234.

117. Sun P, Tropea JE, Austin BP, Cherry S, Waugh DS. 2008. Structural characterization of the *Yersinia pestis* type III secretion system needle protein YscF in complex with its heterodimeric chaperone YscE/YscG. J Mol Biol. 28;377(3):819-30

118. Suzuki S, Franchi L, He Y, Muñoz-Planillo R, Mimuro H, Suzuki T5, Sasakawa C, Núñez G. 2014. *Shigella* type III secretion protein MxiI is recognized by Naip2 to induce Nlrc4 inflammasome activation independently of Pkcδ. PLoS Pathog. 6;10(2):e1003926.

119. Swietnicki W, Powell BS, Goodin J. 2005. *Yersinia pestis* Yop secretion protein F: purification, characterization, and protective efficacy against bubonic plague. Protein Expr Purif. 42:166-172

120. Tenthorey JL, Kofoed EM, Daugherty MD, Malik HS, Vance RE. 2014. Molecular basis for specific recognition of bacterial ligands by NAIP/NLRC4 inflammasomes. Mol Cell. 10;54(1):17-29.

121. Titball RW, Hill J, Lawton DG, Brown KA. 2003. *Yersinia pestis* and plague. Biochem Soc Trans. 31(Pt 1):104-7.

122. Torruellas J, Jackson MW, Pennock JW, Plano GV. 2005. The *Yersinia pestis* type III secretion needle plays a role in the regulation of Yop secretion. Mol Microbiol. 57(6):1719-33.

123. Tosi T, Pflug A, Discola KF, Neves D, Dessen A. 2013. Structural basis of eukaryotic cell targeting by type III secretion system (T3SS) effectors.

124. Toussi DN, Carraway M, Wetzler LM, Lewis LA, Liu X, Massari P. 2012. The amino acid sequence of *Neisseria lactamica* PorB surface-exposed loops

influences Toll-like receptor 2-dependent cell activation. Infect Immun. 80(10):3417-28.

125. Vance RE. 2015. The NAIP/NLRC4 inflammasomes. Curr Opin Immunol. 32C:84-89.

126. Veenendaal AK, Hodgkinson JL, Schwarzer L, Stabat D, Zenk SF, Blocker AJ. 2007. The type III secretion system needle tip complex mediates host cell sensing and translocon insertion. Mol Microbiol. 63(6):1719-30.

127. Viboud GI, Bliska JB. 2005. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu Rev Microbiol. 59:69-89.

128. Wagner S, Stenta M, Metzger LC, Dal Peraro M, Cornelis GR. 2010. Length control of the injectisome needle requires only one molecule of Yop secretion protein P (YscP). Proc Natl Acad Sci U S A. 3;107(31):13860-5.

129. Wang S, Heilman D, Liu F, Giehl T, Joshi S, Huang X, Chou TH, Goguen J, Lu SA. 2004. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. Vaccine. 3;22(25-26):3348-57.

130. Wang S, Joshi S, Mboudjeka I, Liu F, Ling T, Goguen JD, Lu S. 2008. Relative immunogenicity and protection potential of candidate *Yersinia Pestis* antigens against lethal mucosal plague challenge in Balb/C mice. Vaccine. 20;26(13):1664-74.

131. Wang S, Mboudjeka I, Goguen JD, Lu S. 2010. Antigen engineering can play a critical role in the protective immunity elicited by *Yersinia pestis* DNA vaccines. Vaccine. 23;28(8):2011-9.

132. Wang Y, Ouellette AN, Egan CW, Rathinavelan T, Im W, De Guzman RN. 2007. Differences in the electrostatic surfaces of the type III secretion needle proteins PrgI, BsaL, and MxiH. J Mol Biol. 371(5):1304-14.

133. Waterman SR, Holden DW. 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cell Microbiol. 5(8):501-11.

134. Wilharm G, Dittmann S, Schmid A, Heesemann J. 2007. On the role of specific chaperones, the specific ATPase, and the proton motive force in type III secretion. Int J Med Microbiol. 297(1):27-36.

135. Yang J, Zhau Y, Shi J, Shao F. 2013. Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. Proc. Natl. Acad. Sci. U. S. A. 110:14408 –14413.

136. Zhang L, Wang Y, Picking WL, Picking WD, De Guzman RN. 2006. Solution structure of monomeric BsaL, the type III secretion needle protein of Burkholderia pseudomallei. J Mol Biol 359: 322-330.

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

138. Zwack EE, Snyder AG, Wynosky-Dolfi MA, Ruthel G, Philip NH, Marketon MM, Francis MS, Bliska JB, Brodsky IE. 2015. Inflammasome activation in response to the Yersinia type III secretion system requires hyperinjection of translocon proteins YopB and YopD. MBio.17;6(1):e02095-14.