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Analysis Of Yscf And Associated Homologs And The Presence Of An Inflammatory Molecular Pattern

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ANALYSIS OF YSCF AND ASSOCIATED HOMOLOGS AND THE PRESENCE OF
AN INFLAMMATORY MOLECULAR PATTERN

by

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This thesis, submitted by William A. Roughead in partial fulfillment of the requirements for the Degree of Master of Science 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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This thesis meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

_____
Dr. Wayne Swisher
Dean of the Graduate School

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Title   Analysis of YscF and Associated Homologs and the Presence of an
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CHAPTER I

INTRODUCTION

Background

Pathogen-associated molecular patterns (PAMP) are conserved structural motifs recognized by the immune system as foreign. The recognition of these motifs from invading microbes is a first step in priming the immune system and leads to eventual clearance of pathogens. Previous work has shown that an N-terminal truncation of a type three secretion needle, YscF, was pro-inflammatory *in vivo* in a mouse model of *Yersinia pestis* infection (1). Also, macrophages elicited an inflammatory response after exposure to the protein suggesting activity as a PAMP. This thesis shows data expanding on YscF and other needle homolog proteins and their pro-inflammatory effect in macrophage tissue culture. The characterization of the pro-inflammatory effect of these proteins gives potential of use as an adjuvant product in vaccines.

Yersinia pestis

Y. pestis is the causative agent of the plague. This is a disease of particularly severe virulence, with widespread notoriety as a human pathogen. This gram-negative bacterium has acquired its reputation over multiple pandemics through history, with estimates of 200 million deaths in total (2). The bacterium resides in enzootic reservoirs throughout the world, and has potential to be a deadly biological weapon. Despite the threat of bioterrorism, there is no vaccine currently available for the plague (3).

Clinical presentation

        Y. pestis manifests itself in three distinct forms in a human infection; bubonic, septicemic, and pneumonic plague. Naturally occurring plague is spread by flea-borne transmission and most commonly is exhibited in bubonic form. This disease state is characterized by general malaise, fever, headache and the trademark buboes; necrotic, swollen lymph nodes from excessive bacterial proliferation. Without an early diagnosis and antibiotic treatment there is a 60% fatality rate from bubonic plague, primarily from secondary sepsis and accompanying complications. The bacteria grow to remarkable numbers, in a terminal patient up to 10^9 bacteria/ml can be reached in the bloodstream. In primary septicemic plague, which occurs 30% of the time from flea-borne transmission, buboes are absent making diagnosis difficult (4, 5).

        The most lethal form of infection is pneumonic plague, with a nearly 100% fatality rate without treatment within 24 hours of symptom onset. Primary pneumonic plague has a quick time of onset, as early as 24 hours following exposure. Symptoms include fever and malaise, and coughing with difficulty breathing. Death occurs from sepsis or respiratory failure. Again, the infection is marked by an incredible amount of bacteria in the lungs (6, 3).

        All three forms of plague have a noticeable absence or severely mitigated immune response in the early stages of infection. The bacterium uses multiple methods in its biology to stealth past the host innate immune system. This allows *Y. pestis* to grow to the extreme abundance seen in septic patients. It is hypothesized that the bacterium utilizes this strategy for propagation; after growing to high numbers can effectively be

taken back into a flea vector through a blood meal (7). Furthermore, the death of the human host forces the flea vector to seek other hosts, which propagates the bacterium (2).

Plague, the double-edged sword

Y. pestis is an ancient pathogen and has been used in warfare since antiquity. One of the earliest documented examples of biological warfare involving *Y. pestis* was in 1346, two years before the infamous Black Death epidemic in Europe (8). Mongol Tartars were besieging the city of Kaffa, Crimea, which is in present day Ukraine. The Tartars were being ravaged by an outbreak of plague and decided to launch their dead into the city by catapult. Despite the primitive tactics, the strategy may have been successful and the inhabitants of the city were infected by plague as well. Subsequently, the Crimeans fled towards various areas of Europe, which is speculated to have played a role in the start of the Black Death epidemic (8).

As recently as the 1980's, the Soviet Union had an extensive biological weapons program with development of plague for use as an aerosol in long-range missiles (9). The offensive program has ended; however, there is potential for "rogue" scientists to aid in development of weapons to as many as 12 states suspected of biological weapon programs (8, 3).

Y. pestis is currently ranked as a Category A bioterrorism agent by the CDC. The threat from an aerosolized weapon is disturbing given the acute nature of a pneumonic plague infection. In addition, there is legitimate concern of bioengineered antibiotic resistance in the case of a terrorist attack using plague. The narrow window for diagnosis and treatment of pneumonic plague would be exacerbated by the complications that an antibiotic-resistant strain would bring. Because of the threat of bioterrorism, or the

possibility of biological warfare; research into *Y. pestis* biology for alternative methods of treatment and viable vaccine candidates is desirable.

Type Three Secretion System

The type III secretion system (T3SS) is an essential virulence factor of plague (10, 11). It is a syringe-like apparatus encoded by the ~70 kb plasmid CD1 (12, 13). The T3SS delivers effector proteins vectorially into host cells, without leakage into extracellular space (14). These effectors serve to decrease phagocytosis of the bacterium, increase intracellular survival in phagocytes, and dampen the immune response through decreased cytokine production (15). The action of the T3SS is partially responsible for the delayed immune response seen in a *Y. pestis* infection. Other gram-negative pathogens also encode these apparatuses, which have related functions in other pathogen-host interactions (16).

T3SS effectors

There are multiple effectors to accomplish the T3SS-mediated effects. Originally identified by centrifugation techniques, the effectors were termed *Yersinia* outer proteins, or in short, Yops (17). Many Yops have specific chaperones that they interact with in the bacterial cytoplasm (18). The exact function of the chaperones is unknown, although two hypotheses are storage of preformed effectors or possible prevention of folding before secretion (19, 20). Little is known about events just prior to secretion, but a signal is believed to be sent upon eukaryotic cell contact. An ATPase is known to play a role, termed YscN. The ATPase provides energy for dislocation of chaperone from Yop, so the effector can fit into the secretion needle (15). Once delivered into the host cell, they act differentially to exert their function.

YopH

YopH is a potent tyrosine phosphatase that is important for the antiphagocytic function of the T3SS in plague (21). YopH accomplishes this by interfering with actin rearrangements in the phagocyte, and by blocking formation of focal adhesion complexes (22). YopH has also been shown to cause interference of cell signaling through its phosphatase activity, which disrupts cytokine production in phagocytes (23). Outside of innate immunity, YopH can disrupt B-cell receptor expression and damper T-cell proliferation and cytokine production (24). While interesting, the effect on B-cells might play a small role *in* vivo since it has been shown that macrophages, neutrophils, and dendritic cells are the preferred targets of *Yersinia* T3S activity (25).

YopE

YopE is a GTPase activating protein (GAP) (26). After injection into a phagocyte, YopE induces GTP hydrolysis by Rac-1 and RhoA GTPases (27). The GAP activity disrupts modification of the actin cytoskeleton, which contributes to the antiphagocytic property of *Y. pestis* (26, 27). YopE has also been shown to reduce cytokine production through inhibition of caspase-1 in macrophages, preventing maturation of IL-1β and IL-18 cytokines (28).

YopT

YopT is a cysteine protease that synergistically acts with YopE and YopH to inhibit phagocytosis through disruption of the actin cytoskeleton (29). YopT also targets Rho GTPases, through cleavage of a critical modification on the enzyme rendering it unable to interact with essential regulators (30, 23).

YopJ

YopJ is another cysteine protease that induces macrophage apoptosis and disrupts the NF-kB pathway resulting in an inhibition of cytokine release (31, 32). YopJ plays a large role in the enteropathogenic *Yersinia*, but has been shown to only play a minor role in *Y. pestis* pathogenesis (13).

YopM

YopM is an essential effector of *Y. pestis* virulence, but has remained somewhat mysterious in its specific function (33, 15). YopM is known to travel to the nucleus of eukaryotic cells (34). A YopM mutant has a 100,000 fold increase in LD_{50} compared to WT *Y. pestis*, in a mouse model (35). This could possibly be from a systemic depletion of NK cells, through disruption of IL-15R on NK cells, and by lowering IL-15 cytokine production in macrophages (35). Also, recombinant YopM reduced cytokine release in a LPS-induced macrophage model (36). Taken together, YopM is another effector performing the common role of dampening the innate immune response in the host by destroying its ability to communicate distress through cytokine release.

LcrV

While not actively secreted into host cells by the T3SS, LcrV is included here for its role as a soluble effector in *Y. pestis* pathogenesis. LcrV is involved in regulation of the T3SS, as well as being a part of the translocon structure at the tip of the needle apparatus. The protein is a structural component, but is also known for its immunosuppressive properties making it an unusual virulence factor (13). Mice immunized with LcrV showed a systemic downregulation of IFN-γ and TNF-α through action of IL-10 by LcrV binding to TLR-2 (37). This was further proven by using an IL-

10 KO mouse model, where after LcrV administration, higher inflammatory cytokines were elicited. This higher cytokine level was accompanied with an increase in resistance to *Yersinia* challenge. The immunosuppressive role of LcrV was proposed to be a "long-range" function to accompany the cell-directed action of the T3SS (38, 13).

The immunosuppressive effects of LcrV are actively contested however. The resistance to *Yersinia* challenge upon further investigation was shown to be specific to strains of mice used (39). In addition, a study from different authors showed that the immunosuppressive effect is largely dependent upon high molecular-weight multimers of the protein (40).   LcrV does multimerize at the tip of the needle, but in the range of 3 to 5 oligomers in *Yersinia enterocolitica* (41). The higher molecular-weight species of protein that are responsible for the immunosuppressive effect are seen as artificial to over-expression by the authors and not physiologically significant (40).

T3SS structure

The T3SS structure has three domains; a basal body, needle and translocon at the tip of the apparatus. The basal body consists of ten proteins that share significant homology with flagellar basal bodies. In fact, the T3SS is essentially a needle on top of the conserved flagellar basal structure, rather than a hook and filament. This has lead to the speculation of flagella being the evolutionary ancestor to the T3SS, although the point remains controversial (41). As shown in Figure 1, the basal body is composed of several different proteins that span the inner and outer membranes of the bacterium. The exact structure of the cytosolic face is unknown, but YscN putatively sits at the base to fulfill its function as an ATPase for the system. HrcN, the ATPase from *Pseudomonas syringae* is known to associate with the inner membrane. The cryo-electron structure of

HrcN demonstrated a pore in the middle of the protein. From this structure, the authors concluded the ATPase must associate continuously with the base of the apparatus. Substrates of the T3SS would pass through the ATPase, which provides energy for secretion and dissociation of the associated chaperones (42).

YscJ oligomerizes into a ring structure located in the inner membrane of the bacterium (43). YscD connects the inner membrane ring to the N-terminus of YscC, which spans the periplasmic space. The C-terminus of YscC is located in the outer membrane where it is also in a ring shape. YscC is an oligomer with 12-15 monomers forming its structure in the outer membrane (44). The continuous association of YscJ, YscD, and YscC forms a channel that passes through the bacterium's membranes.

The needle portion of the apparatus sits on top of the basal body structure. The needle is composed of approximately 150 subunits of YscF (45, 46). The rod protein YscI passes through the basal body, forming a scaffold on the interior of the basal body, necessary for stability and possibly to aid polymerization of the YscF subunits (47). Polymerization of YscF happens spontaneously, after secretion through the basal body using the T3S machinery (48). The length of the needle is controlled by YscP, which functions as a "molecular ruler" to ensure the proper length of the needle substructure (49).

The translocon is composed of YopB, YopD, and LcrV; these proteins serve the role of inserting themselves through the host membrane so that a pore is formed to deliver Yops into the cell (50). LcrV sits at the tip of the needle until eukaryotic cell contact, when YopB and YopD are recruited (51). These hydrophobic proteins localize

distally to LcrV and form pores in the eukaryotic cell membrane, enabling translocation of effector proteins (50, 52).

Conservation of structure

There is a great deal of homology retained between structural elements of the T3SS among species of bacteria. The structural proteins of the T3SS are retained in genetic blocks within their bacterial constituents. This gives evidence that the T3SS was passed through horizontal gene transfer (53).

The wide-range of strategies employed by bacteria using the system is reflected in the diversity of their effector proteins. While some motifs are retained, effector proteins are variable in their structure to execute their functions (54).

Homologs of needle proteins used in this manuscript are displayed in Figure 1. The gram-negative pathogens *Salmonella enterica* Sv. Typhimurium, and *Shigella flexneri* are in the *Enterobacteriacae* family and cause gastroenteritis in humans. *Vibro parahaemolyticus* also causes gastroenteritis and is in the clade of γ-proteobacteria with the other bacteria listed in Figure 1. Of note, the T3SS is essential to virulence in these pathogens similar to *Y. pestis* (55, 56, 57).

Pathogen-associated molecular patterns

Non-specific defenses are present in all potential hosts for pathogenic bacteria. The innate immune system is almost as ancient as the bacteria themselves, and evolved simultaneously with the pathogens. Consequently, invariant microbial-specific molecular organizations are recognized by the innate immune system and referred to as pathogen-associated molecular patterns (58, 59).

Bacterial PAMP's identified have included molecules such as lipoprotein,
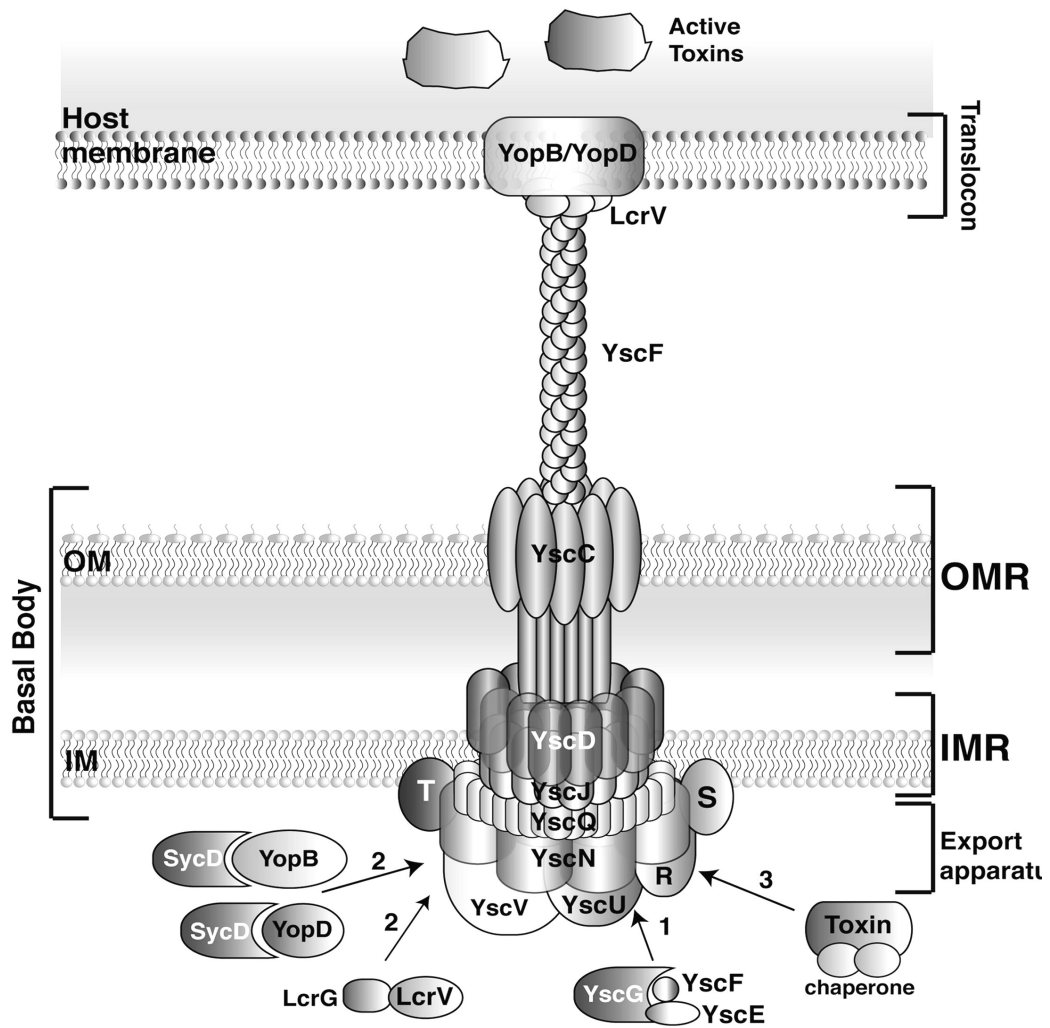
Bacterial species	Needle protein homolog
Yersinia pestis	YscF
Salmonella enterica SPI-1	PrgI
Salmonella enterica SPI-2	SsaG
Shigella flexneri	MxiH

Figure 1. T3SS apparatus and needle protein homologs. Graphic reprinted from Structure, *19*, Izore, T, *et al.* Biogenesis, regulation, and targeting of the type III secretion system, 603-612, 2011, with permission from Elsevier.

lipopolysacchride, peptidoglycan, flagellin, and unmethylated CpG-DNA (60-64). These are prototypical examples of PAMP's because of their unvarying nature and absolute necessity to the bacterium's survival.

<center>*Pathogen recognition receptors*</center>

The host recognizes these PAMP's through germline-encoded receptors termed pathogen recognition receptors (PRR) (65). These receptors are located on immune effector cells such as macrophages, neutrophils, dendritic cells, and mast cells. After recognition of a PAMP through binding to a PRR, the innate immune system activates; alarming and directing the adaptive arm of immunity through cytokine production and upregulation of co-stimulatory molecules.

Toll-like receptors

The Toll-like receptors (TLR) are a family of PRR receptors that are considered the predominant sensors of innate recognition (66). The family was originally identified in *Drosophila*, as a receptor named responsible for dorsal-ventral orientation. Later, the human homologs were discovered and shown to bind to various PAMP's (58). The family of receptors is characterized by an extracellular leucine-rich region and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain (64). Humans have 10 different TLR's that form homodimers of themselves with the exception of TLR2 that forms heterodimers with other TLR's giving the TLR2 complex broader specificity for agonists.

The binding of a TLR agonist to the receptor starts a signaling cascade in its effector cell. An adaptor protein, such as MyD88, is recruited to the TIR domain, subsequently MAP kinase cascades result in NF-kB activation. NF-kB is a transcription factor that upregulates proinflammatory genes ultimately leading to activation of the immune system.

YscF as a PAMP

Previous work in our lab has shown that N-terminally truncated YscF (trYscF) was pro-inflammatory in a *Y. pestis* murine infection model (1). The needle protein was inoculated into C57BL/6N mice as described in Matson, 2005 (67). Serum collected 14 days after inoculation from mice vaccinated with trYscF, showed a significant increase in pro-inflammatory cytokines compared to the full-length YscF. This *in vivo* result was confirmed *in vitro* with THP-1 cells, a human monocyte cell line. 24 hours after exposure to trYscF, the monocytes released significantly more TNF-α compared to the THP-1 cells exposed to full-length YscF (1). This demonstrated an ability of YscF and trYscF to induce pro-inflammatory cytokine release in two different experimental models (1).

The results in this thesis explore treatment with other needle homologs of YscF with *in vitro* macrophage tissue culture, using primarily TNF-α as a marker of activation. Full-length versions of the proteins will be tested alongside an equivalent truncation of the N-terminus compared to YscF. Needle homologs from *S. enterica* Sv. Typhimurium *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2 are used, PrgI and SsaG respectively. *S. flexneri* needle homolog MxiH is also tested. The human monocyte THP-1 tissue culture is used as well as primary macrophages from C57BL/6N mice to

measure cytokine response to the bacterial needle protein. Our hypothesis is that

truncated homolog needle proteins will produce an inflammatory response in macrophage

tissue culture, similar to the effect seen for purified YscF.

CHAPTER II

METHODOLOGY

Cell Culture

THP-1 Monocytes

THP-1 monocytes were maintained in an atmosphere of 5% CO_2 at 37° C.

Medium was RPMI-1640 complete (Mediatech, Manassas, VA); 10% v/v fetal bovine

serum (Atlanta Biological Laboratories; Lawrenceville, GA), 2mM glutamine

(Mediatech), 0.05 µM β-mercaptoethanol (Fisher BioReagents, Fairlawn, NJ), 2mM

Cellgro® antibiotic-antimycotic solution (Mediatech). The monocytes were propagated

according to ATCC guidelines; passaging was accomplished at $8x10^5$ to $1x10^6$ cells/ml,

every 2-3 days to a density of $2x10^5$ cells/ml. Cell viability was assessed by trypan blue

exclusion staining. PMA-treated monocytes were exposed to a concentration of 160nM

PMA for a 24 hour time period. They were verified to have differentiated into an

adherent cell type, with greater than 95% of the monocytes adhering to the culture flask.

The treated THP-1 cells were washed once with complete medium before protein

induction.

B6 Peritoneal Macrophages

Peritoneal macrophages from C57BL/6N mice were obtained from Dr. Nathaniel

Lambert, University of North Dakota. They were maintained in RPMI-1640 complete

(Mediatech) for 48 hours in 24-well plates at a density of $8x10^5$ cells/ml. Before

experimentation, they were washed with RPMI once to remove non-adherent cells. Fresh

RPMI-1640, complete but without FBS, was applied before cytokine induction

experiments.

Cloning

Plasmids used in this thesis were constructed using Champion™ TOPO

expression kits (Invitrogen, Carlsbad, CA). Primers for gene amplification were made by

Eurofins MWG Operon, Inc (Huntsville, AL). Primers used for cloning were as follows:

WT PrgI forward 5'- CAC CAT GGC AAC ACC TTG GTC-3', PrgI reverse 5'- TTA

ACG GAA GTT CTG AAT AAT GGC AG-3', WT SsaG forward 5'-CAC CAT GGA

TAT TGC ACA ATT AGT GGA TAG CTC TCC-3', SsaG reverse 5'-TCA GAT TTT

AGC AAT GAT TCC ACT AAG CAT ATC C-3', Truncated SsaG forward 5'-CAC

CCT CTC CCA CAT GGC GCA C-3'. Template DNA for amplification was generated

using DNeasy kit (Qiagen; Valencia, CA); the manufacturer's instructions were followed.

PCR was conducted using PFU Turbo® polymerase (Agilent Technologies, Santa Clara,

CA). Amplified DNA was then placed in pET200 using the Champion™ TOPO

expression kit, manufacturer's instructions were followed. All of the gene constructs

were sequence verified by Eurofins MWG Operon. Vectors were stored in TOP10 *E. coli*

by chemical transformation. WT and truncated MxiH and truncated PrgI encoding

plasmids are a kind gift from Dr. William Picking, Oklahoma State University.

Recombinant Protein Expression

Protein expression

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, Carlsbad, CA). Protein expression was conducted

as described by Studier, 2005 (68). Auto-induction was found to result in optimal protein

expression at 6 hours of growth. Cells were harvested by centrifugation and resuspended in wash buffer. Wash buffer consisted of 50 mM Na_3PO_4, 300 mM NaCl, and 10% w/v glycerol (Fisher Chemical; Fairlawn, NJ). The cells were passaged two to four times through a French cell press at a pressure of 18,000 lb/in^2 until an opalescent product was obtained. The cell lysate was spun at 20,000 x g for 10 min to clarify the soluble protein. Protein purification was conducted using Talon resin (Clontech; Mountain View, CA), manufacturer's instructions were followed. The purified protein was extensively dialyzed against a solution of PBS and 20% w/v glycerol.

Concentration and Quantification of protein

The purified protein product was concentrated using an Amicon Ultra-15 unit (Millipore; Billerica, MA). Protein was quantified using a Pierce BCA protein assay kit (Thermo Scientific; Rockford, IL), manufacturer's instructions were followed. SDS-PAGE was conducted on a 15% gel as described in (69). Protein was suspended 1:1 with 2x SDS sample buffer and boiled for 5 minutes before loading on gel. Protein was aliquoted and stored at -20°C before experimentation.

Cytokine Induction Experiments

Induction of THP-1 cells was conducted after seeding the cells into 24-well tissue culture plates at a density of $8x10^5$ cells/ml in induction medium, RPMI-1640 without FBS. The cells were acclimated to the wells for 2-8 h before addition of needle protein. Protein was applied at a concentration of 1µg/ml or 105 nM as noted per experiment. LPS positive control was applied at a concentration of 1µg/ml. THP-1 cells were incubated with the respective proteins for 5 or 24 hrs at 37°C in a 5% CO_2 environment. Cells were then pelleted by centrifugation, 250 x g for 10 min at 4°C. Culture

supernatants were collected for cytokine analysis; care was taken to not pipette any

cellular particulates.

Cytokine analysis

Cytokine concentration was measured by ELISA. Human TNF-α was measured

by Quantikine® Colormetric Sandwich ELISA (R&D Systems; Minneapolis, MN).

Mouse TNF-α, and human cytokines IL-4, IL-6, IL-8, IL-15, and IL-17A were measured

by Ready-Set-Go!® Sandwich ELISA (Ebioscience, San Diego, CA). Four-parameter

logistic regression was used to create standard curves for quantification of cytokine

concentrations. Statistical analysis of the data was performed by Prism Graphpad Sigma

v5.0d (LaJolla, CA). One-way ANOVA was used with Tukey's post-hoc test. p values

less than 0.05 were considered to be statistically significant.

CHAPTER III

RESULTS

Previous work showed that the *Y. pestis* T3S needle protein YscF and an N-terminally truncated variant of YscF trigger release of pro-inflammatory mediators from cells (1). This ability was demonstrated with cultured cells *in vitro* and *in vivo* in a mouse model (1). To expand on this observation, homologs of YscF with similar N-terminal modifications were tested to further understand this phenomenon. Additional needle proteins tested were *S. flexneri* MxiH, *S. enterica* Sv. Typhimurium SPI-1 PrgI, and SPI-2 SsaG. The hypothesis is that homolog needle proteins will be recognized by tissue macrophages as pathogenic and elicit an inflammatory response from the cells similar to the YscF needle protein.

Figure 2A shows a CLUSTAL V alignment of the four proteins, with sequence conservation labeled in yellow. The CLUSTAL V alignment algorithm was used because it is well suited to analyze terminal variations like in the case of the highly variable N-termini of these T3SS needle proteins (96). A cursory look at the needle proteins shows that the middle and C-terminal portions of the proteins are relatively conserved in contrast to the highly variable N-terminus. In particular, certain residues are identical in all four proteins, these residues are likely important to form the final needle structure. Importantly, these needle proteins form multimers *in vivo* that have been shown to be abrogated by cleavage of five amino acids on the C-termini of MxiH, PrgI, and BsaL (70, 71). Since needle proteins are so strongly conserved in that region, it is inferred that the region is critical for polymerization in other needle proteins as well (72, 73).

A truncation of each respective protein that would correspond to the YscF 1-22 truncation was found after alignment with YscF. This yielded a deletion of residues 1-18 from MxiH, 1-17 of PrgI, and 1-9 of SsaG. Overexpression of these proteins was conducted as previously described (68), using a T7-based system. Purification was conducted on a cobalt resin column, utilizing a His_6-tag on the N-termini of the recombinant protein. In addition, there is an 8 amino acid epitope marker encoded by the vector for identification with antibodies directly subsequent to the His_6-tag and before the protein sequence. The proteins were kept in a 20% w/v glycerol solution to decrease non-specific protein interactions (74). WT and truncated versions of each protein were overexpressed as described in methods then resolved using SDS-PAGE on a 15% gel for visualization (Fig. 2B). The needle proteins have an approximate weight of 9 kDa, and the resolved gel yielded several discrete bands of protein. This ladder effect was expected as a normal property of the naturally polymerizing protein, and has acted in this way in our hands as well as others (67, 72). The addition of glycerol was successful in limiting non-specific aggregation of protein after a freeze-thaw cycle, demonstrated by lack of visible protein precipitation in the solutions.

To test the potential PAMP activity of these proteins, we used tissue culture models that are known to express receptors capable of detecting these pathogenic motifs. First, primary peritoneal mouse macrophage cells obtained by peritoneal lavage of B6 WT mice were used. TNF-α was used as a marker of macrophage activation, which is extensively characterized as an important inflammatory cytokine produced primarily by macrophages. TNF-α is important due to its role in systemic inflammation, increasing phagocytosis in infections, and having a negative impact in autoimmune

A

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Consensus #1 MS--..-----W.--LD..S.T.D.GAD..Q.AV.DALDKLA.KPSNPALLA.YQSKLSEYSLYRNAQSNTVKVIKD.DA.IIQ.FR
Majority     MS--XX-----WX--LDXXSXTXDXGADXXQXAVXDALDKLAXKPSNPALLAXYQSKLSEYSLYRNAQSNTVKVIKDXDAXIIQXFR

                      10        20        30        40        50        60        70        80

YscF  MSNFSGFTKGTDIADLDAVAQTLKKPADDANKAVNDSIAALKDKPDNPALLADLQHSINKWSVIYNINSTIVRSMKDLMQGILQKFP  87
MxiH  MS--VTVPNDDWT--LSSLSETFDDGTQTLQGELTLALDKLAKNPSNPQLLAEYQSKLSEYTLYRNAQSNTVKVIKDVDAAIIQNFR  83
PrgI  MA--TP-----WSGYLDDVSAKFDTGVDNLQTQVTEALDKLAAKPSDPALLAAYQSKLSEYNLYRNAQSNTVKVFKDIDAAIIQNFR  80
SsaG  MD-------------IAQLVDMLSHMAHQAGQAINDKMN--GNDLLNPESMIKAQFALQQYSTFINYESSLIKMIKDMLSGIIAKI    71
```

B

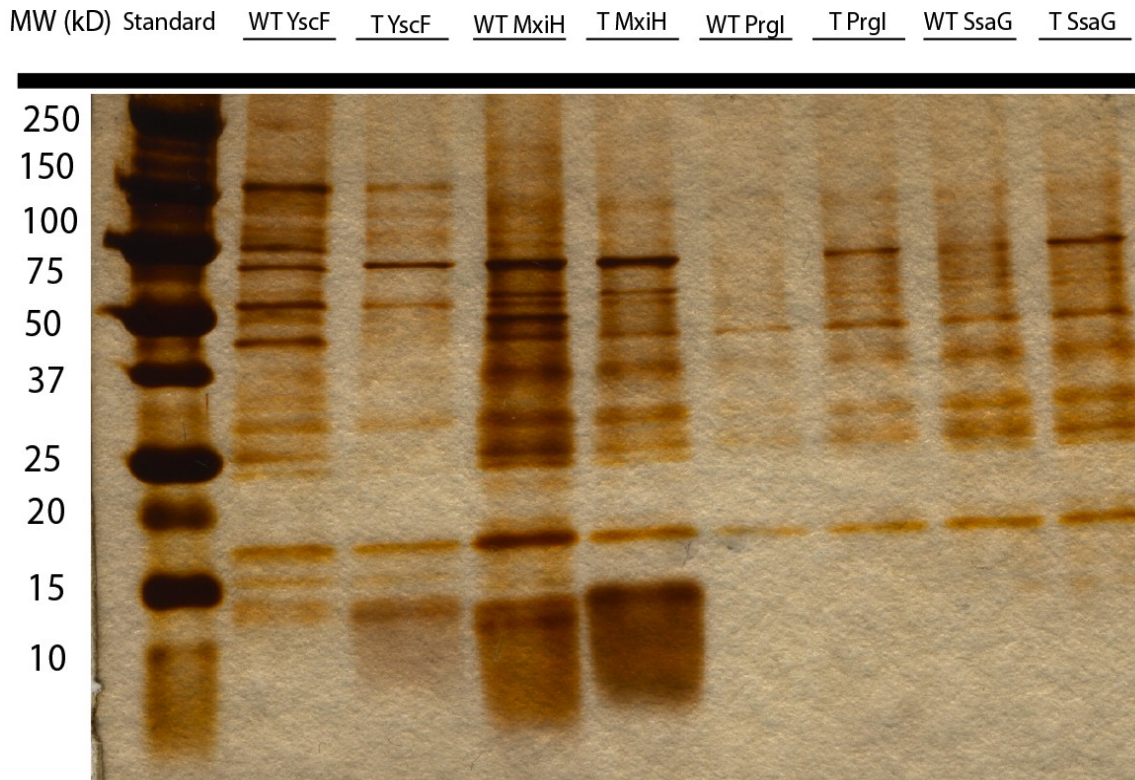Figure 2. Production of homolog proteins. Panel A depicts alignment according to CLUSTAL V method using Megalign software. Yellow coloring indicates identical amino acids in at least two homolog proteins. Panel B shows results of protein purification; 5 µl samples of protein were run on a 15% SDS gel after being solubilized in 2x SDS sample buffer and boiled for 2 minutes. Gel was fixed and silver stained for visualization.

disorders (75). TNF-α is maximally expressed at 4-5 hours after exposure to a PRR

agonist (76). Since TNF-α was the primary marker of activation, we used a 5 h time

point throughout these experiments to maximize the level of TNF-α release providing

clarification of any differences between treatments.

Macrophages were exposed to the needle proteins at a concentration of 105 nM,

the equivalent concentration of 1 µg/ml of YscF, the concentration used in prior

experimentation with YscF (1). The supernatants were analyzed by ELISA and data

normalized according to LPS activation. As shown in Fig. 3, truncated YscF was used as

a positive control and elicited TNF-α release from the murine macrophages. PBS with

20% glycerol w/v (assay diluent) served as a negative control for the experiments. WT

PrgI elicited a small amount of TNF-α release, and was statistically lower than the TNF-α

response from the N-terminally truncated PrgI. The elicited response to WT SsaG was

not statistically different from TNF-α release stimulated by truncated SsaG in this

experiment. Both variants of SsaG elicited TNF-α release from the macrophages, in

contrast to the other needle proteins tested. The response elicited by WT and truncated

MxiH was persistently variable and made conclusions about MxiH's ability to stimulate

TNF-α release difficult in this experiment.

Murine macrophages have been shown to express TLR's differently than human

macrophages (77). Specifically, TLR5 is not expressed by murine bone marrow-derived

macrophages, or peritoneal macrophages (77). This is in contrast to human macrophages

and dendritic cells which do produce TLR5 (78). The induction experiment shown in

appendix A (Fig. 7) shows the response of murine BM-derived macrophages. These

macrophages showed inflammatory responses to the needle proteins. This data taken

together with the results in figure 3 demonstrate independence from TLR5 signaling as a

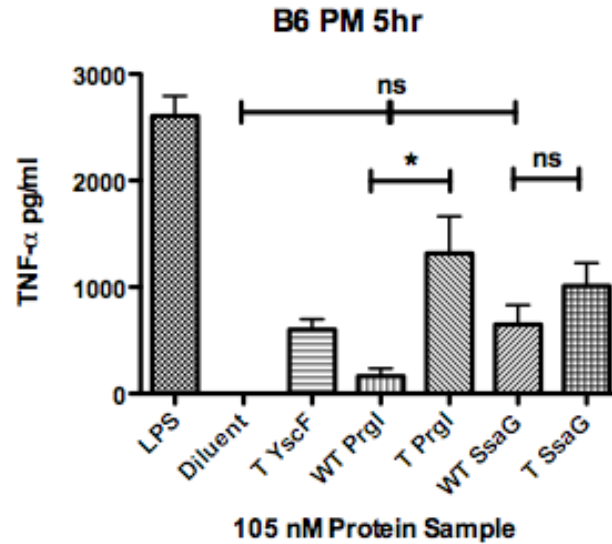potential mechanism of activation for the inflammatory needle proteins.

Figure 3. Protein induction in B6 peritoneal macrophages. Peritoneal macrophages were seeded at a concentration of 8 x 10^5 cells/ml in RPMI-1640. Protein was applied at a concentration of 105 nM and incubated for 5 hours before supernatant was collected for cytokine measurement. Mouse TNF-α ELISA was used to measure cytokine release, and data was normalized compared to LPS control. % activation is defined by TNF-α release in response to treatment. Graph represents the results of 2 experiments with an n=6. Error bars are S.E.M. Statistical significance determined by Tukey's post-test. *, $p < 0.05$.

The monocyte cell line, THP-1, was next used to study the effect of these needle

proteins in a human cell line model. Identical methodology was used in experimentation

as with the murine cell line. Fig. 4A shows the results of TNF-α release from the

monocytes, normalized to LPS activation. Again, truncated PrgI was significantly higher

in activating monocytes compared to its WT counterpart, with the truncated protein

producing approximately 50% of the LPS activation. In THP-1 monocytes, there was a

statistical difference between the responses to WT and truncated SsaG, and it should be

noted that the WT SsaG treatment produced more of a TNF-α response than the other WT

proteins tested. MxiH data is presented in appendix A (Fig.8). Again, the response was variable, however statistically the WT and truncated MxiH produced a non-significant difference from each other. TNF-α cytokine release was also measured at 24 hours (Fig. 9). The quantity of TNF-α was globally diminished at the 24 hour timepont, however trends between WT and truncated protein were comparable to the 5 hour time point. Additionally, *Vibrio parahaemolyticus* needle protein VYscF elicited an inflammatory response of 1,700 pg/ml TNF-α in THP-1 monocytes in one experiment after N-terminal truncation.

Figure 4B shows protein induction in PMA-treated THP-1 cells that have been differentiated into a macrophage cell type. TNF-α induction trends were retained between WT and truncated proteins (Fig. 4A). The PMA-treatment made the THP-1 cells more responsive to the proteins, with the truncated protein phenotype changing from 50% LPS activation to 75% of the LPS activation (Fig. 4). Therefore, in both murine and human macrophage cell types, truncated PrgI reacts in a similar way to truncated YscF in eliciting a response from macrophages. WT SsaG produces a response that is little different from the amount of TNF-α release elicited by truncated SsaG.

To further understand how THP-1 monocytes respond to the needle proteins, we next looked at IL-8 release. IL-8 is an important chemokine produced by macrophages. IL-8's main function is attraction and activation of neutrophils at the site of infection (79). Figure 5 shows that truncated YscF, PrgI, and SsaG treatments resulted in similar amounts of IL-8 production. The truncated PrgI treatment elicited significantly more IL-8 than

A

B
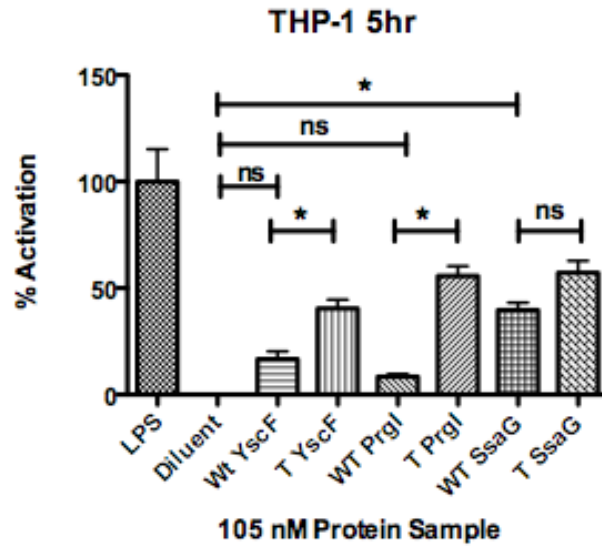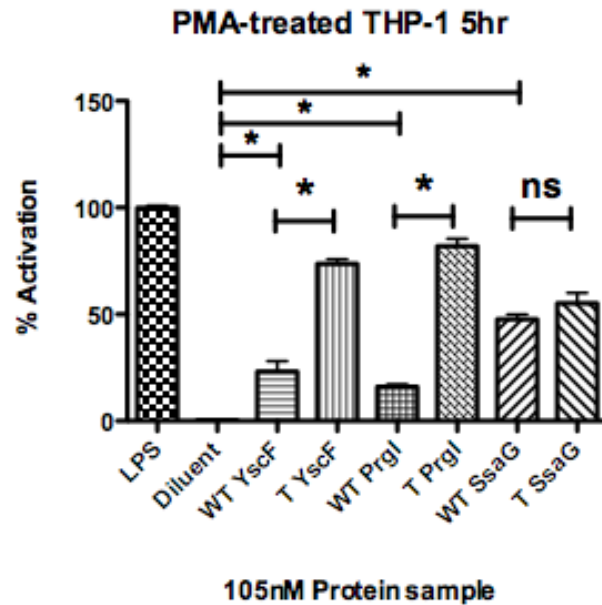
Figure 4. TNF-α release from protein induction in THP-1 culture. Panel A shows the induction experiment in THP-1 non-activated monocyte culture, at a concentration of 8 x 10^5 cells/ml. Panel B is activated THP-1 cells, accomplished through 24 hour incubation with PMA causing the cells to become adherent before application of protein. Protein is applied at 105 nM and incubated for 5 hours, before supernatant was collected for cytokine measurement. % activation is defined by TNF-α release in response to treatment, normalized to the response to LPS. Panel A represents 3 independent

experiments with n=9. Panel B represents 1 experiment, n=3. Error bars are S.E.M. Statistical significance determined by Tukey's post-test. *, p <0.05.

WT PrgI. SsaG is significantly different from the truncated SsaG treatment, while still producing a response. These results carried the trends seen in the TNF-α cytokine data.

Other cytokines were measured to fully characterize the response produced by the macrophages. The cytokines chosen represent different strategies of the immune system for overcoming infection. IL-17A is primarily produced by Th17 T cells and is representative of a neutrophil-driven response from the immune system (80).
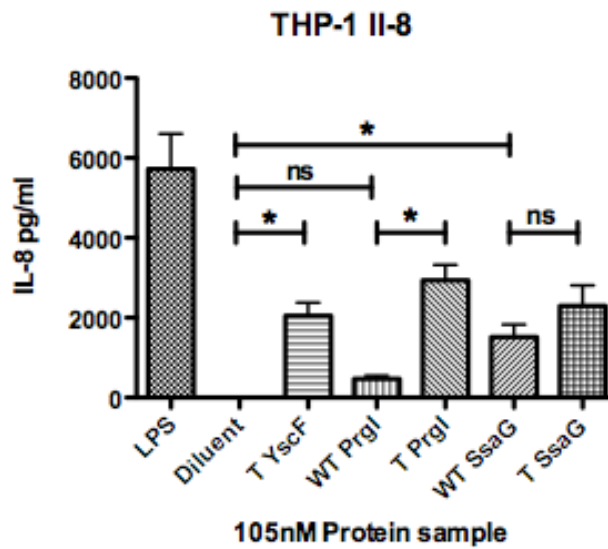
Figure 5. IL-8 release from protein induction in THP-1 culture. Protein induction experiment showing IL-8 cytokine release in THP-1 monocyte culture. Protein is applied at 105 nM and incubated for 5 hours, before supernatant was collected for cytokine measurement. Graph represents 3 independent experiments, n=9. Error bars are S.E.M. Statistical significance determined by Tukey's post-test. *, p <0.05.

IL-15 promotes NK cell and CD8+ T cell proliferation and differentiation (81). IL-6 is another important cytokine for systemic inflammation, long known for its role in the acute phase response and as a lymphocyte stimulator (82).   IL-4 acts on humoral

immunity by increasing B-cell proliferation and by reinforcing a Th2 response from CD4+ T cells (83). As shown in figure 6, there was no significant difference between any of the treatments for IL-17A with the negative control, with all treatments producing a small amount of IL-17A but WT YscF. IL-17A is not a typical cytokine expressed by monocytes, and the results seen may have been a baseline level of expression. IL-15 and IL-6 had similar trends, with treatments not being significantly different from each other or controls. IL-4 did not have a response, which might have been under the sensitivity of the ELISA. For the cytokines tested in figure 6, additional investigation is needed at a later timepoint than 5 hrs. While TNF-α is maximally expressed at this timepoint, other cytokines such as the ones measured in figure 6 are expressed at later timepoints such as 24 hrs.

These results demonstrate that induction of cytokines by YscF, truncated YscF, and other protein homologs is conserved across species of bacteria, indicating that the protein is recognized as a PAMP by the innate immune system. Further, the variable N-terminus that is present on these needle proteins appeares to interfere with this recognition of the PAMP by macrophages in both mouse and human tissue culture models.
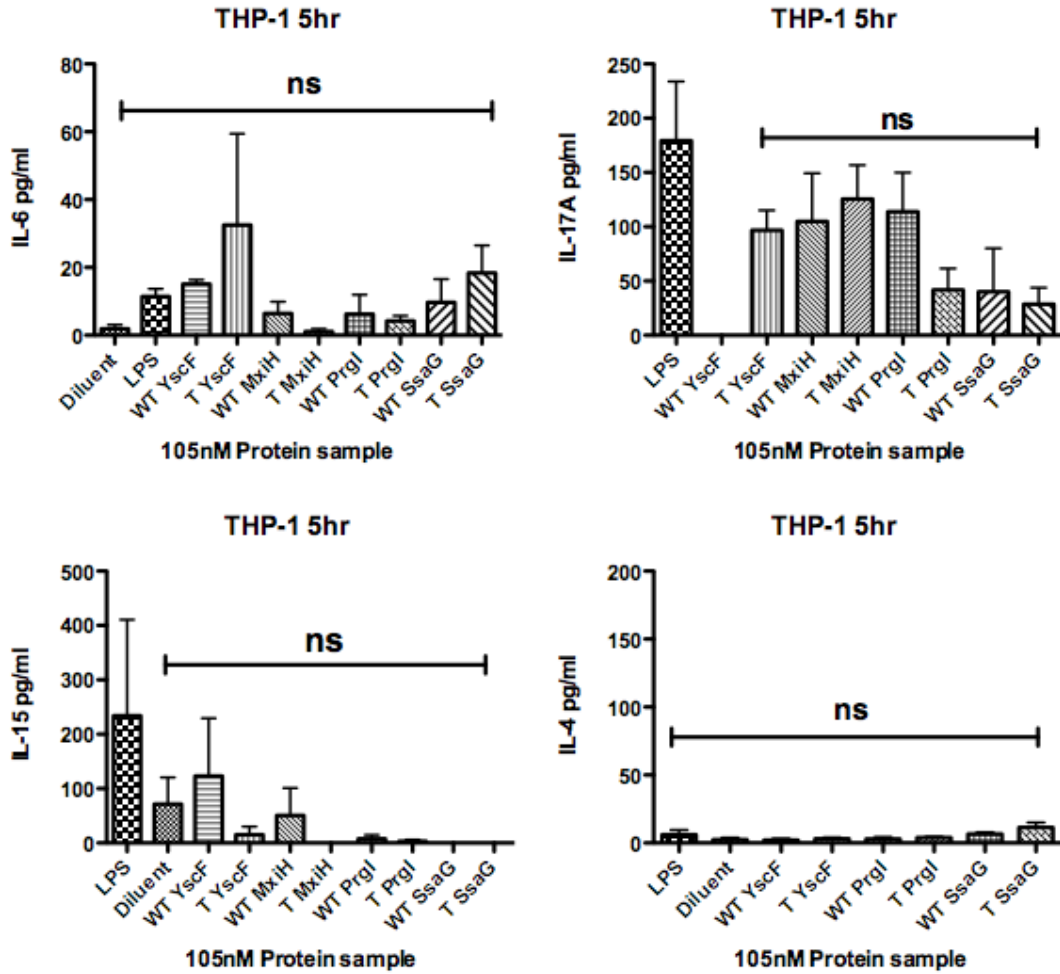
Figure 6. Various cytokines released from THP-1 protein induction. THP-1 cells were seeded at a concentration of 8 x 10^5 cells/ml. The monocytes were induced with 105 nM of respective protein. Supernatant was collected at 5 hours and measured for appropriate cytokine by ELISA. IL-6, IL-17A, IL-15, and IL-4, are the results of one experiment with an n = 3. Error bars are S.E.M. Statistical significance determined by Tukey's post-test. NS is not significant.

CHAPTER IV

DISCUSSION

YscF is a PAMP

Y. pestis is a gram-negative pathogen responsible for multiple pandemics in human history (2). The action of *Y. pestis*' T3SS is essential for virulence (13), and the effectors secreted from this apparatus allow the bacterium to avoid phagocytosis and aid survival in macrophages if phagocytosis is accomplished (14). Use of a T3SS by *Y. pestis* is not unique to the pathogen and is expressed in many other gram-negative pathogens, from the fellow *Enterobacteriaceae S. flexneri*, to plant pathogens such as *Pseudomonas syringae* (51). The structure of the T3S systems is highly conserved and exposed portions of the apparatus, such as the needle, have potential to be recognized by the host as a pathogen-associated molecular pattern and be inflammatory if detected.

This thesis demonstrated that modified T3SS needle proteins are capable of eliciting a strong inflammatory response from macrophage tissue culture. This was demonstrated with N-terminally truncated YscF, PrgI, and SsaG; which belong to *Y. pestis*, *S. enterica* Sv. Typhimurium SPI-1 and SPI-2 respectively. We chose the needle protein SsaG for experimentation because it lacks the variable N-terminus that most T3SS needle proteins possess, essentially making SsaG a natural truncation. Interestingly, the wildtype SsaG protein was also inflammatory, equal or similar to the truncated versions of the protein, which strongly supports the notion that the observed inflammatory effect is a relevant biological response. We saw this effect in both human and mouse macrophages, indicating the PRR responsible for detection of the needle protein is expressed in both organisms.

An exception to the trends displayed by PrgI, SsaG, and YscF was the *S. flexneri* homolog MxiH. MxiH's elicited response in macrophages was variable leading to difficulties in interpretation of the data. The data with THP-1 monocytes, however, showed a repeatable non-significant difference between WT and truncated MxiH proteins, with neither producing an inflammatory response. If the truncation to MxiH does not produce a response in macrophages, this could provide valuable insight into the mechanism for producing inflammatory effects in response to the other homologs by analyzing the limited differences in structure between MxiH and other needle proteins.

The five hour timepoint used in the experimentation was selected for maximal expression of TNF-α in macrophages. It should be noted, however, that 5 hours post-induction is of limited utility for other important cytokines: IL-17, IL-15, IL-6, and IL-4, as evidenced by the minimal expression. A later timepoint, such as 24 hours, would be more optimal for study of those cytokines, if they are induced by needle proteins.

Truncated YscF as a vaccine

The data in this thesis agrees with prior work done in our laboratory on YscF and its pro-inflammatory properties. YscF has been shown to be protective in a mouse model of *Y. pestis* infection (67). In efforts to further study YscF as a potential vaccine candidate, the protein was epitope mapped with antiserum from mice exposed to the WT protein (1). Two areas were found to have antibodies generated against them; a major epitope on the N-terminus at roughly resides 8-15, and a minor epitope in the middle of the protein next to the turn between the two α-helices that would be solvent-accessible, a good antigen candidate (1). The N-terminus, while the major epitope, is relatively non-accessible after polymerization of the needle; it folds back in on the middle and C-

terminal portions and is on the interior of the needle structure, which limits its exposure to the solvent (84). In an effort to push the host antibody response to the more accessible minor epitope at the helix-turn-helix, the N-terminal 22 amino acids were truncated from YscF, which removed the major epitope. Subsequently, immunization of mice was conducted and followed by exposure to *Y. pestis*. Interestingly, average time to death was lower with the mice immunized with truncated YscF, compared to WT YscF immunized mice (1). Analysis of the cytokines in the collected serum indicated that the mice with the truncated YscF immunization had much higher quantities of pro-inflammatory cytokines after *Y. pestis* exposure compared to the WT YscF immunized mice (1). This unexpected result was followed up through *in vitro* experimentation with THP-1 human monocytes, which repeated the observation by seeing a significant pro-inflammatory response with the truncated YscF protein, and relatively little response from the WT protein.

Function of the variable N-terminus?

We wanted to expand on the previous work (1), in this thesis by showing that other needle proteins are also recognized as PAMP's by the host immune system. PAMP's can be predicted based upon three properties of being: essential to the pathogen, conserved in structure across species of bacteria, and distinctly "non-self" in terms of the host defense mechanisms (65). If attempting to predict a PAMP, this makes the T3SS needle a good candidate as it is readily exposed to the host, the solvent-accessible portion of the needle is strongly conserved between pathogens, it is essential to the virulence of the pathogens studied in this thesis, and there are no eukaryotic homologs present in the host.

The data presented in the thesis supports the hypothesis that the needle protein functions similarly across species of bacteria, and reinforces the idea that the inflammatory effect seen after N-terminal truncation in YscF is not unique or artifactual. PrgI is the SPI-1 homologue in *S. enterica* Sv. Typhimurium, which acts in a way that is similar to the T3SS of *Y. pestis* in that it is expressed in the extracellular environment of the host. Structurally, the two T3SS's share homology, but the comparison ends there. *Y. pestis* expresses effectors that actively avoid phagocytosis and has an overarching strategy of subterfuge with the innate immune system (3). The SPI-1 system however, actively induces inflammation to obtain access through the intestinal epithelium by injecting its effectors that induce inflammation (85). After induced phagocytosis, the SPI-2 system takes precedence during *Salmonella* infection. SPI-2 functions control biogenesis of the intracellular *Salmonella*-containing vacuole (SCV) that is crucial for systemic survival of the pathogen (56). The SPI-2 T3SS is expressed in its intracellular niche and is exposed to a very different environment in the vacuole than the extracellular environment of SPI-1 (86). As shown before in figure 2A, SsaG is missing the majority of the highly non-conserved N-terminus that most T3SS needle proteins have.

This raises the question of the function performed by the N-terminus of the needle proteins. The fact that the N-terminus of needle proteins is highly variable in composition and length, even to the point of being absent suggests that it is not critical to the central function of the needle, which is to provide a conduit for effectors from bacterium to eukaryote host. One could make the supposition that the interior of the needle needs to be non-interactive with species-specific effectors to prevent a blockage of the small space on the interior of the needle. Between species of bacteria, the effectors of

the T3SS are very variable compared to the actual structure, so it would be logical for the interior of the needle to have the most variation in the needle structure. This potential function of the N-terminus is countered by evidence showing that effectors from other species of bacteria are able to be secreted in different systems; for example, *Chlamydia* effectors are able to be secreted in the *Yersinia* or *Salmonella* T3SS (87, 88). With this evidence in mind, the N-terminus is likely not important in effector secretion and is present for another function.

The data in this thesis shows that removal of the N-terminus of the needle protein causes YscF and PrgI to go from relatively innocuous, to causing a significant release of TNF-α and IL-8 from macrophages. This leads to the reasoning that the N-terminus either obscures the PAMP-site that a receptor is able to recognize, or is causing a change in conformation in the needle or needle proteins that identifies the needle as a pathogen for the respective PRR.

The choice between these two hypotheses is contingent on a question, is the needle protein stimulating its PRR as a monomer/dimer or in its polymerized needle structure? Unfortunately, our data so far does not give an answer to this question, as the protein purification yields bands of monomeric protein to high molecular-weight multimers. If the protein is stimulating the receptor as a monomer, one could easily see how bacterial evolution of an N-terminus to physically obscure the receptor's recognition could happen. This hypothesis assumes that addition of the N-terminus is an evolutionary recent event, and could account for the variability in the region seen across species of bacteria. Furthermore, even in species of bacteria that actively induce inflammation, such as the strategy of *Salmonella* with its SPI-1 T3SS, one could argue

that the inflammation process is able to be regulated by the bacterium. Whereas an inflammatory needle could be a constitutively active mechanism.

Alternatively, it is possible that the higher molecular weight species of protein from overexpression are activating a PRR after polymerization into a needle structure. In the context of the final needle structure, the idea of the N-termini of these proteins "hiding", as in disallowing spatial access, a PAMP in the needle seems unlikely as the N-terminus is predicted to line the interior of the needle (84). Due to the invariant nature of pathogen recognition receptors, it seems peculiar that creating an artificial change to the interior of the needle structure would suddenly activate one of these germline-encoded receptors. However, if the needle is exposed to the host in more than one conformation of its structure, the truncation to the protein could have forced the needle into a conformation that is recognized by the host as pathogenic, while the *in vitro* wildtype protein is in a constitutively non-inflammatory form. As a counterargument to the truncated needle protein possibly replicating a conformation that is seen *in vivo*, it should be noted that the truncated YscF does not complement a mutant's phenotype when expressed on a plasmid in *Yersinia pestis* (G.V. Plano, personal communication).

The idea that the T3SS needle might have more than one conformation is not novel. The closest crystal structure we presently have for YscF is the MxiH structure, which the authors reported an "A" and "B" form in their study (89, 84). The difference in conformations was largely in a different angle in the region we identified as a minor epitope, which interestingly corresponds to the area that YscF could interact with the needle tip protein, LcrV. Furthermore, it is known that the T3SS does not secrete non-specifically into the environment, but rather translocates exclusively into host cells. The

signaling mechanism for host-cell contact is currently unknown. However, since there are multiple needles on a bacterium's surface, in the range of 10's to 100's depending on the species (45), the control of translocation almost has to be specific to each needle structure. There are no auxiliary structures connecting the host and bacterium on each individual needle, so it is reasonable to assume the signal of host-cell contact must be conveyed through the needle itself.

The possibility of the N-terminus being involved in changing the conformation of the needle structure, and consequently sending a secretion signal is potentially held back by the absence of this region on SsaG, since the SPI-2 system does not constitutively secrete effectors. However, a recent study shows that the SPI-2 system expression and signal for secretion could be controlled in a different manner from most T3SS's, by the very distinct change of pH from the host lumen to the *Salmonella*-containing vacuole (90). This strong signal of pH for appropriate timing of SPI-2 secretion is a mechanism that most bacteria must generate on their own in the absence of a distinct environmental signal. If the N-terminus' function is to change the conformation of the needle to convey the secretion signal, the functionality of this region in hiding the pathogenicity of the needle would be secondary or possibly even coincidental. This topic holds great interest for general T3SS biology, and further investigation on the function of the N-terminus of the T3SS needle bears merit.

Which PRR is stimulated?

The pathogen recognition receptor (PRR) that is activating in response to truncated YscF, PrgI, and SsaG is currently unknown. PRR is a very broad description of multiple classes of receptors that are able to recognize pathogenic proteins, lipoproteins,

lipids, and nucleic acids (66). A good candidate in future studies on this phenomenon would be the TLR class of PRR's, and in particular the externally expressed TLR's, TLR1/2, TLR2, TLR4, TLR5, and TLR2/6. TLR5 was shown in our data to not be a major player in the inflammatory response, mouse bone-marrow and peritoneal macrophages have been shown to produce little or no TLR5 (77), and we still observed a strong inflammatory response in those macrophages. However, TLR2 has been shown to recognize bacterial protein (91), and has shown the broadest specificity to PAMP's through its heterodimer structures. One direction to study the mechanism of activation would be to abrogate TLR activity completely. This could be accomplished by knocking out the vital adaptor molecules necessary for TLR signaling such as MyD88 and TRIF. Using knockout macrophages the protein induction response could be measured and analyzed. This experiment that would give a broad knockout of TLR activity would setup further investigation into either specific TLR receptors or investigation into other classes of PRR's as the source of recognition.

Looking forward: A potential adjuvant candidate

The finding that a modified T3SS needle is a PAMP recognized by the immune system is potentially useful for the design of an adjuvant product. The protein is highly potent, producing 75% of the response to LPS in our PMA-activated human macrophage model despite the needle proteins being an order of magnitude less in molar concentration compared to LPS (Fig. 4B). Stimulators of the innate immune system are important for aiding the adaptive immune system to recognize vaccine candidates as pathogenic through production of co-stimulatory molecules and cytokines (59). An especially important effector between innate and adaptive immunity is the dendritic cell

(DC), and studying how the DC reacts to this PAMP would be especially interesting in further exploration of this protein as an adjuvant product.

Initial studies with DC's and truncated YscF could start with measuring activation through pro-inflammatory cytokines, similar to our macrophage experiments. Further investigation could involve conjugating the needle protein to a FACS-detectable label and measuring binding of the protein to the DC, which would allow quantification of binding as well as monitoring DC cell markers such as CD80 and CD86 to show maturation in response to the T3SS needle protein. A good adjuvant candidate should be a potent inducer of innate immunity, have a highly reproducible response, and be non-toxic to humans. Alum-based products are the only adjuvants approved for human use, which unfortunately generate relatively weak cytotoxic T-cell responses. Novel adjuvants that push the immune system towards cell-based and humoral immunity are needed for more effective vaccines (92). Another bacterial protein, FliC or flagellin, has been shown to stimulate TLR5 and be recognized by the host as a PAMP (62). After clarifying the mechanism of action of the protein, and showing mucosal adjuvant activity and a Th2 response from the immune system, clinical trials are now underway for flagellin-based vaccines (93). The precedent of a bacterial protein PAMP reaching clinical trials as an adjuvant aids in support of further characterization of trYscF and adds interest to its possible role as an adjuvant product.

Structure-function studies of YscF using site-directed mutagenesis should yield some interesting data. The YscF needle protein is relatively small at 87 amino acids, so potential study of the protein in this way is realistic. The most likely areas that are being recognized as a PAMP by the host are going to be highly conserved regions, and surface

exposed. The area adjacent to the turn between the two α-helices of the protein would be one target for mutagenesis. This region is the minor epitope identified in previous work, and is also the region of the needle protein that putatively interacts with the tip of the needle (94).   Additionally, the C-terminal 5 amino acids are vital for polymerization of the protein (84), removal of those residues could give valuable information on the importance of the multimer in its inflammatory activity. Alternatively, high molecular weight species of the needle protein could be isolated in the purification by size-exclusion gel filtration as described in (40). Using size-exclusion chromatography would allow exclusion or isolation of the high molecular weight needle protein, and allow experimentation without further truncation of the sample. Studying the protein further to identify the exact region of PRR recognition would accomplish two things; it would allow experimentation with a truncated, innocuous form of YscF by changing the respective amino acid residues to benign substitutions. This allows investigation into if the protein is more protective with a readily available immunogenic site, without its inflammatory properties. Secondly, understanding the means of activation would support research into the protein as a potential adjuvant for vaccine work.

Besides the potential use as an adjuvant, truncated YscF would have a unique advantage in use as a multivalent recombinant *Y. pestis* vaccine due to its immunogenic and potential adjuvant properties. Recombinant protein vaccines for *Y. pestis* would have the benefit of avoiding the severe side-effects that previous whole-cell killed vaccines, and live-attenuated EV76 vaccines have had on humans (95, 3). A multivalent *Y. pestis* protein subunit vaccine provides some measure of protection against a terrorist

organization engineering a mutant protein around the immunological protection afforded by a recombinant subunit vaccine.

Looking forward, characterization of the modified YscF and its effect on the host bears merit both for its potential use as an adjuvant, and for its immunogenic properties in a *Y. pestis* vaccine. Additionally, expanding this observation to other species that express T3SS opens the door to answering questions of T3SS biology, including yielding clues on the transmission of host-contact signal to the bacterium and evolution in the avoidance of the host immune system.

APPENDICES
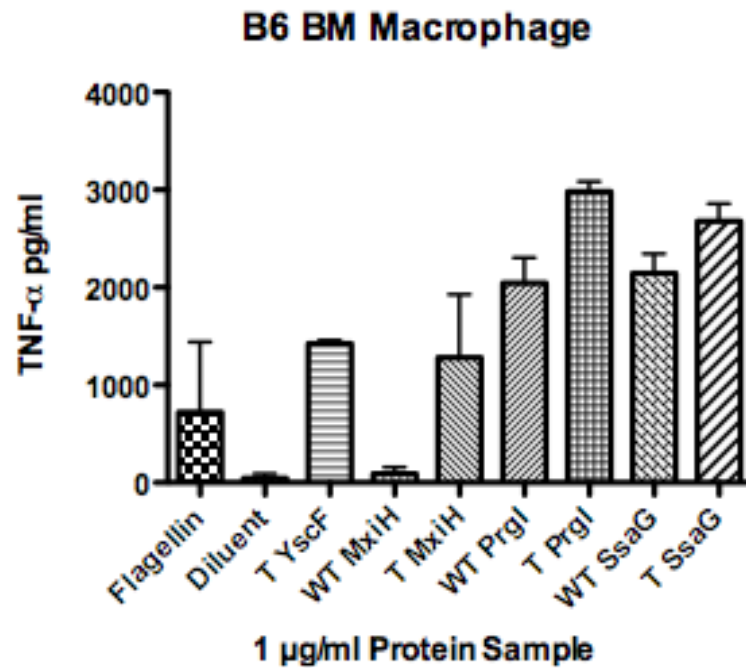
B6 BM Macrophage

Figure 7. Murine bone marrow-derived macrophage protein induction. Macrophages were seeded at a concentration of 2×10^5 cells/ml in DMEM supplemented with colony stimulating factor. Protein induction was conducted with 1 µg/ml protein samples. Macrophages were incubated with protein for 5 hours before supernatant was collected for cytokine analysis by ELISA. Graph is the results of one experiment, n=3. Error bars are S.E.M.
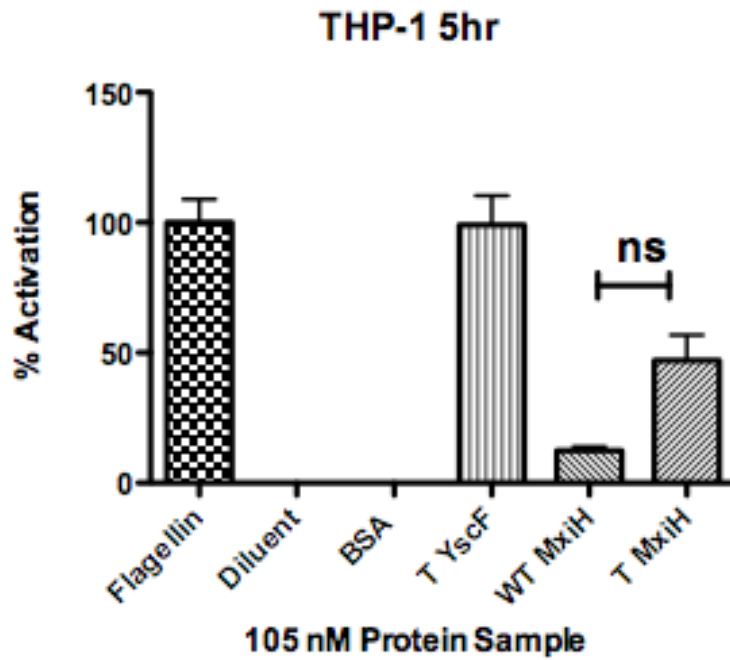
Figure 8. THP-1 monocyte with MxiH induction. Monocytes were seeded at a concentration of 8 x 10^5 cells/ml. MxiH was applied at a concentration of 105nM and incubated for 5 hours before supernatant was collected for cytokine measurement. Human TNF-α ELISA was used to measure cytokine release, and data was normalized compared to flagellin control. Graph represents 3 experiments, n=9. Error bars are S.E.M. Statistical significance determined by Tukey's post-test. *,p <0.05.

THP-1 24hr
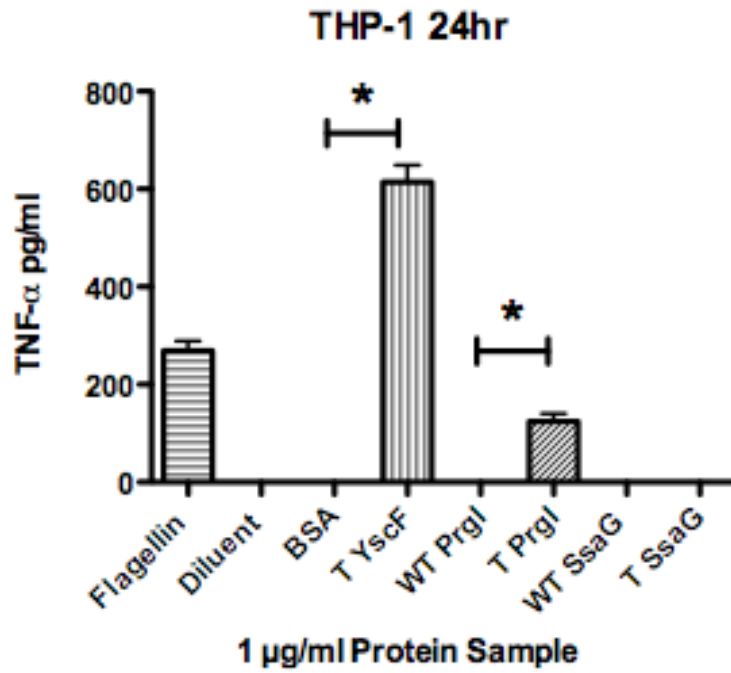
Figure 9. THP-1 monocyte protein induction at 24 hours. Monocytes were seeded at 8×10^5 cells/ml. Protein was applied at a concentration of 1 µg/ml and incubated for 24 hours before supernatant was collected for cytokine measurement. Human TNF-α ELISA was used to measure cytokine release. Graph is the result from one experiment, n=3. Error bars represent S.E.M. Statistical significance determined by Tukey's post-test. *, p<0 .05.

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