

MECHANISMS BY WHICH *SHIGELLA FLEXNERI* IMPAIRS DENDRITIC CELL  
FUNCTION: SILENCING THE LINK BETWEEN INNATE AND ADAPTIVE  
IMMUNITY

by

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## Abstract

*Shigella flexneri* is a Gram-negative bacterial pathogen that causes severe gastroenteritis in humans. Upon infection, *Shigella* invades the host colonic epithelium using a type III secretion system (T3SS) that is encoded by a large virulence plasmid. This secretion system injects protein virulence determinants, or “effectors” directly into the host cytosol where they interfere with host function. The innate immune response to *Shigella* infection has been the subject of intense research. By comparison, little is known about how *Shigella* affects adaptive immunity. *Shigella* infection fails to initiate long lasting immunity; therefore, no effective shigellosis vaccine has been developed. Antigen presenting cells, such as the dendritic cell, represent the link between innate and adaptive immune responses and I hypothesize that *Shigella* impairs dendritic cell function as a means of dampening long term immunity. Little is known about *Shigella*-dendritic cell interactions therefore the goal of this project was to characterize this relationship with a focus on *Shigella*'s main virulence factors- the T3SS effectors. After performing an initial screen with our library of single gene deletion mutants I found that many genes on the virulence plasmid play a role in killing dendritic cells. I chose four mutants from the deletion collection screen for further investigation:  $\Delta ospF$  and  $\Delta ospI$  to investigate manipulation of immune signaling in dendritic cells, and  $\Delta mxiD$  and  $\Delta mxiH$  to investigate programmed cell death mechanisms. I apply a yeast model system to characterize inhibition of immune signaling by outer *Shigella* proteins (Osps) and show that targeting of MAPK signaling is evolutionarily conserved. I use a chemical inhibitor to observe caspase reliance in cell death and measure activation of caspase-1 and IL1 $\beta$  among mutant and Wild-type infection conditions. Gaining a thorough understanding of the processes undergone by *Shigella* and related gastroenteric pathogens to interfere with immune responses paves the road for in vivo studies and targets for rational design in vaccine development against bacillary dysentery.

List of Abbreviations Used:

ABIN1	A20 Binding and Inhibitor of NF $\kappa$ b
APC	Antigen presenting cell
APC	Allophycocyanin
ATP	adenosine triphosphate
BS176	Avirulent <i>Shigella flexneri</i> strain
CFU	colony forming units
DC	Dendritic cell
DH5 $\alpha$	Doug Hanahan Strain 5 $\alpha$
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraaceticacid
EIEC	Enteroinvasive <i>E. Coli</i>
ELISA	Enzyme-linked immunosorbant assay
EPEC	Enteropathogenic <i>E. Coli</i>
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
IFN- $\gamma$	Interferon- $\gamma$
IKK	I Kappa B Kinase
IL	Interleukin
K48	Lysine 48
K63	Lysine 63
LB	Luria Bertani Broth
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
M cells	Microfold cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOI	multiplicity of infection
Mxi	membrane excretion protein
M90T	Wild-type <i>Shigella flexneri</i> strain
NEL	novel E3 ubiquitin ligase
NF $\kappa$ B	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells
NK	Natural Killer
NLR	Nod like receptor
OD <sub>600</sub>	Optical Density at 600nm
ORF	Open Reading Frame
Osp	Outer <i>Shigella</i> protein
PAMP	Pathogen associated molecular pattern

PE	Phycoerythrin
PFA	Paraformaldehyde
PGN	Peptidoglycan
P-Erk	Phospho-Extracellular Signal Regulated Kinase
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween-20
PCR	Polymerase Chain Reaction
P-p38	Phospho-p38
PKC	Protein Kinase C
PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
PSB	Protein sample buffer
PVDF	Polyvinylidene Difluoride
RING	Really Interesting New Gene
RIPA	Radioimmunoprecipitation Assay
RNA	ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute Tissue Culture Medium
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Spa	Surface Presentation Antigen
SUMO	Small Ubiquitin-Like Modifier
TAK1	Transforming Growth Factor $\beta$ -Activated Kinase 1
TCR	T cell receptor
Th1	T Helper 1
TLR4	Toll-like receptor 4
TSB	Trypticase Soy Broth
TRAF	TNF Receptor Associated Factor
T3SS	Type III secretion system
UBC13	Ubiquitin Conjugating Enzyme 13
UI	Uninfected
WT	Wild-type
WHO	World Health Organization

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## **Chapter 1: Introduction**

### **1.1: The Global Impact of Diarrheal Disease**

Diarrheal disease is a leading cause of childhood mortality and morbidity on a global scale (Venkatesan & Van de Verg, 2015). Several large-scale epidemiological studies highlight the importance of both treatment and prevention of enteric disease, particularly in children in developing countries (Venkatesan et al., 2015). In 2010, the Global Burden of Disease Study (GBD) reported nearly one billion episodes of childhood diarrhea with more than 500 000 deaths annually (Murray et al., 2010). The GBD study states that roughly two thirds of diarrheal disease can be attributed to gram-negative bacteria including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), *Shigella*, and *Salmonella* species (Murray et al., 2010).

In 2012, The Global Enteric Multicenter Study (GEMS) of diarrheal disease in infants and young children in developing countries reported *Shigella* as a leading causative agent of moderate-to-severe diarrhea in children 12-59 months of age (Kotloff et al., 2012). This study also provides evidence of the long-term detriment associated with recurring diarrheal disease including stunted growth and decreased cognitive function (Kotloff et al., 2012).

A recent publication by the World Health Organization (WHO) highlights the significance of diarrheal disease globally with the highest incidences being in poor-resource countries where sanitation is poor and access to clean drinking water is limited (WHO, 2014). Currently, the highest incidence of childhood diarrhea is within Africa,

South and South East Asia (Fischer Walker et al., 2010). Heightened awareness of the global burden of diarrheal disease has increased focus toward development of vaccines against prominent bacterial species involved in causing enteric disease, such as *Shigella*, however no licensed vaccine to prevent shigellosis is available despite intense research (Levine et al, 2014).

## **1.2: *Shigella* Pathogenesis**

*Shigellae* species are Gram-negative bacillus-shaped pathogens from the *Enterobacteriaceae* family of bacteria (Schroeder & Hilbi, 2008). These microbes are non-sporulating facultative intracellular pathogens that cause disease in humans or closely related primates. *Shigellae* are non-motile and do not assemble flagella (Niyogi, 2005). However, intracellular *Shigellae* polymerize host actin to propel them from cell to cell during infection (Bernardini et al., 1989). *Shigella* spp. are very closely related to the model enteric pathogen *Escherichia coli* (*E. coli*) but carry a large (~220kb) plasmid to facilitate virulence and pathogenesis (Sansonetti et al., 1981).

### **1.2.1: Discovery and Classification of *Shigellae* Species:**

The *Shigellae* genus is comprised of four species: *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, and *Shigella sonnei*. *Shigellae* are further classified by serotype as defined by the composition of their outer membrane LipoPolySaccharide (LPS) (WHO, 2005). More specifically, serotypes are classified based on the

carbohydrate composition of the O-antigen, the polysaccharide portion of LPS (Lindberg et al., 1991). *Shigella dysenteriae* is referred to as serogroup A and has 15 distinct serotypes, *Shigella flexneri* (serogroup B) has 14 serotypes, *Shigella boydii* (serogroup C) has 20 serotypes, and *Shigella sonnei* (serogroup D) has only one (Levine et al., 2007). The Rohde Laboratory uses *Shigella flexneri* serotype 5a as the parent strain for all experiments and genetic manipulations (Sidik et al., 2014; Onodera et al., 2012).

*Shigella* is the causative agent of a form of bacillary dysentery termed shigellosis, after its founder, Kiyoshi Shiga, (Shiga, 1897). Shigellosis is characterized by acute intestinal enteritis with symptoms ranging from watery diarrhea to severe abdominal cramping, high fever, and blood or mucous in the stool (Niyogi, 2005). Although shigellosis can be self-limiting, aggressive antibiotic therapy is often required and first line drugs such as ampicillin and trimethoprim-sulfamethoxazole are no longer of use due to a rise in antibiotic resistance (CDC, 2013). In 2013 the Centers for Disease Control and Prevention (CDC) declared antibiotic resistant *Shigella* an urgent threat in the U.S. (CDC, 2013).

### **1.2.2: Epidemiology of Shigellosis**

Shigellosis is prominent in developing countries where sanitation is poor and access to clean drinking water is limited (Levine et al., 2007; Camacho et al., 2013). According to a 1999 landmark study the most prevalent species accounting for approximately 60% of cases in developing countries is *Shigella flexneri*, followed by *Shigella sonnei* (15%), with *Shigella dysenteriae* and *Shigella boydii* accounting for approximately 6% of cases

each (Kotloff et al., 1999). Kotloff et al. (1999) estimated that approximately 165 million cases of shigellosis and 1.1 million shigellosis-related deaths occurred annually.

A more recent study shows that although the overall mortality rate from *Shigella* infection is declining, the incidence of shigellosis cases remains high (Bardhan et al., 2010). Bardhan et al. (2010) report that the total number of shigellosis-related deaths has decreased to approximately 14,000 per year. Despite this decrease in mortality shigellosis remains a global burden. A large case controlled study by Kotloff et al. in 2013 reported *Shigella* spp. as one of the top four pathogens associated with moderate-to-severe diarrhea in Africa and Asia. Incidence rates of shigellosis are highest in children under five years of age, followed by the elderly (70+) and immunocompromised individuals (Von Seidlein et al., 2006). Despite intense efforts at prevention and control of *Shigella* infection, shigellosis continues to represent an important cause of bacillary dysentery worldwide. Poor socioeconomic conditions as well as increasing antibiotic resistance continue to represent the driving forces behind formulation of a vaccine against all serotypes of *Shigella*.

### **1.2.3: *Shigella* Route of Infection:**

*Shigella* is transmitted via the fecal-oral route through ingestion of contaminated food or water. These bacteria are highly infectious requiring as few as 10 microorganisms to initiate a productive infection (DuPont et al., 1989). *Shigella* employ acid resistance mechanisms to survive passage through the stomach environment, and down-regulate antimicrobial peptide expression by cells found in mucosal surfaces (Gordon & Small,



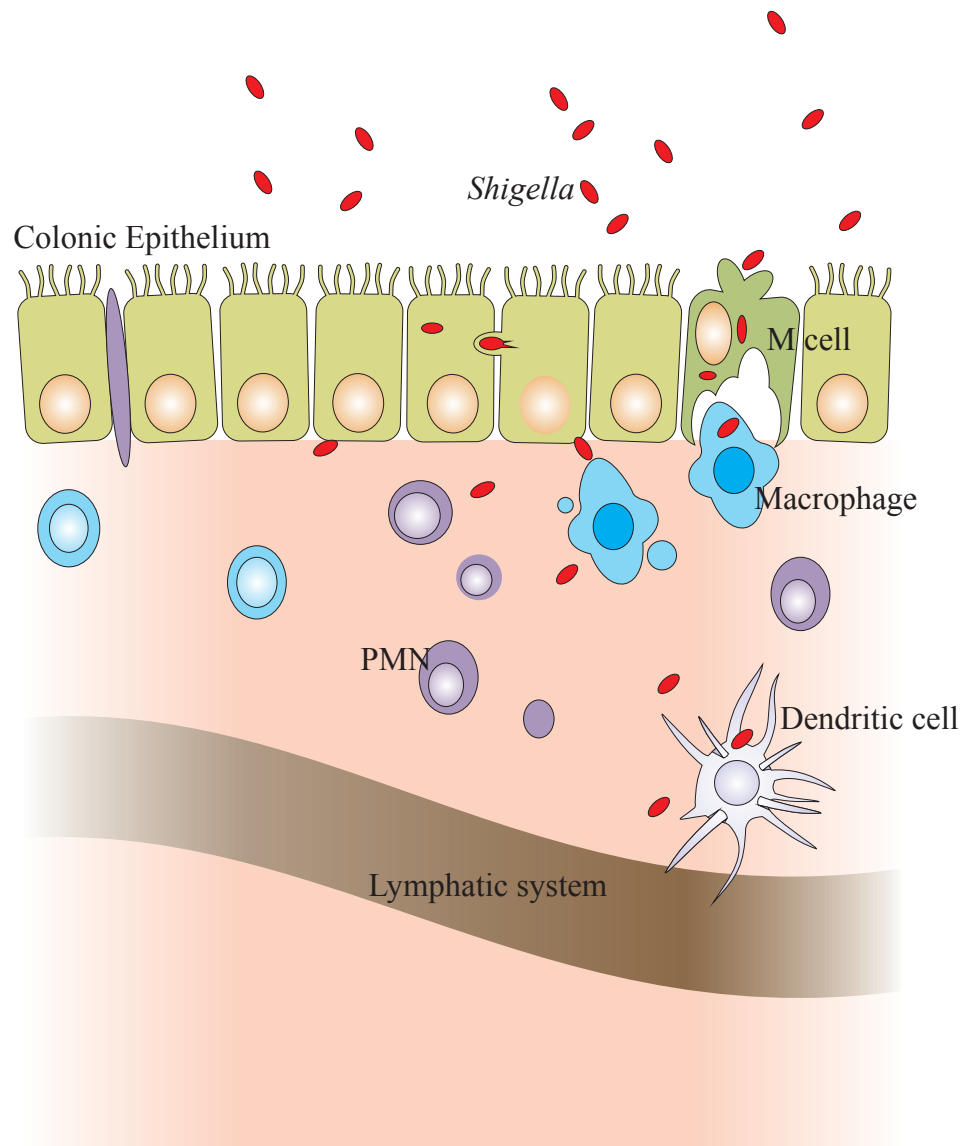
1993; Islam et al., 2001). *Shigella* passes through the stomach and small intestine and establishes infection in the large intestine.

Microfold cells (M cells) are immune related epithelial cells that present potential antigens from the gut lumen to cells of the mucosal lymphoid tissue (Man et al., 2004). In the initial phase of infection *Shigella* cross the intestinal epithelial barrier through M cells in order to transcytose the intestinal layer and infect via the basolateral surface (Figure 1). In the intraepithelial pocket *Shigella* encounter resident macrophages and, potentially other key antigen presenting cells, in which they induce rapid cell death in the form of apoptosis (Islam et al., 1997; Zychlinsky et al., 1992). Immune cell apoptosis results in release of numerous proinflammatory cytokines (ex: IL-1 $\beta$  & IL-18) and related danger signals in addition to the bacterium itself.

Following rupture of infected immune cells, *Shigella* can invade epithelial cells, escape the phagosome, and replicate within the cytoplasm (Sansone et al., 1986). Recent advances in bioimaging technology illustrate the targeting of colonic crypts in the epithelium of a guinea pig early in infection (Sansone et al., 2015). Upon invasion of the colonic epithelium, *Shigella* avoid immune detection by polymerizing host actin to propel themselves from cell to adjacent cell (Bernadini et al., 1989; Monack et al., 2001; Figure 1). Actively secreting *Shigella* have recently been shown to secrete specific effector proteins to escape autophagy during epithelial cell-to-cell spread (Campbell-Valois et al., 2015).

Epithelial cells respond to invasion by recognizing *Shigella* peptidoglycans as Pathogen Associated Molecular Patterns (PAMPs) and utilizing Pattern Recognition

**Figure 1. *Shigella* route of infection.** *Shigella* invades the colonic epithelium by transcytosing through Microfold cells (M cells). *Shigella* are then taken up by resident macrophages and presumably dendritic and other antigen presenting cells. *Shigella* escape antigen presenting cells by inducing cell death and are then free to invade the basolateral surface of the epithelium using their Type III Secretion System (T3SS). Intracellular *Shigella* polymerize host actin to propel from cell to cell thus avoiding immune detection. Proinflammatory signaling from dying antigen presenting cells recruits massive infiltration of neutrophils and related lymphocytes leading to tissue destruction and further bacterial invasion.



Receptors (PRRs), in this case the PRR NOD-1, to activate the Nuclear Factor  $\kappa$ B (NF $\kappa$ B) pathway to up-regulate the innate immune response (Girardin et al., 2003a). Signaling through NF $\kappa$ B results in release of the chemokine IL-8 and recruitment of PolyMorphoNuclear leukocytes (PMNs) to the site of infection (Sansonettil et al., 1999; Philpott et al., 2000; Girardin et al., 2003a).

Massive inflammatory response and infiltration of immune cells results in destruction of the epithelial layer further enabling *Shigella* invasion and propagation in the gut (Perdomo et al., 1994). This severe tissue destruction results in a decrease in absorption of water and nutrients likely accounting for the severe diarrhea associated with shigellosis. The elegance of *Shigellas* cellular pathogenesis showcases that this bacterium is well adapted to battle harmful immune responses in order to further infection.

#### **1.2.4: *Shigella* Virulence:**

The first report of bacillary dysentery was characterized in the late 19<sup>th</sup> century by a Japanese researcher, Kiyoshi Shiga (Kotloff et al., 1999). In the following years descriptions of closely related *E. coli* strains were reported but due to the pathogenicity of *Shigella* spp. the two bacteria were classified in different genres. Now, with the ability to perform comparative genomics on closely related microbes, we know that *Shigella* evolved from multiple *E. coli* strains through convergent evolution (Pupo et al., 2000).

The primary mediator of *Shigella* virulence is its large 220kb plasmid encoding approximately 100 genes, notably, a Type III Secretion System (T3SS) and related effector proteins (Sansonettil et al., 1981; Menard et al., 1996). The virulence plasmid is

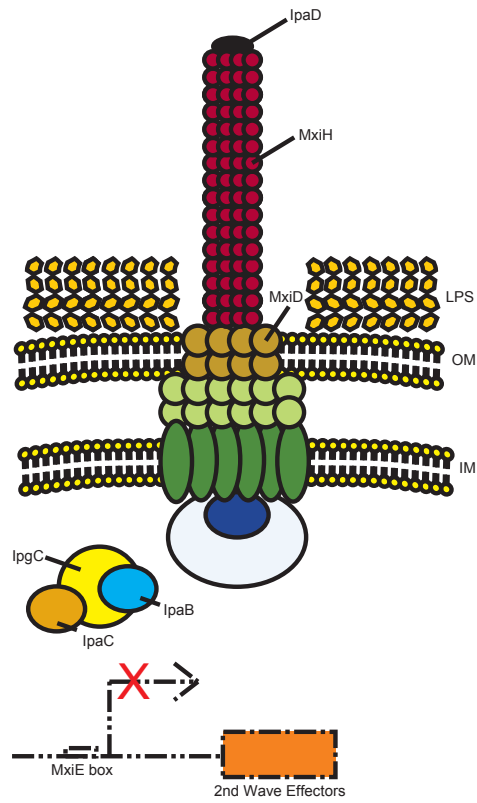
absolutely required for *Shigella* pathogenesis as it is needed to facilitate invasion, survive intracellular environments, and counter host immunity (Sansonetti & Parsot, 2000).

There are currently seven recognized types of secretion systems encoded by gram-negative bacteria, and are categorized based on whether they span both the inner and outer bacterial membrane or the outer membrane alone (Costa et al., 2015). Secretion of proteins can occur through either a one- or two-step process. The T2SS, more commonly known as the general (or “Sec”) secretion system occurs in both gram positive and gram negative bacteria. In the general secretion system, secreted proteins are targeted to the inner membrane by a leader sequence which is recognized by the Sec machinery that transports the protein into the periplasm. The leader sequence is cleaved. The T5SS is a variation of the T2SS where the C-terminal domains of the proteins form a beta barrel structure and insert into the outer membrane, the rest of the protein (the passenger domain) passes through the beta barrel to reach the outside of the cell. In many cases the passenger domain is cleaved. Type 3, 4, and 6 secretion systems do not use leader sequences and directly inject their substrates from the bacterial cytosol into the host (Reviewed in Costa et al., 2015).

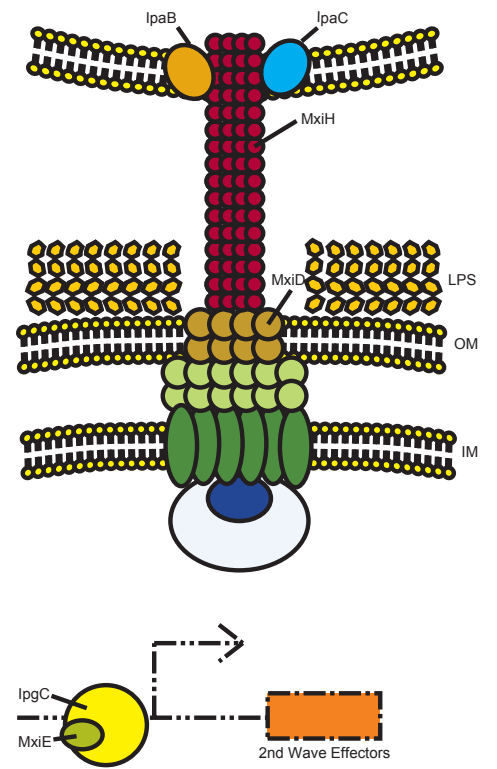
The T3SS encoded by *Shigella* is made up of “mxi”(membrane expression of *ipa*) and “spa” (surface expression of *ipa*) proteins, and is estimated to secrete approximately 25-30 effector proteins (Buchrieser et al., 2000). The T3SS is a needle-like structure that forms in the bacterial membrane and translocates the effector proteins directly from the bacterial cytosol to the host cytoplasm (Kubori et al., 1998; Cornelis, 2006). The needle structure itself is composed of approximately 20 proteins (Figure 2) (Marlovits et al., 2010). The base of the structure (C-ring) is composed of Spa33 and Spa47, proteins

**Figure 2. Overview of Type III Secretion System (T3SS) structure in an active and inactive state.** Needle-like structure spans the inner and outer bacterial membrane and is capped by needle tip protein IpaD. In an inactive secretion state, first wave *Shigella* effectors are stored in the bacterial cytosol in complex with chaperones such as IpgC that maintain them in a secretion-competent state. Upon contact with a host cell (or artificial induction with Congo red dye) the T3SS undergoes conformational changes to allow secretion of effector proteins IpaB and IpaC that form the translocation pore in the host cell. Translocation of first wave effectors including IpaB and IpaC frees IpgC to bind transcriptional activator MxiE and turn on expression of second wave effectors.

A. "Secretion off" state



B. "Secretion on" state



which are found in the bacterial cytosol and regulate effector translocation (Morita-Ishihara et al., 2006). Spa40 interacts with Spa proteins in the C-ring and has recently been reported as critical for the assembly of the secretion complex (Botteaux et al., 2010).

Spanning the inner and outer membranes are proteins MxiD, MxiG, MxiJ, and MxiM, which form the basal body; MxiH and MxiI form the needle structure (Abrusci et al., 2013). The mass ratio of the outer membrane ring to inner membrane ring is 1:2 and the transmembrane regions display 12-fold symmetry (Hodgkinson et al., 2009). The structure is “capped” with proteins IpaB, IpaC, and IpaD when the T3SS is turned off (Menard et al., 1993; Blocker et al., 1999).

There are two “waves” of effector proteins translocated through the T3SS (Demers et al., 1998). The first wave of effectors are secreted upon contact with the host cell to facilitate bacterial entry (Enninga et al., 2005). Effector proteins IpaB and IpaC form a translocation pore complex that allows insertion of the T3SS into the host cell membrane to initiate effector translocation (Page et al., 1999). First wave effectors (ex: IpaA, IpgB1, IpgD, OspB, IcsB) promote host cell actin cytoskeleton rearrangement required for invasion as well as initial immune signal silencing (Hachani et al., 2008; Zurawski et al., 2009). The expression of first wave effectors is under the control of transcriptional regulators VirF and VirB which become activated at a temperature of 37°C, the human internal body temperature (Phalipon et al., 2007).

The second wave of effectors are under the control of the transcriptional regulator MxiE (Mavris et al., 2002). Initially, the chaperone IpgC sequesters IpaB and IpaC in the bacterial cytoplasm; upon activation of the T3SS IpaB and IpaC are translocated into the host leaving IpgC free to bind MxiE (Mavris et al., 2002; Figure 2). MxiE bound to its



IpgC chaperone initiates transcription of second wave effectors including Invasion Plasmid Antigens (Ipas), and Outer *Shigella* Proteins (Osps) (Parsot, 2009). The current understanding is that the second wave of effectors are secreted into the infected cell where they play a significant role in subverting host immune responses for the purpose of survival and propagation (Reddick and Alto, 2014).

Significant progress has been made in elucidating the fine details of the T3SS structure and cutting edge technology has allowed for high-resolution visualization of the T3SS sorting platform at the cytoplasmic surface (Hu et al., 2015). Many questions remain with regard to the vast levels of regulation required for efficient translocation of effectors from bacterium to host. Further investigation and dissection of the mechanisms controlling type III secretion are required to better understand this mediator of pathogenesis in *Shigella*.

### **1.2.5: Type III Secretion System Effectors of *Shigella* Involved in Immune Evasion**

In addition to the Ipa proteins mentioned in Section 1.2.4, which aid in control of translocation, there exists the IpaH family of proteins that are encoded both on the virulence plasmid and on the chromosome. IpaH proteins belong to a Novel group of E3 Ligases termed NELs and are secreted through the T3SS as part of the second wave effectors (Rohde et al., 2007; Schroeder et al. 2008; Quezada et al., 2009). IpaH proteins are a topic of intense research due to the fact that bacteria themselves lack ubiquitin systems and these effectors appear to regulate inflammation-related signaling within the host (Tanner et al., 2015).

In 2007, Rohde et al. utilized a yeast model system to demonstrate E3 ligase activity of IpaH9.8, identifying a target (Ste7) within the yeast pheromone response pathway. The pheromone response pathway is a MAPK pathway very similar to the ERK pathway in humans. Since their initial characterization of IpaHs as E3 ubiquitin ligases, there have been a handful of substrates identified for these enzymes. IpaH9.8 has been shown to target NEMO for proteosomal degradation, a critical component of the NF $\kappa$ B signaling pathway, thus impairing immune signaling in the host (Ashida et al., 2010). IpaH0722 down regulates PKC-triggered NF $\kappa$ B activation by ubiquitinating TRAF2 signaling it for proteosomal degradation (Ashida et al., 2013). IpaH4.5 specifically inhibits the NF $\kappa$ B signaling pathway by ubiquitinating a subunit of the transcription complex (p65) inhibiting expression of immune related genes (Wang et al., 2013). Recently, IpaH7.8 has been implicated in inflammasome activation and subsequent programmed cell death as a means of dampening macrophage responses (Suzuki et al., 2014). The elucidation of all IpaH substrates will reveal mechanisms of *Shigella* pathogenesis that may aid in rational design of live attenuated vaccines. Perhaps creation of an attenuated strain of *Shigella* deleted for certain effectors including select IpaHs would be useful in vaccine studies.

Outer *Shigella* Proteins (Osp) F, G, and I also down regulate immune signaling in infected cells. OspF is a phosphothreonine lyase capable of irreversibly removing the phosphate group from activated MAPKs at specific threonine residues (Li et al., 2007). A pilot study focusing on *Shigella*-DC interactions observed OspF phosphothreonine lyase activity in bone-marrow derived DCs and hypothesized a correlation between OspF

expression and induction of programmed cell death within DCs (Kim et al., 2008). My work supports this hypothesis and further implicates a role for OspF in immune signaling.

OspG is a protein kinase that binds to ubiquitinated E2 enzymes to impair innate immune responses (Kim et al., 2005). Pruneda et al. (2014) solved the crystal structure of OspG in complex with E2-Ubiquitin and demonstrate that this tripartite complex increases the kinase activity of OspG. Further, a mouse model of shigellosis comparing mice infected with either Wild-type or OspG mutant *Shigella* shows that this effector plays a role in inflammation as well as in bacterial clearance in the gut (Pruneda et al., 2014). Targeting E2 enzymes represents an elegant mechanism for regulating effector kinase activity, however the substrates for OspG as a kinase are currently unknown.

OspI is a glutamine deamidase that has been shown to interact with the E2 ubiquitin conjugating enzyme Ubc13 (Sanada et al., 2012). Sanada et al. (2012) demonstrated that OspI dampens the acute inflammatory response through the NF $\kappa$ B pathway. OspI deamidates a glutamine (Q100) residue in the active pocket of Ubc13 disabling ubiquitination of TNF- Receptor Associated Factor 6 (TRAF6) which is required for NF $\kappa$ B activation and translocation into the nucleus (Sanada et al., 2012).

The arsenal of the effectors secreted by the T3SS in *Shigella* have proven to be critical for immune evasion within the host. Identification of all immune related effectors and their full mechanisms of action within the host could further vaccine development by allowing for creation of a live attenuated strain of *Shigella* that is immunogenic and gives rise to a robust adaptive immune response.

### **1.3: Immune Responses Against Shigellosis**

### 1.3.1 Innate Immune Response to *Shigella* Infection

Upon introduction into the gastrointestinal tract *Shigella* employ acid resistance mechanisms to survive the harsh stomach environment in order to colonize within the small intestine (Philapon & Sansonetti, 2007). The intestinal epithelium serves as the first line of defense against infection by acting as a physical barrier between the host and microorganisms in the outside environment (Kuby et al., 2006). Antimicrobial peptides released from the intestinal epithelium are non-specific immune molecules that prove to be important for initial bacterial clearance (Hancock & Scott, 2000). *Shigella* overcomes these first line defense mechanisms by targeting specific cell types for entry (M-cells, Section 1.2) and down-regulating the expression of antimicrobial peptides released from the intestinal tract (Islam et al., 2001; Sperandio et al., 2008). In gaining access to the basolateral surface of the intestinal epithelium *Shigella* invade using the T3SS and move from cell to cell intracellularly to avoid detection by surrounding resident immune cells (Monack et al., 2001).

*Shigella*-infected epithelial cells recognize intracellular bacteria through the Pattern Recognition Receptors (PRRs) Nod1 and Nod2 (Girardin et al., 2003a; Girardin et al., 2003b). These proteins recognize Peptidoglycan (PGN) moieties and mediate non-specific immunity; a mutation in the gene encoding Nod2 results in an increased susceptibility to chronic inflammatory disorders (Carneiro et al., 2004). Epithelial cells produce danger signals such as cytokines (i.e. IL-8) and lipid mediators that recruit innate immune cells (i.e. neutrophils) to the site of infection (Sansonetti, 2004). This has been proposed as an advantage for the bacteria because the resulting tissue destruction allows for easier transmission and dissemination of *Shigella* throughout the body (Sansonetti, 2004).

Interference of immune signaling through the MAPK and NF $\kappa$ B pathway represents an effective mechanism used by *Shigella* and related pathogens to dampen the inflammatory response. T3SS effectors OspG and OspI effectively target components of the host ubiquitin pathway to silence immune signaling through these proinflammatory signaling cascades (Section 1.4). Recent published data reports that *Shigella* has the ability to modify the structure of its LPS intracellularly to decrease its immunopotential and subsequent proinflammatory signaling (Paciello et al., 2013).

### **1.3.2 Adaptive Immune Response to *Shigella* Infection**

In comparison to published data on the innate immune response to *Shigella* infection, very little has been established regarding long term, adaptive immunity against shigellosis (Salgado-Pabón *et al.*, 2013). Natural protective immunity only develops after several repeated infections, lasts only a short time, and poorly limits reinfection (Raqib *et al.*, 2002). It has been suggested that *Shigella* has evolved strategies to dampen the adaptive immune response but few mechanisms have been investigated (Salgado-Pabón *et al.*, 2013).

It has recently been elucidated that CD4<sup>+</sup> Th17 cells are the predominant cell type primed by *Shigella* to initiate an adaptive immune response within the host (Sellge *et al.*, 2011). Experimental evidence also suggests that CD8<sup>+</sup> T cells are not required for protective immunity against shigellosis, and that these cells are not primed upon infection (Jehl *et al.*, 2011). Direct interaction between *Shigella* and T cells has only recently begun to be characterized.

One recent finding with regard to determining the role of the T3SS and effectors in impairing adaptive immunity comes from Konradt and associates (2011). Konradt et al. demonstrate that *Shigella* is unable to invade T cells, but delivers effectors into these cells using

its T3SS (Konradt *et al.*, 2011). One specific effector, IpgD, is injected into activated T cells and inhibits migration by interfering with phosphoinositide metabolism in the lymphocyte (Konradt *et al.*, 2011). IpgD effectively paralyzes T cells, thus impairing their ability to migrate (Konradt *et al.*, 2011). The same group has confirmed that *Shigella* also impairs T cell dynamics *in vivo* as a means of limiting adaptive immunity (Salgado-Pabon *et al.*, 2013).

Given that Antigen Presenting Cells (APCs) represent the link between innate and adaptive immunity it has been postulated that functionally impairing APCs would be a convenient and efficient way for *Shigella* to control long lasting immunity (Phalipon & Sansonetti, 2007). *Shigella* is known to trigger rapid cell death in macrophages, which supports this hypothesis (Islam *et al.*, 1997). Limiting the number of macrophages would decrease antigen presentation and subsequent activation of adaptive immune cells. Lack of cross-talk between these cells makes initiating an adaptive immune response more challenging. Cytokines released in response to *Shigella* infection have been shown to impair the recruitment of dendritic cells (DCs) to the site of infection, further inhibiting antigen presenting cells from phagocytosis of bacteria and antigen presentation to T and B cells (Sperandio *et al.*, 2008). *Shigella* induces rapid cell death of DCs, which are key players in initiating adaptive responses to bacterial infection (Edgeworth *et al.*, 2002; Kim *et al.*, 2008). The involvement of the T3SS and associated effectors in induction of programmed cell death of DCs has not been characterized. One effector, OspF has been shown to down-regulate MAPK signalling by irreversibly removing the activating phosphate group from MAPKs in bone marrow derived dendritic cells (Kim *et al.*, 2008). Given the importance of professional antigen presenting cells in clearing bacterial infection, there has been little characterization of *Shigella*-DC interactions and their outcomes on immune responses against shigellosis.

### **1.3.3. The Role of Dendritic Cells (DCs) in Clearing Bacterial Infections**

Dendritic cells are a subset of innate immune cells that play a critical role as both primary modulators of infection as well as initiators of adaptive immune responses (Ko & Chang, 2015). These cells are named for the long, membranous extensions on their surface that resemble the dendrites of nerve cells (Kuby *et al.*, 2007). There are four types of DCs, all of which are derived from hematopoietic stem cells (Kuby *et al.*, 2007). These include Langerhans cells, interstitial dendritic cells, myeloid cells, and lymphoid dendritic cells, all of which phagocytose bacteria and process antigen for presentation to adaptive immune cells (Kuby *et al.*, 2007).

As part of the innate immune response, DCs use pattern recognition receptors (PRRs) to bind pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), on the surface of bacteria (Ko & Chang, 2015). LPS is recognized by the PRR toll-like receptor 4 (TLR4) and is often used in the lab setting to artificially stimulate maturation of DCs (Granucci *et al.*, 1999). The binding of PAMPs to PRRs on DCs initiates phagocytosis of bacteria so that subsequent processing and antigen presentation can occur (Poltorak & Schrami, 2015). Once activated DCs will migrate to secondary lymphoid sites to interact with naïve adaptive immune cells and initiate signalling cross-talk to prime cells for a lasting immune response (Flannigan *et al.*, 2015; Figure 3).

### **1.3.4 Developing a Vaccine Against Shigellosis**

**Figure 3. Dendritic cell activation by bacterial pathogens stimulates an adaptive immune response.** Upon infection with pathogenic bacteria Pathogen Associated Molecular Patterns (PAMPs) or Danger Associated Molecular Patterns (DAMPs) are detected by Pattern Recognition Receptors (PRRs) expressed on the surface of dendritic cells (DCs) leading to activation, maturation, and migration to secondary lymphoid sites (lymph node depicted here). In the lymph nodes, activated DCs may present antigens to T cells, provide them with co-stimulatory signals and stimulate their differentiation by providing a favorable cytokine environment. Depending on the cytokine environment, CD4<sup>+</sup>T cells may differentiate into various T helper (T<sub>H</sub>) cell subtypes. T<sub>H</sub> cells may also acquire a T follicular helper (T<sub>FH</sub>) cell phenotype to activate naïve B cells promoting differentiation via the plasma cell pathway (antibody producing B cells) or the germinal centre pathway (memory B cells). Image adapted from Desmet & Ishii, 2012.





There is currently no licensed vaccine to protect against all serotypes of *Shigella* infection (Barry et al., 2013). Vigorous oral rehydration strategies and prevention measures such as promotion of hand washing have made a positive impact on the mortality associated with diarrhea related illness, although the morbidity of shigellosis remains unchanged (WHO, 2009; von Seidlein *et al.*, 2006). We are in an era of emerging antibiotic resistance, and *Shigella* are less responsive to the salutary effects of oral rehydration than other enteric pathogens, so the need for a safe and effective vaccine against shigellosis is of higher priority than ever before (Levine *et al.*, 2007; Camacho *et al.*, 2013).

There are several significant barriers impeding the development of a vaccine against shigellosis including knowledge gaps with regard to protective immunity, lack of a robust animal model of infection, and difficulties associated with providing broad coverage against all serotypes of *Shigella* (Barry et al., 2013). In order to overcome these hurdles we must research further the commonality between *Shigella* types (for example the virulence plasmid is common to all *Shigella*), the mechanisms by which *Shigella* impairs adaptive immunity, and generate a small animal model that recapitulates human infection (Camacho et al., 2013).

The first effort toward developing a vaccine against shigellosis was in the 1960s when a live attenuated strain of *Shigella* was administered in skim milk (Dupont *et al.*, 1969). Since this time many strategies have been employed such as the creation of attenuated strains of *Shigella* in live oral vaccines and parenteral conjugative vaccines (muscle injections) that use *Shigella*'s O polysaccharides (Levine *et al.*, 2007). Current efforts toward vaccine development against shigellosis can be divided into two approaches: serotype based vaccines and conserved antigen vaccines (Barry et al., 2013).

### **Serotype based vaccines:**

Live attenuated strains, killed whole-cell, and conjugate vaccines represent serotype-based vaccines (Barry et al., 2013). Conjugate vaccines against *Shigella* are composed of purified O polysaccharide conjugated to a protein carrier; the most successful formulation being *S. flexneri* 2a LPS conjugated to recombinant *Pseudomonas* exoprotein A (rEPA) and *S. sonnei* LPS conjugated to rEPA from the National Institute of Child Health and Human Development (Passwell et al., 2003). Unfortunately this promising formulation did not provide statistically significant efficacy. Instead, efficacy paralleled the age related immune responses induced by the vaccine and it was unable to prime and protect young children (under 2 years) (Barry et al., 2013).

Development of orally administered live attenuated or killed whole-cell formulations as vaccine candidates have shown significant progress in recent years (Venkatesan et al., 2015). Inactivated whole-cell *S. sonnei* vaccine candidates are protective in animal models of infection and are currently undergoing human Phase I trials (McKenzie et al., 2006; Barry et al., 2013). Two attenuating strategies have been used to produce reliable vaccine candidates that continue to progress through clinical trials despite having difficulties balancing safety and immunogenicity (Venkatesan et al., 2006; Barry et al., 2013). The first, CVD1208S, is comprised of a series of *Shigella* strains carrying mutations in various metabolic enzymes and genes encoding Shiga toxin; it is in Phase II clinical trials (Kotloff et al., 2007). The second, SC602, contains mutation in *icsA* a *Shigella* protein required for intracellular spread as well as in *msbB* which is thought to detoxify bacterial LPS. Human vaccine trials yielded varying results dependent on populations tested making this vaccine subpar for global use (Gordon et al., 2012).

### **Conserved antigen vaccines:**

Several new vaccine candidates focus on using a common antigen found in all serotypes of *Shigella* (Barry et al., 2013). The Invasion Plasmid Antigens (Ipas) are highly conserved and essential to pathogenesis therefore many candidates have been developed using these proteins (Turbyfill et al., 2008). One formulation called Invaplex, contains purified LPS as well as IpaB, C, and D, and has passed safety and immunogenicity trials and is currently being optimized for delivery (Riddle et al., 2011). Alternatively, antigenic outer membrane proteins IcsP2 (outer membrane protease) and SigA2 (Serine protease autotransporter, SPATE) are conserved proteins and have been applied toward development of a conserved antigen vaccine that has demonstrated protection in animal models thus far (Czerkinsky et al., 2010).

In determining that all serogroups of *Shigella* harbor a large virulence plasmid with common mediators of pathogenesis it is logical that targeting this aspect of virulence could assist in the creation of an effective vaccine to protect against all species of *Shigella* (Levine et al., 2007). Vaccines are used to stimulate a long lasting immune response against a particular pathogen and *Shigella* effectively impairs adaptive immunity by poorly defined mechanisms, therefore, further research is required to determine the specific function of effector proteins involved in immune system impairment. Perhaps identifying the roles for each gene on the virulence plasmid will assist in development of an immunogenic live attenuated vaccine candidate that will effectively protect against shigellosis on a global scale.

### **1.3.5 Animal models of Shigellosis**

A small animal model that recapitulates the characteristics associated with human *Shigella* infection is of great benefit to researchers interested in studying new vaccine candidates. Given that *Shigella* primarily infect humans and higher primates, development of an animal model has been difficult but there are currently multiple models that have been established as effective for studying *Shigella* infection *in vivo*. The most successful animal models to date include the rabbit ileal loop model, the macaque monkey model, and various mouse models of infection (Schnupf et al., 2012; Kent et al., 1967; Philpott et al., 2000; Zhang et al., 2001; Martino et al., 2005)

Mouse models of *Shigella* infection are favored in the research community as mice are cost effective, easier for experimental repetition, and the infection procedure is less strenuous than in larger animal models. Currently used mouse models include the pulmonary infection model (Voiono-Yasenetsky et al., 1962, van de Verg et al., 1995, Fisher et al., 2014) and the streptomycin mouse model (Martino et al., 2005). Although many mouse models of infection have helped gain insight into *Shigella* virulence and pathogenesis the ideal animal model does not yet exist and the researchers choice of animal infection must instead fit the experiment required. New models, including the guinea pig intracolonic model and the zebra fish model have recently been developed (Shim et al., 2007, Jeong et al., 2013, Mostowy et al., 2013). Each model has both benefits and challenges associated and research continues toward development of better models that can be applied in vaccine challenge studies.

## **1.4: Ubiquitination is an Important Post-Translational Modification in Eukaryotic Systems**

Ubiquitin is a highly conserved polypeptide consisting of 76 amino acids (Goldstein et al., 1975). Ubiquitination of eukaryotic proteins is a reversible post-translational modification and has been implicated in a wide range of cellular processes including cell signaling, localization, and degradation of target proteins by the proteasome (Bhoj and Chen, 2009). Having only three amino acid sequences differ between lower and higher eukaryotes ubiquitin is one of the most conserved proteins in nature (Ozkaynak et al., 1984). An emerging theme in the study of host-pathogen interactions is that the ubiquitin system is a major target of microbial pathogens. Co-evolution of pathogens and their hosts has allowed microbes to develop an arsenal of effector proteins specifically designed to hijack and modulate the ubiquitin system for the purposes of survival and propagation (Tanner et al., 2015).

### **1.4.1: The Ubiquitin Pathway: E1, E2, E3**

The ubiquitin pathway consists of three important enzymes: E1 activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (Rytönen and Holden, 2007). Initiation of the ubiquitination cascade occurs when an E1 hydrolyses ATP and binds a C-terminal glycine residue of ubiquitin to a cysteine residue of the E1 (Rytönen and Holden, 2007). The ubiquitin is subsequently transferred to a cysteine residue of an ubiquitin-conjugating E2 enzyme (Rytönen and Holden, 2007). Finally, the E2-ubiquitin

complex interacts with one of two types of E3 ubiquitin ligases in order to facilitate substrate binding (Tanner et al., 2015). Eukaryotes encode a limited number of E1s, approximately 40 E2s and several hundred E3s (Rohde et al., 2007). In eukaryotes, E3s are subdivided into two groups: HECT (Homologous to the E6-AP Carboxyl Terminus) domain E3s that behave as catalytic intermediates, first binding ubiquitin and then transferring to a substrate and RING (Really Interesting New Gene) domain E3s which directly transfer the ubiquitin from the E2 to the substrate (Huang et al., 1999; Lorick et al., 1999).

#### **1.4.2: Outcomes of Ubiquitination Cascades**

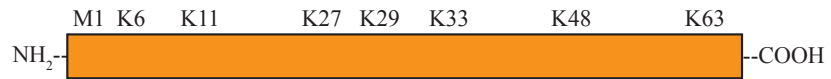
The fate of any ubiquitinated substrate lies in the nature of its ubiquitin linkages. Monoubiquitination, the addition of a single ubiquitin molecule to a lysine residue of a substrate, is most commonly associated with protein localization, DNA repair, and regulation of gene transcription (Ramanathan et al., 2012). Ubiquitin itself contains seven lysine (K) residues (K6, K11, K27, K29, K33, K48, and K63), each of which can be covalently attached to the C-terminal portion of other ubiquitin molecules via the ubiquitin pathway (Ashida et al., 2014) (Figure 4a). Polyubiquitination, the addition of multiple linked ubiquitin molecules, has been the subject of intense research over the past decade (Behrends & Harper, 2011).

The destiny of polyubiquitinated substrates within eukaryotic cells is dependent on ubiquitin chain type; however, the outcomes of such modifications remain incompletely understood (Figure 4b). The best-characterized form of polyubiquitination is at K48 residues. K48 linked ubiquitin chains target substrates for proteosomal degradation. By contrast, K63 linked polyubiquitinated substrates play a role in DNA

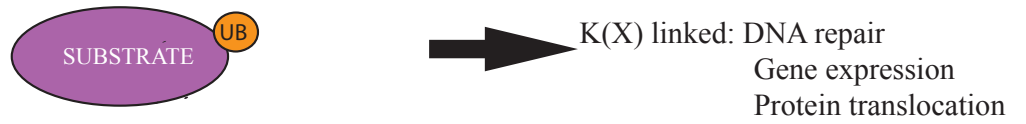
**Figure 4. Types of ubiquitination and their outcomes.** A. Representation of the 76 amino acid ubiquitin molecule: lysine and methionine residues involved in polyubiquitination are labeled. B. Types of ubiquitination and their functional roles within eukaryotic cells. Attachment of a single ubiquitin molecule (monoubiquitination) to a substrate initiates signaling cascades required for DNA repair, gene expression, and protein translocation. Multiple linked ubiquitin molecules can be added to substrates (polyubiquitination) to target proteins for degradation by the proteasome, provide a scaffold for protein interactions required for immune signaling, or act in signaling cascades required for DNA repair and endocytosis.



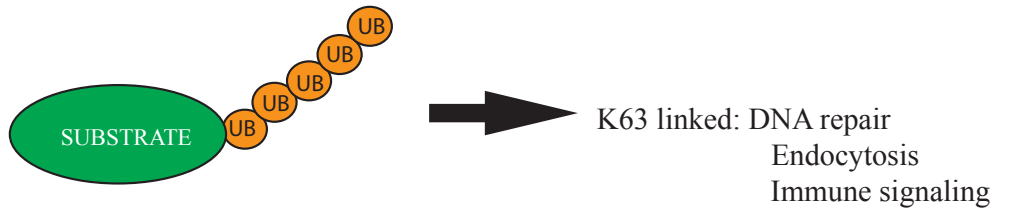
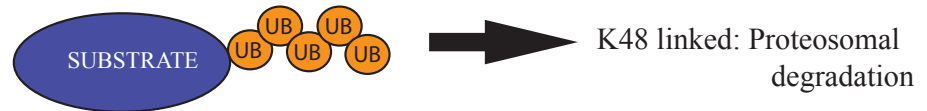
## A. Ubiquitin



## B. Monoubiquitination



## Polyubiquitination



repair as well as signaling required for endocytosis and immune signaling pathways (Behrends and Harper, 2011). Ubc13 is the E2 ubiquitin-conjugating enzyme responsible for formation of K63 linkages (Hoffman & Pickart, 1999). Ablation of Ubc13 *in vivo* dampens mammalian immune signaling through both the NFκB and MAPK pathways (Yamamoto et al., 2006; Wu & Karen, 2015). K63-linked substrates provide a scaffold for which protein interactions may occur, and although recent publications have begun to shed light on purposes of this form of polyubiquitination, many knowledge gaps remain with regard to bacterial manipulation of such linkages.

In addition to lysine based ubiquitin linkages, Met1 (Methionine; 1<sup>st</sup> amino acid of ubiquitin) is able to form linear polyubiquitination chains through a unique multimeric LUBAC (Linear Ubiquitin Assembly Complex) complex (Behrends & Harper, 2011). The LUBAC complex is required for NFκB signaling further implicating polyubiquitin chains in immune signaling (Bianchi & Meier, 2009). Given that ubiquitination is an important and abundant form of post-translational modification and is involved in immune related processes, impairment by pathogens such as *Shigella* represents an effective mechanism of circumventing detection and promoting infection.

Several gram-negative pathogens, including *Shigella*, use a Type III Secretion System (T3SS) to inject effector proteins into host cells. Although T3SSs are conserved among pathogens the repertoire of secreted effector proteins contributing to disease is variable. T3SS effectors can interfere with a plethora of cellular processes to further the infection process including cytoskeletal rearrangement, vesicular trafficking, impairment of immune signaling, cell division, and migration to name a few (reviewed in Tanner et al., 2015; Ashida et al., 2014; Rytönen and Holden, 2007). Bacteria have developed

mechanisms of targeting ubiquitin pathways as a means of regulating host function (Anderson and Frank, 2012).

*Salmonella enterica* serovar Typhimurium is an enteropathogenic bacterium that colonizes the small intestine and is a leading cause of gastroenteritis in humans. Like *Shigella*, *S. Typhimurium* uses a T3SS to inject effector proteins into host cells to modulate the infection process. SopA is a *Salmonella* effector that induces inflammatory responses in the form of PMN recruitment (Rytönen & Holden, 2007). Recent publications identify SopA as a molecular mimic of a HECT- like E3 ubiquitin ligase (Zhang et al., 2006). SopA has potential to ubiquitinate both host and bacterial proteins therefore targets of this effector are of interest to those focused on better understanding its role in *Salmonella* induced enteritis (Rytönen & Holden, 2007).

Like *Salmonella* and *Shigella*, *Escherichia coli* infection represents a major cause of enteric disease globally. The T3SS effector NleE of enteropathogenic *E. coli* (EPEC) interferes with immune signaling cascades through the ubiquitin pathway (Zhang et al., 2012). Specifically, NleE is a methyltransferase that modifies ubiquitin-chain sensory proteins TAB2 and TAB3 involved in the NF $\kappa$ B pathway, effectively inhibiting ubiquitin binding to counteract host defense (Zhang et al., 2012).

*Shigella* is predicted to secrete approximately 50 effector proteins through its T3SS, many of which are thought to be involved in subversion of the host ubiquitin pathway (Tanner et al., 2014). The IpaH family of effectors is the largest class of proteins produced by *Shigella* upon infection (Demers et al., 1998). These proteins are predicted novel E3 ligases (NELs) injected into host cells to manipulate ubiquitin cascades for the

benefit of bacterial survival and propagation. IpaH9.8 was shown to interrupt the MAPK signaling pathway in yeast (Rohde et al., 2007).

Recently, IpaH7.8 has been implicated in induction of macrophage cell death mediated by caspase-1 dependent inflammasome formation (Suzuki et al., 2014). Outer *Shigella* proteins OspG and OspI are also secreted through the T3SS and interact with ubiquitin conjugating enzymes (Tanner et al., 2015). OspG displays weak kinase activity *in vitro* and directly interacts with the E2 ubiquitin-conjugating enzyme UbcH5 when bound to ubiquitin (Kim et al., 2005). Zhou et al. (2013) show that binding of OspG to the UbcH5-ubiquitin complex increases the kinase activity of OspG. The crystal structure of OspG bound to ubiquitinated UbcH5 confirms that this complex increases OspG kinase activity and stabilizes active conformation of the complex, however, the full implications of this interaction remain unclear (Prudena et al., 2014, Tanner et al., 2015).

OspI has recently been shown to display glutamine deamidase activity, specifically deamidating a glutamine residue in the active site of E2 ubiquitin conjugating enzyme Ubc13 (Senada et al., 2012). Ubc13 is critical for formation of K63 linked polyubiquitin chains (Yamamoto et al., 2006). Senada et al. (2012) have shown that inactivation of Ubc13 by OspI inhibits K63 polyubiquitination of TRAF6 (TNF Receptor Associated Factor 6) thus inhibiting protein interactions required for NFκB signaling.

## **1.5: Important immune signaling pathways in Eukaryotes**

Innate immune signaling is generally non-specific and is mediated by phagocytic cells and antigen presenting cells, which rely on their Pattern Recognition Receptors

(PRRs) to recognize Pathogen Associated Molecular Patterns (PAMPs) upon infection (Janeway et al., 2002). Stimulation of PRRs with PAMPs triggers proinflammatory and antimicrobial responses by activating various intracellular signaling pathways resulting in an up regulation in proinflammatory gene expression (Akira et al., 2004).

Toll-Like Receptors (TLRs) are a type of PRR important for bacterial clearance by antigen presenting cells such as DCs and macrophages (Iwasaki & Medzhitov, 2004). Initially it was believed that gram-negative bacterial species are recognized by TLR4 which binds the Lipid A portion of LPS, while gram-positive bacteria are recognized by TLR2 which binds lipoteichoic acid, lipoproteins, and peptidoglycan (Poltorak et al., 1998; Schwandner et al., 1999). We now know that bacteria have the ability to stimulate many different TLRs using alternative PAMPs such as flagellin (recognized by TLR5) or unmethylated CpG DNA (recognized by TLR9 in the endosome) (Hawn et al., 2003; Hemmi et al., 2000).

TLR-independent PRRs are currently a topic of intense research and can be divided into two broad categories: retinoid acid-inducible gene I (RIG-I)- like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Yoneyama et al., 2004; Kanneganti et al., 2007). RLRs play a pivotal role in sensing cytoplasmic RNA and NLRs sense bacterial proteins derived from synthesis and degradation of peptidoglycan (Yoneyama et al., 2005; Kanneganti et al., 2007). The best characterized NLRs are NOD-1 and NOD-2 which sense diaminopimelic acid and muramyl dipeptide (MDP) respectively (Girardin et al., 2003a; Girardin et al., 2003b). Additional NLRs are involved in caspase activation and inflammasome formation and will be introduced in detail in Section 1.6.2.

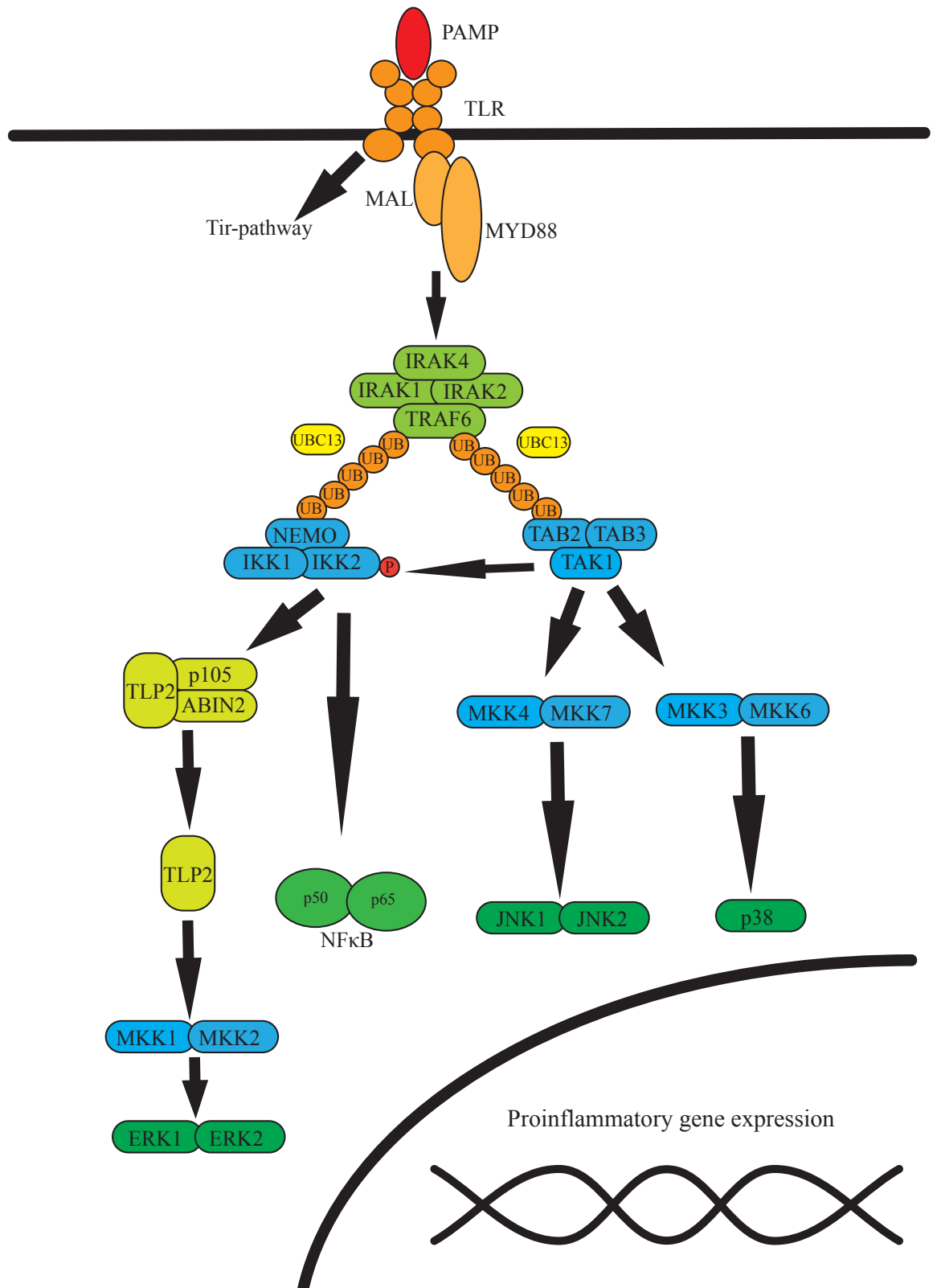
Two major signaling pathways responsible for inducing a proinflammatory response following PAMP sensing by PRRs on antigen presenting cells are the Nuclear Factor  $\kappa$ B (NF $\kappa$ B) and Mitogen Activated Protein Kinase (MAPK) pathways (Akira et al., 2006). Innate immune signaling in response to gram-negative bacterial infection has been the most extensively studied with regard to TLR stimulation and will herein be used to describe initiation of the NF $\kappa$ B and MAPK pathways.

Most TLRs signal via adaptor molecule MYeloid Differentiation primary-response protein 88 (MYD88) or TIR domain-containing adaptor protein inducing IFN $\beta$  (TRIF) (Mogensen, 2009). Following TLR stimulation IL-1 Receptor-Associated Kinase 4 (IRAK4) is recruited to the adapter protein and forms a complex with IRAK1/2, TNF-Receptor Associated Factor 6 (TRAF6; E3 ubiquitin ligase) and the E2 Ubiquitin Conjugating enzyme 13 (Ubc13) (Mogensen, 2009; Figure 5). These E2 and E3 enzymes catalyze formation of K63-linked polyubiquitin chains that facilitate scaffolding of proteins required for activation of the NF $\kappa$ B and MAPK pathways (Arthur & Ley, 2013; Figure 5).

TRAF6 polyubiquitination has been shown to facilitate complex formation of TGF $\beta$ -Activated Kinase 1 (TAK1) with TAK-Associated Binding (TAB) proteins, which stimulates MAKKK activity of TAK1 and initiates MAPK signaling (Arthur & Ley, 2013; Figure 5). MAPK pathways consist of a series of at least three kinases: a MAPK kinase kinase (MAPKKK, like TAK1) activates a MAPK kinase (MAPKK), which activates a MAPK (Mogensen, 2009; Newton & Dixit, 2012). Mammalian cells express 14 MAPKs including the classical Extracellular signal-Regulated Kinase 1 (ERK1) and

**Figure 5. K63-dependent innate immune signaling pathways in mammalian cells.**

Upon Pathogen Associated Molecular Pattern (PAMP) stimulation of Toll Like Receptor (TLR), Myeloid differentiation primary response protein 88 (MyD88) is recruited to the Toll and IL1 Receptor (TIR) domain with the help of adaptor protein MyD88 Adaptor Like protein (MAL). IL1 Receptor Associated Kinase 4 (IRAK4) binds MyD88 which induces complex formation with IL1 Receptor Associated Kinase 1 and 2 (IRAK1, IRAK2), and TNF Receptor Associated Factor 6 (TRAF6). TRAF6 together with Ubc13 catalyzes the formation of K63-linked polyubiquitin chains, providing a scaffold for protein complexes involved in the MAPK and NF $\kappa$ B pathways. TAK1 Binding proteins 2 and 3 (TAB2 and TAB3) recruit TGF $\beta$  Activated Kinase 1 (TAK) to polyubiquitinated TRAF6, which triggers its activation and induction of the p38 and Jun N-terminal Kinase (JNK) MAPK pathways. Activated TAK1 phosphorylates I $\kappa$ B Kinase 2 (IKK2) inducing activation of NF $\kappa$ B signaling. Activated IKK2 degrades p105, which leads to activation of Tumor Progression Locus 2 (TLP2) and subsequent induction of the Extracellular signal Regulated Kinase (ERK) MAPK pathway. K63-dependent innate immune signaling pathways are important for induction of proinflammatory gene expression required to combat bacterial infection. Image adapted from Arthur & Ley, 2013.





ERK2, which are activated by the MAPKKs MKK1 or MKK2 and p38, which is activated by the MAPKKs MKK3 and MKK6 (Arthur & Ley, 2013).

In the canonical NF $\kappa$ B signaling pathway PAMP sensing activates the IKK complex, which is composed of two catalytic subunits IKK1 (IKK $\alpha$ ) and IKK2 (IKK $\beta$ ) and a regulatory subunit NEMO (IKK $\gamma$ ) (Deng et al., 2000). TRAF6 mediated scaffolding of proteins is also relevant to NF $\kappa$ B signaling as NEMO can bind the K63-linked ubiquitin chain in complex with IKK1 and IKK2 which causes activation and nuclear translocation of NF $\kappa$ B; this is due to TAK kinase activity on IKK2 specifically (Deng et al., 2000; Figure 5). Interestingly, IKK2 mediated proteolysis of NF $\kappa$ B precursor p105 leads to activation of MAPKKK Tumor Progression Locus 2 (TLP2), which activates MKK1 or MKK2, which activate ERK signaling (Arthur & Ley, 2013; Figure 5).

### **1.5.1: MAPK signaling in *Saccharomyces cerevisiae***

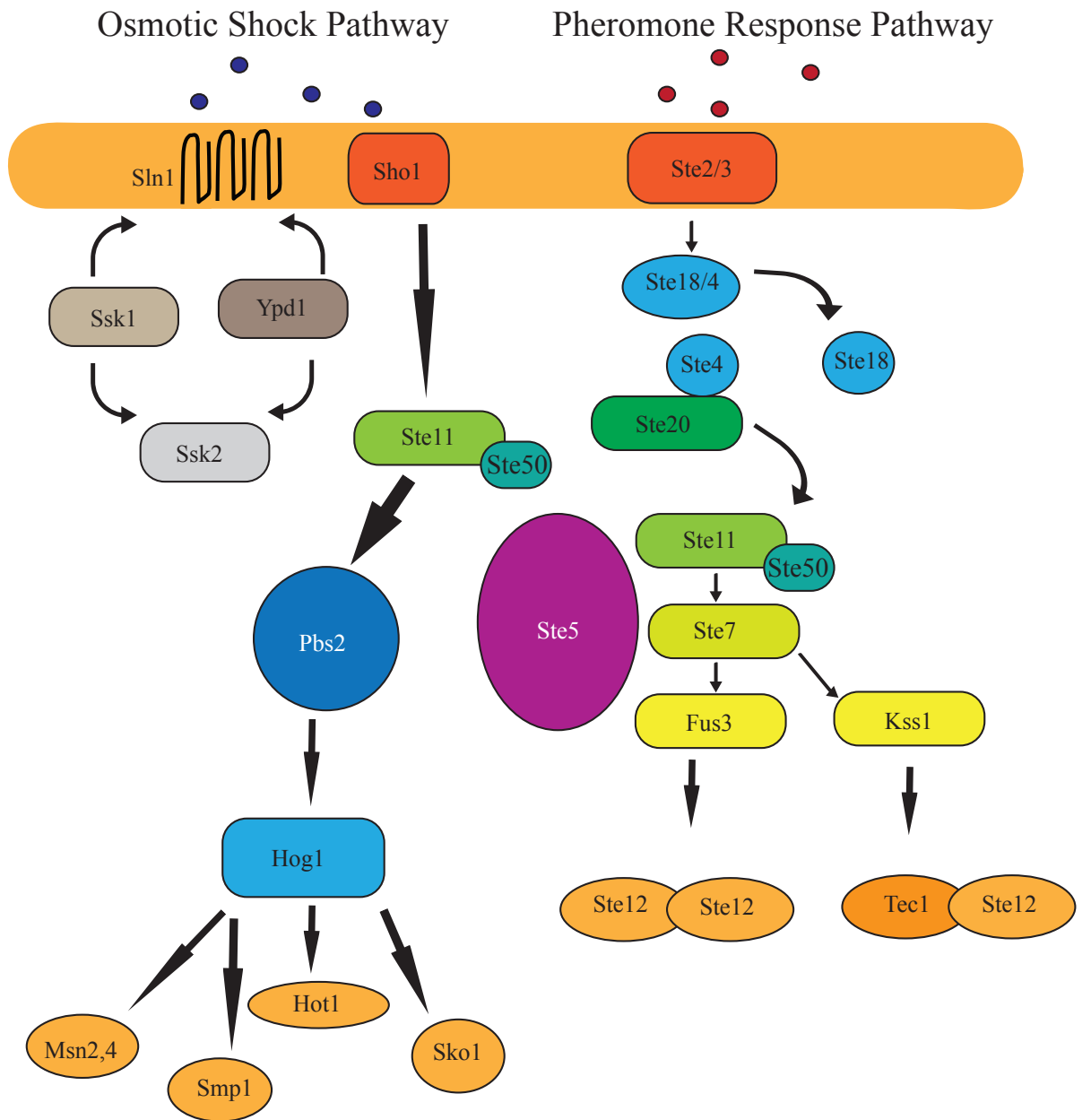
Our understanding of innate immune signaling pathways are ever changing and increasing in complexity and redundancy. Many feedback loops exist and there is extensive overlap within the systems. Studying manipulation of such pathways in the context of infection proves to be challenging, as many questions with regard to biological signaling and protein involvement remain unanswered. The yeast *Saccharomyces cerevisiae* is one of the most intensively studied eukaryotic model organisms in molecular biology and can be used as a tool to study MAPK signaling. *S. cerevisiae* cells have four MAPK signaling pathways but do not encode NF $\kappa$ B (Moore et al., 1993; Srinivasan et al., 2010). *S. cerevisiae* respond to environmental stress by signaling through a related but

more primitive pathway, the Retrograde response Genes (RTG) pathway making it a convenient host for studying NFκB-independent immune signaling impairment (Moore et al., 1993; Klipp & Liebermeister, 2006; Srinivasan et al., 2010). Two pathways, the pheromone response pathway and the osmotic shock pathway, are explored in this study to gain insight into a potential role for *Shigella* effector OspI.

Haploid *S. cerevisiae* yeast cells exist as one of two mating types, *MATa* or *MATα*; these opposite haploid cell types that can fuse upon mating and form a *MATa/MATα* diploid (Bardwell, 2005). Upon stimulation of a haploid cell with pheromone from the opposite mating type the cell will undergo physiological changes to prepare for mating including changes in gene expression and induction of cell cycle arrest (Bardwell, 2005). Signal transduction through the mating pathway is initiated when the pheromone binds a G-Protein Coupled Receptor (GPCR) on the cell surface, which stimulates the dissociation of the Ste4/Ste18 complex from Gpa1 docked at the membrane (Rohde et al., 2007). Ste4 binds Ste20 protein kinase to initiate the signaling cascade. Active Ste20 phosphorylates the MAPKKK Ste11, which phosphorylates the MAPKK Ste7, which in turn phosphorylates the MAPK Fus3 and/or Kss1 (Vaga et al., 2014). Ste11, Ste7, and Fus3 are all bound to the scaffold protein Ste5. Active Fus3 and Kss1 relocate to the nucleus and phosphorylate several target proteins (Rohde et al., 2007; Vaga et al., 2014). Inhibition of signaling through this pathway can be observed in the laboratory using a halo assay as described in M&M Section 2.17.

The osmotic shock pathway, or HOG (High Osmolarity Glycerol) MAPK pathway, is activated in response to osmotic stress placed on a yeast cell and results in glycerol production to regulate cell volume and turgor pressure (Klipp et al., 2005).

**Figure 6. MAPK signaling through the pheromone response pathway and the osmotic shock (HOG) pathway in yeast.** Osmotic Shock Pathway (Left): An increase in external osmolarity is sensed by either Sln1 or Sho1, which separately activate two branches of the MAPK pathway. Sho1 recruits the MAPKKK Ste11 which phosphorylates and activates MAPKK Pbs2. Pbs2 phosphorylates and activates Hog1 to induce an osmotic shock response. Sln1, Ypd1, and Ssk1 are in constant phosphorelay that inhibits Ssk2 during normal osmotic conditions. During a hyper-osmotic shock, Sln1 is dephosphorylated, and Ssk1 is therefore prevented from inhibiting the other MAPKKK of the osmotic shock pathway, Ssk2. Active Ssk2 phosphorylates Pbs2 which again goes on to phosphorylate and activate Hog1, which relocates to the nucleus and phosphorylates several target proteins (Sko1, Hot1, Msn2,4, Smp1, etc.). Pheromone Response Pathway (Right): Pheromone from the opposite mating type binds Ste2/3, which induces the release of the Ste18–Ste4 complex from Gpa1. Ste4 binds Ste20 to initiate the signaling cascade. Active Ste20 phosphorylates the MAPKKK Ste11, which phosphorylates the MAPKK Ste7, which, in turn phosphorylates the MAPK Fus3 or Kss1. Ste11, Ste7, and Fus3 are all bound to the scaffold protein Ste5. Active Fus3 and Kss1 relocate to the nucleus and phosphorylate the CKI Far1 and several transcription factors (including Tec1, Ste12, Dig1, Dig2, and others) Image adapted from Vaga et al., 2014.



Under normal conditions, the transmembrane osmosensor Sln1 is in constant phosphorelay with cytoplasmic proteins Ypd1 and Ssk1 (Klipp et al., 2005). Briefly, changes in osmolarity are sensed by Sln1, the phosphorelay system is interrupted, and Ssk1 is predominately in a non-phosphorylated, active state. Active Ssk1 phosphorylates MAPKKK Ssk2, which then phosphorylates MAPKK Pbs2, which goes on to phosphorylate and activate MAPK Hog1 so that it can enter the nucleus and activate gene expression (Klipp et al., 2005; Figure 6). Inhibition of signaling through this pathway can be observed in the laboratory through artificially inducing cell stress by supplementing growth media with 1M sorbitol (M&M Section 2.18).

## **1.6: Cell Death induced by bacterial infection**

Programming cell death in response to microbial infection is an essential immune response that is evolutionarily conserved (Philip & Brodsky, 2012). Promoting host cell death during infection provides an efficient means of inhibiting pathogen replication and induces proinflammatory signaling required to mount a lasting and effective immune response (Ashida et al., 2011). Multiple cell death pathways are now recognized and understood to have different outcomes with regard to host-pathogen interaction; however, the mechanisms by which these pathways aid in antimicrobial responses remains unclear (Ashida et al., 2014). In addition, bacteria and other pathogens have evolved to utilize such host responses to their advantage by either further promoting or inhibiting host cell death to propagate infection (Ashida et al., 2011).

### **1.6.1: Apoptosis**

Apoptotic cell death can occur in one of two ways: either the extrinsic pathway or the intrinsic pathway (Kerr *et al.*, 1972). The intrinsic pathway involves mitochondrial permeabilisation resulting in release of cytochrome c, an important component of the electron transport chain (Kroemer *et al.*, 2007). Cytochrome c release initiates activation of the caspase signaling cascade through the formation of a multi-protein complex known as the apoptosome (Tsujimoto *et al.*, 1998). The extrinsic pathway is mediated through activation of specific death receptors on the surface of host cells, such as tumor necrosis factor receptor 1 (TNFR1) and Fas receptor (FasR) (Raqib *et al.*, 2002). Signaling through the extrinsic pathway initiates the caspase cascade via caspase-8 (Philip & Brodsky, 2012). Initiator caspases (-2, -8, and -9) proteolytically cleave executioner caspases (-3, -6, and -7, respectively) which when active cleave various substrates resulting in apoptosis (Raqib *et al.*, 2002). Apoptotic cell death is non-inflammatory and is characterized by chromatin condensation as well as fragmentation of the nucleus, cell shrinkage and plasma membrane blebbing (Ashida *et al.*, 2011).

Pathogens have evolved various mechanisms to influence apoptosis via both the intrinsic and extrinsic pathways. Serogroups of *E. coli* (EPEC, EHEC) as well as *Citrobacter rodentium* prevent premature apoptosis intracellularly through the mitochondrial pathway using a subset of T3SS effectors (Holmes *et al.*, 2010). One such effector, EspF, is secreted upon invasion and undergoes trafficking to the mitochondrial membrane where it interferes with membrane potential resulting in inactivation of the intrinsic apoptotic pathway (Nagai *et al.*, 2005). Other pathogens such as *Salmonella*, *Rickettsia*, and *Bartonella* exploit the PI3/Akt prosurvival pathway that prevents cytochrome c release thus inhibiting caspase activation (Knodler *et al.*, 2005).

### 1.6.2: Pyroptosis

While pyroptosis is more closely related to apoptosis than necrosis there are specific differences that have recently been elucidated. Zychlinsky et al. (1992) first described pyroptosis through observation of *Shigella* infected macrophages but this lytic form of programmed cell death was not termed 'pyroptosis' until later (2001) when the discovery of its reliance on caspase-1 made it clearly distinct from apoptosis (Cookson & Brennan, 2001). Caspase-1 is an inflammatory caspase reliant on a multicomplex structure, termed the inflammasome, for activation (Martinon & Tschopp, 2007). Inflammasomes are large signaling complexes that become activated through detection of Pathogen Associated Molecule Patterns (PAMPs) or signals that indicate cellular stress (Danger Associated Molecular Patterns, DAMPS)(Jorgensen & Miao, 2015). Inflammasome activation and subsequent cleavage of caspase-1 to its active form results in proinflammatory downstream effects such as release of cytokines IL-1 $\beta$  and IL-18 (Edgeworth et al., 2002).

The NLRC4 (Nod Like Receptor containing CARD domain 4) inflammasome is known to respond to bacterial flagellin as well as T3SS rod and needle components (Jorgensen & Miao, 2015). Such pathogens as *Salmonella typhimurium* and *Listeria monocytogenes* evade NLRC4 inflammasome activation by repressing PAMP expression (i.e. flagellin) and instead expressing a variant that is not recognized by the inflammasome (Grundling et al., 2004; Way et al., 2004; Miao et al., 2010). Intracellular bacteria such as *Legionella pneumophila* have evolved to maintain their intracellular

vacuole within the host to avoid inflammasome detection and subsequent pyroptosis (Ge et al., 2012).

Caspase-11 is a proinflammatory caspase that recognizes LPS on the surface of intracellular pathogens; gram-negative *Francisella novicida* has recently been shown to modify its LPS structure to avoid detection by this sensor (Hagar et al., 2013). Similarly, *Shigella* remodels its LPS structure inside host cells to avoid immune recognition (Paciello et al., 2013).

*Yersinia pseudotuberculosis* directly interferes with caspase-1 activity by secreting a T3SS effector (YopM) that binds and inhibits enzymatic activity as well as association of caspase-1 with the inflammasome (LaRock et al., 2012). The mechanisms by which *Shigella* interacts with the pyroptotic arm of programmed cell death has not been fully elucidated. A single T3SS effector, OspC3, has been described to directly inhibit caspase-4 (caspase-11 homolog which also recognizes intracellular LPS) mediated pyroptosis in a guinea pig model of infection (Kobayashi et al., 2013). A second effector, IpaB (also forms translocation pore for secretion) binds directly to caspase-1 in macrophages to assist in pyroptosis (Hilbi et al., 1998). Little investigation has been applied to *Shigella* induced dendritic cell death; we know that *Shigella* induces rapid cell death (within 3 hours) and that IL1 $\beta$  and IL18 are released in response (Edgeworth et al., 2002; Kim et al., 2008). Although this cytokine profile suggests death by pyroptosis, the mechanisms exploited and effectors involved are largely unclear.

### **1.6.3: Necrosis**



Necrotic cell death does not rely on the caspase cascade and is extremely proinflammatory (Ashida et al., 2011). The hallmarks of necrotic cell death include membrane rupture and release of cellular contents as well as nuclear swelling (Goldstein & Kroemer, 2007). *Shigella* induced necrotic-like cell death has been observed at high multiplicities of infection (MOI) in primary macrophages (Willingham et al., 2007). This form of bacterial induced cell death is caspase-1 independent but utilizes components of the NLRP3 inflammasome complex, therefore, this mixed form cell death is now referred to as pyronecrosis (Willingham et al., 2007).

Alternatively, regulated necrotic-like cell death that is dependent on signaling through serine/threonine kinase Receptor Interacting Proteins (RIPs) RIP1 and RIP3 is referred to as necroptosis (Cho et al., 2009). Necroptosis is better characterized in virus-host interactions and has been shown to be critical for virus-induced inflammatory responses (Cho et al., 2009).

#### **1.6.4: Balancing Life and Death in Host-Pathogen Interactions**

It is important to note the complexity of balancing life and death within the host cell: this is very much dependent on cell type (Faherty & Maurelli, 2008). *Shigella* hide within epithelial cells to avoid immune detection and propagate infection therefore they prevent programmed cell death in this host (Monack et al., 2001). Antigen presenting cells such as macrophages and dendritic cells have an arsenal of tools to clear pathogens like *Shigella* so *Shigella* have evolved mechanisms to target these cells for destruction (Phalipon & Sansonetti, 2007; Salgado-Pabón *et al.*, 2013). Further defining the mechanisms by which *Shigella* manipulates death pathways will provide insight into

engineering an attenuated strain of *Shigella* for application in vaccine efforts (Levine et al., 2007).

## **1.7: Deletion Collections as a tool for studying bacterial pathogenesis**

Deletion collections have proven to be an invaluable tool in genetics for assigning function to uncharacterized or partially characterized genes in a genome through phenotypic analysis. A deletion collection is comprised of mutant strains of an organism each deleted for a different particular gene in the genome. This tool was pioneered by researchers in yeast genetics who have successfully created and demonstrated the value of deletion collections using the *Saccharomyces cerevisiae* deletion collection (Giaever et al., 2002). Giaever and colleagues first applied the yeast deletion collection to investigate genes involved in stress responses such as osmotic stress, acid stress, as well as glucose and nitrogen starvation (Giaever et al., 2002).

More recently, individual mutants within deletion collections have been tagged with a specific DNA sequence or “barcode” that identifies the mutant of interest. Santiviago and colleagues have constructed a deletion collection of 1,023 *Salmonella enterica* serovar Typhimurium genes each with a different barcode (Santiviago et al., 2009). After verifying the accuracy of their method of detecting barcodes by microarray, Santiviago et al. pooled groups of deletion collection mutants and infected mice to verify their model *in vivo*. Mice were injected intraperitoneally (IP) with an “input” pool of mutant strains from the deletion collection, and were euthanized two days post-infection and mutants were recovered from the spleen (the “output” pool; Santiviago et al., 2009). Genes deleted from mutants included in the input pool but not recovered from the output pool were considered important for virulence as

these mutants were unable to overcome the mouse immune system without these genes (Santiviago et al., 2009).

Given that cell survival is dependent on expression of essential genes, these genes cannot be deleted. To investigate the function of essential genes libraries of mutants that have attenuated gene expression have been created to alter function of these genes whilst enabling survival of the organism. This approach was used to investigate sensitivity of *Staphylococcus aureus* to chemical compounds with potential for antibiotic use as cells become sensitized upon attenuation of essential genes (Donald et al., 2009). This approach assists in identification of novel drug targets and sheds light on the mechanism of action of such compounds within *S. aureus* (Donald et al., 2009). The creation and optimization of such mutant collections allows for generation of rapid and reliable findings and can be applied for high-throughput studies in the field of bacterial pathogenesis.

Baba et al. (2006) have generated a deletion collection (termed the Keio collection) containing roughly 4000 mutants each deleted for a different non-essential gene in the model bacterium *E. coli* K12. Since its creation the Keio collection has been applied to further research in functional genomics studies as well as systems biology. Assessment of genetic interactions can now be rapidly explored through using an organism's deletion collection to generate a double deletion collection. One promising approach has been applied in both *E. coli* and *S. cerevisiae* whereby F-plasmid conjugation facilitates integration of a plasmid carrying a mutated gene of interest into each member of the deletion collection, giving rise to a double deletion collection (Boone et al., 2007; Typas et al., 2008). I attempt to modify this approach (described in M&M Section 2.3-2.6) in an effort to create a repertoire of *Shigella* double deletion mutants.

The Rohde lab has constructed a *Shigella* deletion collection comprised of single gene deletion mutants from each open reading frame (ORF) on the virulence plasmid, pWR100 (Sidik et al., 2014). This study applies the *Shigella* deletion collection as a tool to investigate the involvement of virulence plasmid genes in dendritic cell killing. Further, the deletion collection is applied in an attempt to generate double mutants with the intention of identifying potential genetic interactions.

## **1.8: Research Described in this Thesis**

The primary focus of this research project was to investigate *Shigella*-dendritic cell interaction. Initially, Wild-type *Shigella flexneri* was compared to a T3SS deficient mutant in its ability to kill bone marrow-derived dendritic cells (BMDCs). I found that *Shigella* induced death occurs rapidly and efficiently in BMDCs in a T3SS dependent manner. The *Shigella* deletion collection was applied to assess the importance of each of the genes on the virulence plasmid (pWR100) in induction of dendritic cell death. JAWSII dendritic cell line was infected with each mutant from the deletion collection for a three hour time period and viability was assessed by trypan blue exclusion. Infection conditions where JAWSII cells had low viability indicated that the mutant of interest was still highly virulent and that the gene deleted was not involved in DC impairment. Conversely, conditions where JAWSII cells were almost completely viable indicated a loss of a gene required for DC killing. Many infection conditions had an “intermediate death phenotype” (between 25-75% death of JAWSII cells) indicating some form of involvement in inducing DC death. This screen both validated known virulence factors

encoded on pWR100 and identified a role for previously uncharacterized genes in DC impairment. I focused my research project by choosing four deletion mutants for further investigation: T3SA components MxiD and MxiH and outer *Shigella* proteins OspI and OspF.

I hypothesized that OspI and OspF play roles in DC impairment by inhibiting innate immune signaling through the MAPK pathway. Western blot analysis comparing activated levels of MAPK Erk and p38 among deletion mutant infected JAWSII cells and Wild-type infected JAWSII cells supported my hypothesis as deletion mutant infected cells displayed higher levels of both pErk and pp38. A similar phenotype was observed upon infection of JAWSII cells infected with a double mutant *Shigella* strain deleted for both *ospI* and *ospF*: here, MAPK analysis indicated that levels of activated Erk and p38 were increased over the levels seen in JAWSII cells infected with either of the single mutants or Wild-type *Shigella*. MAPK signaling inhibition was also observed in a yeast model system when OspI or OspF was expressed under a galactose inducible promoter. The E2 ubiquitin conjugating enzyme Ubc13 is confirmed to be a target of OspI and may be involved in the pheromone mating response in *Saccharomyces cerevisiae*.

Deletion collection mutants *ΔmxiD* and *ΔmxiH* were applied in this study to shed light on a potential mechanism of inducing dendritic cell death *in vitro*. I hypothesized that *Shigella* kills JAWSII cells by inducing pyroptosis. Initially a pancaspase inhibitor (ZVAD) was used to pretreat the dendritic cell line prior to Wild-type *Shigella* infection to assess caspase-mediated death mechanisms. A 20% increase in viability was observed in Z-VAD treated JAWSII cells over non-treated Wild-type infected JAWSII indicating

some caspase involvement in inducing programmed cell death in response to *Shigella* infection.

Given that pyroptosis is caspase-1 specific, I infected JAWSII cells with Wild-type *Shigella* and all follow-up mutants:  $\Delta mxiD$  and  $\Delta mxiH$  as well as complemented strains,  $\Delta ospF$  and  $\Delta ospI$  and complemented strains,  $\Delta ospIF$  and complemented strains) to assess levels of caspase-1 activation. I hypothesized that deletion collection mutant strains that were unable to kill JAWSII cells (i.e.  $\Delta mxiD$  and  $\Delta mxiH$ ) would induce less caspase-1 activation in JAWSII cells than those that rapidly induce cell death. This hypothesis was not supported: higher levels of active caspase-1 were observed in T3SS deficient mutant infected JAWSII cells indicating that *Shigella* employs one or more T3SS effectors to specifically target caspase-1 activation *in vitro*. The  $\Delta ospI$  and  $\Delta ospF$  mutants assayed are not responsible for caspase-1 impairment in JAWSII cells as protein levels are not different from those observed in the Wild-type condition.

Understanding that caspase-1 mediated pyroptosis can lead to cleavage and activation of the proinflammatory cytokine IL1 $\beta$ , I hypothesized that JAWSII cells infected with virulent *Shigella* strains would release significantly more cytokines than avirulent strains (i.e.  $\Delta mxiD$  and  $\Delta mxiH$ ). This hypothesis was not supported; JAWSII cells infected with Wild-type *Shigella* display levels of mature IL1 $\beta$  similar to those seen in cells infected with T3SS deficient mutant *Shigella* strains. Additionally, no differences in cytokine levels were observed when comparing Wild-type infected JAWSII to Osp mutants.

This study has established that the *Shigella* deletion collection is a powerful and convenient tool for studying pathogen-host interactions and sets the stage for future

investigation of dendritic cell impairment by *Shigella*. Understanding the role of genes on the pWR100 virulence plasmid in the context of immune impairment paves the road toward novel approaches for vaccine development and design of new therapeutic targets in an era of antibiotic resistance.

## **Chapter 2: Methods and Materials**

### **2.1: Growth and Maintenance of Bacterial Strains**

*Shigella flexneri* serotype 5a (M90T) was used throughout this study as a wild-type strain (Allaoui et al, 1992; Onodera et al. 2012). pWR100 is the virulence plasmid harbored by M90T and was used for all mutational analysis in this study. *Shigella* was grown overnight at 37°C in 30 mg/mL Trypticase Soy Broth (TSB) when liquid cultures were needed, or on 30 mg/mL TSB with 20 mg/mL agar and 0.01% Congo red when a solid matrix was required. Congo red was used as an indicator of T3SS function. The *Escherichia coli* strains DH5 $\alpha$  and S17 were maintained in Luria Bertani (LB) broth (10 mg/mL tryptone, 5 mg/mL yeast extract and 10 mg/mL sodium chloride) with 20 mg/mL agar when a solid matrix was required. Frozen stocks were made by adding 50% glycerol to liquid cultures (1:2 ratio) grown overnight at 37°C, and these stocks were stored at -80°C. Required antibiotics were added to the medium to a final concentration as listed in Table 1.

**Table 1: Final concentrations of antibiotic used for selection**

<b>Antibiotic</b>	<b>Final Concentration</b>
Gentamicin	5 $\mu$ g/mL
Tetracycline	5 $\mu$ g/mL
Chloramphenicol	25 $\mu$ g/mL
Ampicillin	100 $\mu$ g/mL
Kanamycin	25 $\mu$ g/mL

### **2.2: Construction of Deletion Collection Mutants**

The *Shigella* deletion collection was constructed using the  $\lambda$ -red recombination method and is described by Sidik et al. (2014). Briefly, each gene on plasmid pWR100 was



individually deleted and replaced with a tetracycline resistance gene characterized by Karlinsey (2007). Tetracycline knock-out cassettes were generated by performing PCR, with pairs of primers including 50 nucleotides of the sequence flanking the gene that was to be deleted. PCR products were electroporated into wild-type *Shigella* (M90T) containing the plasmid pKD46 (GenBank Accession number AY048746, Datsenko and Wanner, 2000), which encodes the  $\lambda$ -red recombination machinery from  $\lambda$  phage. Bacteria were allowed to recover for two hours in liquid medium at 37°C with shaking at 200 RPM, then plated on medium containing tetracycline to select for bacteria containing the deletion mutation of interest. Gene deletions were confirmed by the ability to amplify a PCR product using a primer inside the tetracycline resistance cassette and a primer in the flanking DNA sequence. Tetracycline resistance cassettes were removed via the introduction of a plasmid encoding flippase machinery capable of promoting recombination between FRT sites flanking the tetracycline resistance cassette (Cherepanov et al, 1995). The *Shigella* deletion collection consists of 103 mutants out of the approximately 110 genes encoded by pWR100 (Sidik et al., 2014).

### **2.3: Construction of Suicide Plasmids**

#### *pJQipgB1:*

Primers were designed as listed in Table 2 to amplify, using PCR, *ipgB1* and portions of the upstream gene, *ipgA*, and downstream gene, *ipgC*, from pWR100. Amplified *ipgB1* fragment was cloned between the XhoI and NotI sites of pBluescript to construct pBSipgB1. A HindIII restriction site near the center of *ipgB1* allowed for insertion of a

chloramphenicol resistance cassette (*cat*) amplified from pCP20 to inactivate *ipgB1*, giving rise to pBSipgB1-Cm<sup>R</sup>. The ipgB1-Cm<sup>R</sup> fragment was cut from pBSipgB1-Cm<sup>R</sup> and cloned between the XhoI and NotI sites of suicide plasmid pJQ200 to make pJQipgB1-Cm<sup>R</sup>. The pJQ200 vector encodes a gentamicin resistance cassette (*aacC1*) and contains the *sacB* gene, that confers sensitivity to sucrose.

*pJQipaD:*

Primers were designed as listed in Table 2 to amplify, by PCR, *ipaD* and portions of the upstream gene, *ipaA*, and downstream gene, *ipaC* from pWR100. The amplified *ipaD* fragment was cloned between the XhoI and NotI sites of pBluescript to construct pBSipaD. A HindIII restriction site near the center of *ipaD* allowed for insertion of a chloramphenicol resistance cassette (*cat*) amplified from plasmid pCP20 to inactivate *ipaD*, giving rise to pBSipaD-Cm<sup>R</sup>. The ipaD-Cm<sup>R</sup> fragment was cut from pBSipaD-Cm<sup>R</sup> and cloned between the XhoI and NotI sites of suicide plasmid pJQ200 to make pJQipaD-Cm<sup>R</sup>.

**Table 2: Primers used for suicide plasmid construction.**

Plasmid	Forward Primer (5'-3')	Reverse Primer (3'-5')
pBSipgB1	CGTTAGCTCGAGTCAA GCCTTTATATTGCTCGA AGAA	TGCCAGGCGGCCGCTT ACTCCTTGATATCCTGA ATTGCG
pBSipgB1-Cm <sup>R</sup>	GCATGTGAGCTCGCAT CACCCGACGCACTTTG CGCCG	GTACTGGAGCTCTTAC GCCCCGCCCTGCCACT C
pBSipaD	CGTTAGCTCGAGGCT AATAGTGCCAAGTCT GTGC	TGCCAGGCGGCCGCCA CACAAGTAGGTATAAC GG
pBSipaD-Cm <sup>R</sup>	GCATGTGAGCTCGCAT CACCCGACGCACTTTG CGCCG	GTACTGGAGCTCTTAC GCCCCGCCCTGCCACT C

#### 2.4: Plasmid Conjugation

Conjugation between donor *E. coli* and recipient *Shigella* strains was performed as described (Sidik et al 2014). Briefly, *E. coli* and *Shigella* cultures were grown to confluence in liquid medium at 37°C with shaking at 200 RPM overnight. Liquid cultures were subcultured 1:100 into fresh medium and grown to late-log phase ( $OD_{600} \approx 1.0$ ). Antibiotic-containing growth medium used to select for plasmids of interest was removed by centrifugation followed by supernatant removal. Pelleted bacteria were washed and resuspended in fresh antibiotic-free LB. Cell density was normalized for each strain spectrophotometrically ( $\text{medium to add } (\mu\text{l}) = (OD_{\text{initial}} \times V_{\text{initial}}) / (OD_{\text{final}} - V_{\text{initial}})$ ) and 20  $\mu\text{l}$  donor *E. coli* was combined with 80  $\mu\text{l}$  of recipient *Shigella* with gentle mixing. The cell mixture was pipetted onto a pre-warmed, antibiotic-free LB agar plate and incubated for 1

hour at 37°C to allow for conjugation to occur. Agar plates were rinsed with fresh medium which was collected for centrifugation at 13 500g for 1 minute at room temperature to pellet the cells. The supernatant was removed and the cell mixture was resuspended in 250 µL antibiotic free LB broth for selection.

### **2.5: Positive-Negative Selection of Transformed *Shigella***

Serial dilutions of the donor-recipient mixture were plated on Congo red agar with selection agents for both the suicide plasmid and the marker of the mutation of interest (usually tetracycline) on the virulence plasmid. Agar plates were incubated at 37°C overnight. Isolated Congo red-positive *Shigella* colonies were chosen for negative selection on agar containing 5% sucrose to select for excision of the suicide plasmid vector. Two similar procedures were attempted to select for excision: positively selected colonies were grown overnight in medium containing antibiotic to maintain plasmid selection and then serially diluted onto sucrose-containing agar, or, colonies were struck directly onto sucrose-containing agar after positive selection. Agar plates were incubated at 37°C overnight and individual Congo-red positive colonies were selected for further investigation.

### **2.6: Confirming Double Mutants of Interest**

Individual colonies from negative selection (sucrose) were patched onto agar plates containing antibiotic for positive selection markers, and grown overnight at 37°C.

Colonies were replica plated onto agar plates containing antibiotic for the selectable marker that should have been lost when the vector of the suicide plasmid was excised. In other words, sensitivity to the marker on the suicide plasmid backbone was used as an indication that the desired mutation had occurred.

## **2.7: Purification and Culture of Bone Marrow Derived Cells (BMDCs)**

C57BL/6 mice (Jackson Laboratories, Bar Harbor, MA) were anaesthetized by inhaled isofluorine and sacrificed by cervical dislocation. BMDC isolation from bone marrow was performed as described in Matheu et al., 2008. Briefly, Bone marrow was collected from both the femurs and tibia of mice using a 27-gauge needle. Isolated bone marrow was centrifuged at 300g for 10 minutes and the cellular pellet was resuspended in 5mL of ammonium chloride for 2 minutes to kill red blood cells. The cell suspension was neutralized in 5mL of Dendritic Cell (DC) medium (96 mL of complete RPMI with 10 % FBS, 500  $\mu$ L  $\beta$ ME, 1 mL 100x amino acids, 1 mL sodium pyruvate; all purchased from Invitrogen) and centrifuged at 300g for 10 minutes. Pellet was resuspended in 30 mL of DC medium and 5 mL of the suspension was plated into each well of a six-well plate. Two microliters of Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF) (0.1 mg/mL, eBioscience, San Diego, CA) was added to each well. The cells were incubated at 37 °C with 5 % carbon dioxide (CO<sub>2</sub>) for 5 days, changing the media at day 3. On day 6, loosely adherent cells were harvested and re-plated in a fresh 6 well plate containing 5 mL DC media and 1  $\mu$ L of GM-CSF. DCs were harvested on Day 8 by mechanically collecting adherent and non-adherent cells mechanically.

## 2.8: *Shigella* infection of Dendritic Cells

DCs were harvested and plated in 6-well plates at a density of 4-5 million cells per well and inoculated with *Shigella* at a Multiplicity Of Infection (MOI) of 10. Control wells were treated with *E. coli* LPS 0127:B8 (10  $\mu$ L/well, Sigma-Aldrich Mississauga, ON). Bacterial strains used are described in Table 3. Plates were centrifuged at 300g for 10 minutes to facilitate interaction between DCs and *Shigella*, and incubated at 37 °C with 5 % CO<sub>2</sub> for an additional 30 minutes to allow *Shigella* to invade. To stop invasion and kill extracellular bacteria, 50  $\mu$ L of gentamicin (15  $\mu$ g/mL) (Fisher Scientific, Ottawa, ON) was added to each well. Plates were then incubated at 37 °C with 5 % CO<sub>2</sub> for 1, 3, 6, 8, 12, or 16 hours. For assays involving caspase inhibition, the pancaspase inhibitor Z-VAD-FMK (InvivoGen catalogue number tlr1-vad) was added to DCs at a concentration of 20 $\mu$ M 1 hour prior to *Shigella* infection.

**Table 3: Bacterial Strains used in this study**

Strain	Description	Reference
M90T	Wild-type <i>Shigella</i> strain	Alloui et al., 1992
pWR100 Deletion Collection Mutants	Collection of 96 <i>Shigella flexneri</i> mutants each lacking one gene from pWR100	Onodera et al., 2012

## 2.9: Trypan Blue Exclusion

Trypan Blue dye was used as an indicator of dendritic cell viability (ThermoScientific catalogue number 15250-061; Kang & Lim, 2012). Infected DCs were mixed with Trypan

blue at a 1:1 ratio and allowed one to two minutes incubation. Ten microliters of cell suspension was loaded onto a hemocytometer for analysis under a light microscope (4X or 10X objective). Non-viable cells stained blue and viable cells resisted infiltration of the dye and remained colorless. Four 1 x 1 mm squares of the hemocytometer were counted and this was repeated three times for each infection condition. The number of live and dead cells was averaged from all hemocytometer quadrants and the following mathematical equation was applied to determine percent viability:  $[(\text{average number of live cells} / (\text{average number of live cells} + \text{average number of dead cells})) * 100]$ . The trypan blue screen with the *Shigella* deletion collection was performed three times to obtain statistical significance.

#### **2.10: Flow cytometry analysis**

Information regarding antibodies used can be found in Table 4. After infection, cells were harvested and centrifuged for 5 minutes at 300g. Supernatants were collected and stored at -80°C and the remaining pellets were resuspended in Fc blocker (1:200 of rat anti-mouse CD16/32 in 2% PBS) for 15 minutes at 4 °C. One hundred microliters of cell suspension from each infection condition was stained with monoclonal antibodies at concentrations listed in Table 3. Samples were vortex mixed and stained for 30 minutes in the dark at 4 °C before washing with 1mL of 2 % PBS. Samples were centrifuged at 300g for 5 minutes and supernatants were removed. Fifty-five microliters of 7-Aminoactinomycin D (7AAD) mix (5 µL 7AAD + 50 µL 2 % PBS) was added to each tube and samples were stained in the dark at room temperature (RT) for 15 minutes. Samples were each washed with 1 mL of 2 % PBS, cells were pelleted at 300g for 5

minutes, and pellets were resuspended in 300  $\mu$ L of 2 % paraformaldehyde (PFA) (Fisher Scientific, Mississauga, ON). Single color controls as well as isotype controls were made using the same method. All samples were analyzed at the Flow Cytometry Core Facility at Dalhousie using a FACSCalibur<sup>TM</sup> (BD Biosciences, San Jose, CA).

**Table 4: Antibodies used for flow cytometry and detailed information.**

Antibody	Product Detail	Purchase Information
7AAD		bioLegend, San Diego, CA
CD11c FITC	Clone HL3	eBioscience, San Diego, CA
CD11c APC	Clone N418	eBioscience, San Diego, CA
CD11c PE	Clone N418	eBioscience, San Diego, CA
I-A/I-E (MHC II) PE	Clone M5/114.15.2	eBioscience, San Diego, CA
CD16/32	Fc receptor blocking antibody; Clone 93	eBioscience, San Diego, CA
rIgG2a APC	Clone R35-95	BD Pharmingen, Mississauga, ON
ArhaIgG APC	Clone ebio299Arm	BD Pharmingen, Mississauga, ON
rIgG2b PE	Clone A95-1	BD Pharmingen, Mississauga, ON
rIgG2a PE	Clone R35-95	BD Pharmingen, Mississauga, ON

**Table 5: Dilutions used for optimal effectiveness of monoclonal antibodies.**

	Antibody ( $\mu$ L)	PBS ( $\mu$ L)
CD11c FITC	15	285
CD11c APC	1	20
CD11c PE	1	20
I-A/I-E (MHC II) PE	1	500
CD16/32	11	2200
rIgG2a APC	1	100
ArhaIgG APC	1	100
rIgG2b PE	1	100
rIgG2a PE	1	100

## 2.11: Tissue Culture

The JAWSII dendritic cell line was maintained in  $\alpha$ -Minimum Essential Medium (MEM, Sigma catalogue number M8042, Oakville, ON) supplemented with 20% Fetal Bovine



Serum (FBS) and 5 ng/mL granulocyte-monocyte colony stimulating factor (GM-CSF; Gibco catalogue number PMC2015). Cells were maintained at 37°C with 5% CO<sub>2</sub>. Non-adherent JAWSII cells were collected in a centrifuge tube and combined with adherent cells which were lifted using 0.25% trypsin-0.03% EDTA (Sigma catalogue number 25200-056, Oakville, ON). Cell suspensions were centrifuged at 1000 rpm for 10 minutes at 37°C, resuspended in fresh medium, and subcultivated at a ratio of 1:2 weekly. Prior to infection, cells were harvested and subcultured into 6-, 12-, 24-, or 96-well tissue culture dishes and incubated overnight at 37°C with 5% CO<sub>2</sub>.

## **2.12: SDS-PAGE and Western Blotting**

Bacterial infections were performed as described in Section 2.8. JAWSII cells were pelleted by centrifugation for 10 minutes at 300g and supernatant was removed and discarded. Culture dishes were rinsed with PBS to collect remaining cells before the addition of RIPA buffer (10% NP40, 1% SDS, 0.5 M Tris-HCl pH7.4, 1.5 M NaCl, 5% sodium deoxycholate, 0.01 M EDTA, dH<sub>2</sub>O) to lyse cells from each infection condition. Lysates were collected in microcentrifuge tubes, 2X Protein Sample Buffer (PSB; 0.375M Tris-HCl pH 6.8, 6% SDS, 36% glycerol, 0.03% Bromophenol blue in 50 mL distilled water) was added to each sample, and all samples were sonicated for 10 seconds to reduce viscosity.

Polyacrylamide Gel Electrophoresis (PAGE) was performed in accordance with Sambrook et al., 2001. Samples were loaded into wells of 10% polyacrylamide gels and proteins were separated at 150V in a running apparatus containing running buffer (Tris 30

g, glycine 144 g, SDS 10 g in 1 L dH<sub>2</sub>O). Bromophenol Blue migration from the top to the bottom of the gel was used monitor optimal protein separation. Broad Range Protein Ladder purchased from New England Biolabs was used as a molecular weight size standard for all experiments (catalogue number 0021405).

For Western blot analysis, a wet transfer apparatus was used to transfer proteins from the polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane. The transfer apparatus was filled with Transfer Buffer (Tris 12g, glycine 57.6g, methanol 800mL, 10% SDS 16mL, dH<sub>2</sub>O 4L) and the transfer was performed overnight at 0.05 A. All subsequent incubation steps were performed with constant agitation on a shaker. PVDF membranes were incubated in blocking buffer (5% skim milk powder in Tris-Buffered Saline with 0.1% Tween (TBST), or 5% BSA in TBST) for 1 hour at room temperature. Primary antibodies were diluted in blocking buffer according to Table 6 and added to PVDF upon removal of the blocking buffer. PVDF membranes were incubated for 1 hour with primary antibody before washing three times with TBST for 15 minutes per wash. Secondary antibodies were diluted in blocking buffer (Table 6) and incubated with PVDF membranes for 1 hour at room temperature. Any excess antibody was removed by washing the PVDF three times with TBST for 15 minutes each. Pierce ECL Plus Substrate kit (Thermo Scientific, Cat# 32132) was used to develop substrate (Horseradish peroxidase, HRP) on membranes, which were then imaged using a KODAK imager (Cat # 864 2985).

**Table 6: Antibodies used in this study for western blot analysis**

<b>Antibody</b>	<b>Dilution</b>	<b>Source</b>
Murine Erk1/2	1:1000	New England Biolabs cat. no. 9101
Murine P-Erk1/2	1:500	New England Biolabs cat. no. 4377
Murine Jnk	1:1000	New England Biolabs cat. no. 9252S
Murine P-Jnk	1:1000	New England Biolabs cat. no. 4668P
Murine p38	1:1000	New England Biolabs cat. no. 9218
Murine P-p38	1:1000	New England Biolabs cat. no. 9211S
Murine IL1-b	1:1000	Abcam cat. no. AB2105
Murine Anti-mouse IgG	1:2000	New England Biolabs cat. no. 7076
Murine Anti-rabbit IgG	1:2000	New England Biolabs cat. no. 7074
Murine GAPDH	1:1000	Life Technologies cat. no. AM4300
Murine caspase-1 (p10)	1:1000	Santa Cruz Biotechnology cat. no. sc-514

### **2.13: Complementation Plasmids**

Four mutants from the deletion collection (MxiD, MxiH, OspF, OspI) were chosen for analysis following the trypan exclusion screen of the *Shigella* deletion collection.

Complementation of these mutants was achieved through introduction of a high copy plasmid (pBluescript) encoding the deleted gene. Primers were designed as listed in Table 7 to amplify the gene of interest from template plasmid pWR100 by PCR. Amplified

fragments were cloned between the BamHI and EcoRI sites of pBluescript and construct identities were confirmed through DNA sequence analysis (performed by Genewiz Inc., South Plainfield, NJ). Purified plasmids were transformed into deletion strains as described in Section 2.19.

**Table 7: Primers used to create complementation plasmids**

Plasmid	Forward Primer	Reverse Primer
pBSMxiD	CGATCGGATCCATGAA AAAATTTAATATTTAA TC	CAGCTCGAATTCTTAGT AATTTAAGTATGAAAC CA
pBSMxiH	CGATCGGATCCATGAG TGTTACAGTACCGAA	CAGCTCGAATTCTTATC TGAAGTTTTGAATAATT
pBSOspF	TAGCCTGGATCCATGG TTCCTCTCGTAAC	CGACTCGAATCCTTAG ATTCTTTGAATGTTG
pBSOspI	CTAGTCGGATCCATGA ATTACCTGAACCGAAC	CTAGTCGAATTCTTAAT GGGGTGTTCGTTACAG

#### **2.14: Construction of OspIOspF double mutant by $\lambda$ -red recombination**

OspF deletion collection mutant was made electrocompetent as described in Section 2.19. The pJR008 plasmid (made from pKD46 described in Section 2.2) was extracted from *E. coli* strain DH5 $\alpha$  as described in Section 2.21 and was transformed into the OspF deletion collection mutant (2.19). The OspF mutant now harboring  $\lambda$ -red recombination machinery from pJR008 was grown overnight at 30°C in TSB supplemented with genatmycin. The following day the culture was diluted 1:100 into 200 mL of TSB supplemented with 1% arabinose to induce expression of  $\lambda$ -red recombination genes on pJR008. The subculture was grown to mid-log phase (OD<sub>600</sub> 0.4-0.6) before cooling on ice to halt growth. Cells

were centrifuged at 4000g for 15 minutes at 4°C, supernatant was decanted, and the pellet was resuspended in ice-cold distilled water. Centrifugation and cold-water wash steps were repeated twice before resuspending in 1 mL of distilled water for use. Cell suspension was aliquoted into 50 µl aliquots in microcentrifuge tubes containing 50% glycerol for each transformation and stored at -80 °C for future use. Kanamycin knock-out cassettes were generated by PCR using primers listed in Table 8 and 10 µl of purified cassette was added to electrocompetent *OspF* mutant. Cells were electroporated as described in Section 2.19 and plated on selective medium. Transformants grown on media containing both tetracycline and kanamycin resistance were selected for confirmation by PCR. Deletion of *ospI* was confirmed by amplification of a PCR product using primers with internal overlap with the kanamycin resistance marker and external overlap with *ospI* (Table 8).

**Table 8: Primers used in construction of  $\Delta$ *OspIF* double mutant by  $\lambda$ -red recombination**

<b>Description</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
“Check” primers: internal overlap with resistance marker, external overlap with <i>ospI</i>	ATGAAATTAAAATTCTTTG TACTTG	CAATTTTTAGCCATGA AAAACGTAAT
Kanamycin resistance cassette + 50bp of homology with <i>ospI</i>	ATGAAATTAAAATTCT TTGTACTTGCACTTTGT GTACCTGCGATCTTTACTAC ACATGATTGAACAAGATGG ATT	CAATTTTTAGCCATGA AAACGTAATCAACTCG GTGAACCAATTCAAAA ATATACTTCAGAAGAA CTCGTCAAGAA

### 2.15: Construction of pA*OspI* for expression in Yeast

Primers were designed as listed in Table 9 to amplify *ospI* from pWR100 by PCR. An amplified *ospI* fragment was cloned between the BamHI and XhoI cut sites of yeast

vector pAO1 to construct pAOspI. The plasmid was analyzed by DNA sequencing to verify that the construct was correct.

**Table 9: Primers used in construction of pAOspI for expression in yeast**

<b>Description</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
OspI primers for yeast expression	CTAGTCGGATCCATGAATTA CCTGAACCGAAC	CTAGTCCTCGAGTTAA TGGGGTGTTCGTTACAG

### **2.16: Yeast Transformation**

Frozen stocks of *S. cerevisiae* strains (listed in Table 10) were plated on YEPD (Yeast Extract Peptone Dextrose) agar and grown by incubation at 30°C for three days. A single fresh colony was isolated from a YEPD plate and was used to inoculate YEPD liquid medium for overnight growth at 30°C shaking at 250 rpm. Starter cultures were diluted the next day in YEPD liquid to approximately  $2 \times 10^6$  cells/mL and were incubated, shaking, for two hours at 30°C. Fifty milliliters of cell culture was pelleted at 1000g for 10 minutes, supernatant was removed, and cells were resuspended in 10 mL of cold distilled water. Cells were centrifuged again at 1000g and pellet was resuspended in 1mL of TE/LiAc (100 µl 10X TE, 100 µl 10X LiAc, 800 µl distilled water). Cell suspension was transferred to a microcentrifuge tube, the samples were centrifuged for one minute at 6K rpm and resuspended in 250 µl of TE/LiAc. Fifty microliter aliquots were pipetted into microcentrifuge tubes for transformation. Carrier DNA (Salmon sperm DNA, BioShop catalogue number DNA999.5) was boiled for five minutes, cooled on ice, and 5 µl was added to each sample to be transformed followed by 2 µl of plasmid DNA. Three hundred microliters of PLATE solution (400 µl 10X TE, 400µl 10X LiAc, 3200 µl 50% PEG) was added to each microcentrifuge tube; the samples were incubated at 30°C for 30 minutes

and then heat shocked at 42°C for 20 minutes. The yeast suspension was centrifuged at 8K rpm for one minute, supernatant was removed, the pellet was washed with distilled water and resuspended in 50 µl of water for plating on selective medium. Dropout medium specific to the plasmid being transformed (SD-URA or SD-URA-HIS) was used to select for transformants that took up plasmid DNA and plates were incubated at 30°C for three days.

**Table 10: Yeast strains used in this study**

<b>Yeast Strain</b>	<b>Description</b>	<b>Source</b>
BY4741	<i>MATa his3Δ leu2Δ met15Δ ura3Δ</i>	Research Genetics
BY4741 pAO1	<i>MATa his3Δ leu2Δ met15Δ ura3Δ</i> transformed with pAO1	This Study
BY4741 pAOspI	<i>MATa his3Δ leu2Δ met15Δ ura3Δ</i> transformed with pAOspI	This Study
BY4741 pYESOspF	<i>MATa his3Δ leu2Δ met15Δ ura3Δ</i> transformed with pYESOspF	This Study
BY4741 pJR001IpaH9.8	<i>MATa his3Δ leu2Δ met15Δ ura3Δ</i> transformed with pJR001IpaH9.8	This Study
SY2625	<i>MATa bar1 ade2 his3::FUS1-HIS3 mfa2::FUS1-lacZ leu2 can1 ura3 trp1</i>	(Evangelista et al., 1997)
SY2625 pAO1	<i>MATa bar1 ade2 his3::FUS1-HIS3 mfa2::FUS1-lacZ leu2 can1 ura3 trp1</i> transformed with pAO1	This Study
SY2625 pAOspI	<i>MATa bar1 ade2 his3::FUS1-HIS3 mfa2::FUS1-lacZ leu2 can1 ura3 trp1</i> transformed with pAOspI	This Study
SY2625 pYESOspF	<i>MATa bar1 ade2 his3::FUS1-HIS3 mfa2::FUS1-lacZ leu2 can1 ura3 trp1</i> transformed with pYESOspF	This Study
SY2625 pJR001IpaH9.8	<i>MATa bar1 ade2 his3::FUS1-HIS3 mfa2::FUS1-lacZ leu2 can1 ura3 trp1</i> transformed with pJR001IpaH9.8	This Study
BY4741 bearing <i>ubc13::G418</i>	<i>MATa his3Δ leu2Δ met15Δ ura3Δ</i>	Research Genetics



### **2.17: Yeast Inhibition Assay/ Halo Assay**

Yeast halo assays were performed essentially as described by Dohlman and coworkers (Hoffman et al., 2002). Frozen stocks of *S. cerevisiae* strains (Table 10) were plated on YEPD agar and grown at 30°C for three days. A single fresh colony was isolated from YEPD plates and was used to inoculate YEPD for overnight growth at 30°C shaking at 250 rpm. A 1% agarose solution was prepared and allowed to cool to 60°C. Ten microliters of yeast starter cultures were inoculated into 2 mL of distilled water and combined with 2mL of molten agarose. This suspension was quickly poured over prewarmed YEPD plates to form an overlay. Paper disks were impregnated with 15 µg of  $\alpha$ -factor and placed on YEPD plates with yeast overlay. Plates were incubated at 30°C for 1-3 days and the diameter of inhibition of yeast cell growth was measured after each day. For yeast strains carrying plasmids with *Shigella* effectors, particular yeast drop-out media were used in place of YEPD to maintain plasmid selection.

### **2.18: Pheromone Response Assay in *Saccharomyces cerevisiae***

*S. cerevisiae* strain SY2625 harbors a *FUS1-HIS3* reporter plasmid that can be used to measure MAPK activity (Evangilista et al., 1997). Yeast strain SY2625 was transformed as described in Section 2.15 with each of the plasmids listed in Table 10. Transformed strains were serially diluted and plated on synthetic complete medium lacking histidine and uracil and containing either glucose or galactose. Medium lacking uracil only was used as a control, and growth on medium containing 1M sorbitol was used to test

inhibition of the osmotic shock MAPK pathway. Serially diluted strains were grown for three days at 30°C and growth was observed and recorded each day.

### **2.19: UV irradiation**

Yeast cultures were grown to stationary phase (overnight at 30°C) before being serially diluted onto YEPD synthetic complete medium to select for plasmids carrying *Shigella* genes (Table 10). Plates were allowed to dry and exposed to 300 µJ of UV light. Plates were incubated for up to 3 days in a 30°C incubator and phenotypes were observed and documented after each day.

### **2.20: Transformation of Bacterial Cells by Electroporation**

Three milliliter starter cultures were grown overnight in nutrient rich medium supplemented with appropriate antibiotic. The next day, cultures were diluted 1:100 and grown to mid-log phase (OD<sub>600</sub> 0.4-0.6). Cells were cooled on ice and centrifuged at 4000g for 15 minutes at 4°C. Supernatant was discarded and pelleted cells were resuspended in ice-cold distilled water. This step was repeated twice and after the third centrifugation step the pellet was resuspended in 1/100 of the original culture volume. Fifty microliter aliquots of the cell suspension were placed in microcentrifuge tubes for use. One microliter of purified plasmid to be transformed was added to electrocompetent cells and mixed gently, keeping cells on ice. Electroporator was set to 2.5V, 200 ohms, 25 µF, 125 µF. Cells were electroporated and quickly resuspended in 1mL of nutrient rich medium (either TSB or LB) and allowed 1 hour recovery at 37°C shaking at 250 rpm.

Bacteria were plated on solid medium containing selective antibiotic to allow for growth of cells that had taken up plasmid DNA. Plates were incubated at 37°C overnight and transformants were confirmed to have taken up plasmid by colony PCR analysis. In cloning steps transformants were confirmed to have correct plasmid by isolating single colonies, growing overnight cultures, extracting the plasmid from the cells, digesting with restriction enzymes that would cut insert from vector, and analyzing product through electrophoresis through 1% DNA gels. Plasmids that were confirmed by this method were then sent for DNA sequence analysis (Genewiz Inc., South Plainfield, NJ).

### **2.21: Transformations of Bacterial Cells by Heat Shock**

*E. coli* cultures were grown overnight at 37°C, shaking at 200 rpm, in nutrient rich medium supplemented with appropriate antibiotic. The next day, cultures were diluted 1:100 and grown to mid-log phase (OD<sub>600</sub> 0.4-0.6). Cells were cooled on ice and centrifuged at 4000g for 15 minutes at 4°C. Supernatant was discarded and pelleted cells were resuspended in ice-cold 100mM CaCl<sub>2</sub>. After a second centrifugation step cell pellet was resuspended in ice-cold 100mM CaCl<sub>2</sub> and allowed 20 minute incubation on ice. Cell suspension was centrifuged at 4000g and resuspended in 100mM CaCl<sub>2</sub> supplemented with 15% glycerol at 1/100 of the original culture volume. One hundred microliter aliquots of the cell suspension were placed in microcentrifuge tubes for use. One microliter of plasmid DNA was used for each transformation and DNA was incubated for one-hour with competent cells before heat shock. Cells with plasmid DNA were heat shocked at 42°C for 45 seconds, incubated on ice for one minute and one milliliter of

nutrient rich medium was added to the microcentrifuge tube. Heat shocked cell suspension was plated on nutrient rich agar containing selectable markers for plasmid DNA and plates were grown at 37 °C overnight. Transformants were confirmed to have taken up plasmid DNA by colony PCR analysis.

## **2.22: Plasmid Extraction**

For high copy plasmids, 1 mL of overnight culture was centrifuged at 13 000 rpm in a microcentrifuge tube to pellet cells (for low copy plasmids, 5-10 mL was pelleted). Cell pellets were resuspended in 100 µl of resuspension buffer (1% ethylene glycol, 1mM sodium azide, 10mM Tris-hydrochloride pH 8). One hundred and fifty microliters of cell lysis buffer (2% SDS, 0.15M sodium hydroxide, .025M sodium iodate, 10mM EDTA) was added and mixture was incubated for five minutes at room temperature. Two hundred microliters of acid iodide precipitate solution (2.5M cesium chloride, 0.25M tataric acid, 0.25M sodium iodide) was added and the microcentrifuge tube was vortexed thoroughly. The mixture was centrifuged at 13 000 rpm for one minute and supernatant was collected and transferred to a fresh microcentrifuge tube. Three hundred microliters of PEG-ethanolamine plasmid precipitation solution (37% polyethylene glycol, 4M monoethanolamine) was added to the supernatant and vortexed thoroughly. The solution was then incubated five-minutes at room temperature or 30 minutes on ice. The mixture was centrifuged at 13 000 rpm for five minutes to pellet plasmid DNA and supernatant was removed. Plasmid DNA was washed with 80% isopropanol three times, centrifuging

at 13 000 rpm after each wash. Plasmid DNA was allowed to air dry on the bench top for 45 minutes to one hour and then resuspended in 20-50  $\mu$ l of distilled water for use.

### **2.23: DNA gel electrophoresis**

One percent agarose gels were used throughout this study. One to two microliters of DNA intercalating agent, ethidium bromide (BioShop catalogue number 4M36777), was added to 70 mL of 1% agarose solution (Bioshop catalogue number 5C37890) that had been boiled and then cooled to 60°C. Ten microliters of sample was loaded into wells and separated at 100V for 30-45 minutes. DNA ladder (1KB) purchased from New England Biolabs (catalogue number 0810906) was run alongside all samples. A Kodak imager was used to visualize all DNA gels with 30-45 second exposure time (Cat # 864 2985).

### **2.24: DC Protein Assay (Lowry Assay)**

Protein concentrations from lysed JAWSII cells were normalized using the BioRad DC Protein Assay kit (cat # 500-0116) prior to western blotting. This colorimetric assay was done in 96-well format and each infection condition was measured in triplicate. Five two-fold dilutions of protein standard (Bovine Serum Albumin, BSA 3mg/mL) were used to generate a standard curve using Excel software. Five microliters of standard and samples was added (in triplicate) to a 96-well plate. Twenty-five microliters of reagent A' (A: alkaline copper tartrate solution + S: 5-10% SDS solution) was added to each well of the microplate, followed by 200  $\mu$ l of reagent B (folin reagent). The microplate was placed in

an Eon spectrophotometer set to agitate the plate, incubated for 15 minutes, and read absorbance of sample from each well at 750nm. Standard/sample free controls were used to eliminate any background fluorescence. Protein concentrations were quantified using the standard curve generated in Excel and were standardized for each infection condition so that equal concentrations of protein would be loaded into a protein gel for western blot analysis (generally 25-40  $\mu$ g).

### **2.25: Statistical Analyses**

All statistical analyses were run through Microsoft Excel and Graphpad Prism. When comparing only one condition to a control T-tests were used; when comparing multiple conditions unpaired one-way ANOVAs were performed through Graphpad. Significance is denoted graphically using asterisks where “\*” =  $p < 0.05$ , “\*\*” =  $p < 0.01$ , “\*\*\*” =  $p < 0.001$ , and “\*\*\*\*” =  $p < 0.0001$ .

## **Chapter 3: Results**

### **3.1: Wild-type *Shigella flexneri* kill dendritic cells in a T3SS dependent manner**

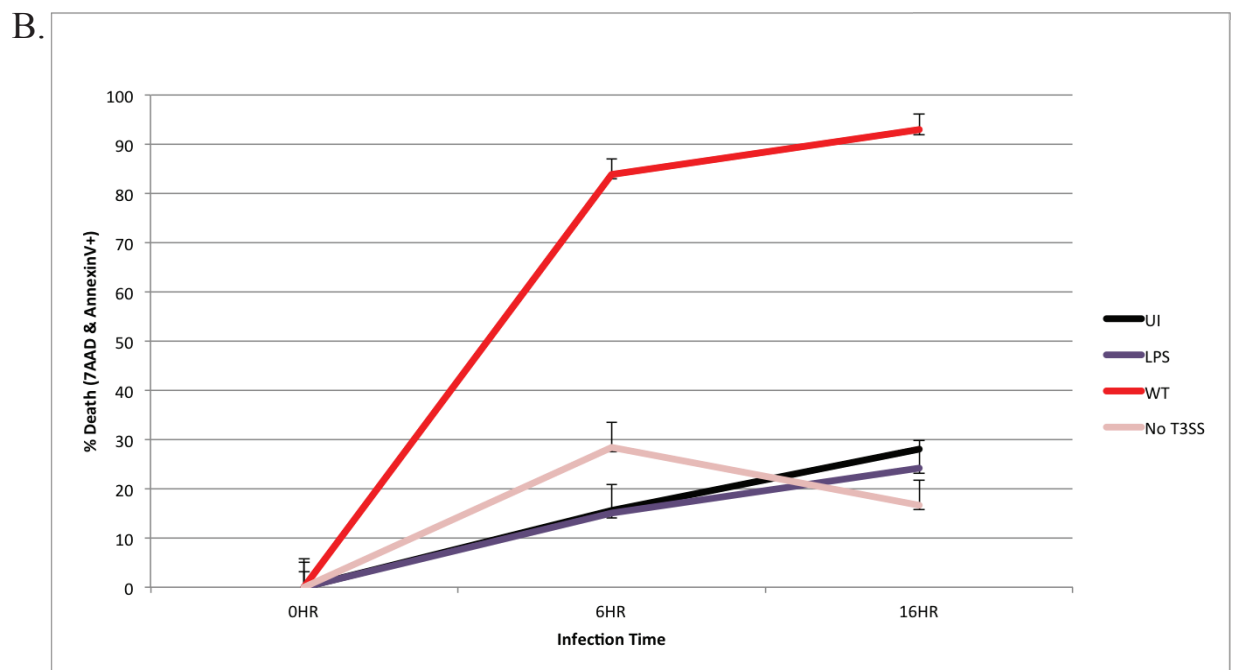
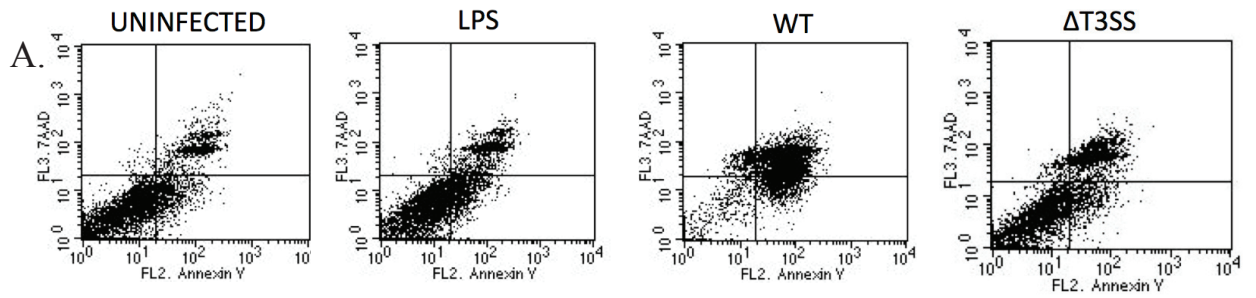
#### **3.1.1: Bone marrow derived dendritic cells die upon infection with Wild-type *Shigella flexneri***

Consequences of *Shigella*- dendritic cell (DC) interaction are largely unexplored. The role of specific *Shigella* T3SS effectors has only recently been examined (Edgeworth et al., 2002; Kim et al., 2008). I used murine bone marrow-derived dendritic cells to examine induction of cell death upon exposure to Wild-type *Shigella* and compared this to the response of the same cells to a *Shigella* T3SS-deficient mutant. Uninfected dendritic cells were used as a negative control for cell death, and purified LPS was used as an artificial positive control. Bone marrow-derived dendritic cells were isolated from C57/BL6 mice and infected with *Shigella* strains at a multiplicity of infection of 10 for a short (six hour) or long (16 hour) infection time.

Flow cytometry was used to measure *Shigella*-induced cell death over time. *Shigella*-induced death was measured by 7AAD and Annexin V staining, and double positive populations were recorded to measure viability. DCs infected with Wild-type *Shigella* were killed rapidly with an average of 83.9% dying by six hours and 92.9% dying by 16 hours of infection. By contrast, DCs infected with a mutant lacking a structural component of the T3SS (MxiD) were not rapidly killed (Figure 7). The frequency of cell death in uninfected control DCs was comparable to the T3SS mutant having less than 30% cell death 6 or 16 hours post-infection. These data indicate that rapid killing of DCs by *Shigella* is T3SS-dependent.

**Figure 7. *Shigella* kills Bone Marrow Derived Dendritic Cells (BMDCs) in a T3SS-dependent manner.** *Shigella* strains were struck onto TSB agar (tetracycline containing agar to select for mutant strains) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB liquid at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). Bone marrow-derived dendritic cells (BMDCs) were isolated from C57/BL6 mice and infected with *Shigella* strains (Uninfected, UI; lipopolysaccharide: LPS; Wild-type: WT; Type 3 Secretion System deficient:  $\Delta$ T3SS) at a multiplicity of infection of 10 for a short (six hour) or long (16 hour) infection time. A. Representative dot plots illustrating gating strategies used for analysis. B. Time course of infection with *Shigella* strains, as determined by 7AAD AnnexinV staining. Mean values from three independent experiments (n=3) are plotted with error bars representing standard deviation.





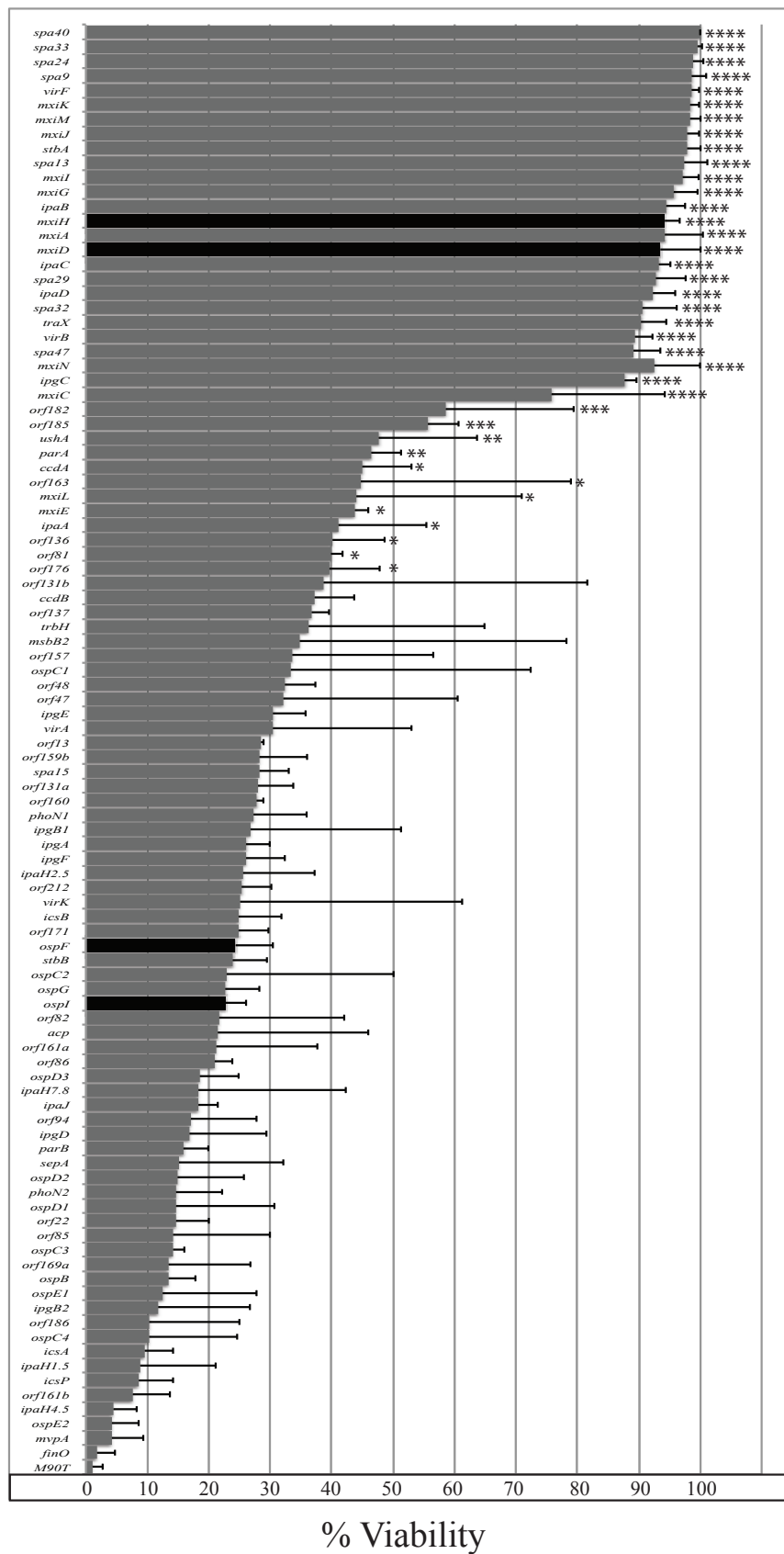
### **3.1.2: Dendritic cell line JAWSII cells display variable death phenotypes upon infection with *Shigella* deletion collection mutant strains**

To identify mechanisms of the rapid killing of DCs by *Shigella*, I screened a collection of 99 mutants of the pWR100 virulence plasmid (Sidik et al., 2014). Figure 8 illustrates the ability of various mutants to induce DC death three hours post-infection. All infections were carried out using a multiplicity of infection of 10, as with BMDC infections. Dendritic cell viability was measured by trypan blue exclusion. Mutants lacking structural components of the T3SS or one of the key regulators of secretion (transcriptional activators, chaperones etc.) did not kill JAWSII cells and were classified as mediating a “low” death phenotype (Figure 8). The majority of deletion collection mutants display an impaired ability to rapidly induce DC death, having an “intermediate” death phenotype (Figure 8). The function of many of these genes/proteins are unknown but many have been implicated in various stages of pathogenesis, primarily in non-phagocytic cell types (Philapon & Sansonetti, 2007). Some deletion collection mutants did not show an impaired ability to kill DCs and, thus, the deleted genes of interest were assumed to not play a role in DC killing; these mutants were classified as “high” death phenotype (Figure 8). Four mutants from the deletion collection, *ΔmxiD*, *ΔmxiH*, *ΔospF*, and *ΔospI* were chosen for further study (highlighted in black, Figure 8). The *ΔmxiD* and *ΔmxiH*, mutants are representative of the low death phenotype, the *ΔospF* and *ΔospI* mutants are representative of the intermediate death phenotype.

### **3.2: T3SS effectors OspF and OspI impair MAPK signaling in JAWSII dendritic cells**

**Figure 8. *Shigella* deletion collection mutants display drastic differences in their ability to kill JAWII cells three hours post-infection.** *Shigella* deletion collection strains were struck onto tetracycline-containing TSB agar and grown overnight at 37°C. A single colony from each strain was then grown in nutrient rich TSB at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). The JAWSII dendritic cell line was infected at a multiplicity of infection of 10 for three hours with each mutant from the *Shigella* deletion collection and cell viability was measured using trypan blue exclusion. Results are representative of three independent experiments. One-way ANOVA comparing mutants to Wild-type (M90T) was performed to obtain statistical significance. Bars represent standard deviation and asterisks represent significance (\*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001). Black bars represent mutants of interest chosen for further study.

Deletion Mutant



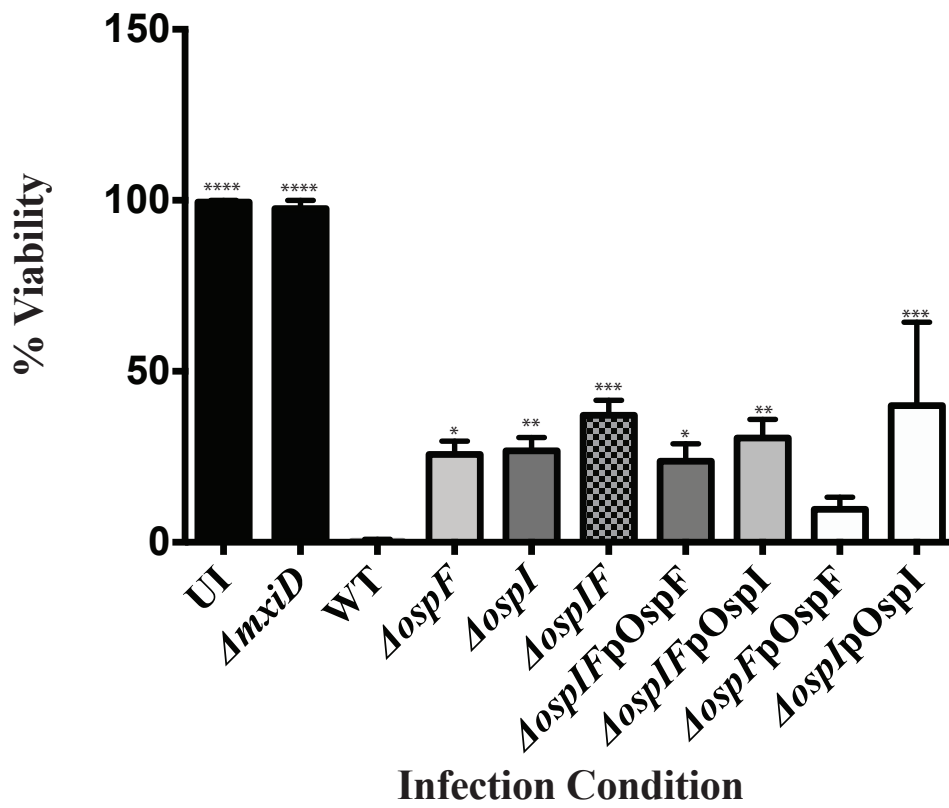
### **3.2.1: Infection with *Shigella* mutant lacking both *ospF* and *ospI* displays an increase in JAWSII viability compared to single mutant infected JAWSII cells**

Screening the *Shigella* deletion collection for mutants with an impaired ability to kill DCs revealed that Outer *Shigella* Proteins F and I (OspF and OspI) play a role in inducing cell death in JAWSII cells (Figure 8). The degree of impairment was not considered statistically significant, as dendritic cell viability in these conditions was approximately 25%. Both of these effector proteins have been implicated in immune signaling impairment: therefore, I hypothesized that a double mutant lacking both OspF and OspI would further increase viability of JAWSII infected cells. Three hour infections with single mutants  $\Delta ospI$  and  $\Delta ospF$  consistently resulted in approximately 25% viability (Figure 9). Infection with  $\Delta ospFospI$  double mutant revealed a further increase in viability to approximately 35% (Figure 9). Complementing the  $\Delta ospFospI$  double mutant with either OspI or OspF resulted in a death phenotype comparable to that of the single mutants. Complementation of the single mutants resulted in a phenotype comparable to Wild-type for OspF, however complementation efforts for  $\Delta ospI$  resulted in an increased viability of approximately 30% (Figure 9). Uninfected (UI) control as well as a T3SS deficient control ( $\Delta mxiD$ ) were used as controls throughout these experiments and display approximately 100% viability consistently.

### **3.2.2: OspF and OspI target MAPK signaling pathways to impair immune response in JAWSII cells 30 minutes post infection**

OspF irreversibly removes the activating phosphate from MAPKs in various cell types (Li et al., 2007). Kim et al. (2008) used human peripheral blood derived DCs to demonstrate this phosphothreonine lyase activity 30 minutes post *Shigella* infection. OspI

**Figure 9. JAWSII cells infected with the *Shigella* double mutant lacking both *ospI* and *ospF* displays an increase in viability as compared to single mutant-infected JAWSII cells.** *Shigella* strains were struck onto antibiotic-containing TSB agar (containing tetracycline for single mutants, tetracycline and kanamycin for double mutant) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB supplemented with antibiotic at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). Trypan blue exclusion was used to measure JAWSII dendritic cell viability three hours post-infection with *Shigella* mutants of interest. Uninfected JAWSII (UI) cells and T3SS mutant strain (*ΔmxiD*)-infected JAWSII were used as negative controls and display full viability. Results are representative of three independent experiments. An unpaired T-test was used to compare viability between mutant infected JAWSII cells and Wild-type (WT) infected JAWSII cells. Error bars represent standard deviation and asterisks represent significance (\*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001).



has been shown to inactivate the ubiquitin conjugating enzyme Ubc13, resulting in decreased NF $\kappa$ B signaling (Sanada et al., 2012). I tested if  $\Delta ospF$  and  $\Delta ospI$  mutants altered levels of phosphorylated MAPK in JAWSII cells. Uninfected JAWSII cells showed little or no activated MAPKs and was used as a negative control (Figure 11). GAPDH was used as a loading control and all quantification was measured as a ratio of MAPK to GAPDH using ImageJ software to quantify band intensities. Infection of JAWSII cells with an  $\Delta ospF$  mutant resulted in an increased amount of phosphorylated MAPK compared to that seen in Wild-type infected cells (Figure 10). Infection of JAWSII cells with an  $\Delta ospI$  mutant also resulted in an increased amount of phosphorylated MAPK as assessed by western blot analysis (Figure 11).  $\Delta ospF$  and  $\Delta ospI$  mutants displayed increased band intensities for activated phosphorylated Erk and p38 compared to proteins visualized from Wild-type infected JAWSII cells 30 minutes post infection (Figure 11).

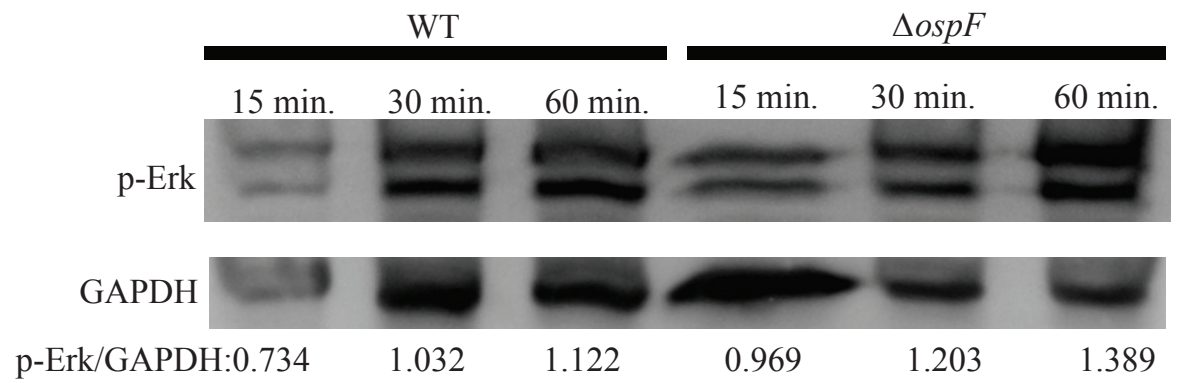
Complementation efforts partially restored the levels of phosphorylated MAPK in JAWSII cells infected with the  $\Delta ospF$  to levels comparable with that of Wild-type infected cells (Figure 11). Curiously, attempts to complement the  $\Delta ospI$  mutant (using an identical strategy to that employed for complementation of the  $\Delta ospF$  mutant) resulted in a further increase in MAPK phosphorylation (Figure 11). Taken together, my results indicate that OspF and OspI act on multiple MAPK signaling programs to down regulate their activity.

### **3.2.3: OspF and OspI target MAPK signaling independent of one another and by different mechanisms**

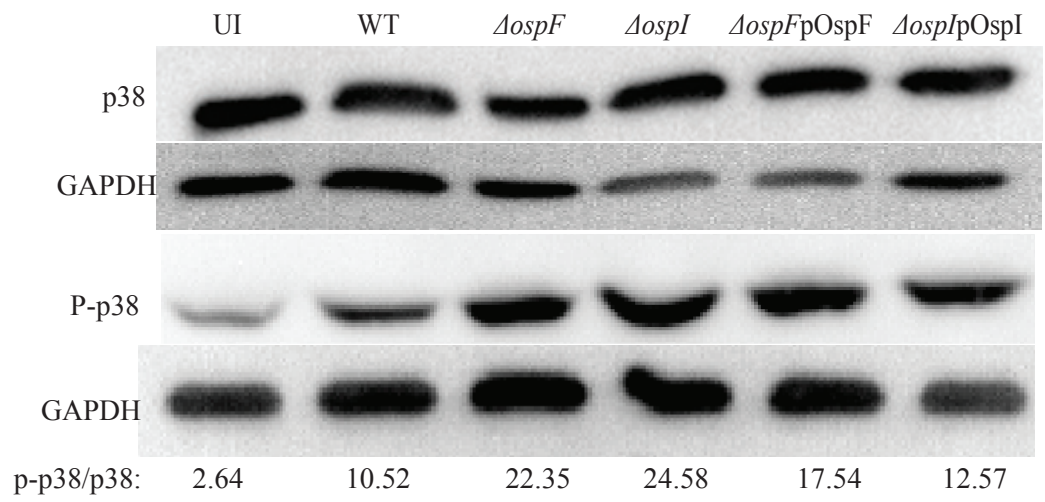
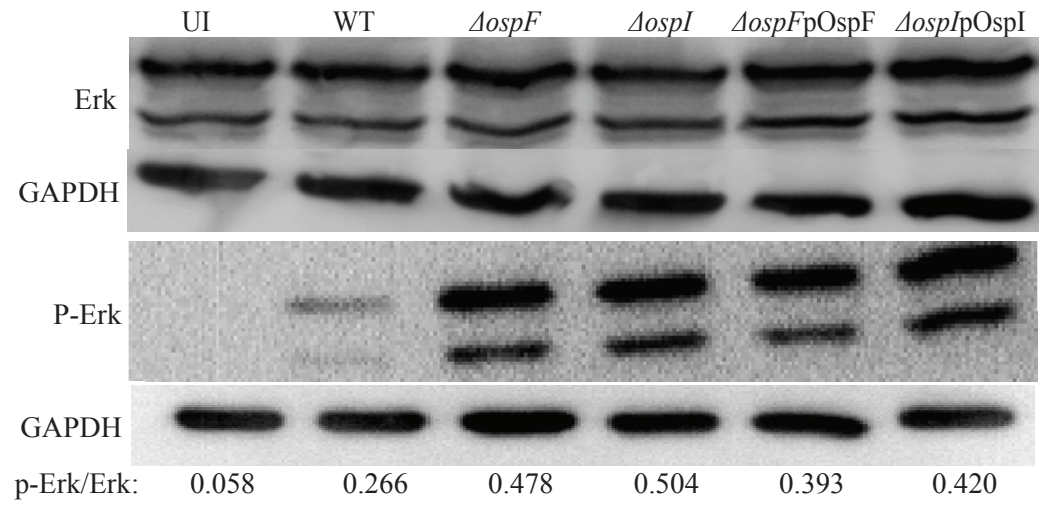


**Figure 10. *ΔospF* infected JAWSII cells display an increase in p-Erk activation**

**compared to Wild-type infected cells 30 minutes post-infection.** *Shigella* strains were struck onto TSB agar (tetracycline containing to select for *ΔospF*) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB supplemented with antibiotic at 37°C overnight. Cultures were diluted grown to log phase (OD<sub>600</sub> between 0.4-0.6). JAWSII dendritic cells were infected with either Wild-type (WT) *Shigella* or a mutant deleted for *ospF* for 15, 30 or 60 minutes. Cells were collected and harvested in Laemmli buffer, boiled for 5 minutes and protein concentration was determined using a DC protein assay. Equal amounts of protein for each sample were separated by migration through 10% polyacrylamide gels. Immunoblotting was performed using primary rabbit antibody against phosphorylated Erk. Quantification of western blots was performed through ImageJ software and a ratio of activated MAPK to total MAPK band intensity is listed.



**Figure 11. *Shigella* deletion collection mutants  $\Delta ospF$  and  $\Delta ospI$  target Erk and p38 MAPK signaling in JAWSII dendritic cells.** *Shigella* strains were struck onto TSB agar (tetracycline containing to select for deletion mutants) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB supplemented with antibiotic at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). JAWSII dendritic cells were infected with Wild-type (WT),  $\Delta ospF$  mutant,  $\Delta ospI$  mutant and mutant-complemented strains,  $\Delta ospFpOspF$  and  $\Delta ospIpOspI$  for 30 minutes. Cells were collected and harvested in Laemmli buffer, boiled for 5 minutes and protein concentration was determined using a DC protein assay. Equal amounts of protein for each sample were separated by migration through 10% polyacrylamide gels. Immunoblotting was performed using primary rabbit antibodies directed against phosphorylated Erk (p-Erk) and p38 (p-p38) as well as total Erk and p38. Quantification of western blots was performed through ImageJ software and a ratio of activated MAPK to total MAPK band intensity is listed.

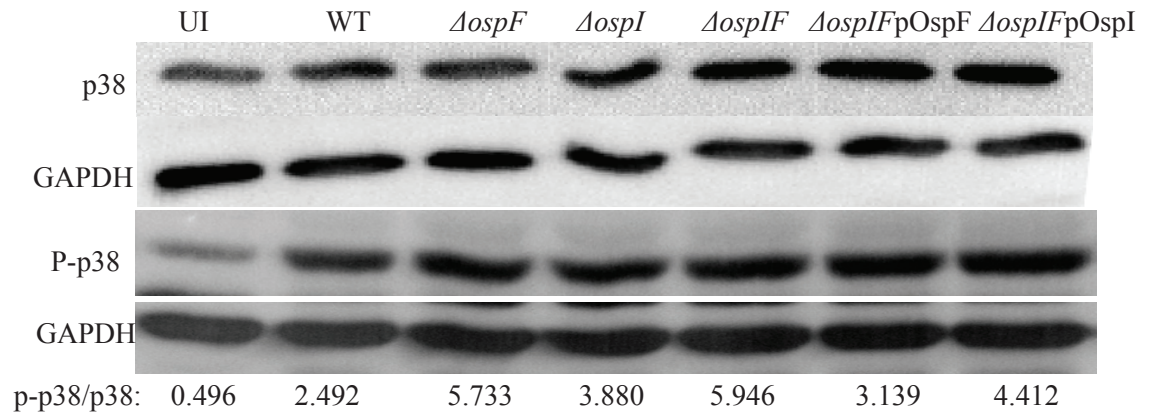
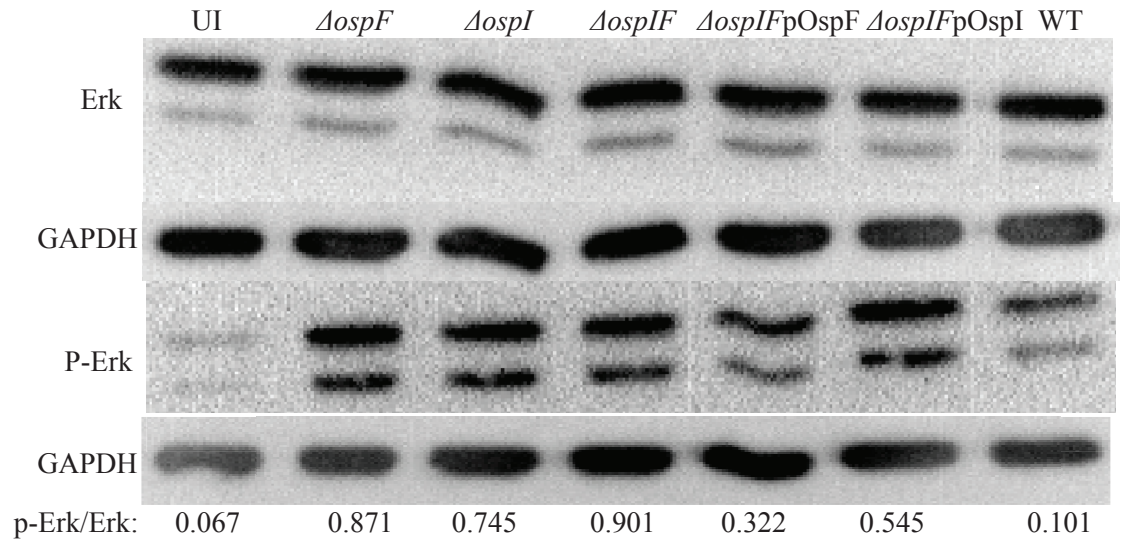


In finding that OspF and OspI both target MAPK signaling in JAWSII dendritic cells and a double mutant deleted for OspI and OspF displays increased JAWSII cell viability in the context of infection, I hypothesized that an increase in MAPK activation would be observed following infection with the *ΔospIF* double mutant. JAWSII were infected at a multiplicity of infection of 10 with Wild-type (WT), *ΔospF* deletion mutant, *ΔospI* deletion mutant and complemented strains *ΔospFpOspF* and *ΔospIpOspI*, double mutant *ΔospIF*, and complemented strains *ΔospIFpOspF* and *ΔospIFpOspI* for 30 minutes. Infected JAWSII cells were harvested and lysates were collected. Western blots were performed using antibodies against MAPKs Erk and p38 as well as their activated forms p-Erk and p-p38 (Figure 12). GAPDH was used as a loading control throughout and protein levels were normalized by performing a Lowry assay as described in M&M Section 2.24.

ImageJ analysis revealed that JAWSII cells infected with double deletion mutant *ΔospIF* display an increase in phosphorylated MAPKs Erk and p38 compared to JAWSII cells infected with strains mutant for *ΔospF* or *ΔospI* alone (Figure 12). Complementation of the double mutant with a plasmid harboring *ospF* partially restored protein levels to that of the single *ΔospI* and *ΔospF* mutant. Uninfected JAWSII cells were used as a negative control and display low levels of activated MAPKS. All infection conditions display relatively equal levels of total MAPK and ratios in Figure 6 represent levels of activated (phosphorylated) MAPK to total MAPK after being normalized to the GAPDH loading control.

### **3.3: *Saccharomyces cerevisiae* expressing OspI or OspF inhibit MAPK signaling through the pheromone response pathway**

**Figure 12. *Shigella* T3SS effectors OspF and OspI down-regulate MAPK signaling in JAWSII cells through the Erk and p38 innate immune signaling pathways, independent of one another.** *Shigella* strains were struck onto antibiotic-containing TSB agar (tetracycline for single mutants, tetracycline and kanamycin for double mutant) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB supplemented with antibiotic at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). JAWSII dendritic cells were infected with Wild-type (WT),  $\Delta ospF$  deletion mutant,  $\Delta ospI$  deletion mutant and complemented strains  $\Delta ospFpOspF$  and  $\Delta ospIpOspI$ , double mutant  $\Delta ospIF$ , and complemented strains  $\Delta ospIFpOspF$  and  $\Delta ospIFpOspI$  for 30 minutes. Cells were collected and harvested in Laemmli buffer, boiled for 5 minutes and protein concentration was determined using a DC protein assay. Equal amounts of protein for each sample were separated by migration through 10% polyacrylamide gels. Immunoblotting was performed using primary rabbit antibodies directed against phosphorylated Erk and p38 as well as total Erk and p38. Quantification of western blots was performed through ImageJ software and a ratio of activated MAPK to total MAPK band intensity is listed.



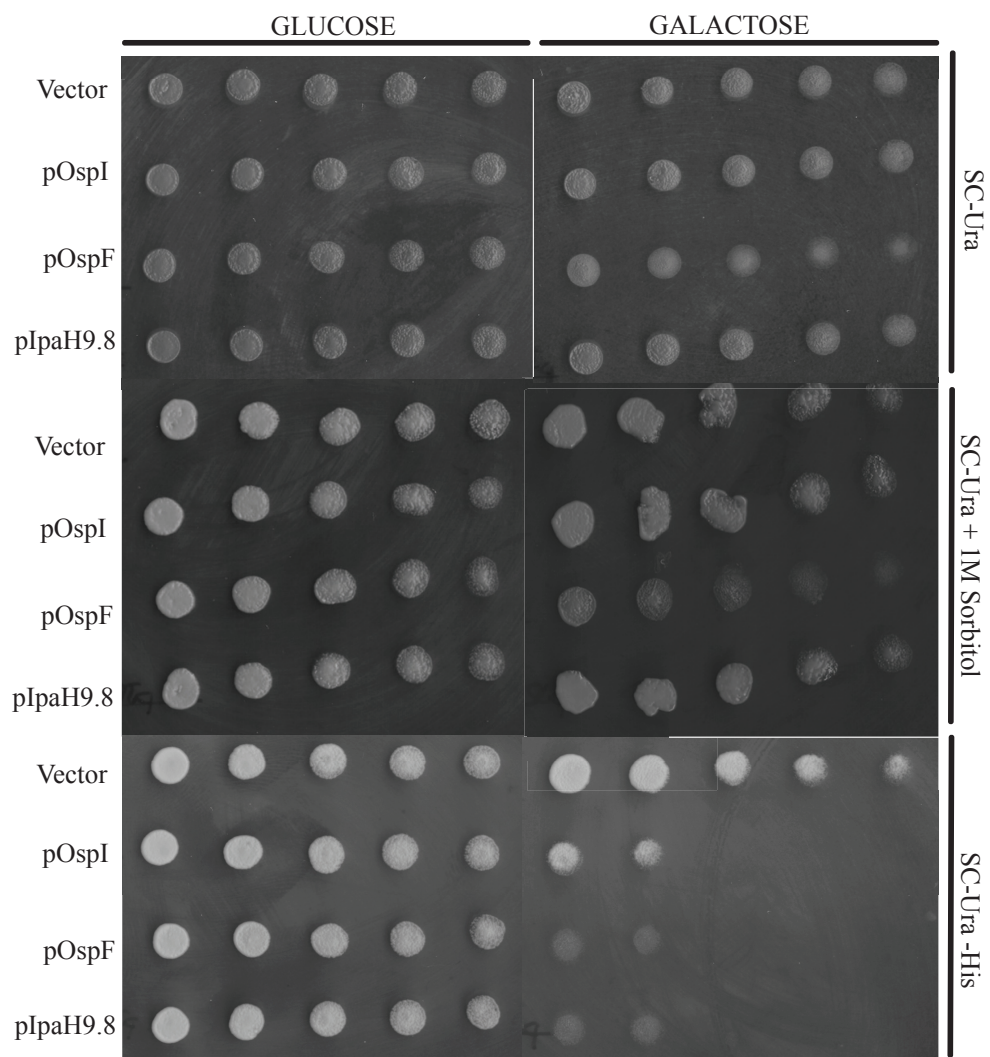
### **3.3.1: OspI expressed in *Saccharomyces cerevisiae* strain SY2625 inhibits MAPK signaling through the pheromone response pathway**

Yeast MAPK signaling pathways have been well characterized and are now exploited for the purpose of determining mechanisms of bacterial interference (Rohde et al., 2007). The robust phenotypes associated with MAPK signaling in yeast can be used to identify proteins that inhibit MAPK function using a highly conserved mechanism. In this study I use two different approaches to investigate potential mechanisms by which OspI might impair MAPK signaling.

*Saccharomyces cerevisiae* strain SY2625 harboring a *FUS3-HIS1* reporter is dependent on the pheromone response pathway for growth on medium lacking the amino acid histidine because Fus3 is a transcriptional activator of the pheromone response (Evangelista et al., 1997). Strain SY2625 was transformed with empty vector pAO1, pAOspI, pYESOspF, or pJR001IpaH9.8. Each of these vectors use the GAL1-10 promoter that is strongly repressed during growth on medium containing glucose and strongly induced during growth on medium containing galactose in order to control effector expression. Strain SY2625 transformed with pAO1 was used as a negative control while SY2625 transformed with pJR001IpaH9.8 was used as a positive control as it has been reported to dampen MAPK signaling through the pheromone response pathway by targeting the MAPKK Ste7 (Rohde et al., 2007). The experimental cultures were strain SY2625 transformed with pAOspI or pYESOspF. Cultures were grown in liquid media overnight at 30°C in nutrient rich broth and serially diluted onto selective media lacking uracil and histidine, and supplemented with either glucose or galactose as the sole source of carbon. All four transformed strains were His<sup>+</sup> when grown on



**Figure 13. *Saccharomyces cerevisiae* strain SY2625 expressing *ospI* or *ospF* inhibit MAPK signaling through the pheromone response pathway.** Serial dilutions of transformed SY2625 strains harboring *FUS1-HIS3* reporter and expressing *Shigella* effectors as indicated. Growth was measured on glucose (non-inducible condition) or galactose (inducible condition) 24 hours post incubation at 30° C. Transformed yeast strains grown on: synthetic defined medium lacking uracil (Top panels, control); synthetic defined medium lacking uracil and supplemented with 1M sorbitol (middle panels); synthetic defined medium lacking both uracil and histidine (bottom panels). Results are representative of three independent experiments.



glucose-containing medium, and all but the empty vector control were His<sup>-</sup> when grown on galactose containing medium (Figure 13). This indicates that expression of both *ospI* and *ospF* inhibits signaling through the pheromone response pathway in yeast.

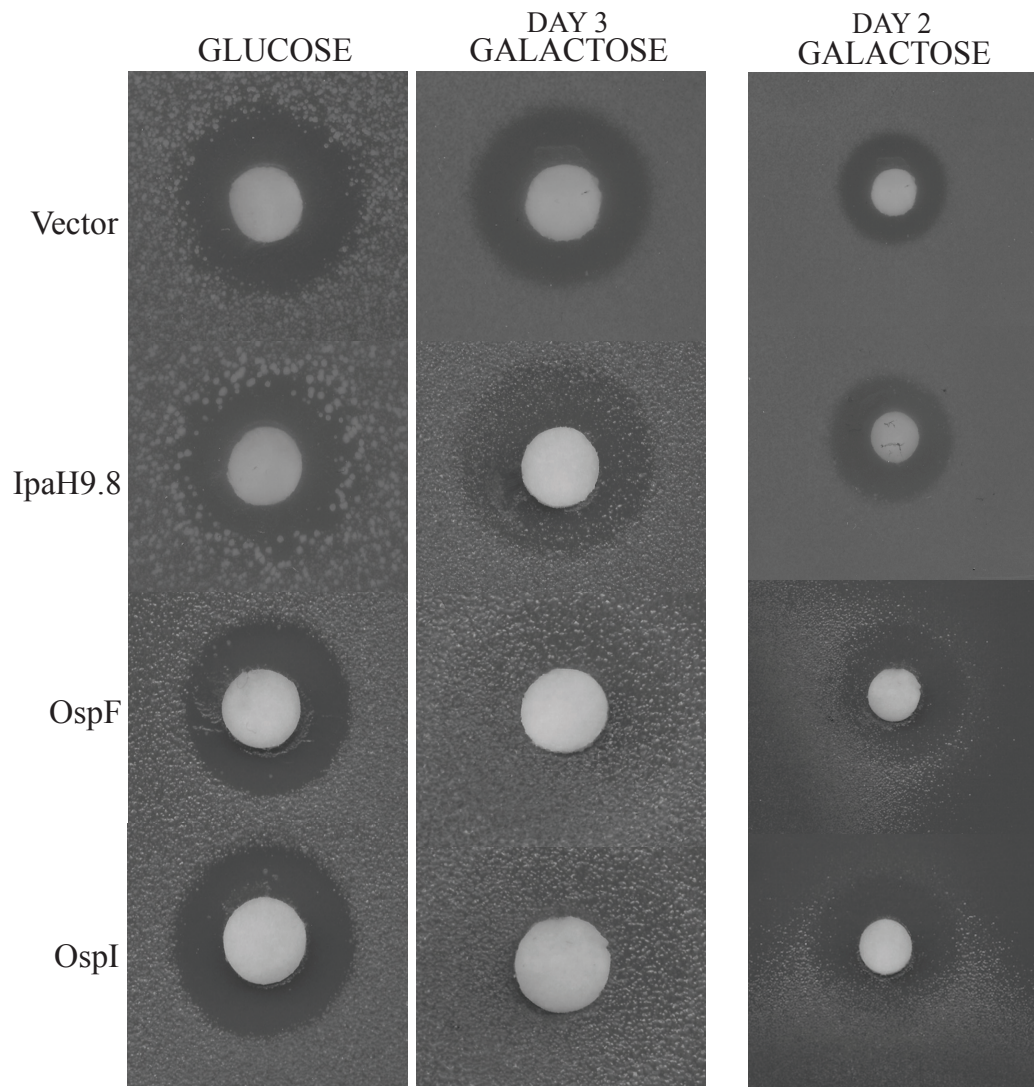
To ensure that expression of the *Shigella* effectors alone does not inhibit yeast cell growth, cells were inoculated onto synthetic defined medium lacking uracil only. All transformed strains of SY2625 grew equally well when supplemented with either glucose or galactose (Figure 13). These data indicate that expression of the *Shigella* effectors alone does not inhibit growth of transformed SY2625 cells.

To test if multiple yeast MAPK pathways were altered by expression of OspF or OspI I examined the ability of yeast to grow on medium containing 1M sorbitol (high osmolarity growth condition). Growth in high osmolarity conditions requires a functional HOG MAPK signaling cascade, and cross-talk between the pheromone response and the HOG pathway has been identified (O'Rourke & Herskowitz, 2004). Although some components of the pheromone pathway and the HOG pathway are shared, I predicted that the pheromone response alone would be affected. Transformed strains (same as above) were serially diluted onto synthetic defined media lacking uracil and supplemented with 1M sorbitol (as well as glucose or galactose) to induce the osmotic shock MAPK pathway. All transformed strains grew equally well on glucose containing medium supplemented with sorbitol and only OspF-expressing SY2625 displayed a limited growth phenotype on galactose-containing medium (Figure 13). These results indicate that expression of OspF interferes with MAPK signaling in both the pheromone and osmotic shock response whereas OspI and IpaH9.8 inhibition is specific to the pheromone response.

### 3.3.2: *Saccharomyces cerevisiae* strain BY4741 expressing OspI or OspF display MAPK inhibition through the pheromone response pathway

The *S. cerevisiae* mating pheromone  $\alpha$ -factor induces cell cycle arrest via the pheromone response pathway in *MATa* yeast strains (Hoffman et al., 2002). Upon exposure to a paper disk impregnated with  $\alpha$ -factor, a lawn of proliferating Wild-type *MATa* yeast display a clear zone of inhibition where cell cycle arrest has occurred (Hoffman et al., 2002). The *MATa* strain BY4741 was transformed with empty vector pAO1, pAOspI, pYESOspF, or pJR001IpaH9.8 to test the hypothesis that *Shigella* proteins OspF and OspI inhibit MAPK signaling through the pheromone response pathway. IpaH9.8 was used as a positive control as it is known to inhibit the pheromone response pathway by targeting the MAPKK Ste7 for degradation by the proteasome (Rohde et al., 2007). In all cases, a clear halo was observed for all yeast when grown on glucose-containing medium (Figure 14). A clear halo was also observed for yeast harboring an empty vector when grown on galactose-containing medium (Figure 14). For reasons that I cannot explain, yeast expressing IpaH9.8 also exhibited a clear halo in response to alpha factor when grown on galactose medium. This result is in discord with the published results that have demonstrated that IpaH9.8 interrupts the pheromone response pathway (Rohde et al. 2007) (Figure 14). For yeast harboring the OspF plasmid, a partial double halo was observed after two days of incubation and no halo was observed by the third day (Figure 14). A similar result was observed for OspI expressing yeast: a partial double halo with some growth on day two, and no halo observed by day three (Figure 14).

**Figure 14. *Shigella* T3SS effectors OspF and OspI inhibit MAPK signaling through the pheromone response pathway in *Saccharomyces cerevisiae* strain BY4741.** The *MATa* strain BY4741 was transformed with empty vector pAO1, pAOspI, pYESOspF, or pJR001IpaH9.8. Each transformed strain was grown in a 1% agarose overlay on selective medium supplemented with either glucose (left panels) or galactose (mid and right panels) and exposed to a paper disk impregnated with 1mg/mL  $\alpha$ -factor. Results are representative of three independent experiments.



### **3.4: OspI expressing *Saccharomyces cerevisiae* display impaired ability to recover from UV induced DNA damage**

#### **3.4.1: Ubc13 is involved in the DNA repair pathway in *Saccharomyces cerevisiae***

The E2 ubiquitin conjugating enzyme Ubc13 is critical for formation of K63 linked polyubiquitination chains required for DNA repair in *Saccharomyces cerevisiae* (Hofmann & Pickart, 1999). A *ubc13::kanMX* mutant (obtained from the EGAD CORES Facility, Dalhousie University) was compared to Wild-type BY4741 for its ability to recover from exposure to 3000  $\mu\text{J}/\text{cm}^3$  UV radiation. Without exposure to UV radiation Wild-type BY4741 and the  $\Delta\textit{ubc13}$  mutant grew equally well on nutrient rich YEPD medium (Figure 15). Wild-type BY4741 exposed to UV radiation grew as well as the no UV control. By contrast, the  $\Delta\textit{ubc13}$  strain displayed an impaired growth phenotype compared to its no UV control (Figure 15). This result confirms that Ubc13 is indeed involved in the DNA damage response in yeast.

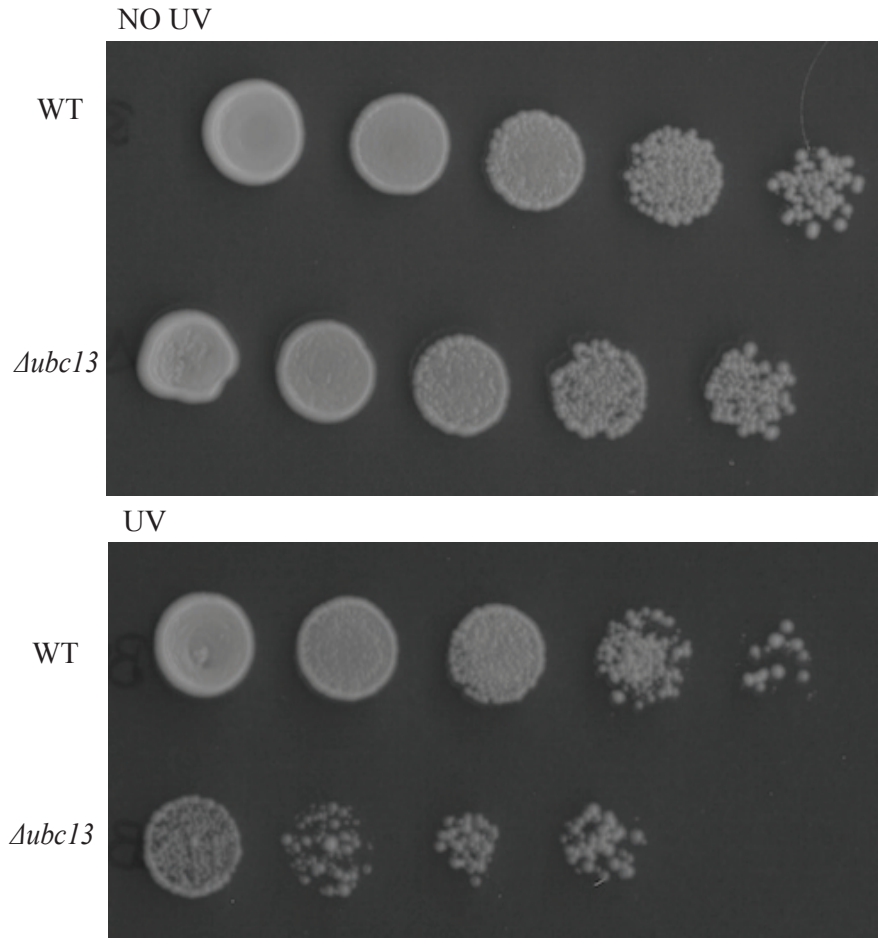
#### **3.4.2: OspI expressing *Saccharomyces cerevisiae* display an impaired ability to recover from UV radiation**

In mammalian systems OspI has been shown to target Ubc13 as a means of dampening immune signaling through the NF $\kappa$ B pathway (Senada et al., 2012). To investigate whether Ubc13 is also targeted by OspI in yeast, inhibition of the DNA repair pathway was assessed. Without exposure to UV radiation, Wild-type strain BY4741 harboring empty vector or pAOspI grow equally well on selective media containing glucose or galactose (Figure 16). Following exposure to UV radiation, vector-containing

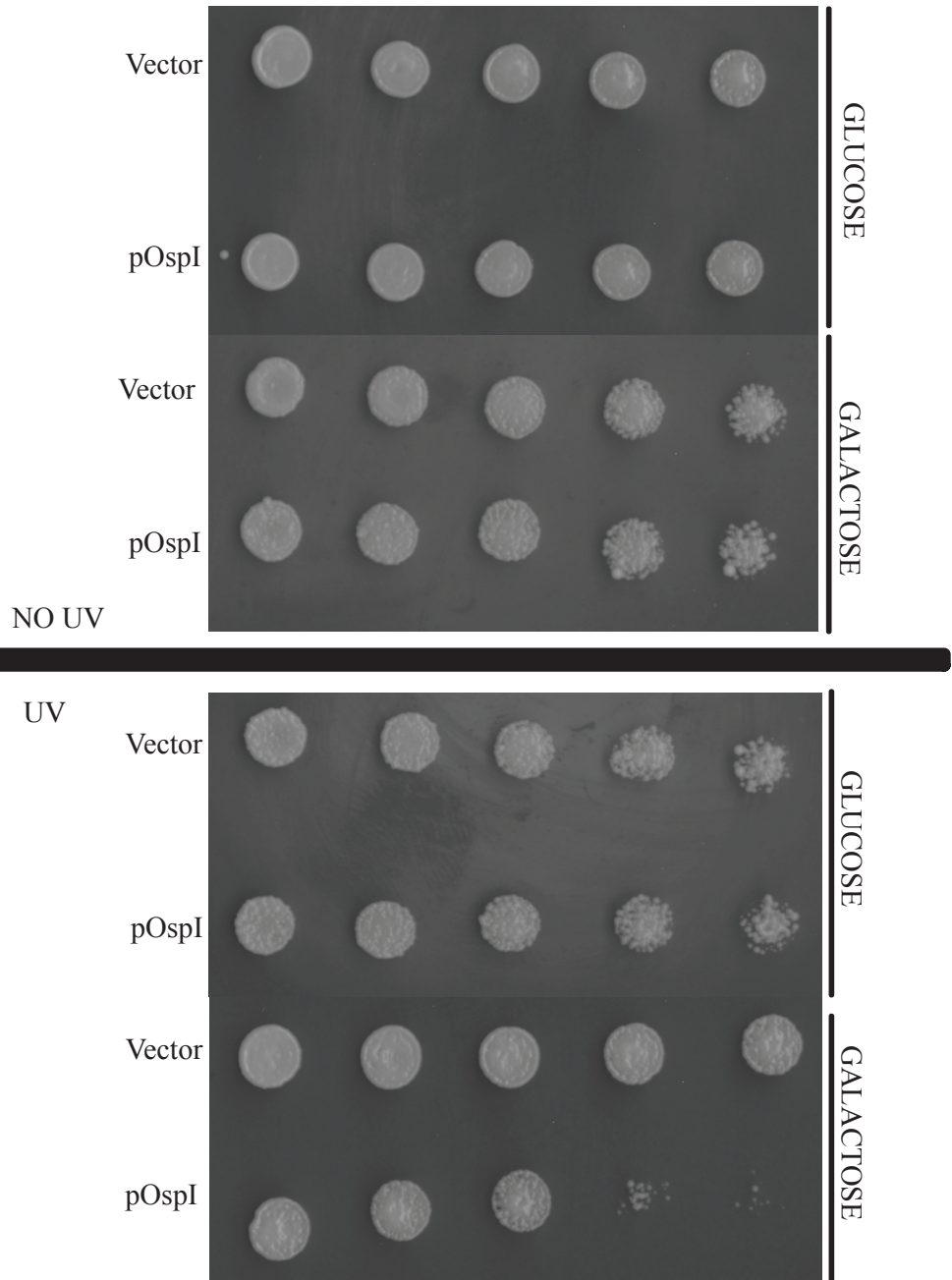
**Figure 15. Ubc13 is involved in the DNA repair pathway in *Saccharomyces***

*cerevisiae*. Top panels: BY4741 Wild-type (WT) strain of *S. cerevisiae* compared to mutant strain deleted for Ubc13 (E2 ubiquitin conjugating enzyme;  $\Delta ubc13$ ) without UV exposure. Bottom panels: Upon exposure to 3000 uJ/cm<sup>3</sup> of UV radiation WT BY4741 employs a DNA repair pathway to overcome UV induced damage. Ubc13 mutant strain recovers from UV damage to a lesser degree than WT. Serial dilutions of each yeast strain were plated on YEPD prior to UV radiation using a DNA Stratalinker and plates were incubated for 24 hours to recover at 30°C. Results are representative of at least three independent experiments.





**Figure 16. *ospI* expressing *Saccharomyces cerevisiae* cells display impaired ability to recover from UV radiation.** *S. cerevisiae* strain BY4741 was transformed with pAO1 empty vector or pAO*ospI* expressing *ospI* under a galactose inducible promoter. Serial dilutions of each yeast strain were plated on selective media supplemented with either glucose or galactose and allowed 48-72 hours to recover at 30°C post-UV exposure. Upon exposure to 3000 uJ/cm<sup>3</sup> of UV radiation BY4741 cells harboring empty vector employs the DNA repair pathway to overcome UV induced damage. *OspI*-expressing cells display an impaired growth phenotype compared to cells bearing the empty vector. Results are representative of at least three independent experiments.



BY4741 cells grew comparably to those of the no UV control. Strain BY4741 bearing an OspI expressing plasmid and grown in galactose were more resistant to UV radiation than the same cells grown on glucose (Figure 16). This result suggests that OspI targets Ubc13 activity in yeast.

### **3.5: Ubc13 is involved in the pheromone response pathway in *Saccharomyces cerevisiae***

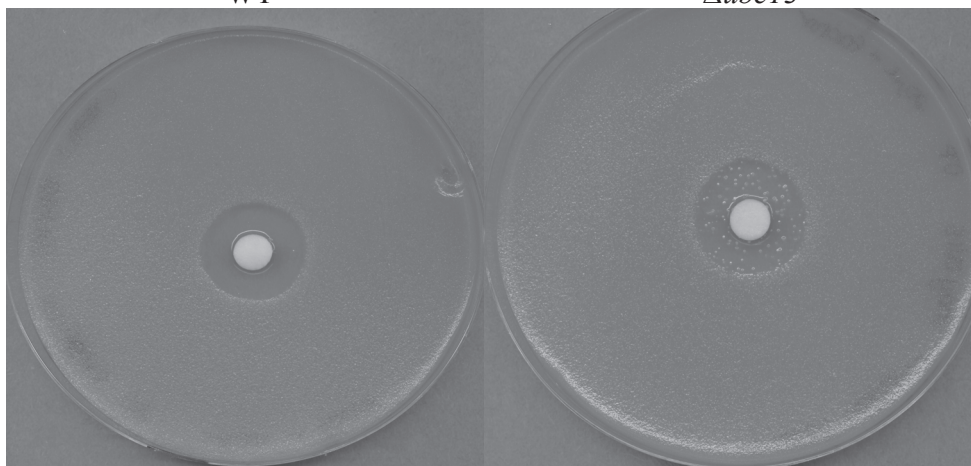
Results from this study indicate that OspI targets the pheromone response pathway in *S. cerevisiae* and may also act through inactivation of *ubc13* akin to its mechanism of action in mammalian systems. I hypothesized that OspI targets Ubc13 as a means of impairing MAPK signaling through the pheromone response pathway, however, Ubc13 has not previously been linked to this signaling cascade. To investigate whether Ubc13 plays a role in the pheromone response pathway I again used the halo assay described in Section 3.3.2 (M&M 2.17). The zone of inhibition, indicative of cell cycle arrest by the mating MAPK pathway, was compared between Wild-type *MATa* strain BY4741 and an Ubc13 mutant ( $\Delta$ *ubc13*). Following exposure to a paper disk impregnated with  $\alpha$ - factor Wild-type BY4741 displays a clear zone of inhibition (Figure 17). Following exposure to a paper disk impregnated with  $\alpha$  mating factor, the  $\Delta$ *ubc13* also displayed a clear halo, however there were a number of colonies growing within the halo (Figure 17). These data indicate a possible role for Ubc13 in the pheromone response pathway.

### **3.6: *Shigella flexneri* induced dendritic cell death is partially dependent on caspase activity**

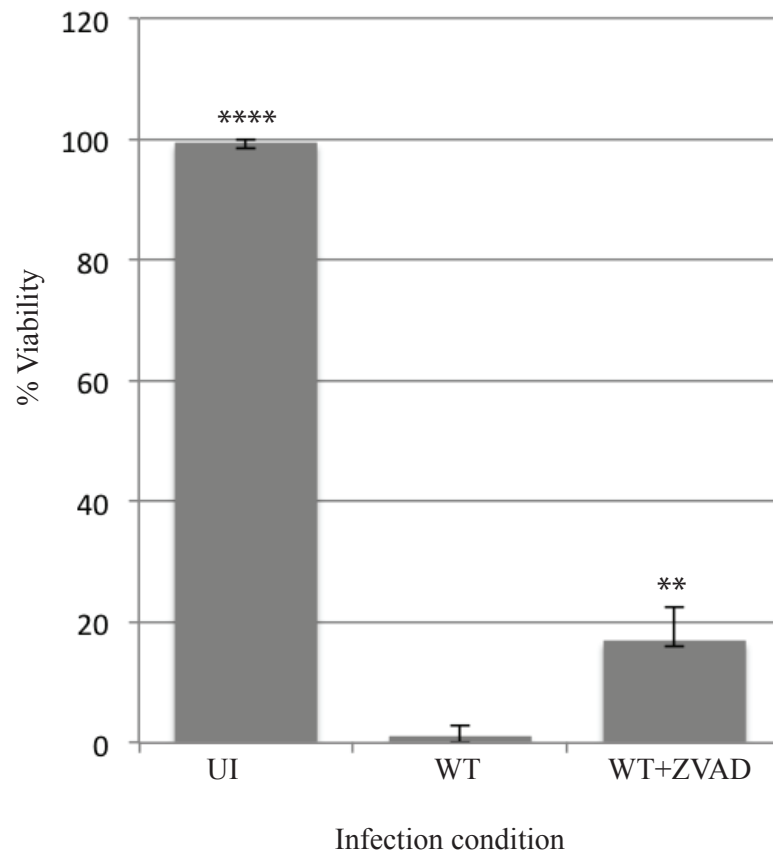
**Figure 17. Ubc13 is involved in MAPK signaling through the pheromone response pathway in *Saccharomyces cerevisiae*.** Wild-type (WT) BY4741 strain of *S. cerevisiae* displays a strong zone of inhibition due to cell cycle arrest induced by exposure to 15 mg/mL of  $\alpha$ -factor. BY4741 *ubc13* $\Delta$ :kanMX strain displays reduced ability to inhibit growth through the pheromone response pathway as visualized by colony formation within the initial zone of inhibition. Images were taken 24 hours after addition of  $\alpha$ -factor. Results are representative of at least three independent experiments.

WT

*Δubc13*



**Figure 18. One hour pretreatment of JAWSII dendritic cells with the pancaspase inhibitor Z-VAD significantly increases cell viability three hours post-*Shigella* infection.** *Shigella* strains were struck onto TSB agar (tetracycline containing to select for  $\Delta mxiD$ ) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB (supplemented with tetracycline for  $\Delta mxiD$ ) at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). JAWSII dendritic cells were exposed to 20µM Z-VAD 1 hour prior to *Shigella* infection. Cell viability following infection of Z-VAD pretreated cells was compared to infected non-pretreated and uninfected JAWSII cells. Results are representative of three independent experiments: mean values are expressed and bars represent standard deviation. Unpaired T-tests were performed to determine statistical significance (\*\*, p<0.01; \*\*\*, p<0.0001).





Evidence exists to support caspase-1 dependent and independent DC death induced by *Shigella* in human monocyte-derived dendritic cells (Edgeworth et al., 2002). I hypothesized that pretreatment of JAWSII dendritic cells with the pancaspase inhibitor Z-VAD would block *Shigella* induced JAWSII cell death. JAWSII cells were infected with Wild-type *Shigella* at an MOI of 10 for three hours with or without Z-VAD exposure. One hour pretreatment with Z-VAD increased viability by approximately 20% three hours post-infection (Figure 18). Uninfected control JAWSII cells were almost completely viable with less than 3% cell death, and JAWSII cells not exposed to Z-VAD but infected with Wild-type *Shigella* had cell viability less than 2% (Figure 18).

### **3.7: Wild-type *Shigella flexneri* targets caspase-1 in JAWSII dendritic cells**

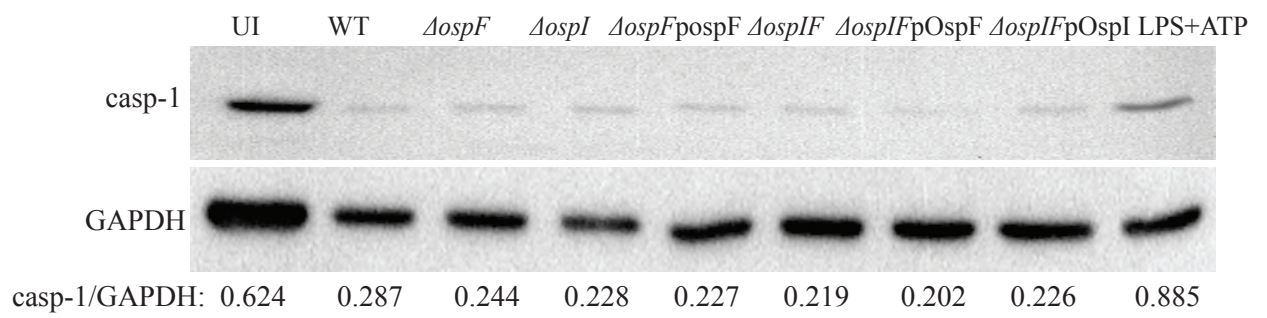
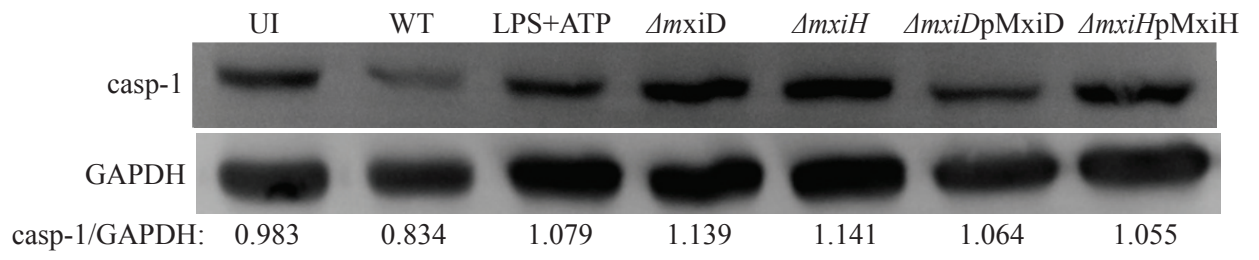
*Shigella* induces pyroptosis in macrophages through activation of caspase-1; this form of programmed cell death is accompanied by release of the proinflammatory cytokine IL-1 $\beta$  (Hilbi et al., 1998). I hypothesized that pyroptotic cell death was occurring in JAWSII dendritic cells three hours post *Shigella* infection and therefore measured caspase-1 and IL-1 $\beta$  levels by western blot.

Immunoblotting for the active p10 subunit of caspase-1 revealed that Wild-type *Shigella* infection decreases caspase-1 expression in JAWSII cells 3 hours post-infection (Figure 19). Results indicate that down-regulation of caspase-1 activation is dependent on the T3SS as cells infected by mutants lacking structural components (MxiD or MxiH) display an increase in the levels of caspase-1 compared to Wild-type *Shigella* infected or uninfected control JAWSII cells. Complementation of  $\Delta$ mxiD or  $\Delta$ mxiH partially restores levels of caspase-1 activity to a value close to that seen in Wild-type *Shigella* infected cells (Figure 19).

**Figure 19. Wild-type *Shigella* targets caspase-1 in JAWSII dendritic cells.** *Shigella*

strains were struck onto nutrient rich TSB agar (containing tetracycline to select for single mutants, tetracycline and kanamycin for double mutant) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB supplemented with antibiotic at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). JAWSII dendritic cells were infected with indicated *Shigella* deletion mutants of interest as well as the complemented strains (MOI=10) for three hours. Cells were collected and harvested in Laemmli buffer, boiled for 5 minutes and protein concentration was determined using a DC protein assay. Equal amounts of protein for each sample were separated by migration through 10% polyacrylamide gels.

Immunoblotting was performed with primary rabbit antibodies directed against the p10 subunit of caspase-1 (casp-1). Purified LPS (0.1mg/mL) and ATP (5mM) were used as a bacteria-free positive control to stimulate inflammasome-mediated pyroptosis. Anti-GAPDH was used to detect the loading control. ImageJ software was used to quantify band intensity and a ratio of casp-1 to GAPDH intensity is listed.

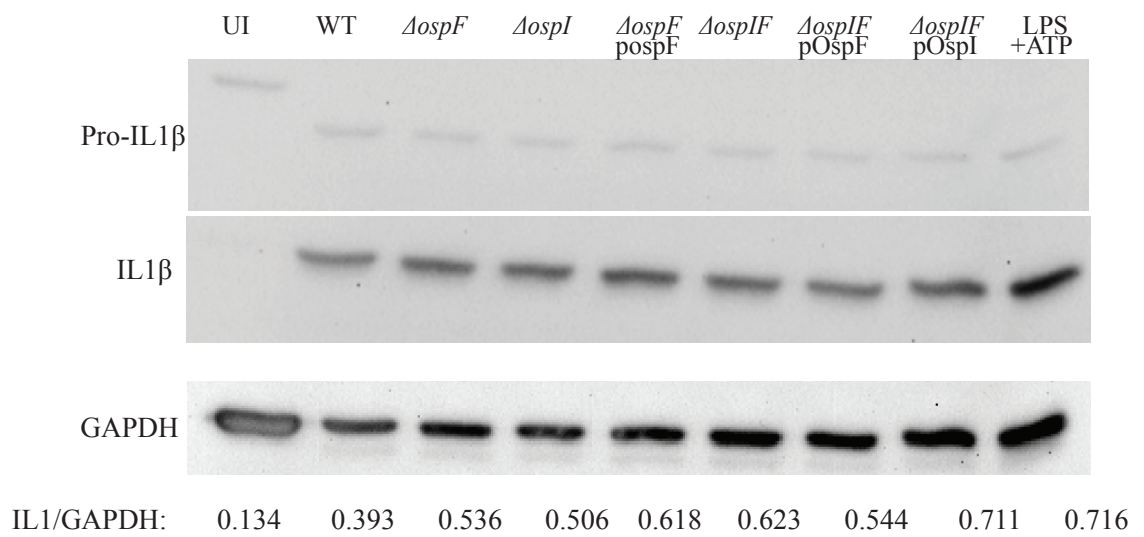
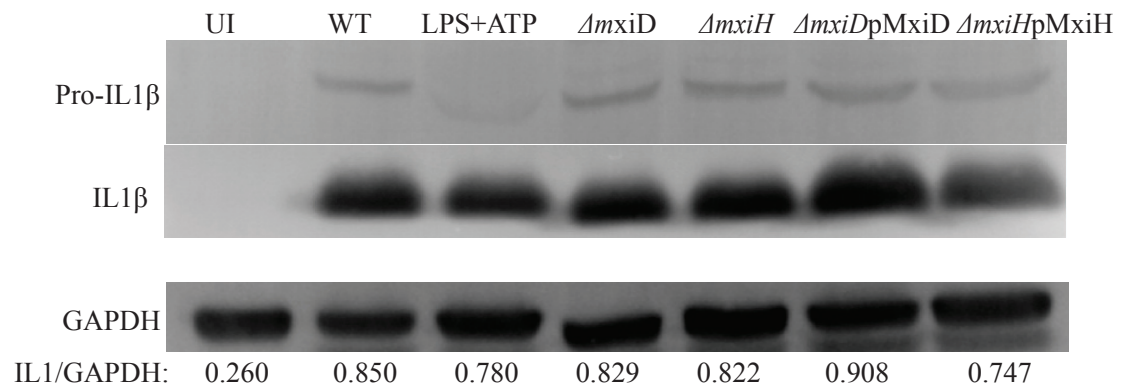


No significant differences in levels of caspase-1 were observed when Wild-type *Shigella* infected JAWSII cells were compared to  $\Delta ospF$  or  $\Delta ospI$  mutant infected DCs. There was no difference in levels of caspase-1 observed in the  $\Delta ospIF$  double mutant infected cells when compared to those infected with the Wild-type control. I observed that uninfected JAWSII cells had basal levels of active caspase-1 that were decreased upon infection with Wild-type *Shigella*. Taken together, these data indicate that *Shigella*-induced JAWSII cell death it is independent of caspase-1 activation (Figure 19).

### **3.8: Dendritic cell death by *Shigella* infection results in release of IL1 $\beta$**

Given that induction of programmed cell death by pyroptosis can result in cleavage and release of mature IL-1 $\beta$  I hypothesized that JAWSII cells infected with secretion-competent mutants would release high levels of IL-1 $\beta$  due to the cytotoxic effect of *Shigella* on JAWSII cells, and that IL-1 $\beta$  release would be inhibited in T3SS mutants as they are unable to kill JAWSII cells. Uninfected JAWSII cells were used as a negative control and produce very little IL-1 $\beta$  (Figure 20). Control JAWSII cells treated with LPS (0.1mg/mL) and ATP (5mM) produced more mature IL-1 $\beta$  than uninfected controls (Figure 20). No significant differences in IL-1 $\beta$  levels were observed between Wild-type (WT) infected cells and mutant infected cells indicating that neither Osp proteins nor T3SS components are required for IL-1 $\beta$  release from JAWSII cells three hours post-infection (Figure 20). This result suggests that infection of JAWSII cells alone is sufficient to trigger maturation of IL1 $\beta$  in JAWSII cells.

**Figure 20. JAWSII dendritic cells release mature IL-1 $\beta$  three hours post *Shigella* infection.** *Shigella* strains were struck onto nutrient rich TSB agar (containing tetracycline to select for single mutants, tetracycline and kanamycin for double mutant) and grown overnight at 37°C. A single colony from each strain was isolated and grown in nutrient rich TSB supplemented with antibiotic at 37°C overnight. Cultures were diluted and grown to log phase (OD<sub>600</sub> between 0.4-0.6). JAWSII dendritic cell line was infected with all *Shigella* deletion mutants of interest (MOI=10) as well as their complemented strains for three hours. Cells were collected and harvested in Laemmli buffer, boiled for 5 minutes and protein concentration was determined using a DC protein assay. Equal amounts of protein for each sample were separated by migration through 10% polyacrylamide gels. Immunoblotting was performed with primary rabbit antibodies directed against pro- and mature IL-1 $\beta$ . Purified LPS (0.1mg/mL) and ATP (5mM) were used as a bacteria-free positive control to stimulate inflammasome mediated pyroptosis. Anti-GAPDH was used as a loading control. ImageJ software was used to quantify band intensity and a ratio of IL-1 $\beta$  to GAPDH intensity is listed.



## **Chapter 4: Discussion**

### **4.1: *Shigella* effectively kills BMDCs and JAWSII DCs in a T3SS-dependent manner**

Antigen presenting cells represent an important line of immune defense to protect against bacterial infection (Mogensen, 2009). Although significant research has been devoted to *Shigella*-macrophage interactions, *Shigella*-dendritic cell interactions are largely unexplored. Over a decade ago, Edgeworth et al. (2002) published a description of cytotoxicity and IL1 $\beta$  processing following *Shigella* infection of human monocyte derived dendritic cells. Rapid DC death was found to occur in an IpaB-dependent manner, but the role of the T3SS and associated effectors was not assessed (Edgeworth et al., 2002).

I hypothesized that dendritic cell killing by *Shigella* occurs early in infection and is dependent on a functional T3SS. To test this, I first infected bone marrow derived dendritic cells isolated from C57/BL6 mice with *Shigella* strains M90T (Wild-type strain) and  $\Delta$ *mxiD* (T3SS deficient; lacking structural component MxiD). I used uninfected BMDCs as a negative control and purified LPS as a positive control. I used flow cytometry to quantify the proportion of cells dying upon *Shigella* infection for a short (3 hour) or long (16 hour) time, as measured by Annexin V Propidium Iodide staining. Using a multiplicity of infection of 10 I observed that Wild-type *Shigella* effectively killed BMDCs at both time points (Figure 7). Significantly less cytotoxicity was observed in the T3SS mutant infection conditions, which was comparable to both the uninfected

and LPS control conditions (Figure 7). This result supports my hypothesis that dendritic cell killing by *Shigella* is dependent on the T3SS.

Using flow cytometry to measure the effects of *Shigella* infection of BMDCs proved to be difficult. Granulocyte-monocyte colony stimulating factor (GM-CSF) and IL4 were used to differentiate naïve stem cells into DCs however this environment also facilitates differentiation of macrophages that share identifying cell surface markers (Poltorak & Schrami, 2015). Due to the nature of the process involved in differentiating bone marrow into dendritic cells, gating relevant populations from a contaminated population (many different cell types with similar identifying markers) of cells was challenging. Populations of interest (DCs) were gated based on CD11c expression, and MHCII expression (CD11c<sup>hi</sup> MHCII<sup>hi</sup>). In order to avoid collecting data that could be skewed by contaminating populations of cells, and to increase availability of cells for future experimentation I chose to continue investigating using the dendritic cell line, JAWSII. JAWSII cells are an immortalized DC line established from bone marrow cultures of p53-deficient mice. These cells have been shown to behave like BMDCs and display similar characteristics (Daw et al. 2012; Senba et al., 2013).

#### **4.2: *Shigella* deletion collection mutants display various degrees of virulence in JAWSII cells**

Upon establishment of JAWSII as a reliable cell line for studying *Shigella* induced cytotoxicity I screened the *Shigella* deletion collection to assess which virulence plasmid genes were required for rapid killing of dendritic cells. To remain consistent with previous efforts I infected JAWSII cells with Wild-type *Shigella* and deletion collection



mutants at a multiplicity of infection of 10 for a short time period (three hours). I used trypan blue exclusion to measure JAWSII cell viability post-infection and performed the screen three times to obtain statistical significance.

Not surprisingly, all structural components of the T3SS are required to kill JAWSII cells as are transcription factors (for example VirF and VirB) required for regulation of T3SS activity (Figure 8). Mutants lacking virulence plasmid-encoded chaperones or effectors required for invasion of epithelial cells displayed reduced cytotoxicity in JAWSII cell infection (Figure 8). Given that chaperones are required for translocation of effectors in a secretion competent state, some insight can be gained as to the requirement of the chaperoned effectors in DC killing. For example, IpgC is the chaperone for the pore forming IpaB and IpaC proteins; deletion of *ipgC* disallows secretion of IpaB and IpaC. The trypan exclusion screen in Figure 8 shows that IpgC is required for JAWSII cell killing, further emphasizing the importance of its effectors IpaB and IpaC. This is supported by results obtained from infecting cells with either  $\Delta ipaB$  or  $\Delta ipaC$ , both of which resulted in loss of JAWSII cell killing function (Figure 8).

In contrast, Spa15 is the chaperone for proteins IpgB1, IpgB2, IpaA and OspC3;  $\Delta spa15$  does not significantly increase viability in JAWSII cell infection suggesting that its effectors do not play a significant role in DC killing (Figure 8). This is supported by results obtained from infecting cells with any one of these deletion mutants (Figure 8).

Interestingly, infection of JAWSII cells with *Shigella* mutants lacking one of many uncharacterized genes (Open Reading Frames; ORFs) displayed various phenotypes. For example, mutant  $\Delta orf161b$ -infected JAWSII cells did not display changes in cytotoxicity compared to cells infected with Wild-type *Shigella* so this gene is

not considered to be important for dendritic cell killing (Figure 8). Alternatively, mutant *Δorf176*-infected JAWSII cells display an intermediate cytotoxicity profile, having approximately 40% viability after three hours of infection, and *Δorf182*-infected cells were roughly 60% viable, indicating that these genes could play a role *Shigella* virulence as it relates to immune cell killing (Figure 8).

Four mutants from this screen were chosen for further investigation. Type three secretion component mutants *ΔmxiD* and *ΔmxiH* and outer *Shigella* protein mutants *ΔospF* and *ΔospI* were applied to further characterize dendritic cell responses to *Shigella* infection.

#### **4.3: OspF and OspI target MAPK signaling in JAWSII dendritic cell line**

OspI and OspF are T3SS effectors known to interfere with immune signaling through the NF- $\kappa$ B and MAPK signaling pathways respectively (Li et al., 2007; Senada et al., 2012). OspF is a phosphothreonine lyase with the ability to irreversibly remove the phosphate group from activated MAPK proteins such as Erk, JNK, and p38 (Li et al., 2007). OspI is a glutamine deamidase that selectively deamidates a glutamine residue on the ubiquitin conjugating enzyme (UBC) Ubc13 (Senada et al., 2012). This deamidation results in the inability of Ubc13 to transfer ubiquitin to its target, TRAF6, thus inhibiting NF $\kappa$ B activation (Senada et al., 2012).

I hypothesized that OspI and OspF play a role in inducing programmed cell death in infected DCs by inhibiting their respective pathways. To confirm the validity of the mutant phenotypes, I built complementing plasmids to transform into their respective

*Shigella* mutant strains to rule out the possibility of polar mutations (data not shown). I also engineered, by  $\lambda$ -red recombination and accompanying complementation plasmids, a double mutant lacking both *ospI* and *ospF*, to observe any synergistic or antagonistic effects these effectors may have. I infected JAWSII cells with each mutant or complemented mutant for three hours at a multiplicity of infection of 10 and measured cell viability by trypan blue exclusion. Cytotoxicity among JAWSII cells infected with  $\Delta ospF$  or  $\Delta ospI$  was consistent with that observed in the deletion collection screen- having an approximate 25% increase in viability over Wild-type infected cells (Figure 9). Complementation of  $\Delta ospF$  with  $\Delta ospFpOspF$  partially restored the viability phenotype to that comparable with Wild-type infected cells (mean = 8.6%; Figure 9). Complementation of  $\Delta ospI$  with  $\Delta ospIpOspI$  resulted in a further increase in JAWSII cell viability, perhaps because *ospI* was inserted into a high copy vector and levels of expression were high enough to be recognized by PRRs that allow the dendritic cells to prime for defense (Figure 9). It is possible that cloning *ospI* into a low copy vector might resolve this issue.

OspF has been shown to display phosphothreonine lyase activity to facilitate inhibition of MAPK signaling in human dendritic cells (Kim et al., 2008). I was able to replicate these findings in the context of JAWSII cell infection (Figure 10). Dendritic cells were infected with either Wild-type *Shigella* or a mutant deleted for *ospF* ( $\Delta ospF$ ) for 15, 30 or 60 minutes. Western blot analysis confirms that WT infected JAWSII cells have lower levels of activated Erk at each time point tested compared to  $\Delta ospF$  mutant infected cells (Figure 10). This result confirms that the function of our mutant is consistent with what is published in the literature regarding its activity within JAWSII dendritic cells.

Glutamine deamidase activity by the T3SS effector OspI has been shown to dampen the NF $\kappa$ B pathway by targeting Ubc13 and effectively blocking polyubiquitination of TRAF6, which provides a scaffold for protein interactions (Senada et al., 2012). It has recently been elucidated that polyubiquitinated TRAF6 also provides a scaffold for proteins required to initiate MAPK signaling (Arthur & Ley, 2013). Ubc13 ubiquitinates TRAF6 resulting in complex formation of TAK1/TABs complex and subsequent activation of protein kinase TAK1 which initiates the MAPK signaling cascade in response to PRR stimulation (Arthur & Ley, 2013). I hypothesized that OspI-mediated inhibition of TRAF6 limits TAK1 activation thereby dampening signaling through the MAPK pathway.

I infected JAWSII for a short (30 minute) time period with a low multiplicity of infection (10) and compared levels of activated Erk and p38 seen in uninfected, Wild-type, mutant  $\Delta ospF$  and  $\Delta ospI$ , and complemented mutant-infected cells. Western blot results indicate that OspI, in addition to OspF, may play a role in inhibiting MAPK signaling as determined by increased levels of p-Erk and p-p38 compared to Wild-type infected cells (Figure 11). Uninfected JAWSII cells were used as a negative control and display very little activated MAPK despite having equal amounts of total MAPK as all other *Shigella* infected JAWSII cells (Figure 11). Complementation of deletion mutants slightly decreased levels of activated p38 and Erk but did not completely restore the Wild-type phenotype (Figure 11).

Given that *Shigella* bearing mutations in  $\Delta ospF$  and  $\Delta ospI$  both display an increase in activated MAPKs over Wild-type *Shigella* in the context of JAWSII infection, I hypothesized that the  $\Delta ospIF$  double mutant would be further inhibited in its ability to

dampen immune signaling and I would observe an increase in activated Erk and p38 compared to single mutant infections. Interestingly, there was no real difference among mutants  $\Delta ospI$ ,  $\Delta ospF$ , or  $\Delta ospIF$ - all three mutant infected conditions resulted in similar levels of activated MAPKs with  $\Delta ospIF$  having a slightly higher band intensity when quantified using ImageJ (Figure 12). I would argue that the change in MAPK activation is reflective of the different mechanisms of action between the two effectors, OspF working at the MAPK level (removing activating phosphate) and OspI working upstream of the signaling pathway (UBC13-TRAF6-TAK1). Perhaps if the effectors displayed the same mechanism of action a more distinct synergistic increase would have been observed in double mutant infected cells.

JAWSII cells infected with double mutant complemented strains  $\Delta ospIFpOspF$  or  $\Delta ospIFpOspI$  displayed levels of activated MAPKs comparable to that of their respective single mutants ruling out the possibility of polar mutations (Figure 12). Total levels of Erk and p38 did not differ among infection conditions (Figure 12).

Bacterial manipulation of proinflammatory pathways is not uncommon. Several *Shigella*-related pathogens impair MAPK and/or NF $\kappa$ B signaling using T3SS effectors. *Yersinia* T3SS effector YopJ (*Yersinia* Outer Protein J) is a potent inhibitor of innate immune signaling in phagocytic cells, acting as an acetyltransferase and transferring acetyl groups to serine and threonine residues of MAPKKKs (TAK1) and MAPKKs (MKK2) preventing their activation and inducing cell death (Mills et al., 1997; Monack et al., 1998; Mettal et al., 2006; Paquette et al., 2012). Similarly, Enteropathogenic *E. coli* (EPEC) secrete a T3SS effector, NleE, which possesses methyltransferase activity and targets the ubiquitin binding activity of TAB2/3 (Zhang et al., 2012). Lys63 linked

polyubiquitin chains formed on TRAF6 provide the scaffold needed for complex formation of TAB2/3 with TAK1. NleE inhibits complex formation as a mechanism to inhibit NF $\kappa$ B and presumably MAPK signaling (Zhang et al., 2012).

#### **4.4: OspF and OspI inhibit the pheromone response pathway in *Saccharomyces cerevisiae***

Measuring immune signaling impairment by western blotting methods proved challenging and laborious as phospho-kinases are present in relatively low abundance within a cell and can become dephosphorylated upon processing for analysis. To verify my hypothesis that OspI targets MAPK signaling I used *Saccharomyces cerevisiae* as a test system. MAPK signaling in yeast is comparable to that in mammalian cells and components of the yeast MAPK signaling pathway have homologs in mammalian cell types. Rohde et al. (2007) used *S. cerevisiae* to demonstrate that the *Shigella* T3SS effector IpaH9.8 is an E3 ubiquitin ligase that targets MAPKK Ste7 for destruction by the proteasome. I applied two assays from this publication using IpaH9.8 expressing yeast as a control for MAPK inhibition. Empty vector (pAO1) is used throughout as a negative control and does not inhibit signaling through yeast MAPK signaling pathways.

The first assay I performed upon building plasmids to express *ospI* or *ospF* was a reporter assay using *S. cerevisiae* strain SY2625 harboring a *FUS1-HIS3* reporter. Fus1 expression is dependent on MAPK signaling through the pheromone response pathway (required for fusion of haploid yeast cells) therefore growth on medium lacking histidine represents activation of the pathway. *Shigella* effectors were expressed using a galactose inducible promoter so each transformed strain was grown on both glucose (effector “off”

state) and galactose (effector “on” state) while lacking uracil to maintain plasmid selection, and lacking histidine as a readout for MAPK inhibition. All transformed strains grew equally well on synthetic complete medium lacking uracil and histidine with glucose and all but the empty vector control did not grow synthetic complete medium lacking uracil and histidine with galactose, indicating that all effectors assayed inhibit the pheromone response in *S. cerevisiae* SY2625 (Figure 13). Synthetic complete medium lacking uracil supplemented with glucose or galactose was used as a negative control to ensure that expression of effectors alone did not induce cell death (Figure 13). Additionally, the osmotic shock-sensing MAPK pathway was tested to investigate whether the growth defect observed was or was not specific to the pheromone response as some MAPK components are shared between the two pathways (Saito, 2010). Supplementing plates of synthetic complete medium (lacking uracil) with sorbitol activates the osmotic shock pathway in *S. cerevisiae*; strain SY2625 expressing OspF was the only yeast strain impaired in the osmotic shock signaling cascade (Figure 13). This is logical since OspF is known to act directly on activated MAPKs and impair the pathway by irreversibly removing the activating phosphate group (Li et al., 2007). Neither IpaH9.8 expression nor OspI expression in SY2625 affected the osmotic stress pathway as growth was not impaired in these conditions.

The second assay performed was a halo assay using the *MATa* strain BY4741 transformed with empty vector pAO1, pAOspI, pYESOspF, or pJR001IpaH9.8. Upon exposure to  $\alpha$ -factor impregnated on a sterile paper disk, BY4741 cells will induce cell cycle arrest via the pheromone response pathway (Hoffman et al., 2002). This reaction can be observed as a halo of inhibited growth around the paper disk on an otherwise

uniform lawn of yeast grown in an agarose overlay (Hoffman et al., 2002). Rohde et al. (2007) exploited this MAPK pathway to define IpaH9.8 as an E3 ligase for Ste7 by transforming BY4741 cells with a plasmid expressing *ipaH9.8* under control of a galactose inducible promoter. A clear zone of inhibition was observed when transformed BY4741 cells were grown with glucose as a carbon source, and no zone of inhibition was observed when grown with galactose as a carbon source, indicating inhibition of the pheromone response pathway by IpaH9.8 (Rohde et al., 2007).

I was able to replicate this result by transforming the plasmids above (pAO1, pAOspI, pYESOspF, and pJR001IpaH9.8) into BY4741 cells and exposing strains to  $\alpha$ -factor under non-inducible (glucose) or inducible (galactose) growth conditions. Empty vector pAO1 was used as a negative control and displayed a clear zone of inhibition upon exposure to  $\alpha$ -factor on selective medium supplemented with either glucose or galactose (Figure 14). BY4741 cells expressing IpaH9.8 was used as a positive control for MAPK inhibition: a zone of inhibition was observed when grown on medium containing glucose but not on medium containing galactose consistent with Rohde et al., 2007 (Figure 14).

Given that OspF is already documented to impair classical MAPK signaling I predicted that BY4741 cells expressing OspF would impair the pheromone response pathway and the zone of inhibition observed on glucose containing medium would not be observed on galactose containing medium (same phenotype as IpaH9.8 expressing). This hypothesis was supported; a lawn of growth was observed on galactose containing medium where a clear zone of inhibition was observed on glucose containing medium (Figure 14).



Results obtained from western blotting experiments with  $\Delta ospI$  and growth inhibition assays with *ospI*-expressing SY2625 cells lead me to predict that MAPK signaling through the pheromone response pathway would be inhibited in *ospI*-expressing BY4741 cells. A clear zone of inhibition was observed on glucose containing medium but not on galactose containing medium (Figure 14). As with *ipaH9.8*- and *ospF*-expressing BY4741 cells, a lawn of yeast grown up to the sterile disk containing  $\alpha$ -factor was observed on galactose containing medium, indicating interference with MAPK signaling (Figure 14). It is interesting to note that while the growth phenotypes of each of the effector-expressing yeast appear to be the same three days after exposure to  $\alpha$ -factor, differences can be observed after the second day of incubation. A weak “secondary” zone of inhibition was observed on day two for *ospF*- and *ospI*-expressing yeast that was not seen in the *ipaH9.8*-expressing cells. In each case, the  $\alpha$ -factor seemed to temporarily inhibit cell growth before effector-expressing colonies would arise (Figure 14). I predict that the mechanisms by which the different effectors act on the pheromone response pathway mediates the rate of yeast cell growth- if this prediction is correct, *IpaH9.8* is more effective than *OspF* or *OspI* at inhibiting MAPK signaling.

Ubc13 is involved in assembly of polyubiquitin chains required for the DNA repair pathway in yeast (Hoffman & Pickart, 1999). I hypothesized that *OspI*-expressing *S. cerevisiae* would be impaired in their ability to recover from UV radiation due to Ubc13 inactivation by *OspI*. BY4741 cells and a mutant strain deleted for *ubc13* ( $\Delta ubc13$ ) were grown on nutrient rich agar as a negative control; both strains grew equally well indicating that  $\Delta ubc13$  does not display an impaired growth phenotype without exposure to UV (Figure 15). Upon exposure to UV radiation, yeast cells employ a

DNA repair pathway to overcome UV induced damage (Figure 15). The  $\Delta ubc13$  mutant strain recovers from UV damage less well than WT validating a role for *ubc13* in UV-induced DNA repair (Figure 15). Growth phenotypes on medium lacking uracil were consistent between cells bearing empty vector or expressing OspI when grown without UV exposure (Figure 16). Upon exposure to UV radiation and under inducible conditions (growth on galactose) OspI-expressing BY4741 cells display an impaired growth phenotype comparable to that of  $\Delta ubc13$ . This result suggests that Ubc13 is also a target for OspI in *S. cerevisiae*.

Having found that OspI targets the pheromone response pathway in yeast, and that Ubc13 appears to be a target, I hypothesized that Ubc13 may play a previously uncharacterized role in the pheromone response pathway. To test this prediction I assessed the ability of my  $\Delta ubc13$  mutant to induce cell cycle arrest as indicated by the halo assay described previously (Figure 17). If Ubc13 is involved in MAPK signaling through the mating pathway then the zone of inhibition observed in Wild-type yeast would be impaired in the mutant. Wild-type BY4741 cells consistently display a clear zone of inhibition, indicating cell cycle arrest through the pheromone response pathway. BY4741 cells deleted for *ubc13* displayed colony formation within the zone of inhibition after just 24 hours incubation (Figure 17). This result suggests Ubc13 may play a role in the yeast pheromone response. This is perhaps not surprising since mice deleted for *ubc13* in immune cells displayed defects not only in activation of the NF $\kappa$ B pathway but also in MAPK activation (Yamamoto et al., 2006).

Given that K63-linked polyubiquitin chains have recently emerged as regulatory elements for immune signaling it is logical that Ubc13, the only known E2 involved in

K63 formation, may function in MAPK signaling in yeast. The role of ubiquitin in the pheromone response pathway has only begun to be characterized; Dohlman and colleagues have recently shown that the MAPKK Ste7 is ubiquitinated upon pheromone stimulation in order to direct MAPK specificity (Hurst & Dohlman, 2013). I hypothesize that K63 linkages could be involved in creating a scaffold for protein interactions required for MAPK signaling through the pheromone response pathway. Perhaps Ste7 or an upstream signaling factor such as Ste20 becomes polyubiquitinated to facilitate induction of the pheromone response. An *in vitro* ubiquitination assay using purified ubiquitin components (Ubc13 as the E2) and potential substrates may reveal a novel regulation mechanism by which a K63 protein scaffold facilitates MAPK signaling in yeast.

#### **4.5: *Shigella* induced cell death likely does not occur by classical pyroptosis in JAWSII dendritic cell line**

*Shigella*-induced macrophage death occurs by pyroptosis and is recognized as a crucial step in *Shigella* pathogenesis (Schroeder & Hilbi, 2008). A knowledge gap still remains with regard to the role of dendritic cells in shigellosis; we know that *Shigella* targets dendritic cells for destruction but the mechanisms by which this phenomenon occurs have not been fully elucidated.

Initial studies investigating *Shigella*-dendritic cell interaction reported IpaB dependent cell death of *Shigella* infected human monocyte derived dendritic cells that can be partially blocked with chemical inhibitor YVAD (caspase-1 specific inhibitor; Edgeworth et al., 2002). I used a similar inhibitor, ZVAD (pancaspase inhibitor), with the prediction that pretreatment of JAWSII cells would inhibit cell death by Wild-type

*Shigella*. One hour pretreatment with ZVAD significantly increased JAWSII cell viability in Wild-type infection conditions to approximately 20% indicating only partial reliance on caspase activity (Figure 18). I conclude that *Shigella*-induced JAWSII cell killing is largely caspase independent three hours post-infection.

I have investigated the parameters required for efficient killing of DCs by *Shigella* using a variety of experimental methods. I was able to confirm that killing is dependent on the virulence plasmid-encoded T3SS and associated effectors. After screening the *Shigella* deletion collection, I chose four mutants for further analysis: two of which,  $\Delta mxiD$  and  $\Delta mxiH$ , are required for assembly of the T3SA. MxiH has been published to be recognized by murine PRR NAIP1 within antigen presenting cells and induce NLRC4-inflammasome activation that results in caspase-1 activation and release of mature IL1 $\beta$  (Yang et al., 2013).

I hypothesized that *Shigella*-induced dendritic cell death occurs via inflammasome-mediated pyroptosis and this is why viability is not affected upon infection with a T3SS deficient mutant. Western blot analysis suggests that this hypothesis is not fully supported. I infected JAWSII cells for three hours at a multiplicity of infection of 10 with all four mutants of interest ( $\Delta ospF$ ,  $\Delta ospI$ ,  $\Delta mxiD$ ,  $\Delta mxiH$ ), as well as their complemented strains, the double mutant ( $\Delta ospIF$ ) and its complemented strains (Figures 19 & 20). Following infection I expected to observe significant levels of mature caspase-1 and IL1 $\beta$  in Wild-type infected JAWSII cells but not in  $\Delta mxiD$  and  $\Delta mxiH$  infected cells. Additionally, I expected to visualize a difference between Wild-type and *osp* mutants, given the change in viability observed by trypan exclusion. Instead, Wild-type *Shigella*-infected JAWSII cells appeared to have less caspase-1 than even uninfected

JAWSII cells (Figure 19). This result suggests that Wild-type *Shigella* actively inhibit caspase-1 activation in DCs; *osp* mutants and their complemented strains also had less caspase-1 than the uninfected control indicating that these effectors are not responsible for caspase-1 inhibition.

Caspase-1 activation was slightly higher in  $\Delta mxiD$  and  $\Delta mxiH$  infected JAWSII cells than the uninfected control, and were comparable with the ATP+LPS positive control (artificially induces pyroptosis; Figure 19). Attempts at complementation partially reduced caspase activation but levels were still higher than that seen in Wild-type infected cells (Figure 19). These results are perplexing as they conflict with the data published by Yang et al. (2013). In their work, the dendritic cell line (DC2.4) was infected with Wild-type or  $\Delta mxiH$  mutant *Shigella* strains and caspase-1 activation was measured by western blot (Yang et al., 2013). Yang et al. found that Wild-type-infected JAWSII cells promote caspase-1 activation and  $\Delta mxiH$  mutant-infected cells do not. This group did not directly compare IL1 $\beta$  maturation between Wild-type and *mxiH* mutant-infected cells but they did show that Wild-type infection (accompanied by caspase-1 activation) results in release of IL1 $\beta$  that is not observed in an infection-free control (Yang et al., 2013). I observed a significant increase in IL1 $\beta$  maturation in Wild-type *Shigella*-infected JAWSII cells compared to uninfected cells, however, this same phenotype is observed in all other *Shigella* infection conditions, including  $\Delta mxiD$  and  $\Delta mxiH$ , and the positive control (LPS+ ATP; Figure 20). This result suggests that infection alone, regardless of the level of pathogenesis is sufficient to promote an IL1 $\beta$  response in JAWSII cells.

There are several potential explanations for the discrepancies between my data sets and those published. First, I used complete cell lysates in my immunoblotting

procedure and Yang et al. isolated protein from the supernatants of each infection condition by TCA (TriChloroacetic Acid) precipitation. Second, although the infection times were the same (three hours) Yang et al. used a high multiplicity of infection (50) and I used a low multiplicity of infection (10), which has been published to affect the nature of inflammasome activation and reliance on caspase-1 (Willingham et al., 2007; Suzuki et al., 2007; Miao et al. 2010). Where DC2.4 cells initiate caspase-1 dependent pyroptosis via recognition of MxiH by the NLRC4 inflammasome, JAWSII cells could be undergoing an alternative form of inflammasome dependent (caspase-1 independent) pyronecrotic cell death.

One major downfall associated with using a cell line to study host-pathogen interaction is that there is potential for changes in behavior from primary cells and other cell lines. Although JAWSII cells have been studied in the context of infection, inflammasome activation has only been described for the NLRP3 complex so whether or not NLRC4 becomes activated in response to pathogen stimulation remains to be investigated (Hua et al., 2013). Knockdown of potential NLR complexes may help resolve which types of inflammasome complexes are being activated upon *Shigella* infection of JAWSII cells. Alternatively, co-immunoprecipitation of MxiH or MxiD with potential inflammasome complexes might further elucidate a specific mechanism of inflammasome activation in JAWSII cells. Immunofluorescence could also be used to visualize colocalization of T3SS components with inflammasome proteins by tagging each with a different fluorophore.

#### **4.6: Suicide plasmids as a tool for introducing secondary gene deletion into the *Shigella* deletion collection**

Development of high-throughput genetic interaction screens in bacteria may prove invaluable to our understanding of gene function and pathway organization (Typas et al., 2008). One research objective for this project was to determine a strategy for generating double gene deletions in a high throughput manner using specialized suicide plasmids. The goal of this project was to engineer a plasmid carrying an inactivated copy of one of *Shigella*'s effector proteins and mate this plasmid into each of the mutants from the *Shigella* deletion collection where allelic exchange could occur and give rise to double deletion collections. Allelic exchange generally occurs as a two-step process: in the first step the suicide plasmid is integrated into the genome (or in this case the virulence plasmid) by homologous recombination (Maloy et al., 1996). In the second step the backbone of the suicide plasmid is excised leaving only the inactivated copy of the gene of interest (Maloy et al., 1996).

In my first attempt, I built a suicide plasmid with an inactivated version of the *ipgB1* gene (that codes for the effector IpgB1), which is involved in T3SS-dependent invasion of epithelial cells. After mating *E. coli* containing the suicide plasmid with *Shigella* deletion mutants I selected for integration of the suicide plasmid by growth on medium containing gentamicin (antibiotic resistance cassette found in the backbone of the suicide plasmid) or both gentamicin and chloramphenicol (resistance cassette used to inactivated *ipgB1*). I then selected for excision of the backbone by growing bacteria on medium containing 5% sucrose; the backbone of the suicide plasmid encodes a *sacB* gene which when expressed is lethal to the cell therefore bacteria that have to excised the backbone survive and replicate. I selected for successful excision by growth on medium

containing chloramphenicol and tetracycline (resistance cassette used for primary gene deletion). I subsequently attempted to grow the mutants of interest on gentamicin containing medium; lack of growth ensured that the backbone was excised.

Although I was able to find pJQIpgB1Cm<sup>R</sup> integrants in plasmid pWR100 I could not pressure the backbone to excise and leave the inactivated version of *ipgB1*. An unanticipated challenge with this suicide plasmid was the absence of an observable phenotype for  $\Delta ipgB1$  *in vitro*. In hopes of verifying this method of double mutant creation, I constructed a suicide plasmid (pJQIpaDCmR) that harbors an inactivated allele of *ipaD* (codes for IpaD), the needle tip protein of the T3SS. Mutants for *ipaD* have a strong Congo red phenotype that can be observed on selective agar (Sidik et al., 2014). Despite increasing sequence homology between the DNA fragments, inactivating a gene with a readily observable phenotype, and screening a larger number of bacterial colonies, I was unable to isolate double mutants of *Shigella* using this approach. The rarity of the genetic events required for this method to be successful rendered this approach ineffective for generating high-throughput mutations among bacterial strains in *Shigella*.

In 2008 Typas et al. used a similar strategy to generate double deletions in *E. coli* by exploiting the Keio collection. This group robotically generated a collection of double mutant *E. coli* that they termed GIANT-coli (Genetic Interaction ANalysis Technology for *E. coli*). Typas and colleagues were successful in their attempt and were able to identify novel protein interactions using this high-throughput strategy. Parameters stated to be important for successful double mutant creation that may have affected the outcome of my trials include the efficiency of mating, efficient transfer of the suicide plasmid, and



the rate of recombination. Creation of the OspF OspI double mutant was thereby created using the standard method of  $\lambda$ -red recombination as described in M&M 2.14.

#### **4.7: Conclusions**

*Shigella* is a clinically relevant pathogen for which no licensed vaccine currently exists (Venkatesan et al., 2015). The focus of this study was to investigate *Shigella*-DC interaction as it relates to impairment of immunity against shigellosis. Significant research has been devoted to understanding the innate immune response to *Shigella* infection, however the mechanisms *Shigella* employ to impair adaptive immunity have only begun to be investigated. Leaders in the field suggest that impairment of antigen presenting cells would be an efficient means of silencing long-term immune responses (Philapon & Sansonetti, 2007; Salgado-Pabón et al., 2013).

I propose that *Shigella* targets dendritic cells for destruction early in infection to eliminate the potential for activation of adaptive immune cells such as T cells, B cells, and NK cells. Initial studies focused on the role of the T3SS in dendritic cell infection. Flow cytometry revealed that dendritic cell killing is dependent on a functional T3SS as a mutant lacking a structural component required for assembly of the T3SS was unable to induce DC death. I then assessed the importance of each of the genes on the *Shigella* pWR100 virulence plasmid by screening the deletion collection for mutants that affected viability in an infected dendritic cell line (JAWSII).

I chose two mutants ( $\Delta ospI$  and  $\Delta ospF$ ) whose gene products were reported in the literature to impair immune signaling and assessed their function within the JAWSII

dendritic cell line. I was able to confirm inhibition of MAPK activation by T3SS effector OspF, and identify a novel role for OspI in MAPK inhibition. I showed that the activity of these effectors is conserved in *Saccharomyces cerevisiae* and identify a novel role for the E2 ubiquitin-conjugating enzyme Ubc13 in the pheromone response pathway. Deletion collection mutants  $\Delta ospF$  and  $\Delta ospI$  display an impaired ability to kill JAWSII cells three hours post-infection suggesting that impairment of immune signaling is involved in induction of cell death. I constructed a double mutant,  $\Delta ospIF$ , to investigate potential synergistic or antagonistic effects on dendritic cell impairment. Although a slight increase in viability was observed in JAWSII infected cells, the increase in MAPK signaling was comparable to that observed in the single mutant infection conditions.

Two additional mutants ( $\Delta mxiD$  and  $\Delta mxiH$ ) were used in this study to investigate the mechanisms of cell death induction of JAWSII dendritic cells. Mutants lacking either structural component MxiD or MxiH were unable to kill JAWSII cells. I hypothesized that virulent *Shigella* strains were killing dendritic cells by classical pyroptosis. Western blot analysis for active caspase-1 and IL1 $\beta$  did not support my hypothesis. It appeared that virulent *Shigella* strains were down-regulating caspase-1 despite a significant release of mature IL1 $\beta$ . Caspase-1 activation was higher in avirulent mutants but IL1 $\beta$  levels were similar to virulent conditions. These results highlight the emerging complexity of bacteria-mediated programmed cell death.

Further investigation and elucidation of the mechanisms by which *Shigella* impairs dendritic cell function using its arsenal of T3SS and effectors could aid in creation of a live attenuated vaccine. An attenuated *Shigella* strain that does not kill

antigen-presenting cells but rather primes them to initiate cross talk with adaptive immune cells would be key to developing long lasting immunity against shigellosis.

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