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Development of in vitro models to study the rapid extraintestinal dissemination of salmonella.

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DEVELOPMENT OF *IN VITRO* MODELS TO STUDY THE RAPID EXTRAINTESTINAL DISSEMINATION OF *SALMONELLA*

By

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M.Sc., Biology, Valdosta State University, 2013

B.Sc., Biochemistry, University of Leeds, 2009

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ABSTRACT

DEVELOPMENT OF *IN VITRO* MODELS TO STUDY THE RAPID EXTRAINTESTINAL DISSEMINATION OF *SALMONELLA*

Adarsh Gopinath

April 7, 2020

Salmonella appears in the bloodstream of mice in as little as 15 minutes after oral inoculation and establishes persistent colonies in the spleen and liver. While its pathway to blood is undetermined, this phenomenon is dependent on the activity of *Salmonella* pathogenicity island 2 (SPI-2) coded type III secretion system (T3SS) and CD18+ phagocytes. We hypothesize that dendritic cells associated with the basal face of the gut epithelium, that are naturally migratory and known to sample for luminal antigens directly transport *Salmonella* to the bloodstream. This process comprises of at least two phases, dissociation and reverse transmigration. We define dissociation as the process were intraepithelial dendritic cells separate from the epithelium after picking up *Salmonella* from the gut. This is followed by reverse transmigration, a normal host process in which dendritic cells can reenter the bloodstream by traversing the vascular endothelium in the basal to apical direction. *In vitro* models of the two processes were developed to help identify T3SS effectors that could affect rapid extraintestinal

dissemination of *Salmonella* in mice. Chronologically the *in vitro* reverse transmigration assay was developed first and allowed dendritic cells to migrate from the basal to apical face of endothelial cell monolayers cultured on filters with small pores. The T3SS effector SpvC was found to play an important role in the SPI-2 mediated *in vitro* reverse transmigration of dendritic cells and along with SrfH was demonstrated to greatly promote early extraintestinal dissemination of *Salmonella* in mice. However, SrfH failed to stimulate reverse transmigration *in vitro*. We were able to demonstrate that SrfH triggered dissociation of dendritic cells bound to the basal side of epithelial cell monolayers cultured on tissue culture inserts with small pores. The presence of a glycine residue at position 103 of SrfH was vital for dissociation and could potentially trigger this process through its association with the host adaptor protein TRIP6. These results combined with the observation that the *srfH* Gly103 and the *spv* operon are conserved amongst strains of non-typhoidal *Salmonella* capable of causing bacteremia in people suggests that this pathway to the bloodstream could be important for understanding human infections.

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CHAPTER 1

GENERAL INTRODUCTION

Bacteria belonging to the genus *Salmonella*, are facultative anaerobic gram-negative rods that possess multiple flagella arranged in a peritrichous fashion. Ninety nine percent of *Salmonella* mediated disease in mammals is propagated by the species *Salmonella enterica*. *S. enterica* can be subdivided into six subspecies and around 2600 serotypes based on variations in the somatic O antigen, capsular K antigen and flagellar H antigens (Brenner et al., 2000). Three major diseases are caused by *S. enterica* in humans namely non-typhoidal salmonellosis, invasive non-typhoidal salmonellosis and typhoid or enteric fever.

Epidemiology and clinical manifestations

Non-typhoidal salmonellosis is a broad term that encompasses all the diseases caused in humans by all *S. enterica* serovars but for those implicated in enteric fever. Non-typhoidal *Salmonella* (NTS) serovars usually result in enterocolitis, an acute inflammation of the small intestine and colon and inflammatory diarrhea. It is transmitted via the fecal oral route through the consumption of contaminated food and water and around 50,000 bacteria are enough to manifest disease in as little as six hours (Blaser & Newman 1982). The disease mediated by NTS is self-limiting and often resolves itself without medical

intervention in less than a week in adults. Seemingly healthy individuals can shed virulent *Salmonella* in their feces for up to three months and in rare cases for a year.

Globally NTS mediated disease affects at least 1.3 billion individuals and contributes to the death of at least a million people per year (Majowicz et al., 2010). Mortality associated with salmonellosis is the highest in the developing world where infrastructure pertaining to healthcare, sanitation and water supply are inadequate. However even in the developed world NTS are the most commonly isolated pathogens in fresh fruits and vegetables (Hanning et al., 2009).

Invasive non-typhoidal *Salmonella* (iNTS) cause a systemic disease characterized by high fever and bacteremia that can eventually develop into extra-intestinal focal infections such as septic aortitis, meningitis, pneumonia, septic arthritis, osteomyelitis or cholangitis (Chen et al., 2005). Unlike NTS infections, iNTS infections are marked by a distinct absence of enterocolitis. iNTS infections usually occur in immunocompromised individuals with an estimated 3.4 million cases recorded each year (Balasubramanian et al., 2019). There is a significant link between iNTS disease and adults suffering from AIDS, indicating a reliance on the immune response mediated by CD4+ T helper cells to clear infection. However, iNTS infections have been recorded in children suffering from malnourishment or infected with malaria, pneumonia and tuberculosis prior to the spread of the AIDS epidemic. Based on clinical data pertaining to iNTS infections in Sub-Saharan Africa between 2000-2010, Feasy and colleagues conclude that for every 100,000 individuals, iNTS affects as much as 388 children and up to 7500 HIV positive adults (Feasey et al., 2012). Twenty to Twenty-five percent of these cases are fatal.

In humans, non-typhoidal bacteremia is caused by a host of *S. enterica* serovars including Typhimurium, Enteritidis, Choleraesuis and Dublin. Of these, emergent multidrug resistant (MDR) strains derived from serovar Typhimurium sequence type 313 (ST313) have resulted in an ongoing pandemic in sub-Saharan Africa. ST313 and its various geographical offshoots within the African continent exhibit genomic degradation patterns associated with host restriction and most have acquired resistance to first line antibiotics ampicillin, trimethoprim and chloramphenicol with some strains in Malawi and Kenya acquiring resistance towards extended spectrum β-lactamases (Van Puyvelde et al., 2019). There are currently no vaccines targeting antigens in a broad spectrum of NTS serovars.

Typhoid or enteric fever is caused by the human restricted *S. enterica* serovars Typhi and Paratyphi transmitted via the fecal-oral route through contaminated food and water (Goldberg & Rubin, 1988). Annually around 21 million individuals worldwide are infected by typhoidal *Salmonella* and at least one percent of these cases results in fatality (Crump & Mintz, 2010). The bulk of these infections are restricted to regions endemic to this disease that include parts of Asia and Africa. A feature unique to typhoidal strains of *Salmonella* is an extracellular polysaccharide capsule known as the Vi antigen that helps the bacterial cell survive the acidic environment of the gastric tract (Waddington et al., 2014). Typhoidal *Salmonella* invade and survive within cells of the reticuloendothelial system (RES) such as mononuclear phagocytic cells during the acute phase of infection and colonizes RES organs such bone marrow, spleen and liver (Gunn et al., 2014). Initially the patient typically suffers from a brief bout of fever, bacteremia, rash, weight loss and

headache. In most patients Typhi infections resolves itself within six months but in 2-5% of the cases the individual fails to clear the bacteria within a year and end up maintaining a carrier state where the typhoidal *Salmonella* is constantly shed into the blood and excrement from persistent colonies in the bile duct and gallbladder (Levine et al.,1982). Due to the systemic nature of infection, *Salmonella* Typhi infections are treated immediately with antibiotics. Similar to iNTS, several MDR strains of typhoidal *Salmonella* have emerged preventing the use of first line antibiotics and in some cases, bacteria have been isolated that are even resistant to fluoroquinolone derivatives such as ciprofloxacin (Qamar et al., 2014). There are currently two vaccines in use, namely Ty21a, a live attenuated vaccine, and another targeting the capsular Vi antigen. Ty21a is effective in preventing disease in 50% of cases but cannot be used in children under the age of six, which is a demographic that is highly susceptible to enteric fever (Loetcher et al., 2012). The vaccine targeting the Vi antigen protects 60% of recipients but has a limited efficacy of three years, with immunity unable to be boosted through supplementary immunizations (Anwar et al., 2014).

Mouse model of *Salmonella* pathogenesis

The prevalence of MDR strains of both typhoidal and iNTS serovars and the lack of an effective long lasting broad spectrum vaccine for both diseases have prompted the significant investment of time and effort into the development of *in vivo* and *in vitro* models to better understand the minutiae of host-pathogen interactions at the molecular, cellular and organismal levels. Several animal models have been developed that try to emulate the symptoms associated with all three diseases caused by *Salmonella*

species in humans. Non-human primates and bovine species are examples of models where *Salmonella* pathogenesis is the closest to that of their human counterparts. At extremely high doses, human-restricted *S.* Typhi can cause systemic illness in chimpanzees, while iNTS infections observed in AIDS patients can be simulated through co-infection of *Salmonella* in simian immunodeficiency virus infected rhesus macaques (Raffatellu et al., 2008). Unfortunately, the ethical and financial constraints associated with the general upkeep of primate and bovine species coupled with their poor genetic tractability make them unsuitable model organisms. A reasonable compromise is the use of widely available inbred lab mice strains such as C57BL/6 and BALB/C.

S. enterica serovar Typhimurium causes a systemic illness in mice that is reminiscent of typhoid fever in humans. The acute phase of enteric fever can be simulated in "susceptible" mice strains carrying a loss of function SNP in the natural resistance-associated protein 1 (Nramp1) gene. Loss of Nramp1, a transport protein involved in iron metabolism, predisposes C57BL/6 and BALB/C mice strains to infection with *S.* Typhimurium and other intracellular pathogens such as *Leishmania donovani* and *Mycobacterium bovis* (Blackwell et al., 2000). Virulent Typhimurium strains replicate unchecked in the macrophages of susceptible mice and result in rapid systemic spread and death within a week or two from infection. The chronic phase of *S.* Typhi can be simulated in resistant mice carrying functional copies of Nramp1. The mouse strains 129Sv and C57Bl/6×129x1/sv can assume a carrier state where they survive the initial acute phase of disease and shed virulent *Salmonella* into the gut and feces from colonies in the spleen and liver (Govoni et al., 1996; Monack et al., 2004). Mice do not exhibit

inflammation of the gut associated with NTS disease. This has been attributed to colonization immunity afforded by a robust gut microflora. However, acute inflammation of the small intestine characterized by neutrophil invasion of the lumen and lamina propria can be achieved by pretreating mice with streptomycin (Barthel et al., 2003).

Molecular pathogenesis

Salmonella codes for a host of virulence factors spread across six pathogenicity islands that help manifest various aspects of its virulence traits. The most important of these are effector proteins secreted by independently regulated type III secretion systems (T3SS) coded for on *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2). *In vitro* and *in vivo* studies in mouse models have indicated that functional SPI-1 T3SS is essential for the manifestation of symptoms reminiscent of the enterocolitis associated with NTS while SPI-2 is required for the establishment of extraintestinal disease similar to typhoid fever (Galan & Curtiss, 1989; Hensel et al., 1999).

Type III secretion system

The T3SS apparatus otherwise referred to as an injectisome is highly conserved in Gram-negative bacteria. These include human pathogens such as *Pseudomonas aeruginosa*, *Shigella flexneri*, *Yersinia pestis*, enteropathogenic *Escherichia coli* (EPEC) and those that infect plants such as *Pseudomonas syringae* and *Xanotomonas* species (Buttner 2012). The T3SS injectisome complex is a secretion apparatus that forms part of an elaborate basal structure made up of several pairs of rings that span the inner and outer membranes of the bacterial cell wall. After assembly of the cytoplasmic components, protein subunits are then secreted that form a hollow needle like structure that protrudes

outwards. This is followed by the secretion of translocon proteins through the channel formed by the needle like filament. Translocon proteins form pores on the host membrane, facilitating the secretion of effector proteins.

Effector proteins possess a highly variable 10 to 25 amino acid long N-terminal signal sequence for T3SS export that is rich in polar residues and sparse in charged and hydrophobic amino acids (Samudrala et al., 2009). The signal sequence is flanked by a domain that helps bind effector to a specific chaperone protein (Stebbins & Galan, 2001). The chaperones play an important role in not just directing the effector proteins to the T3SS injectisome but also prevent the degradation of the effector by bacterial cytosolic proteases and prevents the accidental translocation of effectors within the bacteria by binding to and masking signal sequences and transmembrane domains meant for subcellular localization inside the host cells (Tucker & Galan, 2000; Krampen et al., 2018). Effector molecules are injected into the host cell through the filament and translocon channel in an unfolded state through a single step ATP and proton-motive force driven mechanism.

Salmonella pathogenicity island 1 (SPI-1)

SPI-1 is 40kb in length and codes for a cluster of 39 genes (Hansen-Wester & Hensel, 2001). These range from components for a complete T3SS injectisome complex, effector proteins secreted by T3SS, chaperones associated with T3SS effectors and proteins that regulate the expression of T3SS effectors located on SPI-1 and those on genetic loci independent of SPI-1 (Hansen-Wester & Hensel, 2001; Zhang K. et al., 2018). Several roles have been assigned to the SPI-1 T3SS effectors and the most important of these is its

ability to mediate the invasion of host epithelial cells and phagocytes of the innate immune system. A combination of 10 T3SS effectors are injected directly into the cytoplasm of the host cell, triggering actin cytoskeletal rearrangement that causes membrane ruffling that leads to engulfment and internalization off bacterial cells into epithelial and phagocytic cells. This process along with pathogen associated molecular patterns (PAMPS) such as components of the bacterial flagella and cell wall trigger an inflammatory response in an NF-κB dependent manner (Lawrence et al., 2009). The resulting localized inflammation helps *Salmonella* thrive and outcompete gut microflora in several ways.

SPI-1 mediated recruitment of neutrophils to the site of infection results in damage to the gut microflora which would have otherwise competed for electron acceptors such as iron and substrates for fermentation and in the process provided colonization resistance (Sekiro et al., 2010; Chiu et al., 2017). Secondly inflammation damages the gut epithelium resulting in the generation of tetrathionate which can be selectively used by *Salmonella* as an electron acceptor and substrates such as mucin and phosphatidylinositol from the enterocytes that are broken off the microvilli (Winter et al., 2010; Thiennimitr et al., 2011). An important strategy employed by the mammalian innate immune system is the use of proteins such as lipocalins to target bacterial iron scavenging molecules known as siderophores. Most *Salmonella* serovars code for the iron gene cluster (*iroBCDE*) which encodes a lipocalin-resistant siderophore, yet another advantage in the inflamed gut (Fishbach et al., 2006). Enterocolitis results in diarrhea, which further helps with the

clearance of microflora and their preferred substrate and helps propagate NTS to new hosts.

Salmonella pathogenicity island 2 (SPI-2)

The term *Salmonella* pathogenicity island 2 (SPI-2) was first coined by Shea and colleagues in 1995. It was used to describe a segment of DNA 40kb in length located 30 centisomes on the chromosomal DNA. Mutations at this locus resulted in the generation of strains that require ID50 value 100 times greater than wild type Typhimurium to establish systemic infection in mice (Hensel et al., 1995, Shea et al., 1999). SPI-2 was found to have a net GC content of 44%, which is significantly lower than the 52.2% associated with the rest of the genome. SPI-2 also shares a sequence homology of around 50% with SP-1 (Shea et al., 1996). This supports the hypothesis that the entire pathogenicity island was acquired independently of SPI-1 through horizontal gene transfer (HGT) (Shea et al., 1996). It is postulated that SPI-2 was acquired in two phases. The first event involved the acquisition of one large segment of DNA comprising the genes coding for the T3SS apparatus, translocon proteins and the effector proteins SseF and SseG (Hensel et al., 1999). A second HGT event led to the acquisition of the rest of SPI-2, which includes genes that help *Salmonella* utilize tetrathionate (Hensel et al., 1999).

While SPI-1 is required for the invasion of host cells and triggers enterocolitis in the process, it is negligible for the establishment of extra-intestinal disease in mice (Galan & Curtiss, 1989). SPI-2 plays a role in the gastrointestinal phase and is essential for intracellular survival, growth and dissemination to distal sites (Hensel et al., 1995; Carnell et al., 2007). Invasion of *Salmonella* into host macrophages results in the formation of a

membrane bound compartment containing the bacteria called the *Salmonella* containing vacuole (SCV). Initially the SCV acidifies to a pH around 4-5 (Rathmann et al., 1996). This sudden drop in pH combined with scarce nutrients and other signals within the phagolysosomal compartment triggers the shutdown of SPI-1 gene expression and the activation of SPI-2 genes through the actions of two component regulatory systems OmpR-EnvZ and the SPI-2 coded SsrA-B (Feng et al., 2004; Garmendia et al., 2003). The newly assembled SPI-2 T3SS apparatus secretes a host of SPI-2 effector molecules directly across the SCV membrane. These effectors remodel the surface of the SCV and through their manipulation of the cytoskeleton prevent the fusion of the SCV to lysosomes, traffic nutrients to the SCV and facilitate the intracellular replication of *Salmonella* (Bakowski et al., 2008, Haraga et al., 2008).

Salmonella is known to secrete 48 effector proteins into the host cell. Of these 10 are exclusively secreted by SPI-1 T3SS, 23 by the SPI-2 coded T3SS and 10 by both systems (Kidwai et al., 2013). The remaining eight effectors are translocated into the host cell by outer membrane vesicles (Kidwai et al., 2013). Of the 33 effectors that are secreted by the SPI-2 T3SS, seven are consistently found in all *S. enterica* serovars and have thus been dubbed core effectors (Jennings et al., 2017). The distribution of the other effectors is limited by host range and the type of disease inflicted. For instance, a set of effectors termed accessory effectors that include SrfH and SpvC are coded for on mobile genetic elements or around remnants of mobile genetic elements that are distributed sporadically amongst various serovars.

The *spv* locus

The *spv* (*Salmonella* plasmid virulence) locus codes for the *spvABCD* operon and one upstream transcriptional regulator SpvR. The *spv* locus, though absent in most typhoidal strains is found in *S. enterica* subspecies I, II, IIIa, IV, and VII (Boyd & Hartl, 1998). In recent years a strong correlation has been established between the *spv* locus and iNTS serovars causing extraintestinal disease in immunocompromised individuals (Montenegro et al., 1991; Fierer et al., 1992; Guiney et al., 1995).

Despite its link to iNTS mediated disease, the molecular mechanisms or the functions of all four structural genes is yet to be fully elucidated. SpvR is a transcriptional activator that binds to inverted repeats upstream to *spv* structural genes (Krause et al., 1995; Grob et al., 1997). SpvC and SpvD are secreted by the T3SS coded in both pathogenicity islands, while SpvB is exclusively secreted by the SPI-2 T3SS (Kidwai et al., 2013). SpvA is predicted to be an outer membrane protein. Unfortunately, its contribution towards bacterial virulence has not been reproduced consistently and is the least understood of the *spv* proteins. SpvD, a cysteine hydrolase contributes towards the establishment of persistent infection in the Nramp1 positive 129/SvJ mice strain (Monack et al., 2004). Unfortunately, SpvD is not required for virulence in BALB/c mice strain that are acutely sensitive to *Salmonella* owing to the lack of Nramp1 (Matsui et al., 2001). However, SpvD was shown to contribute to an anti-inflammatory effect by downregulating the effects of p65-mediated transcription of inflammatory cytokines in Tlr-4 negative macrophages (Rolhion et al., 2016).

P65 belongs to NF-κB family of dimeric transcription factors that are involved in the upregulation of genes that have an influence on a broad range of processes including immune response, stress response, cell survival and development (Stewart., 2007). Usually found in an inactivated state bound to IκBα proteins that masks its nuclear localization sequence. Inflammatory cytokines or extracellular triggers of PAMPs such as bacterial LPS trigger a signaling cascade that results in the degradation of the IκBα protein attached to p65 (Stewart., 2007). These proteins then form a complex with adaptor proteins importin-α and importin-β, which facilitate transport of the transcription factor to the nucleus via the nuclear pore complex (Stewart., 2007). Once within the nucleus, p65 is released from the import complex with the interaction of importin β and importin α with RanGTP and a complex comprising RanGTP and β-karyopherin exportin-2 (Xpo2). The importin proteins are recycled back to the cytoplasm to facilitate another round of p65 import (Stewart., 2007). SpvD sequesters Xpo2, preventing the export of importin α back to the cytoplasm. This in turns reduces the amount of p65 imported into the nucleus, contributing towards the decrease of inflammatory cytokines (Rolhion et al., 2016).

Of the four structural proteins, SpvB and SpvC are the two *spv* effectors that have been consistently shown to be required for the bacterial virulence phenotype associated with the *spv* locus (Roudier et al., 1992). SpvB codes for a N-terminal domain (NTD) that is separated from the C-terminal domain (CTD) by a space of 7 proline residues. The NTD shares some sequence homology with *Photorhabdus Luminescens* Tc toxin, however its function is yet to be determined (Otto et al., 2000). The CTD has ADP ribosylase activity. At the molecular level SpvB covalently modifies G-actin monomers and abrogates the

formation of F-actin filaments in a CTD-dependent manner (Otto et al., 2000). The conversion of actin from monomer to filament and back again is a constantly occurring process. The loss of F-actin filament formation results in the loss of F-actin cytoskeleton (Libby et al., 2000, Lesnick et al., 2001; Browne et al., 2002). F-actin cytoskeleton acts as a support structure during the assembly of the integral membrane protein NADPH oxidase (Tamura et al., 2006). NADPH oxidase gene expression is upregulated by inflammatory cytokines and in phagocytic cells such as macrophages. This protein localizes to the phagolysosome where it catalyzes the conversion of oxygen to superoxide, which is a reactive oxygen species that contributes to oxidative killing (Vazquez-Torres et al., 2001). Loss of F-actin results in decreased recruitment of NADPH oxidase to the SCV and a loss in actin cytoskeleton. These factors combined with an increase in SpvB-mediated activation of Caspase-3 is believed to contribute towards the detachment and late stage apoptosis of macrophages (Libby et al., 2000; Lesnick et al., 2001; Browne et al., 2008; Guiney & Fierer, 2011).

SpvC codes for a phosphothreonine lyase that shares 63% amino acid sequence identity with *Shigella*-encoded T3SSE OspF (Li et al., 2007). It can be secreted by both the SPI-1 and SPI-2 T3SSs during the intestinal phase of infection; however, once engulfed by a phagocytic cell, it is secreted exclusively through the SPI-2 T3SS (Mazurkiewicz et al., 2008; Haneda et al., 2012). SpvC inactivates MAP kinases such as p38, ERK 1/2 and JNK through dephosphorylation of threonine residue from a TYX motif (Li et al., 2007; Mazurkiewicz et al., 2008). However, in rat caecum, the effects of the SpvC catalyzed phosphothreonine lyase activity could only be detected in ERK 1 and 2 (Haneda et al.,

2012). Loss of SpvC was also shown to result in increased production of proinflammatory cytokines such as TNFα, IL8, Kc, MCP1 and MIP2 which in turn resulted in a net reduction in the weight of the cecum resulting from submucosal edema, epithelial erosion, loss of goblet cells and an increased levels of invasion by neutrophil (Haneda et al., 2012). Activated MAP kinases trigger signaling cascades that result in the upregulation of several anti-apoptotic factors and proinflammatory cytokines and chemokines. By inactivating a subset of MAP kinases, SpvC contributes to an anti-inflammatory effect that mollifies the effects of inflammatory SPI-1 T3SS effectors such as SptP, AvrA and SspH1 that have evolved to establish an inflamed environment in the gut, which in turn helps *Salmonella* outcompete gut microbiota that offer colonization resistance (Fu & Galán, 1999; Murli et al., 2001). Haneda and colleagues believe this reduction in inflammation at the site of infection helps *spv* positive *Salmonella* to evade the initial immune response and help establish a systemic infection (Haneda et al., 2012).

How does *Salmonella* rapidly disseminate to distant tissue?

In mouse models, *Salmonella* acquired orally can enter the bloodstream in less than 30 minutes and establish colonies in the spleen and liver in less than 60 minutes (Vazquez-Torres et al., 1999, Spadoni et al., 2015). Three possible routes of entry have been postulated to explain this phenomenon. The commonly accepted mode of entry is through its invasion of specialized epithelial cells known as Microfold cells (M cells) that line the Peyer's patches of the distal ileum (Neutra et al., 1996). Most epithelial cells in the cecum are unfavorable towards the fimbriae-mediated attachment and subsequent invasion of *Salmonella* owing to a microvilli rich brush border covered in mucus and

individual cells separated through tight junctions. M cells are specialized cells that transport antigens through the process of transcytosis to be sampled by underlying phagocytic cells such as dendritic cells. As a result, they have a relatively flatter luminal surface with sparse microvilli, distinct glycocalyx and are surrounded by fewer mucus producing goblet cells. Once attached invasion of *Salmonella* occurs in a SPI-1 dependent manner. The bacterial cells are then picked up by phagocytic cells in the upper dome of the Peyer's patches and are eventually transported to secondary lymphoid organs such as mesenteric lymph nodes (MLN) for the presentation of antigen to T cells. In a SPI-2 dependent manner *Salmonella* can survive and replicate inside these phagocytic cells and eventually enter the bloodstream from MLNs through thoracic ducts. This is a process that relies largely on the passive dissemination of dendritic cells and is estimated to take 12-24 hours. Several observations prevent this model from being the most likely route of systemic *Salmonella* dissemination. Firstly, the removal of MLNs significantly increases bacterial burden and decreases the amount of time it takes for *Salmonella* to migrate to the spleen and liver (Voedish et al., 2009). This indicates that MLNs play an important role in containing the spread of *Salmonella*. While the presence of migratory dendritic cells is a factor that limits migration, the modulation of dendritic cell number and migratory kinetics within the lymphatic system does not prevent the establishment of *Salmonella* colonies in the liver and spleen (Vasques-Torres et al., 1999; Voedish et al., 2009). The loss of *Salmonella's* ability to invade M cells through SPI-1 T3SS secreted invasion genes or the complete lack of Peyer's patches does not diminish the ability of *Salmonella* to colonize the spleen and liver (Barnes et al., 2006; Coburn et al., 2005).

A second route of entry depends on the capacity of *Salmonella* to perturb β-catenin-dependent signaling in gut endothelial cells, disrupting a gut vascular barrier to gain access to the bloodstream through the portal vein (Spadoni et al., 2015). The authors suggest that blood vessels associated with the gut mucosa function similar to the blood-brain barrier and demonstrate its capacity to prevent the translocation of bacteria and solutes larger than 70kda. SPI-2 T3SS activity was correlated with the deactivation of the endothelial cell associated adherens junction protein β-catenin and the up-regulation of plasmalemma vesicle–associated protein-1 (PV-1). PV-1 is associated with increased permeability of the blood-brain barrier and this led the authors to conclude that *Salmonella* facilitates systemic spread by penetrating this gut vascular barrier through a yet undiscovered T3SS effector. The third pathway involves *Salmonella* ferried to the bloodstream in CD18+ phagocytic cells.

The rapid dissemination phenotype of *Salmonella* was shown to be dependent on the activity of both SPI-2 and the host CD18 protein (Vazquez-Torres et al., 1999; Worley et al., 2006). CD18 is a cell surface marker found on phagocytic cells such as macrophages, dendritic cells and neutrophils and the bulk of *Salmonella* isolated from systemic tissue have been found within macrophages (Mastreoni et al., 2009). This pathway to the bloodstream is not conventionally thought to enhance microbial virulence (Vazquez-Torres & Fang 2000; Vazquez-Torres et al., 1999). Rather it was proposed that this is a host-controlled process that takes *Salmonella* cells to the spleen, which filters the bloodstream, to engender a systemic immune response against the bacteria to combat subsequent, delayed invasion of deeper tissue through the lymphatic system (Vazquez-

Torres & Fang 2000, Vazquez-Torres et al., 1999). We have uncovered evidence however, which suggests that *Salmonella* can actively exploit reverse transmigration to bypass the lymphatic system, expediting its colonization of internal organs (Worley et al., 2006; Thornbrough & Worley, 2012). The reliance of *Salmonella* on its intracellular phase for systemic dissemination is further highlighted by the fact that mice are more susceptible to *Salmonella* infection when they are depleted of T cell producing inflammatory cytokines such as IFNγ than with B cells incapable of producing antibodies (Nanton et al., 2012). This has also been observed in humans were iNTS infections are prevalent in AIDS patients who are naturally depleted of CD4+ T cells. IFNγ triggers the IFNγR receptor on phagocytic cells and leads to the activation of JAK/STAT signaling that results in the regulation of antimicrobial molecules toxic to intracellular bacteria such as iNOS (Blanchette et al., 2003). We propose that *Salmonella* is trafficked to systemic tissue through phagocytic cells that bypass the lymphatic system and enter the blood stream immediately after uptake of *Salmonella* from the lumen of the small intestine. Owing to their distribution and migratory properties, dendritic cells are an excellent candidate to facilitate this process.

Dendritic cells play an important role in the mediation of innate, adaptive immunity and tolerogenic responses to self and oral antigens. The ability to freely migrate between their site of origin to disparate peripheral tissue such as the skin, lungs and intestines and several secondary lymphoid organs through the use of the circulatory and lymphatic system plays an important role in their ability to mediate immune responses. The classical dendritic cell was discovered in 1973 and is described to possess stellate morphology,

express copious amounts of MHC-II molecule on its surface and functions as an antigen presenting cell to T and B cells. However, there is a huge overlap in function, morphology and surface markers between classical dendritic cells and various other cells of the mononuclear phagocyte system (MPS) such as macrophages and monocytes. The plasticity of MPS cells, specifically monocytic lineages that can differentiate to dendritic cells is, largely affected by tissue type and inflammatory stimuli. For decades this has muddied what constitutes a dendritic cell and the trend in immunology seems to migrate towards classifying the various classical dendritic cell and dendritic cell like lineages based on ontogeny (Guilliams et al.,2014).

Dendritic cells are distributed throughout the gut mucosa and are not just restricted to Peyer's patches. Of interest are a subset of immature CD11c+ dendritic cells that migrate to the lamina propria. These cells can insert themselves between intestinal epithelial cells and have been shown to express a complement of tight junction proteins that help project dendrites into the lumen of the intestine and sample for antigens without disrupting the epithelia (Rescigno et al., 2001). Given the migratory nature of dendritic cells and the surface area covered by the gut epithelia, there is huge potential for dendritic cells to be infected by *Salmonella* either accidentally or in a process mediated by bacterial effectors. We hypothesize that the cellular machinery of infected dendritic cells gets reprogrammed by SPI-2 effectors to dissociate from the epithelia and reverse transmigrate directly into the bloodstream. This is partly supported by the fact that monocytes that differentiate to dendritic cells positive for CD11c and HLA-DR are capable of reverse transmigrating *in vitro* across primary blood vessel endothelial cell monolayers

but those that differentiate to macrophages are not (Randolph & furie 1996; Randolph et al.,1998).

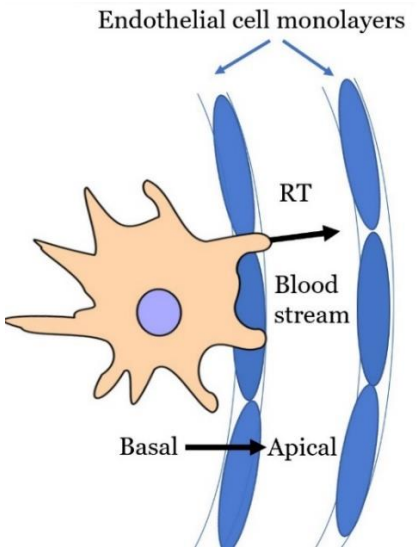

Figure 1.1 Reverse transmigration. Reverse transmigration is the movement of dendritic cells from the basal to the apical face of a vascular endothelial cell monolayer.

Reverse transmigration

Reverse transmigration, intravasation and reverse transendothelial migration are interchangeable terms used to describe the movement of leukocytes or metastatic cells across blood or lymphatic endothelia from the side facing the connective tissue known as the basal face into the lumen containing the apical face of endothelial cells. It is a process regularly associated with growth, development, inflammation and metastasis. In succeeding chapters of this work, we use the term reverse transmigration to describe the process of dendritic cells reentering the bloodstream by traversing endothelium in the basal to apical direction (Figure 1.1).

Reverse transmigration of dendritic cells

While an exact mechanism is yet to be determined, reverse transmigration of dendritic cells seems to involve three phases. The early stages of reverse transmigration are induced by cytokines such as tumor necrosis factor α (TNFα) that trigger the upregulation of chemokines, chemokine receptors and adhesion molecules on the surface of both endothelial cells and dendritic cells. Chemokines promote the migration of dendritic cells towards a vessel layered with endothelial cells. This is followed by binding of the dendritic cell to the basal face of the endothelium and the last phase involves invasion of the dendritic cell into the lumen of the endothelium in a paracellular manner. Throughout this process the integrity and structure of the endothelial vessel is maintained through the preservation of cellular junctions.

Several chemokines and their corresponding receptors expressed on the surface of dendritic cells and endothelial cells play an important role in the early stages of reverse transmigration. For leucocytes to reverse transmigrate, they need to initially migrate towards the blood or lymphatic vessel from deeper tissue. This important first step towards reverse transmigration is regulated by a well-studied chemokine-receptor pairing. Dendritic cells, T and B lymphocytes express several combinations of sphingosine-1 phosphate receptors (S1PR) on their cell surface that interact with the chemokine Sphingosine-1 phosphate (S1P). Owing to constant degradation, S1P concentration is lower in tissue compared to that of the blood stream. This results in a chemokine gradient towards the endothelial vessel, that contributes towards an S1P dependent migration and subsequent reverse transmigration of dendritic cells resident to the lamina propria of the

gut mucosa to the mesenteric lymph nodes (Rathinasamy et al., 2010). S1P aids in the motility of dendritic cells by activating the Rac/Cdc42 pathway that promotes the generation of filopodia and lamellipodia (Rathinasamy et al., 2010).

Activation of immature dendritic cells usually contributes towards the upregulation of the chemokine receptor CCR7 on its surface. CCR7 and its ligands play an important role in directing the migration of classical dendritic cell, monocyte derived dendritic cell and plasmacytoid dendritic cell lineages from the skin, lungs and intestines into afferent lymphatic vessels (Worbs et al., 2017). However, CCR7 also plays a role in the reverse transmigration of dendritic cells into the bloodstream (Roufeil et al., 2016). Myeloid cells with dendritic cell-like features local to the arterial intima were shown to reverse transmigrate into circulating blood in a CCL19-CCR7 dependent manner. CCL19 was secreted by intimal myeloid cells in response to systemic *Chlamydia* infection in mice, this chemokine activated CCR7 on the surface of other myeloid cells in the intima in an autocrine manner and resulted in their transmigration into the blood. This eventually contributed towards the clearing of the bacterial infection in the intima and highlights the importance of reverse transmigration with regards to the rapid mobilization of leukocytes associated with the innate immune system (Roufeil et al., 2016).

Several cell surface proteins have been shown to facilitate either the adhesion of dendritic cells to the endothelia or the process of invasion into the lumen of the endothelia or in some cases both. Tissue factor and CD99 are examples of proteins that only facilitate adhesion. CD99 is a ubiquitous membrane spanning type 1 glycoprotein

expressed on the surface of monocyte derived dendritic cells and either the cell junction or the luminal surface of endothelial cells (Mamadouh et al., 2009; Torzicky et al., 2012). A protein previously implicated in the process of diapedesis, Torzicky and colleagues speculate that CD99 might facilitate the adhesion of dendritic cells to endothelial cells through homotypic interactions. Tissue factor (TF) is an important component of the blood coagulation cascade and is expressed on the surface of mononuclear phagocytes such as dendritic cells. TF mediates the adhesion of mononuclear cells through an unidentified receptor on the basal surface of the endothelia and facilitates exit from the inflamed tissue through reverse transmigration (Randolph et al., 1998).

Several cellular adhesion molecules including vascular cell adhesion protein-1 (VCAM-1), L1 cell adhesion molecule (LICAM) and Intercellular adhesion molecule-1 (ICAM-1) play an important role in both adhesion and the reverse transmigration of dendritic cells across vascular endothelia. ICAM1 is one of six ligands to the integrin protein lymphocyte function-associated antigen-1 (LFA-1). Usually found on both apical and basal faces of endothelial cells, the interaction between ICAM-1 and LFA-1 contributes to the migration of lymphocytes and mononuclear cells from tissue to blood (Randolf & furie, 1996). ICAM-1 and VCAM-1 were shown to be upregulated on the surface of lymphatic endothelial cells in response to stimulation with inflammatory cytokines TNFα, TNFβ and IL1 and blocking of these CAMs resulted in a significant decrease in the cytokine mediated reverse transmigration of dendritic cells across endothelial cells (Johnson et al., 2006). LICAM is expressed on the surface of certain subsets of dendritic cells and on endothelial cells in response to inflammatory stimuli. LICAM is capable of engaging with other CAMs, growth

factor receptors and activating signaling cascades through their C-terminal tail. Homotypic interaction between LICAM molecules is postulated to facilitate the adhesion and invasion of dendritic cells across lymphatic and blood vessel endothelial cells (Maddaluno et al., 2009).

Other proteins associated with the regulation of reverse transmigration include MDR-1 and semphorin 3A. Antibody blocking of P-glycoprotein otherwise known as MDR-1, an ATP binding cassette transport protein associated with multiple drug resistance in cell culture and cancerous cells, prevented the migration of dendritic cells across HUVECs and dendritic cells and T lymphocytes from skin explants into lymphatic vessels (Randolph et al., 1998). A defect in the reverse transmigration of dendritic cells into draining lymph nodes was observed in double knockout mutations for the axonal guidance molecule semaphorin 3A and its receptor plexin A1. This led the authors to conclude that Semaphorin 3A might induce the reverse transmigration of dendritic cells through the induction of myosin II contractions at its trailing edge (Takamatsu et al., 2010).

Reverse transmigration in disease

Reverse transmigration of neutrophils

The process of inflammation can be equally damaging to both host tissue and pathogen. Inflammatory responses are usually resolved by reducing the activated state of lymphocytes such as T and B cells or through apoptosis and clearance by macrophages in the case of granulocytes. An exception to the latter seems to be neutrophils high in ICAM-1 and low in CXCR1. These cells are capable of dispersing from the site of inflammation through reverse transmigration into the bloodstream. It was shown *in vitro*

with HUVEC monolayers that ICAM-1 ^{high}CXCR1 ^{low} neutrophils could migrate in both directions. In the same study it was verified that neutrophils with this phenotype were found in relatively large numbers in the blood of patients suffering from chronic inflammatory conditions such as rheumatoid arthritis and atherosclerosis (Buckley et al., 2005).

Reverse transmigration of metastatic cancer

Reverse transmigration plays an important role in the dissemination of metastatic tumors to distal sites. Given the sheer diversity of metastatic cell types, several disparate mechanisms of reverse transmigration have been delineated. Some of the most noteworthy include the reliance on chemoattractants, creation of leaky blood vessels and the secretion of proteases that cleave endothelial cell junctions (Weis & Cheresh, 2011; Escribese et al., 2007; Kveiborg et al.,2008). Epidermal growth factor (EGF) secreted by endothelial cell associated macrophages serve as a chemoattractant that recruits cancerous cells. EGF also stimulates the formation of invadopodia through its activation of phosphatidylinositol 3-kinase (PI3K)-Akt cascade and its downstream effector proteins such as neural Wiskott–Aldrich syndrome protein (NWASP) and Ras homolog family member A (RhoA) that have been known to affect the migration of leukocytes (Roh-Johnson et al.,2014; Gligorijevic., et al., 2012).

Research goals

We hypothesize that one or several SPI-2 effectors could potentially reprogram gut epithelia associated dendritic cells to disassociate from the epithelium and migrate towards gut vasculature. In a potentially SPI-2 mediated process the dendritic cell with

intracellular *Salmonella* binds to the basal face of the blood vessel and reverse transmigrates into to the bloodstream and rapidly disseminates to systemic tissue such as the spleen and liver that are rich in nutrition and devoid of endogenous microflora. This allows *Salmonella* to set up persistent colonies that constantly shed bacteria into the gastrointestinal tract and feces through the lymphatic system before the infection is potentially cleared through the development of a pathogen specific adaptive immune response.

The goal of this project was to develop *in vitro* assays that would help delineate SPI-2 T3SS effectors and host factors involved in the SPI-2 dependent dissemination of *Salmonella* from the gut to systemic tissue in mice. Two assays were developed. Chronologically the first to be developed was the *in vitro* reverse transmigration assay to emulate the process of dendritic cells reverse transmigrating across an endothelial cell monolayer into the bloodstream. The second assay dubbed the *in vitro* dissociation assay was developed to model the process of dissociation of infected intraepithelial dendritic cells from the basal face of the gut epithelia.

CHAPTER 2

THE *SALMONELLA* TYPE III EFFECTOR SpvC TRIGGERS THE REVERSE TRANSMIGRATION OF INFECTED CELLS INTO THE BLOODSTREAM[1]

Introduction

A key component in the virulence of many successful pathogens is the ability to spread from the initial site of infection to deeper tissue. Following oral ingestion, *Salmonella* can disseminate from the gastro-intestinal (GI) tract to the blood and subsequently internal organs through three independent pathways. In what is believed to be the primary pathway, *Salmonella* as well as numerous other enteropathogens adhere to and invade the M cells of Peyer's patches and are subsequently internalized by the underlying phagocytes. The infected phagocytes can then migrate to the mesenteric lymph nodes, where they can orchestrate immune responses against the microbes. As *Salmonella*, like many pathogens, can withstand the microbicidal activities of these cells, the bacteria are conventionally thought to passively spread throughout the host after the infected cells drain from the mesenteric lymph nodes through the thoracic duct into the bloodstream. As ingrained as this model is, there is recent evidence, which indicates that the mesenteric lymph nodes act as a firewall, largely containing oral infections, allowing

[1] As appears in: Gopinath et al., 2019

for the generation of a local immune response, while shielding the host from systemic, microbial dissemination (Voedisch et al., 2009; Barnes et al., 2006). In fact, even though the availability of migratory dendritic cells is the rate-limiting step in mesenteric lymph node colonization, modulation of dendritic cell numbers or migratory properties within the lymphatic system does not affect colonization of the spleen and liver (Voedisch et al., 2009). Moreover, *Salmonella* and *Yersinia* colonize these tissues in mice that completely lack Peyer's patches with nearly identical kinetics as they do in congenic control mice (Barnes et al., 2006; Coburn et al., 2005). Thus, it is curious that passive, ordered dissemination through the lymphatic system to the bloodstream remains the prevailing model to explain the spread of enteropathogens to deeper tissue. In another recently described pathway, *Salmonella* perturbs β-catenin-dependent signaling in gut endothelial cells, disrupting a gut vascular barrier to gain access to the bloodstream (Spadoni et al., 2015).

In an alternative pathway, CD18 expressing phagocytes, presumably dendritic cells, can ferry *Salmonella* directly into the bloodstream, also bypassing the lymphatic system (Vazquez-Torres et al., 1999; Worley et al., 2006). These cells send processes across the epithelium to engage in intestinal antigen sampling (Rescigno et al., 2001). Normally, after microbe internalization, they presumably mature, become responsive to CCL19 and CCL21 via up-regulation of CCR7 and follow the chemotactic gradients into the lymphatic system. When *Salmonella* enters them however, the infected cells sometimes rapidly penetrate the bloodstream, through an as of yet, largely uncharacterized mechanism. Traversing the blood vascular endothelium in the basal to apical direction is referred to

as reverse transmigration. This pathway to the bloodstream is not conventionally thought to enhance microbial virulence (Vazquez-Torres & Fang 2000; Vazquez-Torres et al., 1999). Rather it was proposed that this is a host-controlled process that takes *Salmonella* cells to the spleen, which filters the bloodstream, to engender a systemic immune response against the bacteria to combat subsequent, delayed invasion of deeper tissue through the lymphatic system (Vazquez-Torres & Fang 2000; Vazquez-Torres et al., 1999). We have uncovered evidence however, which suggests that *Salmonella* can actively exploit reverse transmigration to bypass the lymphatic system, expediting its colonization of internal organs (Worley et al., 2006; Thornbrough & Worley, 2012).

Reverse transmigration is likely relevant to numerous infectious processes including the spread of pathogenic microbes from the GI tract, lung tissue and the oral mucosa to the systemic circulation. Here we report on our studies of how *Salmonella* manipulates reverse transmigration. *Salmonella* infection leads to millions of deaths world-wide every year (Pang et al., 1995).

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) causes gastrointestinal illness in humans, which often is not serious, but can be fatal in infants, the elderly and the immunocompromised. Also, *S.* Typhimurium can sometimes cause bacteremia and even septicemia in otherwise healthy individuals, which is a growing public health threat, especially in immunodeficient people, such as those infected with HIV. *Salmonella enterica* serovar Typhi (*S.* Typhi) on the other hand can cause typhoid fever, a serious systemic illness. S. Typhimurium causes a typhoid fever like disease in mice as S. Typhi does in humans. In addition to public health concerns, *Salmonella* is also studied because

it is a convenient model pathogen. *Salmonella* harbors two primary pathogenicity islands, termed *Salmonella* pathogenicity island one (SPI-1) and *Salmonella* pathogenicity island two (SPI-2). SPI-1 is used to invade host cells and invoke an inflammatory response and can kill host cells (Groisman et al., 1993; Galyov et al., 1997; Hobbie et al., 1997; Van der Velden et al., 2003). SPI-2 on the other hand promotes intracellular growth (Cirillo et al., 1998; Hensel et al., 1998; Ochman et al., 1996) and we have previously demonstrated that it is required for the rapid appearance of infected cells in the bloodstream following oral inoculation of mice (Worley et al., 2006). We showed that one allele of the SPI-2 associated type III effector SrfH/SseI accelerates the appearance of *Salmonella*-infected cells in the bloodstream potentially through an interaction with the host protein TRIP6 (Thornbrough & Worley, 2012).

Some *Salmonella* serovars carry plasmids, which share a highly conserved locus called the *spv* (*Salmonella* plasmid virulence) operon (Boyd et al., 1998). It has been suggested that *spv* genes are important for human pathogenesis as *spv*-carrying strains dominate among clinical isolates from patients with non-typhoidal bacteremia (Montenegro et al., 1991; Fierer et al., 1992). SpvC is a phosphothreonine lyase that dephosphorylates Erk1/2, p38 and JNK (Mazurkiewicz et al., 2008; Li et al., 2007). An *spvC* mutant is not defective in replication within macrophages but is attenuated in mice (Mazurkiewicz et al., 2008). Here, we describe the development of an *in vitro* reverse transmigration assay to model the rapid colonization of the bloodstream by *Salmonella*-infected cells following oral ingestion. We demonstrate that microbial components down regulate reverse transmigration and that *Salmonella* overcomes this by secreting SpvC and at least one

additional unknown SPI-2 associated type III effector into infected dendritic cells that stimulate reverse transmigration. These results suggest that the reverse transmigration pathway to the bloodstream could be an important component of *Salmonella* pathogenesis.

Results

Development of an *in vitro* reverse transmigration assay

We used blind wells to establish an *in vitro* reverse transmigration assay adapted from Bianchi et. al. (Bianchi et al., 2001) that models how dendritic cells can reenter the bloodstream in the basal to apical direction (Figure 2.1). Blind wells consist of two compartments that you can sandwich membranes in between. In our assay, C166 murine endothelial-like cells were grown into monolayers on PVP-free polycarbonate membranes with 5µm pores. The formation of confluent monolayers was confirmed with diff-kwik staining. One membrane was placed upside-down over the top of the bottom compartment of the blind well, which was filled with media. Another membrane with a monolayer of cells was stripped, revealing a natural extracellular matrix and was placed right side up on top of the first membrane. The device was then screwed together. Murine bone marrow-derived cells were differentiated into dendritic cells with GMCSF, IL-4 and TGF-ß. The cytokine treatment routinely produced a heterogenous population of cells of which 30%-40% were CD11c+, presumably a mixture of macrophages and dendritic cells. Dendritic cells are the only cell type capable of reverse transmigration (D'Amico et al., 1998; Randolph et al.,1998). After seven days of cytokine treatment, we added the cells

to the top compartment and after one hour of incubation, the device was disassembled and the liquid withdrawn from the bottom compartment and dendritic cells were counted with a hemocytometer. One hundred thousand cells were added to the top compartment and in 1 hour, about 20% had reverse transmigrated through the endothelial monolayer (Figure 2.2).

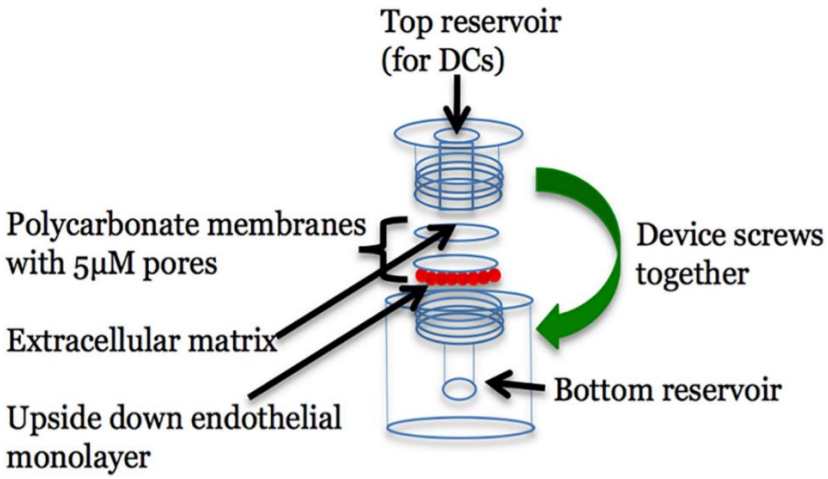

Figure 2.1. An *in vitro* reverse transmigration assay. In our *in vitro* reverse transmigration assay, two PVP-free polycarbonate membranes with 5μm pores are sandwiched in between the upper and lower compartments of a blind well. Media is placed in the bottom compartment and then the bottom membrane is placed over the liquid which contains an upside-down confluent monolayer of endothelial cells. The upper membrane is then placed on top of the bottom membrane right side up. It is coated with extracellular matrix. The device is then screwed together and dendritic cells are added to the upper compartment. The devices are incubated at 37˚C for 1 hour. Then the liquid is carefully aspirated from the top compartment, the device disassembled, and the membranes discarded. The media from the bottom compartment is withdrawn and reverse transmigration measured by either counting uninfected dendritic cells with a hemocytometer or lysing infected dendritic cells and recovering CFU on agar plates.

The presence of bacteria deters reverse transmigration

The rapid appearance of *Salmonella* in the bloodstream following oral ingestion was initially proposed to be passive on the part of the bacteria (Vazquez-Torres et al., 1999).

However, it seems as though the host would have a vested interest in denying bacteria access to the bloodstream and deeper tissue. Accordingly, we tested whether heat-killed *Salmonella* might deter the reverse transmigration of dendritic cells. We incubated cells with or without heat killed *Salmonella* for thirty minutes and then applied them to the endothelial monolayers in the blind wells. After one hour of incubation, we disassembled the blind wells and enumerated the number of dendritic cells that migrated through the endothelial monolayer into the bottom compartment with a hemocytometer. Treating the dendritic cells with dead *Salmonella* inhibited reverse transmigration five-fold (Figure 2.2).

SPI-2 stimulates reverse transmigration

We next tested whether or not live *Salmonella* could up-regulate reverse transmigration. In these experiments we infected dendritic cells with either wild type bacteria, a SPI-2 structural mutant that could not secrete any SPI-2 effectors, or one with a transposon disruption in *srfH*. We deleted *sipB* from all three strains as it was previously reported to kill dendritic cells (Van der Welden et al., 2003). In agreement with this report, in initial experiments using a wild type background, we observed that *Salmonella* killed some of the dendritic cells (unpublished observation). We chose to perform the experiments with strains lacking *sipB* simply to reduce the number of dendritic cells required for our assays. In subsequent mouse experiments, we did not find it necessary to use the *sipB* mutant background. The inclusion of gentamicin in our *in vitro* assay

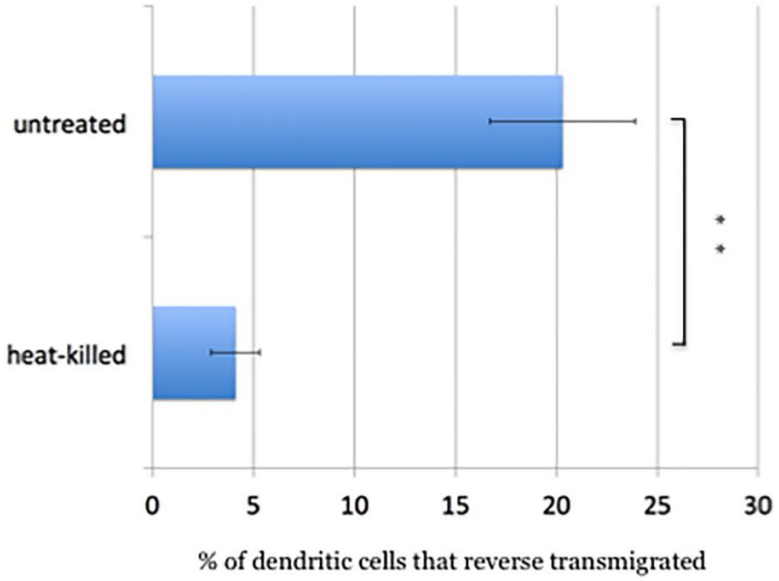

Figure 2.2. Bacterial components inhibit reverse transmigration. Dendritic cells were treated with either LB or LB containing heat-killed *S.* Typhimurium and the reverse transmigration assay performed. Heat-killed bacteria decreased reverse transmigration five-fold. This experiment was performed in triplicate on three different occasions. Error bars represent the standard deviation. ** p-value <0.01.

selectively killed the extracellular bacteria. We infected dendritic cells separately with the three strains and incubated them for six hours prior to performing the reverse transmigration component of the assay. While *in vivo*, SPI-2 associated genes can be expressed in as little as 15 minutes post-infection prior to penetrating the intestine, *in vitro*, in cell culture models of infection, it takes four hours for them to be induced and expression peaks at six hours post infection (Brown et al., 2005). Unlike the experiments with uninfected dendritic cells, we could not count the number of dendritic cells that reverse transmigrated with a hemocytometer because the majority of the cells were not infected and the uninfected ones would also reverse transmigrate and dilute the phenotypes. Accordingly, in order to specifically look at infected dendritic cells, we lysed

the dendritic cells with dilute detergent and recovered CFU on agar plates. We tried to detect reverse transmigrating cells infected with strains of bacteria expressing the green fluorescent protein but found it was not feasible to scale up to the point where we had enough reverse transmigrating cells to enter the linear range of a flow cytometer. CFU is actually more informative anyway since it readily distinguishes between live and dead bacteria. Prior to adding the infected dendritic cells to the blind wells, we lysed a small aliquot and plated for CFU to determine the input. After the reverse transmigration assay, we lysed the host cells present in the bottom compartment and similarly recovered CFU on agar plates. In control experiments we did survival assays on the three strains within dendritic cells for the entire length of the assay (including the one hour they were in the blind wells) and observed no differences (Figure 2.S1), indicating that differences in CFU recovered with the three strains was not due to differences in persistence.

We were unable to detect a defect in our reverse transmigration assay for the *srfH* mutant, which we previously reported has about a five-fold defect in penetrating the bloodstream of mice following oral infection (Worley et al., 2006) (Figure 2.3). In fact, the *srfH* mutant was more efficient than wild type at triggering reverse transmigration, although the difference was not statistically significant. We observed a very large 10.5-fold defect however in our reverse transmigration assay for the mutant that could not secrete any SPI-2 effectors (Figure 2.3). This result indicated that *Salmonella* actively manipulates reverse transmigration by secreting at least one type III effector into infected dendritic cells that stimulates the process. We cannot rigorously exclude the possibility that the monolayers lost confluence during the course of the assay as there is no way to

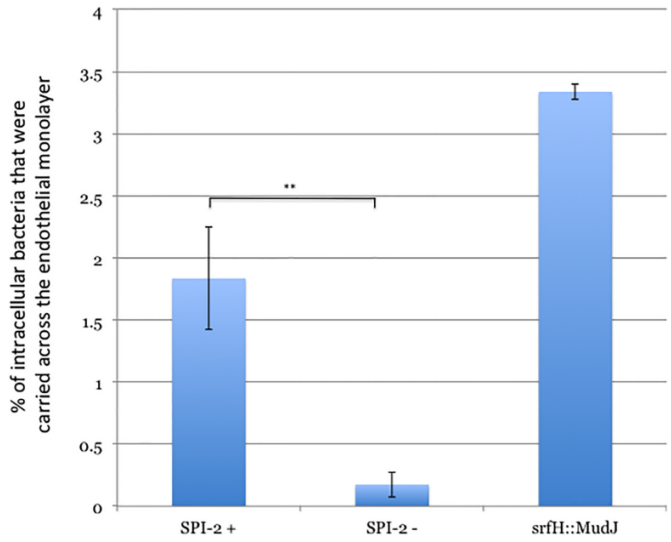

Figure 2.3. SPI-2 stimulates reverse transmigration. Dendritic cells were infected with either SPI-2 + or SPI-2 – bacteria or a *srfH* mutant. Surprisingly, the *srfH* mutant had no defect in triggering reverse transmigration and in fact was a little more efficient at it than wild type but the difference was not statistically significant. The strain defective in all SPI-2 secretion on the other hand triggered reverse transmigration over an order of magnitude less efficiently than wild type. This experiment was performed in quadruplicate on two independent occasions. Error bars depict the standard deviation. ** p-value <0.01.

get an electrode into the bottom compartment of an assembled blind well. However, this possibility seems very unlikely because if the dendritic cells were going through holes in the monolayer, you would expect them to go through at roughly the same rate, regardless of what strain of bacteria they were infected with. Regardless, we excluded the possibility that the dendritic cells were getting though holes in the monolayer with FITC-labeled dextran beads. In figure 2.S2, we demonstrate that greater than 99.9% of the beads are excluded from the bottom compartment of a blind well by the endothelial monolayers. Even though we killed the extracellular bacteria with gentamicin prior to adding the infected dendritic cells to the blind wells, as an additional control we incubated eight blind wells with 10,000 non-invasive *Escherichia coli* cells and observed that the endothelial

monolayers excluded greater than 99.9% of the bacteria from the bottom compartment. Cumulatively, it seems safe to conclude that the vast majority of the bacteria that we recovered from the bottom compartment of the blind wells in our *in vitro* reverse transmigration assays were carried there by reverse transmigrating dendritic cells. Even if a minority of the dendritic cells got through holes in the monolayer this would only cause us to underestimate the magnitude of our phenotypes and would not actually alter our conclusions since again, presumably dendritic cells would get through holes in the monolayer at the same rate regardless of what strain of bacteria they were infected with.

SpvC stimulates reverse transmigration *in vitro*

In order to detect a potentially subtle effect of SrfH on reverse transmigration, we developed a competition assay that took advantage of the fact that the *srfH* mutant as well as the other ones we tested are resistant to the antibiotic kanamycin. In these experiments, we infected cells separately with either wild type *Salmonella* or a mutant, killed the extracellular bacteria with gentamicin and combined them and plated an aliquot onto Lura-Bertani (LB) agar and onto LB agar supplemented with kanamycin to determine the input ratio. We then performed the reverse transmigration assay with the remainder of the mixture and plated the output onto LB agar and LB agar augmented with kanamycin. Surprisingly, even in this assay, we could detect no effect of SrfH on reverse transmigration (Figure 2.4). We next turned our attention to the *spv* operon, which was reported to be conserved amongst strains of non-typhoidal *Salmonella* that cause bloodstream infections of humans (Fierer et al., 1992). We first tested an in-frame

deletion of *spvB* as SpvB was reported to depolymerize actin (Lesnick et al., 2001; Otto et al., 2001; Tezcan-Merdol et al., 2001) and thus could potentially play a role in facilitating

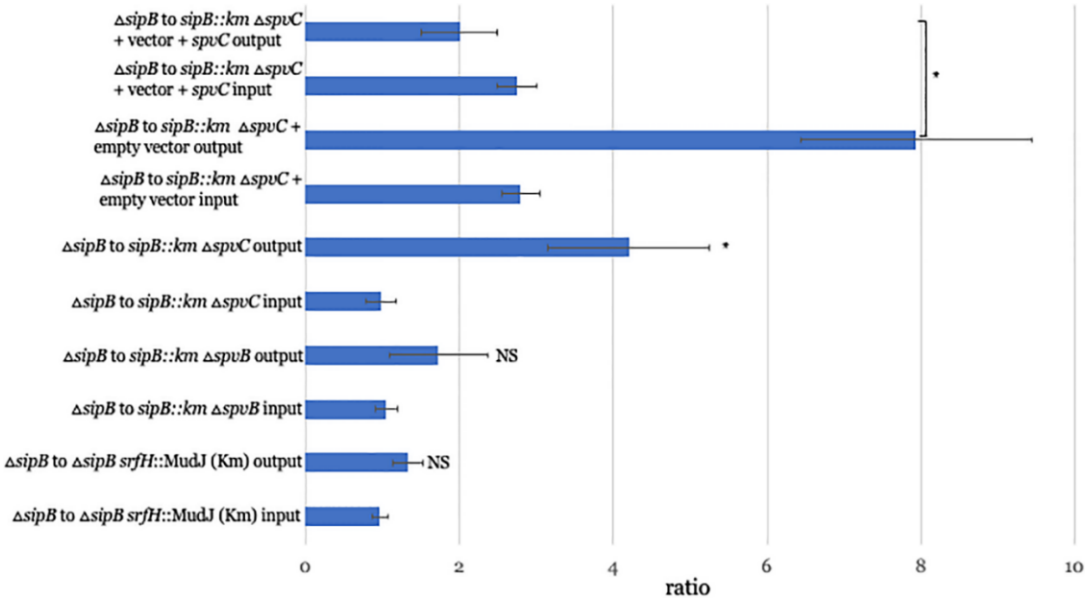

Figure 2.4. *spvC* triggers reverse transmigration in a competition assay. Dendritic cells were separately infected with wild type bacteria or a kanamycin resistant mutant. After six hours, the two populations of infected cells were combined and a small aliquot lysed and CFU recovered on LB versus LB kanamycin plates to determine the input ratio, which was always close to 1:1. The remainder of the mixture was added to the top compartment of a blind well and incubated. After one hour, the blind well was disassembled and the infected cells in the bottom compartment lysed and CFU recovered again on LB versus LB-kanamycin plates. In this competition assay, *srfH* and *spvB* had a negligible effect. *ΔspvC* however displayed a 4.2-fold defect. Five replicates of each competition assay were performed on four independent occasions. Error bars depict the standard error of the mean. * p-value <0.05.

reverse transmigration. Surprisingly, as with SrfH, SpvB had a negligible effect on reverse transmigration. We proceeded to test an in-frame deletion of *spvC*, as SpvC was reported to deactivate signal transduction pathways whose activation might discourage reverse transmigration. An *spvC* in-frame deletion mutant displayed a 4.2-fold defect versus wild

type in a reverse transmigration competition assay (Figure 2.4). We were able to complement the phenotype with ectopic expression of SpvC in the mutant background.

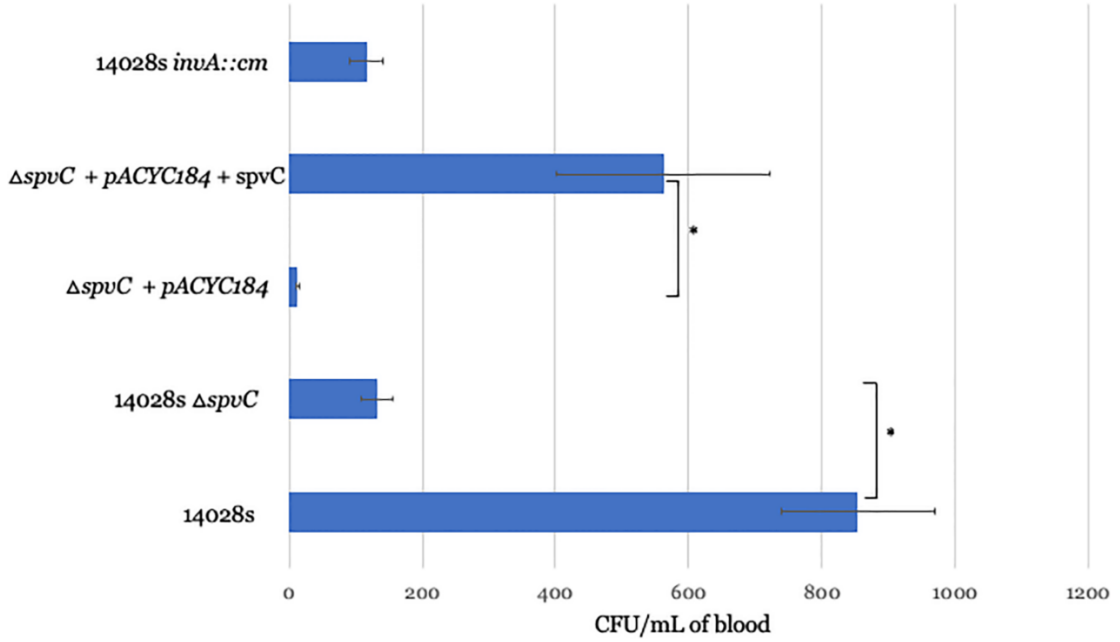

Figure 2.5. *spvC* triggers reverse transmigration in mice. Groups of 5–8 mice were orally inoculated by gavage with the indicated strains and peripheral blood recovered by heart puncture 30 minutes later. Δ*spvC* displayed a 6.5-fold defect versus wild type *S.* Typhimurium. The phenotype is complemented with ectopic *spvC* expression from a plasmid. In contrast, to the *in vitro* assay, in vivo, SPI-1 appears to enhance reverse transmigration, presumably by facilitating the invasion of the dendritic cells associated with the GI epithelium. These infections were performed on at least three independent occasions. Error bars depict the standard error of the mean. * p-value <0.05.

SpvC promotes early extraintestinal dissemination

We orally infected mice with either wild type *Salmonella* or a derivative that differed only in containing an in-frame deletion of *spvC* and thirty minutes later withdrew peripheral blood. We and others have previously shown that all bloodborne *Salmonella* at this time point are within CD18-expressing phagocytes. In fact, no *Salmonella* can be recovered from the bloodstream of CD18 deficient mice at 30 minutes post-infection

(Vazquez-Torres et al., 1999, Worley et al., 2006). CD18 is one of the host molecules required for reverse transmigration (D'Amico et al., 1998). We lysed the host cells with detergent and recovered *Salmonella* on XLD-agar plates. Consistent with *in vitro* experiments, we observed a 6.5-fold defect for a *spvC* deletion in early travel from the GI tract to the bloodstream (Figure 2.5). The phenotype is complemented with plasmid-borne expression of spvC in the mutant background (Figure 2.5). We also tested a SPI-1 mutant to explore how *Salmonella* overcomes SPI-1 mediated killing of dendritic cells. Surprisingly, impairing SPI-1 significantly reduced reverse transmigration suggesting that it functions differently *in vivo* than it does *in vitro*. Under our *in vitro* conditions SPI-1 seemed predisposed towards killing the dendritic cells and impairing reverse transmigration. *In vivo*, however, SPI-1 appears to enhance reverse transmigration, presumably by facilitating the invasion of the dendritic cells associated with the GI epithelium (Figure 2.5).

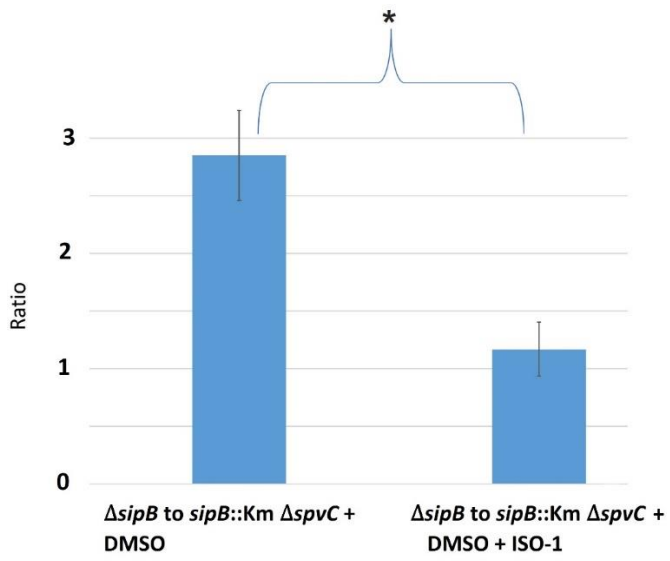

Figure 2.6. ISO-1 rescues *in vitro* reverse transmigration phenotype in Δ*spvC* mutant. Dendritic cells were separately infected with wild type *Salmonella* or a kanamycin resistant *spvC* mutant. After five hours of incubation half the dendritic cell population infected with each strain received 25mM ISO-1 and the remaining cells received DMSO. After a one-hour incubation at 37ºC, competition reverse transmigration assays were conducted. An equal number of wildtype and Δ*spvC* infected dendritic cells reverse transmigrated when treated with ISO-1 indicating a link between SPI-2 mediated reverse transmigration and the MMF induced pro-inflammatory signaling. Five replicates of each competition assay were performed on four independent occasions. Error bars depict the standard error of the mean. * p-value <0.05.

ISO-1 rescues *in vitro* reverse transmigration phenotype in Δ*spvC* mutant

SpvC functions as a phosphothreonine lyase that decreases MAP kinase signaling by preventing the activation of Erk1/2, JNK, and p38 kinases (Mazurkiewicz et al., 2008). In mice SpvC has been correlated with an anti-inflammatory effect at the site of infection in the colon through a reduced production of inflammatory cytokines such as TNFα (Haneda et al., 2012). Macrophage migration inhibitory factor (MIF) is expressed by a variety of eukaryotic cells including monocytes, macrophages and dendritic cells in response to

exposure to bacterial antigens and contributes towards the upregulation of inflammatory cytokines such as TNFα and IL-β1 in a CD74-dependent activation of ERK1/2 MAPK (Calandra et al., 1994). A cell permeable MIF antagonist (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1) was used to suppress the production of inflammatory cytokines and test if the anti-inflammatory nature of SpvC correlates with its capacity to stimulate reverse transmigration *in vitro*.

ISO-1 stock suspended in cell culture grade DMSO was diluted to 25µM in RPMI and added to the dendritic cell mixture infected with 14028s Δ*sipB* to *sipB*::*km* and 14028s Δ*sipB* to *sipB*::*km*, Δ*spvC* five hours post infection. The reverse transmigration assay was then finished as described previously. An equal number of wild type (14028s Δ*sipB* to *sipB*::*km*) and *spvC* mutant (14028s Δ*sipB* to *sipB*::*km*, Δ*spvC*) was recovered when dendritic cells received ISO-1 (figure 2.6). This indicates that there is a significant link between SpvC mediated reverse transmigration and the MIF mediated activation of ERK1/2 MAPK. The major downside to this approach was the three-fold decrease in the reverse transmigration phenotype observed in the control samples that received DMSO only. This has been attributed towards the DMSO being cytotoxic towards dendritic cells. One way this experiment could be improved upon is to directly target the activity of MIF or its receptor CD74 through the use of neutralizing monoclonal antibodies or SiRNAs. The results could also be verified *in vivo* through the oral inoculation of 14028s Δ*spvC* into MIF or CD74 knockout mice, both of which have been documented to grown into adulthood and available for purchase from retailers such as The Jackson Laboratory (ME, USA) (Bikoff et al., 1993; Bozza et al., 1999).

Discussion

This study identifies reverse transmigration as the likely process responsible for the rapid appearance of *Salmonella*-infected cells in the bloodstream of mice following oral infection that we previously observed (Worley et al., 2006; Thornbrough et al., 2012). Our results indicate that dendritic cells down regulate reverse transmigration in the presence of bacteria and that *Salmonella* in part overcomes this by secreting at least two SPI-2 effectors into infected cells that encourage reverse transmigration. Enhancing our understanding of reverse transmigration is medically important for a variety of reasons. This process likely plays a role in pathologic conditions including the invasion of the bloodstream by cancerous cells and the resolution of excessive inflammation in addition to the inadvertent dissemination of intracellular pathogens from infected tissue into the bloodstream. This inadvertent dissemination may not only be from the GI tract to the blood but may also play a role in numerous infectious processes including the spread of pathogens from lung tissue and the oral mucosa to the systemic circulation (Kawakami et al., 2002; Zeituni et al., 2010). The potential role of reverse transmigration in cancer and infectious diseases raises the possibility of designing drugs that inhibit the process. The corollary is that drugs, which augment the process, might be useful in cases of chronic inflammation such as autoimmune or graft versus host disease. Remarkably, for all the implications for human pathological processes, reverse transmigration is very poorly understood.

It is interesting to consider why *S.* Typhimurium seeds internal organs quickly. The speed with which the bacteria penetrate the liver and gallbladder of its animal reservoir

may be a component of its virulence. These are privileged sites of infection that are rich in nutrients and generally free of endogenous flora and thus can support the extensive replication of *Salmonella*, before the bacteria return to the GI tract through the lymphatics (connected to the liver) or the bile duct (connected to the gallbladder) for extra-host dissemination. The gallbladder is an extremely beneficial niche for *Salmonella* as 2–6% of the time, the bacteria become asymptomatic here and can potentially be shed intermittently for the lifetime of the host (Levine et al., 1982; Vogelsang & Boe 1948; Brooks 1996; Marr 1999). As *Salmonella* infections are often, however, ultimately resolved by an adaptive immune response (Vogelsang & Boe, 1948), the bacteria may be under time pressure to reach these organs as quickly as possible, and in essence, be in a race with the immune system. As penetrating the bloodstream through the lymphatic system can take days (MacPherson et al., 1995; Westermann et al., 1988), manipulating reverse transmigration may be a clever strategy through which *Salmonella* bypasses mesenteric lymph node confinement, accelerating its intra-host dissemination. This may increase the transmission rate by providing the bacteria with additional time to grow in their preferred sites of replication and also with more opportunities to establish a chronic, asymptomatic infection. SpvC is found in nontyphoidal strains of *Salmonella* and is likely a virulence factor in its animal reservoir where it does cause systemic disease. The fact that it can also rapidly colonize the bloodstream of humans where it does not typically cause systemic disease may be unintentional on the part of the bacteria.

The pathway is inefficient at 30 minutes post-infection anyway with only about one in a million inoculated bacteria being translocated from the GI tract to the bloodstream in

this timeframe. Reverse transmigration could still play an important role in *Salmonella* pathogenesis however as the bacteria presumably disseminate to the bloodstream from the GI tract through this pathway throughout the course of infection. Also, very few *S.* typhi founder cells are needed to seed the spleen and liver to cause problems as they can grow extensively there and any non-typhoidal *Salmonella* organisms in the bloodstream can potentially cause health problems. The fact that the *spv* operon is conserved among non-typhoidal *Salmonella* strains that cause bacteremia in humans further suggests that this pathway to the bloodstream could be important for understanding human infections.

It is interesting to consider potential molecular mechanisms underlying the ability of SpvC to promote reverse transmigration. SpvC deactivates, Erk1/2, p38 and JNK (Mazurkiewicz et al., 2008; Li et al., 2007). SpvC has been linked to having an anti-inflammatory effect in the cecum. This coupled will the increased reverse transmigration of the SpvC mutant infected dendritic cells associated with the suppression of MIF activity indicates that SpvC might act downstream of the MIF initiated signaling cascade (Haneda et al., 2012). Another possible mechanism is that targeting the JNK pathway abrogates endothelin signaling. Endothelin is a ligand produced by vascular endothelial cells that upon binding its G protein coupled receptor transduces a signal through the JNK pathway that discourages migration (Yamauchi et al., 2002; Kedzierski et al., 2001; Christiansen et al., 2000). Endothelial cells likely secrete endothelin to discourage metastasis and perhaps also to regulate reverse transmigration.

It was surprising that we did not observe a defect in reverse transmigration for dendritic cells infected with a *srfH* mutant, as we have shown in the past that *srfH* is

involved in the early penetration of the bloodstream by *Salmonella*-infected cells following oral inoculation of mice (Worley et al., 2006; Thornbrough & Worley, 2012). It is possible that SrfH is involved in a step prior to reverse transmigration. Perhaps SrfH is involved in disassociating the dendritic cells from the GI epithelium before the cells become available for reverse transmigration. It is also of course possible that our model does not capture everything that occurs during reverse transmigration *in vivo.*

Our results demonstrate that dendritic cells down regulate reverse transmigration in the presence of microbial components and that *Salmonella* in part overcomes this inhibition by secreting at least two SPI-2 effectors into them that stimulates the process by over an order of magnitude. This work provides some suggestive evidence that the CD18 expressing phagocyte pathway to the bloodstream involves reverse transmigration and that this may be an important component of the extraintestinal dissemination of *Salmonella***.** The model described here may be useful in studying the dissemination of other pathogens and could also be used for studying metastasis and excessive inflammatory disorders.

Materials and methods

Ethics statement

Animals were housed, cared for, and used strictly in accordance with the USDA regulations and the NIH guide for the care and use of laboratory animals (NIH publication no. 85–23, 1985). The University of Louisville is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. A full-time, specialty-trained

veterinarian directs the program of animal care. The protocol was approved by the University of Louisville Institutional Animal Care and Use Committee (protocol # 12–090). All reasonable efforts were made to alleviate discomfort.

Mice, cell culture and bacterial strains

Six to eight-week-old female C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Bone marrow was harvested, and monocytes cryopreserved as previously described (Marim et al., 2010). For extraintestinal dissemination assays, groups of 5–8, 4–6-week-old female C57BL/6J mice were orally infected by gavage with 1 X 10^9 bacterial cells suspended in 100µl of phosphate buffered saline (PBS). Food was withdrawn 12 hours prior to infection. Thirty minutes following infection, mice were euthanized with CO_2 and blood recovered by heart puncture with a 25G needle attached to a 1mL syringe. Blood was collected in microtubes on ice containing 50 units of heparin sodium salt (Sigma Aldrich) in 100µl of water to prevent coagulation.

Triton X-100 was added to a final concentration of 1% to lyse host cells and the tubes incubated at 4˚C with end over end rotation on a rotisserie for ten minutes. CFU were then recovered on xylose lysine deoxycholate agar plates, which are selective for *Salmonella*. C166 murine endothelial-like cells were cultured in DMEM (VWR) supplemented with 10% FBS (Sigma Aldrich) and sodium pyruvate (Life technologies) and passaged 1:10 every 4–5 days. Monocytes were cultured at a concentration of 1 X 10^6 cells/mL in RPMI supplemented with 10% FBS and sodium pyruvate. They were differentiated into dendritic cells by culturing them in the presence of GM-CSF (Life

technologies) (20ng/mL) and IL-4 (Life technologies) (20ng/ mL) for three days. The media also included 55µM ß-mercapoethanol (Life technologies). On the third day, an equal volume of media supplemented with fresh GMCSF (40ng/mL), IL-4 (40ng/mL) and 110µM ß-mercapoethanol was added. On the sixth day, TGF-ß1 (R&D systems) was added to a final concentration of 10ng/mL to induce expression of CD16, which is associated with an enhanced ability to reverse transmigrate (Randolph et al., 2002). Assays were performed 24 hours later. *S.* Typhimurium 14028s with an in-frame deletion of *sipB* described previously (Kidwai et al., 2013) was the parent strain for testing the effects of SPI-2 and *srfH*. We separately transduced with P22 HT int a *srfH*::MudJ allele (Worley et al., 2000) into this background and an *ssaK*::*km* allele (Geddes et al., 2005) with established techniques (Maloy et al., 1996). *ssaK* is part of an operon of structural genes that compose the type III secretion system. This mutant cannot secrete any effectors associated with SPI-2 (Geddes et al., 2005). Into previously described strains that separately contained in frame deletions of *spvB* and *spvC* (Kidwai et al., 2013), we transduced a previously described *sipB*::*km* allele (Kidwai et al., 2013). *spvC* was PCR amplified from the virulence plasmid of *S.* Typhimurium 14028s and cloned into the EcoRV and SalI sites of pACYC184, under the control of the constitutive tet promoter. The construct was sequence verified.

Growing endothelial cells on membranes

PVP-free polycarbonate membranes with 5µm pores were sterilized by autoclaving and individual membranes were submerged in 1mL of PBS (Life technologies) supplemented with 8µg of fibronectin from bovine plasma (Sigma Aldrich) in 24 well plates. The membranes were coated with fibronectin overnight at 4˚C. The following day,

the fibronectin solution was aspirated from the wells and 5 X 10^5 endothelial cells were added to each well. The endothelial cells were given 5 days to form confluent monolayers. Twenty-four hours before assays began, endothelial cell media was replaced with fresh media supplemented with TNFα (Life technologies) (20ng/mL). The formation of confluent monolayers under these conditions was confirmed with diff-kwik staining.

Infections and individual reverse transmigration assays

The three bacterial strains were grown overnight at 37˚C in LB. Approximately 1 X 10^6 dendritic cells were infected at an MOI of 25 in quadruplicate with the three bacterial strains. The bacteria were given one hour to invade. Under the conditions used, 1–2% of the bacteria were internalized. Next, gentamicin (Life technologies) was added to a final concentration of 100µg/ mL to selectively kill the extracellular bacteria and the cells were incubated for one hour at 37˚C. After the one-hour incubation the mixture was dialyzed against PBS with Slidalyzer mini dialysis devices with a 2kDa molecular weight cutoff (Thermo Fischer). Following dialysis, the mixture was incubated in media supplemented with 10µg/mL gentamicin for five hours at 37˚C. After five hours, the mixture was again dialyzed against PBS and a small aliquot lysed with 1% triton X-100 for ten minutes and CFU recovered on agar plates to determine the input. Two-hundred microliters of media containing gentamicin was added to the bottom compartment of the blind wells. Then, a membrane coated with a confluent monolayer of endothelial cells was placed upside down in the device. A second membrane was then stripped by dipping it into a solution of PBS containing 0.5% triton X-100 and 20mM ammonium hydroxide (Sigma Aldrich) for 30 seconds and then rinsed in DMEM, revealing a natural extracellular matrix (Bianchi et

al., 2001). This membrane was placed right side up on top of the first membrane. The device was then screwed together, and the infected dendritic cells added to the top compartment. The blind wells were incubated at 37˚C for one hour. Then, the liquid in the upper compartment was aspirated, the devices were disassembled, the membranes discarded and the media in the bottom compartment withdrawn and dialyzed against PBS as described for the input. The dendritic cells were then lysed in 1% triton X-100 and CFU recovered on agar plates.

Reverse transmigration assay with heat killed bacteria

In this experiment, an equal volume of either LB or a saturated LB overnight culture of *S.* Typhimurium was diluted 100-fold in cell culture media and heated to 95˚C for 15 minutes. 1 X 10^5 dendritic cells were then resuspended in either the LB or the heat-killed *Salmonella* and incubated at 37˚C for 30 minutes. The dendritic cells were then counted on a hemocytometer to determine the input and applied to the blind wells. They were processed as described above except instead of recovering CFU, the number of dendritic cells that traversed the endothelial monolayer was determined by concentrating the media in the bottom compartment and counting the cells with a hemocytometer.

Competition assays

One million dendritic cells were infected with either wild type bacteria or a *srfH*, *spvB*, or *spvC* mutant. The infections were processed as described for the individual assays. After the five-hour incubation, the wild type bacteria and one of the mutants were combined and a small aliquot lysed and CFU recovered on LB-agar plates or LB-agar plates

supplemented with 60µg/mL kanamycin to determine the input ratio. The reverse transmigration assay was then performed with the remainder of the mixture as described for the individual assays except after lysis of the cells in the bottom compartment, CFU were recovered on LB-agar plates or LB-agar plates supplemented with kanamycin. The number of CFU present on kanamycin plates was subtracted from the number present on LB plates to determine how many wild type bacteria were present. The CFU counts of the kanamycin plates revealed how many mutant bacteria were present.

Competition assays with ISO-1

A 100mM stock of (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1) purchased from TOCRIS (Minneapolis, MN) was prepared in cell culture grade DMSO (VWR). One million dendritic cells were infected with either wildtype or *spvC* mutant. Five hours post invasion, ISO-1 stock solution was serially diluted in complete RPMI and added to the dendritic cell mixture at a final concentration of 25µM. The reverse transmigration assay was then performed as described for the competition assay.

Survival assay

In this experiment, 1.7 X 10^5 dendritic cells were infected at an MOI of 25 with the three bacterial strains and a gentamicin protection assay performed. The number of intracellular CFU at seven hours post-infection was determined by dialyzing the gentamicin and lysing the dendritic cells with triton X-100 and recovering bacteria on agar plates.

Supporting information

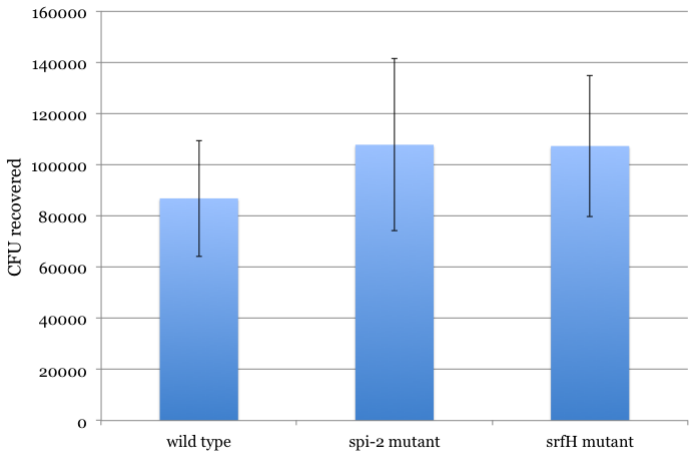

Figure 2.S1. The three strains survive similarly within dendritic cells. Dendritic cells were infected separately with the three strains and a gentamicin protection assay performed. This assay was performed in triplicate on two independent occasions. There was no significant difference in the number of bacteria present with the different strains at seven hours post-infection.

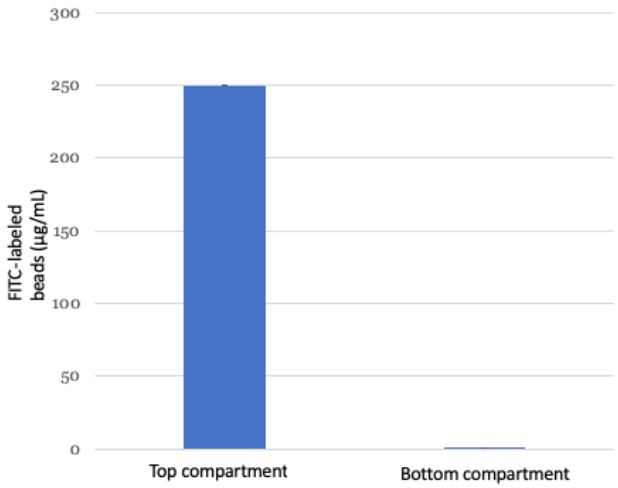

Figure 2.S2. Monolayers exclude FITC beads. On three occasions, blind wells with endothelial monolayers were incubated for one hour with 250µg/mL of FITC-labeled dextran beads (MW 70,000) and the fluorescence of the bottom compartment determined. The monolayers excluded greater than 99.9% of the beads.

CHAPTER 3

THE *SALMONELLA* TYPE III EFFECTOR SrfH TRIGGERS THE DISSOCIATION OF INFECTED INTRAEPITHEIAL DENDRITIC CELLS

Introduction

Salmonella enterica strains cause a wide range of illnesses in humans than range from acute self-limiting gastroenteritis to long term systemic infections such as typhoid fever and invasive nontyphoidal salmonellosis that result in the death of millions of people around the world. Key aspects of its virulence are regulated by effector proteins secreted by type III secretion systems (T3SS) encoded by pathogenicity islands 1 (SPI-1) and 2 (SPI-2). In brief SPI-1 mediates the induction of localized inflammation in the small intestine and helps *Salmonella* out compete local microflora. SPI-2 on the other hand plays an important role in the intracellular survival and systemic dissemination of the bacteria.

SrfH is a SPI-2 T3SS effector found in the Gifsy-2 prophage of *S.* Typhimurium (Figueroa-Bossi & Bossi, 1999). Once translocated into the host cell SrfH is palmitoylated at its cysteine 9 residue by host lipid transferases and localizes to the plasma membrane (Hicks et al., 2011). The SrfH C-terminal domain exhibits deamidase activity and is a key determinant of its virulence. SrfH was shown *in vitro* to deamidate the 205th glutamine residue of the α-subunit of hetero-trimeric G_{i1-3} proteins. This results in the inhibition of the GTPase activity of the α-subunit and the release of the β and γ subunits that then

contribute to a decrease in cAMP levels and the activation of PI3 kinase (Brink et al., 2018).

Loss of *srfH* severely restricts the SPI-2 mediated rapid dissemination of *Salmonella* within CD18+ phagocytic cells from the gut to the bloodstream in C57BL/6 mice (Worley et al., 2006). However, the effects of SrfH in an *in vitro* reverse transmigration assay that studied the movement of *Salmonella* infected dendritic cells from the basal to the apical face of an endothelial cell monolayer was negligible (Gopinath et al., 2019). The rapid dissemination of *Salmonella* from the lumen of the gut to systemic sites such as the spleen and liver is a complex process and SrfH could easily play an important role in a step that is yet to be characterized *in vitro*.

Dendritic cells are a subset of CD18+ phagocytic cells that serve as sentinels in tissue that form an interface with the external environment. They play an important role in mediating innate and adaptive immune responses and tolerogenic responses to self and oral antigens. Dendritic cells are distributed throughout the gut mucosa. Of interest to this study are a subset of immature dendritic cells that migrate to the lamina propria. These cells can insert themselves between intestinal epithelial cells and have been shown to express a complement of tight junction proteins that help project dendrites into the lumen of the intestine and sample for antigens without disrupting the gut epithelia (Rescigno et al., 2001). In this study we demonstrate that SrfH plays an important role in the dissociation of CD11c+ dendritic cells associated with the gut epithelia and that this process is dependent on the presence of a glycine residue at the 103rd position of the SrfH amino acid sequence. Dissociation could be the first step in the process of early extra-

intestinal dissemination of *Salmonella* were an intraepithelial dendritic cell infected with *Salmonella* separates from the gut mucosa and eventually migrates towards a blood vessel and reverse transmigrates into the bloodstream.

Results

Development of an *in vitro* competition dissociation assay

The *in vitro* dissociation assay was developed based on an experiment designed to demonstrate the capacity of CD11c+ immature intraepithelial dendritic cells to sample for antigens in the lumen of the small intestine without disruption of the epithelial monolayer (Rescigno et al., 2001). Corning Costar tissue culture inserts suspended in standard 24 well tissue culture plates were used to simulate the environment surrounding the epithelial cell monolayer of the gut mucosa (Figure 3.1). An insert with a fully developed monolayer acts as a barrier that separates the contents of the 24 well plate from that of the insert. The goal of this assay was to compare the relative effects of two competing *Salmonella* strains on the apical side of an epithelial cell monolayer on the dissociation of dendritic cells tightly bound to the basal face of the monolayer.

Upside down inserts were seeded with human colorectal carcinoma cell line Caco2 and transferred to a 24 well tissue culture plate with complete DMEM after a day of incubation. This allowed the cells to adhere to the bottom of the insert and let the apical face of the developing Caco2 monolayer face the media aliquoted into the 24 well plate. Confluency of the slow growing monolayer was determined through periodic Transepithelial electrical resistance (TEER) measurements. After a period of 11-15 days,

monolayers exhibiting a target resistance of 330ohms/cm^2 were deemed confluent and selected for use in the assay (Rescigno et al., 2001).

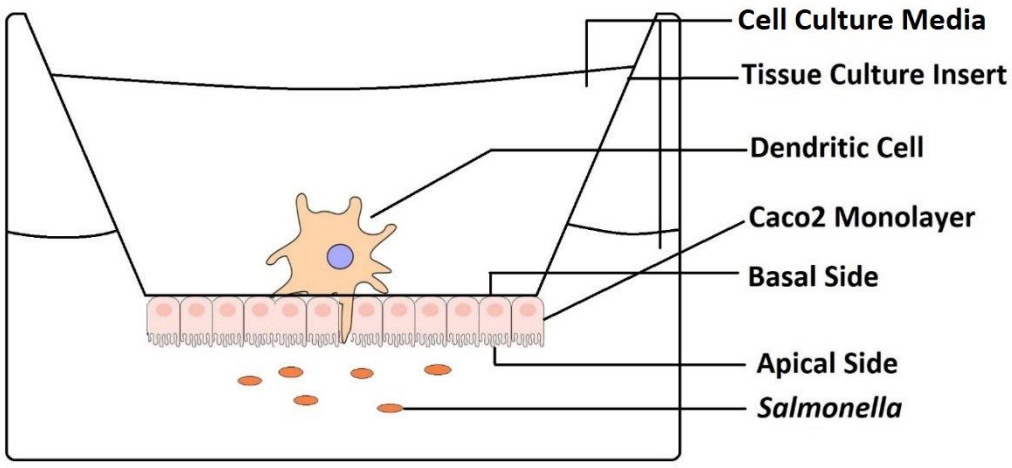

Figure 3.1. **Competition *in vitro* dissociation assay**. Caco2 epithelial cell monolayers were cultured on the bottom face of 3µm pore sized cell culture inserts. Primary dendritic cells were added to the basal face of this monolayer four hours prior to the start of the assay. The inserts were washed thoroughly to remove cells that did not bind and were transferred to 24 well plates that contained 10^8 CFU of two separate *Salmonella* strains suspended in cell culture media per well. The plates were incubated for an hour at 37^{o}C and dendritic cells that dissociated were recovered through gentle wash steps. The effects of T3SS effectors on dissociation was quantified through the recovery of *Salmonella* CFU on LB-agar and LB-agar plates supplemented with antibiotics.

On the day of the assay the media in the inserts was replaced with 400,000 immature CD11c+ dendritic cells that were derived from the treatment of mice bone marrow derived monocytes with recombinant mouse GMCSF and IL4 for a week. After a period of four hours the cells that did not bind to the monolayer were removed through rinsing of the inserts with cell culture media. The bottom compartment was replaced with DMEM resuspended with 10^8 CFUs of two *Salmonella* strains in triplicate. Prior to this step the *Salmonella* were incubated for one hour in MGM media at pH 7. MGM media induces the

expression of SPI-2 by simulating the milieu inside the SCV and is generally poor in divalent cations and nutrients (Deiwick et al., 1999). The 24 well plates were incubated in a water-jacketed cell culture incubator for an hour. At the end of the incubation period the inserts were rinsed with extreme caution to only pick dendritic cells that had dissociated naturally and not disturb cells that were still bound to the monolayer. The supernatant was treated with dilute detergent that selectively lysed dendritic cells and this was followed by plating an equal volume of the lysate on LB agar and LB agar plates supplemented with a strain specific antibiotic to acquire *Salmonella* colony forming units (CFU). Transepithelial electrical resistance (TEER) measurements were recorded in between every wash step to determine if the integrity of the Caco2 monolayer was maintained.

The amount of *Salmonella* CFU recovered is directly proportional to the amount of dendritic cells that dissociated. This was demonstrated by the control for this assay which comprised of cell culture inserts with confluent Caco2 monolayers that did not receive dendritic cells but were otherwise subject to the same conditions and placed in wells containing two *Salmonella* strains, one of which was the highly virulent *S.* Typhimurium strain 14028s. No *Salmonella* CFUs were recovered from the supernatant isolated from these inserts after the final set of washes (Figure 3.2). The gut epithelia is a natural barrier that contains the spread of gut microflora. Caco2 monolayers fulfilled this role and with no dendritic cells present to sample for bacteria through the projection of dendrites across the monolayer, *Salmonella* could not breach the barrier set by the Caco2 cells.

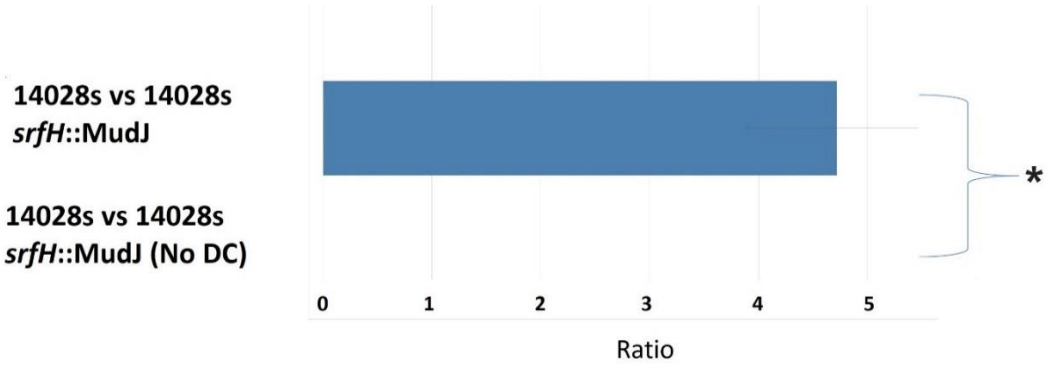

Figure 3.2. SrfH stimulates the dissociation of intraepithelial dendritic cells. *In vitro* competition dissociation assays were performed with wild type and *srfH* mutant in the presence and absence of dendritic cells on the basal face of the Caco2 epithelium. *Salmonella* could not breach the epithelium in the absence of dendritic cells. Four times as many wild type bacteria were shuttled across the epithelium by dendritic cells relative to the *srfH* mutant. This experiment had three replicates and was repeated on four independent occasions. Error bars represent standard error of mean, * indicates a p value <0.05 for the Student's t-test.

SrfH stimulates the dissociation of intraepithelial dendritic cells

Orally inoculated *Salmonella* disseminates to the blood of C57BL/6 mice within host CD18+ phagocytic cells in less than 30 minutes in a SPI-2 dependent manner (Worley et al., 2006). In the same study it was demonstrated that the SPI-2 T3SS effector SrfH was shown to play a huge role in the extraintestinal dissemination of *Salmonella* in mice. However, SrfH failed to stimulate the reverse transmigration of CD11c+ dendritic cell in our *in vitro* reverse transmigration assay (Gopinath et al., 2019). Hence, we tested to see if SrfH contributed to the process of reverse transmigration by stimulating the dissociation of intraepithelial dendritic cells sampling for antigens across the Caco2 monolayer. The expression of *srfH* was abrogated through transposon disruption with the bacteriophage p22HT *int* in 14028s. 14028s is a highly virulent strain of *S. enterica*

Typhimurium that causes a typhoid fever like disease in mice and carries the genetic material for all known T3SS effectors. A competition *in vitro* dissociation assay was performed in triplicate where 24 well plates were replaced with DMEM containing 10^8 CFU each of 14028s (wild type) and the *srfH* mutant (Figure 3.2). The loss of *srfH* contributed towards a 4.5 fold decrease in the amount of *Salmonella* CFU recovered compared to wild type.

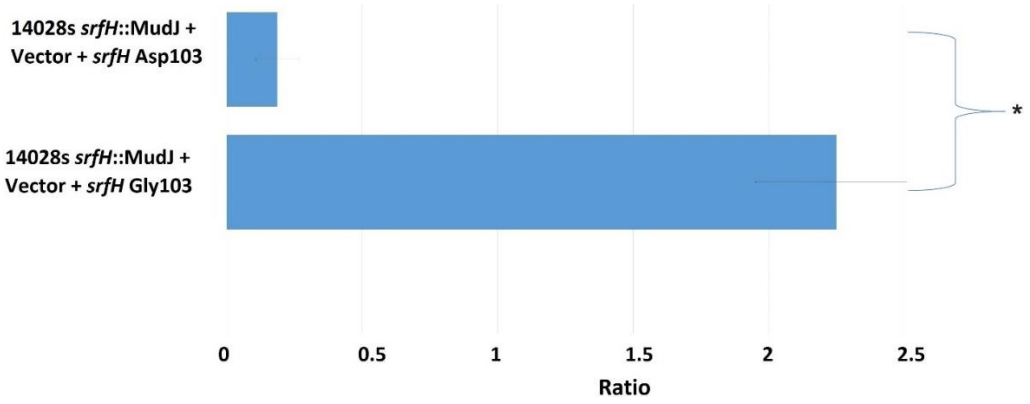

Figure 3.3. SrfH Gly103 promotes dissociation of dendritic cells while SrfH Asp103 has the opposite effect. The *srfH* mutant was complemented with a PCR amplified copy of the *srfH* allele from 14028s (Gly103) or SL1344 (Asp103). The *srfH* complemented strains were then used in *in vitro* competition dissociation assays against the *srfH* mutant parent strain. Complementation of *srfH* Gly103 resulted in the dissociation of twice as many dendritic cells relative to the *srfH* mutant, on the other hand dendritic cells picking up *Salmonella* secreting SrfH Asp103 exhibited a fourfold reduction in dissociation. This experiment had three replicates repeated on four independent occasions. Error bars represent standard error of mean, * indicates a p value <0.05 for the Student's t-test.

SrfH Gly103 promotes dissociation of dendritic cells

In their original work Rescigno and colleagues used *S.* Typhimurium strain SL 7207 (Rescigno et al., 2001). When compared to the non-virulent intestinal commensal

Streptococcus Gordonii, SL 7207 had a negligible effect on the dissociation of intraepithelial dendritic cells (Rescigno et al., 2001). SL 7207 is a derivative of the commonly used lab strain SL 1344 that is auxotrophic for ρ-aminobenzoic acid and 2,3-dihydroxybenzoate resulting from a 1kb deletion in *aroA* (Denich et al., 1993). Despite having a reduced capacity to replicate, SL 7207 retains SL 1344's capacity to colonize the gut mucosa (Rescigno et al.,2001). When compared to 14028s, SL 1344 carries a *srfH* allele with a SNP at position 103 of its amino acid sequence. The presence of either a glycine or an aspartic acid residue at this position was shown to affect the rapid dissemination of *Salmonella* into the bloodstream of C57BL/6 mice (Thornborough & Worley, 2012). SrfH Gly103 was shown to promote this process while SrfH Asp 103 inhibited it. (Thornborough & Worley, 2012). We performed the competition *in vitro* dissociation assay to determine if this SNP influenced the dissociation of intraepithelial dendritic cells (Figure 3.3). To achieve this, we complemented the 14028s *srfH* mutant with PCR amplified copies of each *srfH* allele cloned into a pACYC184 back bone. Dissociation assays comparing the effects of the *srfH* mutant with that of a similar strain complemented with either *srfH* Gly 103 or *srfH* Asp103 allele were performed in triplicate. *srfH* Gly103 contributed towards a two-fold increase in dissociation relative to the *srfH* mutant. This reduction in phenotype compared to the wildtype 14028s strain could be a result of the plasmid vector having a metabolic burden and hence being toxic towards the bacterial strain. SrfH Asp103 had an adverse effect on the dissociation of CD11c+ dendritic cells. Four times as many of the *srfH* mutant infected dendritic cells dissociated compared to the ones infected with the same strain complemented with the *srfH* Asp103 allele.

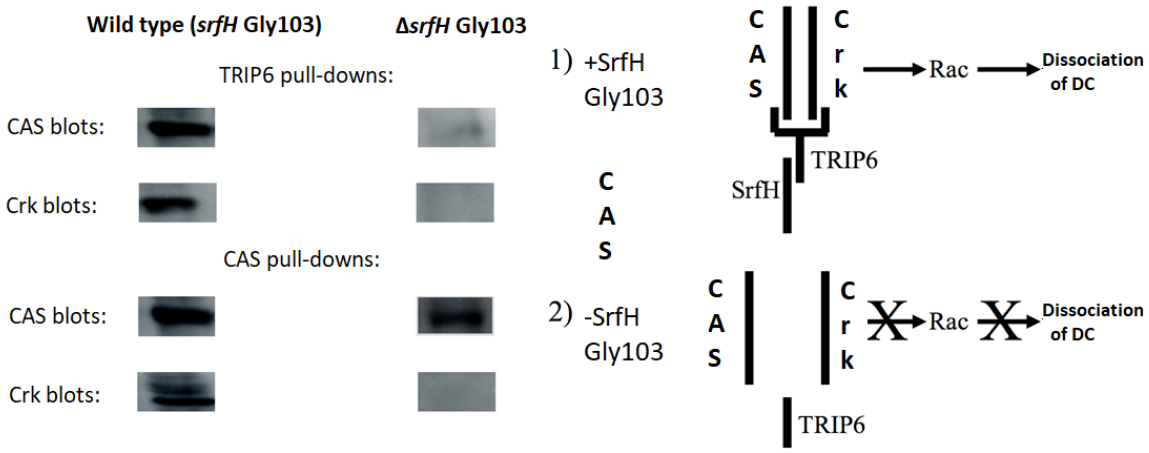

Figure 3.4. **TRIP6 recruits CAS and Crk proteins in the presence of SrfH**. Macrophages were infected with wild type *Salmonella* positive for *srfH* Gly103 or a *srfH* mutant. The cells were lysed, and the lysate was subjected to pull down assays with either TRIP6 or CAS antibodies. This was followed by western blotting with either CAS or Crk antibody. Crk was not detected when SrfH was not produced by *Salmonella*. On the other hand, the blots were positive for CAS and Crk in the presence of SrfH Gly103. This indicates that CAS and Crk proteins are downstream of TRIP6 in the SrfH mediated dissociation of dendritic cells.

TRIP6 recruits CAS and Crk proteins in the presence of SrfH

SrfH Gly103 was shown to bind to the adaptor protein TRIP6 and colocalize with it to focal adhesions (Worley 2006; Thornborough & Worley, 2012). Pull down assays indicate that TRIP6 recruits $p130^{cas}$ (CAS) and Crk proteins in the presence of SrfH (Figure 3.4). In brief, the lysate from macrophages infected with 14028s wild type bacteria or the *srfH* mutant were subjected to affinity purification with either TRIP6 or CAS antibodies. Pull down was followed by western blots with CAS or Crk antibodies. The TRIP6 pull down for wild type infected macrophages gave a strong signal for both CAS and Crk blots but was negative for both proteins in the case of *srfH* Gly103 mutant. A similar result was observed for the CAS pull downs, the only exception here being that the SrfH mutant was positive

for CAS while negative for Crk. This indicates that TRIP6 promotes CAS-Crk coupling in the presence of SrfH Gly103.

TRIP6 belongs to the zyxin family of adaptor proteins that contain a proline rich N-terminal domain and three LIM binding domains towards their C-terminal. TRIP6 localizes to the plasma membrane at focal adhesion sites in a lysophosphatidic acid (LPA) dependent manner and participates in cellular signaling that promotes adhesion and motility. SrfH has been previously shown to localize to the plasma membrane after host cell mediated palmitoylation of its cysteine 9 residue (Hicks et al., 2011). We hypothesize that SrfH Gly103 acts as a molecular tether that binds TRIP6 and recruits a complex that drives pro-migration signaling from the focal adhesion (Figure 3.5). As SrfH Gly103 is secreted into the dendritic cell, it binds TRIP6. Palmitoylation of SrfH tethers the TRIP6-SrfH complex to the host cell's focal adhesion. TRIP6 serves as a scaffold that recruits the adaptor protein CAS and the regulatory protein Crk. CAS and Crk are abundant in focal adhesion sites and this CAS-Crk coupling has been previously associated with stimulation of motility through the activation of the RAC signaling pathway (Kain & Klemke, 2001). We propose this same pathway could trigger the dissociation of dendritic cells bound to the basal face of the gut epithelia.

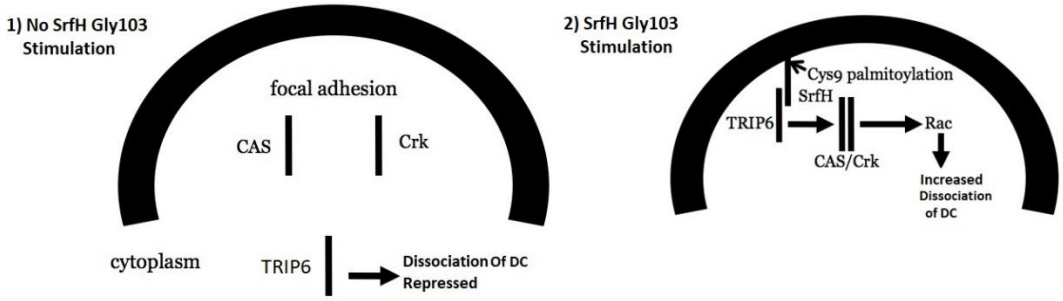

Figure 3.5. **Molecular tether model**. SrfH is palmitoylated at its cysteine 9 residue on translocation into the dendritic cell cytoplasm. This modification helps anchor SrfH Gly103 bound to TRIP6 at focal adhesions. TRIP6 helps recruit focal adapter protein CAS and focal regulatory protein Crk and this in turn triggers dissociation of the dendritic cells through the activation of the Rac signaling pathway.

Discussion

To date two SPI-2 T3SS effectors have been discovered that contribute towards early extraintestinal dissemination of *Salmonella* in mouse model. While SpvC was shown to facilitate reverse transmigration *in vitro*, SrfH did not seem to have an effect in stimulating the basal to apical migration of *Salmonella* infected dendritic cells across an endothelial cell monolayer (Gopinath et al., 2019).From the data presented here it is clear that SrfH plays an important role in an early phase of reverse transmigration, namely the dissociation of immature CD11c+ dendritic cells that associate with the epithelial cell monolayer and will serve as a vessel for the rapid dissemination of *Salmonella* to systemic tissue. This function is affected by the presence of a glycine residue at the 103rd position on the SrfH amino acid sequence. SrfH Gly103 was shown to bind TRIP6 while the Asp103 isoform bound IQGAP1, a large scaffold protein that regulates cellular migration (Thornborough & Worley, 2012; McLaughlin et al., 2009). SrfH's interaction with IQGAP1

results in the inhibition of the motility of *Salmonella* infected dendritic cells and macrophages *in vitro* and a significant reduction in the rapid dissemination phenotype in mice (McLaughlin et al., 2009, Thornborough & Worley, 2012). The coupling of SrfH Gly103 with TRIP6 could contribute towards the process of dissociation through the activation of the Rac signaling pathway. While the Gly103 allele is rare, it is found in highly virulent *S.* Typhimurium strains such as 14028s and Universal Killer-1 (Thornbrough & Worley, 2012). SrfH Gly103 is also found in the emergent invasive NTS strain serovar 4 and on the Gifsy 2 prophage carried by the human adapted *S.* Typhi Dakar (Cirillo et al., 1998; Hansen-wester et al., 2001; Thornbrough & Worley, 2012).

Materials and Methods

Generation of dendritic cells

Bone marrow derived mononuclear cells (BMDM) were isolated from the femur of 6-10-week-old C57BL/6. BMDMs were seeded at a ratio of 500,000 cells per ml of RPMI 1640 (Corning) supplemented in 10% FBS, 2mM l-glutamine, 1mM sodium pyruvate, 20ng/ml of recombinant mouse IL4 and GMCSF (Shenandoah Biotechnology) 55µM/ml 2-mercaptoethanol and 100units/mL of penicillin and 100µg/mL of streptomycin. Fresh media with twice the concentration of cytokines and 2-mercaptoethanol was added after three days. One day prior to the reverse transmigration assay recombinant mouse TGF-β1 (Biolegend). was added to the cells at a concentration of 20ng/ml of growth media. Loosely adherent dendritic cells were harvested on day seven. In brief, the media was pipetted up and down several times and pelleted in a 10-minute spin at 200g, 4^{o}C. Ice

cold 2mM PBS-EDTA was added to the dish and agitated at 4°C for 15 minutes. The buffer was pipetted up and down several times and the cells were pelleted in a 10-minute spin at 200g, 4°C. The pellets from the two washes were resuspended in 1ml of RPMI and pooled together. A tenfold dilution of the resulting cell slurry was stained with trypan blue and enumerated on a Reichert Bright-Line hemocytometer (Hausser Scientific).

Generation of Caco2 monolayers

Human epithelial cell line Caco2 (ATCC) was raised in DMEM (Corning) supplemented with 20% FBS, 1mM sodium pyruvate, 2mM l-glutamine, 1X non-essential amino acids (Corning) and 100units/mL of penicillin and 100µg/mL of streptomycin to 80% confluency in tissue culture treated T175 flasks. Caco2 cells were passaged by rinsing the growth surface with 1xPBS (Corning) followed by treatment with 0.25% porcine trypsin for 5-15 minutes. This was followed by quenching of the trypsin with complete growth media. 10% of the resulting cell slurry was placed back into flask with fresh complete DMEM.

Two hundred thousand Caco2 cells were seeded onto upside down tissue culture treated Corning Costar transwell polycarbonate inserts with 3 µm pores (Corning) and incubated in a water-jacketed cell culture incubator set to 37°C, 5% CO_2 for an hour. The next day tissue culture inserts were transferred to 24 well plates containing 600µL of complete DMEM and a further 100µL was added to the top compartment. Media was renewed every three days and transepithelial electrical resistance (TEER) resistance was measured using an EVOM^2 volt/ohm meter (World Precision Instruments). Media renewal continued till the Caco2 monolayers exhibited a resistance of 330Ω/cm^2.

Bacterial culture

The wild-type and subsequently the parent strain used to test the loss of SPI-2 T3SS and SrfH was *S. enterica* serovar Typhimurium 14028s (ATCC). 14028s was transduced with P22 HT-*int* a *srfH*::MudJ allele (Worley et al., 2000) with established techniques (Maloy & Stewart, 1996).

The *srfH* allele from *S.* Typhimurium strains 14028s and SL1344 were PCR amplified and cloned separately into the EcoRI site of pACYC184. One of each was electroporated into the 14028s *srfH*::MudJ to generate two new strains that complemented each *srfH* allele.

Bacteria were cultured overnight in luria broth (VWR) and one hour prior to the start of the dissociation assay were rinsed thoroughly in MGM adjusted to pH 7 (100mM Tris-Cl, 5mM KCl, 7.5mM (NH4)2SO4, 0.5mM K2SO4, 1mM KH2PO4, 8µM MgCl2, 38mM glycerol and 0.1% casamino acid).

Dissociation assay

Four hundred thousand monocytes were suspended in RPMI and cultured on the basolateral side of Caco2 monolayer for four hours. Bacterial o/n cultured in LB were washed and resuspended in SPI-2 inducing MGM media at pH 7.4. Right before the start of the assay the cell culture inserts were washed extensively to remove dendritic cells that did not attach and were transferred to a 24 well plate containing $1x10^8$ CFU of 14028s *srfH*::MudJ and a second bacterial strain resuspended in 600µl of complete DMEM in triplicate. The plates were incubated for 60 minutes in a water jacketed cell culture

incubator set to 37°C, 5% CO_2. At the end of the incubation period, the inserts were rinsed gently thrice to remove any dendritic cells that dissociated. The supernatant from these washes were lysed using 1% triton-X 100, serially diluted in PBS and plated on LB agar and LB agar plates supplemented with tetracycline.

Generation of primary macrophage monolayers

Bone marrow derived macrophages were generated as described previously (Marim et al., 2010). In brief monocytes were harvested from the femur of six to eight week-old C57BL/6 mice and seeded at a density of 1x 10^6 cells/ml into 24 well plates and differentiated into macrophages using the bone marrow differentiation media comprising of RPMI 1640 (Corning) supplemented with 20% FBS (Sigma Aldrich) and 30% L929 (ATCC) conditioned media and 2mM L-glutamine (Corning). An equal amount of bone marrow differentiation media was added to each well after four days of incubation. Cells were grown to confluency over a period of seven days.

Pull down assay

Bone marrow derived macrophages were infected with 14028s or 14028s *srfH*::MudJ at an MOI of one for a period of 10 hours. Cells were lysed using NP-40 lysis buffer (Sigma Aldrich) and the lysate used in a pull-down assay. Lysates were hybridized with anti-mouse monoclonal antibodies for either TRIP6 (Santa Cruz Biotechnology), CAS (Santa Cruz Biotechnology) or an isotype control. The hybridized lysate was co-immunoprecipitated with Protein G Sepharose 4 Fast Flow suspension (Sigma Aldrich) following the manufacturer's directions, which can be found online:

https://www.sigmaaldrich.com/technical-documents/protocols/biology/purifying-challenging proteins/pull-down-assays.html.

Co-immunoprecipitated lysates were subjected to sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels. The proteins on the SDS- PAGE gels were transferred to nitrocellulose membranes and hybridized with anti-mouse primary antibodies for either CAS (Santa Cruz Biotechnology, USA) or Crk (Santa Cruz Biotech) followed by secondary antibodies conjugated to alkaline phosphatase in triplicate. Samples that were initially co-immunoprecipitated with CAS antibodies were directly subjected to western blotting with an isotype specific secondary antibody in triplicate.

CHAPTER 4

DISCUSSION

The primary goal of our research project was to better understand the molecular mechanisms behind the rapid dissemination of *Salmonella* from the gut to systemic sites in mice. We hypothesized that *Salmonella* sampled by CD11c+ dendritic cells associated with the gut epithelia trigger the reverse transmigration of the host phagocytic cell across blood vessels to gain direct access to the bloodstream. To test our hypothesis, we initially developed the *in vitro* reverse transmigration assay described in chapter 2. Dendritic cells have been previously documented to be naturally capable of reverse transmigrating (D'Amico et al., 1998; Randolph et al., 1998). We were able to replicate this phenomenon *in vitro* with primary mice bone marrow derived dendritic cells and endothelial monolayers composed of the C166 mice endothelial cell line. Dendritic cells stopped migrating in the presence of heat killed *Salmonella,* but this was reversed when infected with live *Salmonella*. Intracellular *Salmonella* triggered reverse transmigration of dendritic cells in a SPI-2 dependent manner. SpvC a T3SS effector coded for on the virulence plasmid was identified to stimulate the process of reverse transmigration of dendritic cells *in vitro* and *in vivo*.

SpvC functions as a phosphothreonine lyase that decreases MAP kinase signaling by preventing the activation of Erk1/2, JNK, and p38 kinases (Mazurkiewicz et al., 2008). In

mice SpvC has been correlated with an anti-inflammatory effect at the site of infection in the colon through a reduced production of inflammatory cytokines such as TNFα (Haneda et al., 2012). MIF is expressed by a variety of cells including monocytes, macrophages and dendritic cells in response to exposure to bacterial antigens and contributes towards the upregulation of inflammatory cytokines such as TNFα and IL-β1 in a CD74-dependent activation of ERK1/2 MAPK (Calandra et al., 1994). We used a cell permeable MIF antagonist (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1) to down regulate the activity of MIF in dendritic cells used in the *in vitro* reverse transmigration assay. The use of ISO-1 increased the rate of reverse transmigration of dendritic cells infected with *spvC* mutant *Salmonella*, matching it to that of dendritic cells infected with wild type *Salmonella*. SpvC seems to act downstream of the MIF-CD74 initiated signaling cascade. This establishes a link between SpvC mediated reverse transmigration to the down regulation of the production of inflammatory cytokines.

The SPI-2 mediated reverse transmigration of dendritic cells is a complex process and loss of *spvC* alone does not completely inhibit this process. This indicates that at least two or more SPI-2 effectors are implicated in this process. Our current study was set up to test for the effects of individual T3SS effectors on reverse transmigration, future studies could be modified to include *Salmonella* strains that have at least two effectors knocked out. The assay in its current form can also be used to discover mammalian factors implicated in the SPI-2 mediated reverse transmigration of dendritic cells. Neutralizing monoclonal antibodies or an SiRNA approach could be used to perturb a host of cell surface proteins expressed by dendritic cells and endothelial cells previously implicated in metastasis and

reverse transmigration such as ICAM-1, Tissue factor, PECAM-1, CD99, CXCR4 and Endothelin (Yamauchi et al., 2002; Trozicky et al., 2012; Kedzierski et al., 2001; Christiansen et al., 2000).

While *in vivo* studies had previously associated the T3SS effector SrfH with the rapid dissemination phenotype, we were unable to establish a link between SrfH and reverse transmigration of dendritic cells *in vitro*. However, we were able to establish that SrfH had a significant effect over the dissociation of intraepithelial dendritic cells associated with the gut epithelia that had picked up *Salmonella* while sampling for antigens through epithelial cell junctions. SrfH has two prominent alleles distinguished by a single nucleotide polymorphism at amino acid position 103. The presence of a glycine residue at position 103 promotes the rapid dissemination of *Salmonella in vivo* while the presence of an aspartic acid residue at the same position results in an eight-fold decrease in the process (Thornbrough & Worley, 2012). Through the *in vitro* dissociation assay we were able to demonstrate that SrfH Gly103 is essential for the dissociation of CD11c+ intraepithelial dendritic cells while the Asp103 allele significantly reduced the process of dissociation. SrfH Asp103 was previously shown to bind with the scaffold protein IQGAP1 and this interaction has been speculated to contribute towards an inhibitory effect on the motility of dendritic cells and phagocytes *in vitro* (McLaughlin et al., 2009). SrfH Gly103 on the other hand binds the adaptor protein TRIP6 and colocalizes with this protein to the focal adhesion (Worley et al., 2006; Thornbrough & Worley, 2012). Through pull down assays we were able to demonstrate that TRIP6 is capable of recruiting CAS and crk proteins in the presence of SrfH Gly103. Dissociation of dendritic cells could be facilitated

by SrfH Gly103 acting as a molecular tether that anchors TRIP6 to the focal adhesion and promoting the coupling of CAS a focal adhesion adaptor protein with Crk, a focal adhesion regulatory molecule. Both proteins are abundant at the focal adhesion and have been shown to activate the RAC signaling pathway that promotes cellular migration (Kain & Klemke, 2001). We hypothesize this same pathway could trigger the dissociation of dendritic cells bound to the basal face of the gut epithelia. An siRNA knockdown approach can be used to test if TRIP6, CAS and Crk are indeed required for the SrfH stimulated *in vitro* dissociation of dendritic cells.

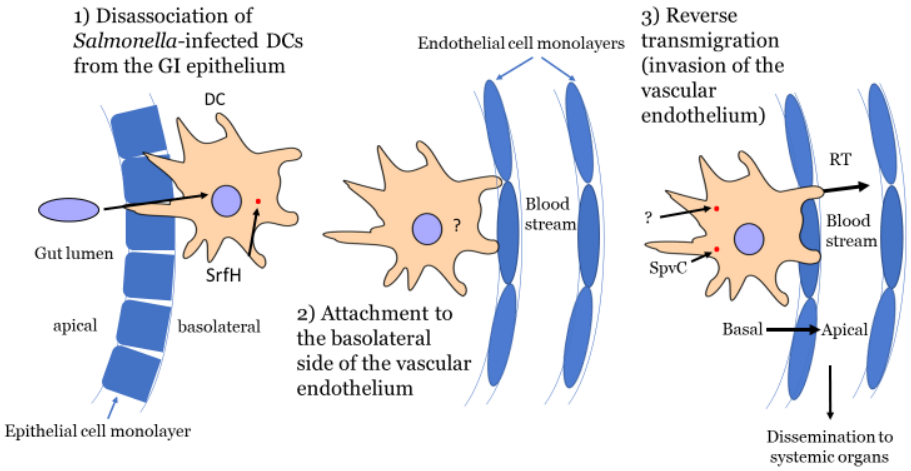

Figure 4.1. The rapid dissemination of *Salmonella* in mice. We postulate that the SPI-2 and CD18 dependent rapid dissemination of *Salmonella* in mouse model comprises three phases, namely, dissociation, adhesion and reverse transmigration. T3SS effectors translocated into infected intraepithelial dendritic cells manipulate cellular signaling and trigger dissociation of dendritic cells from the GI epithelium and promote migration towards a blood vessel. This is followed by adhesion of the dendritic cell to the basal face of vascular endothelial cells and the invasion of bloodstream via reverse transmigration.

Based on our work and current information available on the reverse transmigration of mammalian cells, the CD18 dependent rapid extraintestinal dissemination of *Salmonella* in mice potentially comprise of three phases, dissociation, adhesion and reverse transmigration (Figure 4.1). The first phase involves an immature CD11c+ dendritic cell associated with the lamina propria picking up *Salmonella* through the projection of dendrites across epithelial cell junctions into the lumen of the small intestine. SPI-2 effectors including but not restricted to SrfH will trigger dissociation of the *Salmonella* containing dendritic cell from the intraepithelial space and trigger migration towards the nearest blood vessel through the activation of the Rac signaling pathway. Migration could also be influenced through the modulation of gene expression of chemokine receptors such as CXCR4 that would otherwise guide the dendritic cell towards the lymphatic system. Phase 2 of this process involves adhesion of the *Salmonella* infected dendritic cell to the basal face of vascular endothelia. *In vitro* this process could be simulated by culturing endothelial cell monolayers on a 24 well plate and stripping the cells using a weak detergent, leaving behind an extracellular matrix layer. We are yet to determine any SPI-2 effector that could potentially affect this process, but a host of cellular factors found on the surface of dendritic cells and endothelial cells such as tissue factor, PECAM-1 and CD99 could potentially affect this process. The final phase is reverse transmigration and results in the invasion of bloodstream by the infected dendritic cell. This process involves the movement of the dendritic cell from the basal to apical face of the endothelial monolayer lining the blood vessel. SpvC and an unidentified set of effectors

could potentially manipulate host signaling pathways such as MAP kinase signaling to facilitate this process.

The rapid dissemination of *Salmonella* through reverse transmigration is an inefficient process with only one in a million *Salmonella* making it to the bloodstream of mice 30 minutes following oral inoculation. However, rapid entry into the bloodstream allows *Salmonella* to infect tissue types that are rich in nutrition and devoid of endogenous microflora such as the gallbladder, spleen and liver. This allows *Salmonella* to set up persistent colonies that constantly shed bacteria into the gastrointestinal tract and feces through the lymphatic system before the infection is potentially cleared through the development of a pathogen specific adaptive immune response. The speed with which the bacteria penetrate the liver and gallbladder of its animal reservoir may be a component of its virulence.

Bacteremia caused by *Salmonella* Typhi and invasive NTS in humans is poorly understood. The emergence of multidrug resistant strains for both diseases have nullified the effectiveness of several first and second-line antibiotics (Van Puyvelde et al., 2019; Chatham-Stephens et al., 2019). Our *in vitro* model provides a genetically tractable and cost-effective way of discovering T3SS effectors that are potentially conserved amongst the various *Salmonella* serovars and host factors that could potentially contribute to the process of systemic dissemination of *Salmonella*. This could help pave the way for the discovery of novel drug targets that could perturb the process of reverse transmigration and contain the establishment of chronic infection of not just *Salmonella* but other intracellular pathogens capable of T3SS mediated systemic infection such as *Yersinia*

(Barnes et al., 2006; Coburn et al., 2005). A better understanding of reverse transmigration could also help develop strategies to minimize inflammation in patients suffering from chronic diseases such as rheumatoid arthritis or atherosclerosis positive for ICAM1^{high} reverse transmigrating neutrophils or stem the metastasis of certain types of tumors (Buckley et al., 2005; Roh-Johnson et al.,2013; Gligorijevic., et al., 2012).

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Education

BACHELOR OF SCIENCE | 2009 | UNIVERISTY OF LEEDS, LEEDS, UK

- Major: Biochemistry, Minor: Biotechnology, Microbiology and Molecular Biology

MASTER OF SCIENCE | 2013 | DEPARTMENT OF BIOLOGY, VALDOSTA STATE UNIVERISTY, VALDOSTA, GEORGIA, USA

- Major: Biology

DOCTOR OF PHILOSOPHY | 2020 | DEPARTMENT OF BIOLOGY, UNIVERSITY OF LOUISVILLE, LOUISVILLE, KENTUCKY, USA

- Major: Biology

Experience

GRADUATE TEACHING ASSITANT | DEPARTMENT OF BIOLOGY, VALDOSTA STATE UNIVERISTY, VALDOSTA, GEORGIA, USA

- Period: Aug 2010- Dec 2013
  Job Description: Taught Biol 1020 Biodiversity Biology Lab, Biol 1040 Organismal Biology lab to non-major biology undergrads.
  Assisted with set-up and grading the lab component of Bio -1107 Principles of Biology

LAB ASSISSTANT | DEPARTMENT OF BIOLOGY, VALDOSTA STATE UNIVERISTY, VALDOSTA, GEORGIA, USA

- Period: Jan 2014- March 2014
- Job Description: Volunteered as Lab Technician in Dr. Emily Cantonwine's Research Lab

QC LAB TECHNICIAN, BADISCHE ANILIN- UND SODA-FABRIK (BASF), SPARKS, GA, USA

- Period: March 2014- May 2014
- Job Description: Loaned by contracting firm NIC INFOTEK (Tampa, FL, 813-985-3630) to BASF, sparks, GA to work as Quality Control Lab Technician. Performed analytical and physical chemistry techniques to measure levels of active ingredients in batch samples for a suite of herbicides and fungicides

GRADUATE TEACHING ASSITANT | DEPARTMENT OF BIOLOGY, UNVERSITY OF LOUISVILLE LOUIS VILLE, KENTUCKY, USA

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Publications

- Gopinath, A., Allen, T. A., Bridgwater, C. J., Young, C. M., & Worley, M. J. (2019). The Salmonella type III effector SpvC triggers the reverse transmigration of infected cells into the bloodstream. PloS one, 14(12).
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