Disruption of Toll-like Receptor 4 Signaling Pathway by Salmonella Effector SigD

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Cell and Systems Biology
University of Toronto

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2011

Abstract

The enteropathogenic bacteria *Salmonella* are the main cause of food borne gastroenteritis worldwide. The activation of Toll-like receptor 4 (TLR4) by LPS triggers an immune response to counter infection. Signaling by TLR4 requires the adaptor proteins, TIRAP and TRAM. Recruitment and activation of these molecules is dependent on the membrane lipid, PIP$_2$. The *Salmonella* effector, SigD, is a 4-phosphatase that depletes PIP$_2$ from the host plasma membrane during invasion. Thus, we investigated if SigD could lead to the interruption of the TLR4 pathway. We observed that SigD expression caused the disappearance of TIRAP from the *Salmonella* containing vacuoles (SCVs) in HeLa cells. Furthermore, we demonstrated that SigD attenuates NF-κB activation, implicating SigD in the disruption of the MyD88 dependent pathway. In addition, the observed inhibition of PKCε phosphorylation suggests SigD may also block the other branch of the TLR4 signaling cascade, the MyD88 independent pathway.
Acknowledgments

This work is the culmination of a memorable and enriching lab experience, one made possible by those who have both taught and supported me along the way. I am deeply thankful to my supervisor, Dr. Mauricio Terebiznik, who has never failed to inspire, motivate and teach me. His patience, vast knowledge, advice and, of course, his undeniable witty humor were all pivotal to my growth as a student and researcher.

I want to express my sincerest gratitude to my lab mate, Akriti Prashar. In retrospect, we have come a long way. From our humble beginnings, to setting up the lab, and throughout many a scary committee meeting day, you have been someone that I could always count on. I can say that because of your strong will, kindness and willingness to help, our “lab of two” never felt that small. I also want to thank JC Szamosi for her hard work and contribution to this study.

And, of course, what to say of our friendly neighbours next door, Dr. Rene Harrison’s lab, but that they have all left an indelible mark on my experience here at UTSC. With her expertise, Dr. Harrison has been an invaluable resource and has guided my work to reach its potential. To the various members of the Harrison lab, Cara, Ed, He song, Krissy, Noushin, Prerna, Raed and Sofia: many thanks for your help and all the laughs that we shared. I want to extend a special thank you to Raed, who on more than one occasion has been an uplifting presence during those bleak days of incessant thesis writing.

This journey has undeniably been an arduous one, at times seeming insurmountable. But I’ve always known that not too far behind, I have had a very loyal and loving companion, Daniel Petrozziiello. Your support, patience and motivating words have kept me determined all this time. Although cliché, it is true when I say that I could not have done this without you.

Of course, it is without question that the completion of this work would also have not been possible without the love and support of my parents and sister. To my mom, for whom a simple thank you will never suffice for all her unwavering support: gracias por todo.
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SigD mediated disruption of TLR4 signaling
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<td>API</td>
<td>Activator protein 1</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CD14</td>
<td>Cluster of differentiation 14</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosomal antigen 1</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>GALT</td>
<td>Gut associated lymphoid tissue</td>
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<td>GAP</td>
<td>GTPase-activating protein</td>
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<tr>
<td>GARG16</td>
<td>Glucocorticoid-attenuated response response gene 16</td>
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<td>GEF</td>
<td>Guanine exchange factor</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
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<td>Description</td>
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<tr>
<td>GSK</td>
<td>Glycogen synthase kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
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<td>Inhibitor of κ light chain gene enhancer in B cells</td>
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<td>IL-1 receptor-associated kinase</td>
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<td>IRF3</td>
<td>IFN regulatory factor 3</td>
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<td>JNK</td>
<td>c-jun NH\textsubscript{2}-terminal kinase</td>
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<tr>
<td>LBP</td>
<td>LPS binding protein</td>
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<td>LRR</td>
<td>Leucine rich repeat</td>
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<td>lipopolysaccharide</td>
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<td>Mal</td>
<td>MyD88-adaptor-like</td>
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<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>MAMP</td>
<td>Microbial associated molecular pattern</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MD2</td>
<td>Myeloid differentiation protein-2</td>
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<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>M6PR</td>
<td>Mannose-6-phosphate receptor</td>
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<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
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<td>NAK</td>
<td>NF-κB-activating kinase</td>
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<td>NAP1</td>
<td>NAK-associated protein 1</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PIP₂</td>
<td>Phosphotidylinositol 4,5-bisphosphate</td>
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<tr>
<td>PKCε</td>
<td>Phosphokinase ε</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PMB</td>
<td>Polymixin B</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
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<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
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<tr>
<td>RILP</td>
<td>Rab7-interacting lysosomal protein</td>
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<td>RIP1</td>
<td>Receptor-interacting protein 1</td>
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<td>SARM</td>
<td>Sterile alpha and HEAT/Armadillo motif</td>
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<td>SCV</td>
<td><em>Salmonella</em> containing vacuole</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<td>SNP</td>
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<td>SPI</td>
<td><em>Salmonella</em> pathogenicity island</td>
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<td>ssRNA</td>
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<td>Transforming growth factor-β-activated kinase 1</td>
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<td>TBS Tween-20</td>
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<td>Toll/IL-1 receptor</td>
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<td>TIR-domain-containing adaptor protein</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>Acronym</td>
<td>Full Name</td>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor protein-inducing IFN-β</td>
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<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<td>T3SS</td>
<td>Type 3 Secretion System</td>
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<td>Live cell imaging of HeLa cells expressing TIRAP-GFP being invaded by RFP-expressing Δ<em>sigD</em> <em>Salmonella</em></td>
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1 Introduction

1.1 Salmonella: a current global health concern

*Salmonella* is an enteropathogenic, gram-negative bacterium. Ingestion of contaminated food or water results in two main pathologies: gastroenteritis or enteric (typhoid) fever (PHAC. Infectious Diseases Information). The former, caused by *S. enteriditis*, is characterized by diarrhea that usually dissipates within 5-7 days. However, in some cases, as with immuno-compromised people, the bacteria cause typhoid fever, a systemic dissemination of the infection which is carried from immune cells, like macrophages, to lymph nodes and eventually the bloodstream (Gerold et al., 2007). The spreading of the infection can sometimes lead to Reiter’s syndrome, a debilitating condition known as “reactive arthritis” (PHAC Infectious Diseases Information). The most virulent, *S. typhi*, ordinarily produces systemic infections in humans and is the etiological agent of typhoid fever which, in 16% of cases, proves fatal (http://www.phac-aspc.gc.ca/id-mi/index-eng.php, Public Health Agency of Canada, 2008).

According to the World Health Organization (WHO), the prevalence of *Salmonellosis* has risen over the past 25 years. This increase in incidence has made non-typhoidal *Salmonellosis* (NTS) the most common cause of death from diarrheal disease related to viruses, parasites or bacteria and are the leading cause of food borne disease outbreaks in the USA (Santos et al., 2009). In addition, the WHO also raises concerns about the increasing incidence, since the 1990s, of *Salmonella* strains that are resistant to antimicrobials often used to treat humans (WHO. Foodborne diseases, emerging. http://www.who.int/mediacentre/factsheets/fs124/en/, World Health Organization, 2008). Considering that *Salmonella* infections are emerging as the leading cause of food borne disease worldwide, current research is making great strides in discovering the complexities of its pathogenesis.
1.2 The host intestinal environment

The surface of the intestine is composed of finger-like projections called villi and tubular invaginations called crypts. Various cell types line the intestine, the most common being enterocytes or intestinal epithelial cells (IECs) which themselves have membranous extensions called microvilli on their apical surface. Key to maintaining the integrity of the epithelium, IECs are bound together by adherent tight junctions, characterized by transmembrane proteins like claudin and occludin. IECs are constantly being renewed from stem cells situated within the basal region of the crypts (Lotz et al., 2007). Importantly, IECs are one of the main cell types involved in Salmonella invasion. The remainder of the cell population is divided among endocrine, goblet and Paneth cells. Endocrine cells play a part in regulating intestinal processes by secreting hormone-like intermediates. As mucin-secreting cells, goblet cells are distributed throughout the intestine and are crucial in maintaining a thick layer of mucus covering the epithelial lining (Kindon et al., 1995). Paneth cells are localized solely in the lower regions of the crypts, surrounding the stem cells with a protective layer of antimicrobial peptides, such as α-defensins, that they secrete (Hornef et al., 2004).

Within the intestinal lumen, commensal bacteria compose a microbial ecosystem consisting of $10^{14}$ organisms (Guarner and Malagelada, 2003). Apart from their role in metabolism and synthesis of some vitamins such as vitamin K, B$_{12}$ and folic acid (Hooper et al., 2002), commensal bacteria also participate in the maintenance of the IEC population by stimulating cell differentiation. This was demonstrated in germ-free mice that displayed reduced proliferation rate and impaired development of IECs in comparison to colonized mice (Falk et al., 1998; Hooper and Gordon, 2001).
The intestinal epithelium needs to avoid unnecessary activation of an immune response by commensal bacteria, which may lead to intestinal inflammation. However, the host also needs to be able to detect pathogenic bacteria as well. This delicate balance is achieved via the gut immune system. The gut-associated lymphoid tissue (GALT) is specialized lymphoid tissue situated beyond the epithelial surface. Within the GALT, there are regions called Peyer’s patches, composed of microfold (M) cells that are specialized for high endocytic activity. M cells sample for antigens in the gut lumen, transporting them across the intestinal cell layer to underlying lymphocytes and macrophages (Lotz et al., 2007). This causes lymphocytes to differentiate into mature effector lymphocytes and also induces B cells to express immunoglobin A (IgA) which controls the populations of commensal flora (Lotz et al., 2007). Dendritic cells (DCs) also participate in host defense by sampling from within the GALT, through the epithelial layer and into the intestinal lumen. Once activated, DCs will migrate to lymphatic tissue and present antigens to T cells, stimulating an adaptive immune response (Neutra et al., 1996).

1.3 Salmonella as an invasive pathogen

The process of invasion involves bacteria-induced uptake into non-phagocytic cells. Invasion into host cells occurs either through the ‘zipper’ or ‘trigger’ mechanism (Isberg, 1991). In the ‘zipper’ scenario, the binding of a bacterial surface protein with a host receptor molecule causes the bacterium to be tightly enveloped by the host cell membrane, as in the case of Yersinia enterolitica in which the outer membrane protein, Invasin (Inv), binds to human integrin proteins (Isberg and Leong, 1990). The ‘trigger’ mode of entry, used by Shigella and Salmonella, is mediated by the Type III Secretion System (T3SS), a needle-like appendage composed of more than 20 proteins that traverses the inner and outer membranes of the bacterial envelope (Galan and Zhou, 2000). The T3SS allows the injection of bacterial effector proteins into the host cell;
these effectors activate small GTPase proteins that initiate cytoskeletal changes, causing extensive host membrane ruffling and subsequent internalization of the bacteria. Internalization of the bacteria occurs in a process that is similar to the formation of macropinosomes such that the bacteria are packaged into membrane bound compartments within the host (Cossart and Sansonetti, 2004).

*Salmonella* employs the trigger mechanism to invade host cells. The bacterial effectors are delivered through two distinct T3SSs encoded on *Salmonella* pathogenicity islands (SPI), SPI-1 and SPI-2 (Galan, 2001). To date there are 12 SPI in addition to SPI-1 and SPI-2, however, only SPI-1 to SPI-5, inconclusive, have been studied extensively (Sanchez-Jimenez *et al.*, 2010). There are approximately 30 effectors translocated by the SPI-1 and SPI-2 T3SS. These effectors have been shown to assist during the process of invasion, signal transduction and the manipulation of other host cell functions (McGhie *et al.*, 2009). Specifically, SPI-1 and SPI-2 T3SS effectors act during different stages of *Salmonella* infection; SPI-1 T3SS effectors participate in the initial stages of the invasion process such as the extensive ruffling of the host plasma membrane, internalization of bacteria, and formation of a membrane bound compartment called the *Salmonella* containing vacuole (SCV). SPI-2 T3SS effectors come into play after the SCV has formed; these effectors are necessary for intracellular survival of the bacteria within the SCV and bacterial proliferation in the host cell (Watson *et al.*, 1995). The first step of *Salmonella* invasion into non-phagocytic cells involves the injection of SPI-1 T3SS effectors, SipA, SipC, SopE and SopE2, into the host (Figure 1). SipA and SipC are codified on SPI-1 while the SopE and SopE2 genes are on bacteriophage remnants within the genome (McGhie *et al.*, 2009). Together these effectors induce massive membrane ruffling and cytoskeletal rearrangements. SipA functions to promote actin polymerization while at the same time it facilitates SipC in actin nucleation and bundling (McGhie *et al.*, 2004). Most importantly, SipA
Figure 1. Salmonella invasion. 1, Salmonella use the Type 3 Secretion System (T3SS) to forcibly invade intestinal epithelium by injecting Salmonella pathogenicity island (SPI) 1 (SPI-1) effectors (Galan, 2001). During the initial stages of the invasion process, SPI-1 T3SS effectors induce membrane ruffling and bacteria internalization. 2, Later, engulfment of bacteria into the Salmonella containing vacuole (SCV) occurs. The maintenance of the SCV and survival of bacteria within are mediated by the SPI-2 T3SS effectors which are injected into the host cytoplasm from the SCV (Watson et al., 1995). 3, As the SCV matures, it avoids association with lysosomal proteins and therefore, does not become degraded. The SCV migrates towards the perinuclear region where the formation of Salmonella induced filaments (Sifs) occurs (Steele-Mortimer, 2008). This marks the beginning of the replicative stage for the bacteria.
plays a key role in the invasion process by inhibiting the actin depolymerizing proteins of host cells (McGhie et al., 2004). Unlike SipA and SipC, SopE and SopE2 do not directly bind actin but instead act as guanine exchange factors (GEFs) that stimulate the GTP binding proteins, Rac-1 and Cdc42, of the Rho family (Patel and Galan, 2005). Another key SPI-1 effector is the inositol phosphatase SopB/ SigD (herein called SigD), which is codified on SPI-5. SigD aids in the rapid closure of the membrane invaginations to form the SCV (Terebiznik et al., 2002). SigD is the focus of our study and will be discussed at length later. Actively cooperating with SigD to facilitate membrane fission is SopD, another SPI-1 T3SS effector (Bakowski et al., 2007).

Shortly after bacterial invasion, another effector, SptP, comes into play. SptP is a GTPase-activating protein (GAP) that acts to reverse the initial cytoskeletal changes by mediating the hydrolysis of the GTPases, Rac-1 and Cdc42, switching them into their inactive state (Fu and Galan, 1999). SCV maturation occurs as it migrates through the cell (Figure 1). Initially, the SCV resembles an early endosome with characteristic cellular markers such as early endosomal antigen 1 (EEA1) and Rab5 that guide the SCV along the endocytic pathway (Garcia-del Portillo et al., 1993). SopE and the dephosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) by SigD have been shown to be necessary for the recruitment of Rab5 (Mukherjee et al., 2001; Mallo et al., 2008). However, within an hour, these markers are replaced by proteins associated with late endosomes like Rab7 and lysosomal glycoproteins such as lysosomal-associated membrane protein 1 (LAMP1) (Garcia-del Portillo et al., 1993; Steele-Mortimer et al., 1999). At this point, SCVs transiently acquire Rab7-interacting lysosomal protein (RILP) which binds to microtubule motor, dynein, facilitating the migration of the SCV towards the perinuclear region (Harrison et al., 2004). Key to the survival of Salmonella is the SCVs ability to halt its association with distinct lysosomal proteins such as hydrolases, like cathespin, and mannose-6-phosphate receptors (M6PR) which sort lysosomal hydrolases to the endosomal system (Garcia-
del Portillo and Finlay, 1995; Garvis et al., 2001). The proximity of the SCV to the Golgi may help in obtaining nutrients and/or membrane components by intercepting transport vesicles (Ramsden et al., 2007). Effectors SifA, SseG and SseF have been implicated in this role due to their involvement in re-directing exocytic transport vesicles to the SCV (Kuhle et al., 2006). SseG and SseF have also been shown to keep the SCV localized in the perinuclear region by assembling a complex between the Golgi and the SCV (Deiwick et al., 2006) or by controlling the activity of dynein (Ramsden et al., 2007). Once the SCV has reached the perinuclear area, bacterial proliferation commences, a stage characterized by the appearance of tubular extensions called Salmonella induced filaments (Sifs) (Steele-Mortimer, 2008). The most important role of SifA is the formation of Sifs and the maintenance of SCV integrity (Ruiz-Albert et al., 2002). These Sifs connect the SCV to the microtubule network however, their exact function has not been determined yet.

1.4 Host recognition of microbial organisms by Toll-like receptors (TLRs)

Host cells have an arsenal of tools they use to identify microbes. Microbes are distinguishable because they express specific, highly conserved surface molecules known as microbial associated molecular patterns (MAMPs) such as lipids, lipoproteins, proteins, and nucleic acids (Kawai and Akira, 2010). MAMPs are recognized by host cell’s pattern recognition receptors (PRRs) (Kawai and Akira, 2010). There are many types of PRRs such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs).

TLRs are a family of evolutionary conserved transmembrane receptors found among plants, insects, worms and invertebrates. The first Toll receptor was discovered in Drosophila
melanogaster (Anderson et al., 1985). To date, 13 TLRs have been found; 10 TLRs are expressed in humans (TLR1-10) while 12 TLRs are found in mice (TLR1-9, TLR11-13) (Rock et al., 1998). All TLRs share the same basic structure: an extracellular domain composed of leucine rich repeats (LRRs) and a cytoplasmic domain that is homologous to the interleukin-1 receptor (IL-1R) referred to as the Toll/IL-1 receptor (TIR) (Gerold et al., 2007). However, the extracellular domain is not as highly conserved as the TIR domain, and shows variation in the LRRs, contributing to each TLRs ligand specificity. The LRR domain contains 19-25 tandem repeats, where each repeat is 24-29 amino acids in length (Kawai and Akira, 2006). TLRs are differentiated based on their location in the cell and the MAMPs they detect. TLR1, 2, 4, 5 and 6 are localized on the surface of the cell while TLR3, 7, 8, and 9 are present intracellularly (Albiger et al., 2007). TLR2 can form a complex with either TLR1 or TLR6 to detect triacylated or diacylated lipoproteins, respectively (Sugawara et al., 2003). TLR3 can recognize bacterial or viral double stranded RNA (dsRNA) and TLR4 detects lipopolysaccharide (LPS) (Hoshino et al., 1999; Sen and Sarkar, 2005). TLR5 is specific for flagellin, while both TLR7 and TLR8 can detect single stranded RNA (ssRNA) (Hayashi et al., 2001; Crozat and Beutler, 2004; Triantafilou et al., 2005). Lastly, TLR9 recognizes CpG-DNA (Coban et al., 2005).

As MAMP recognition receptors, TLRs form part of the innate immune system. Ligand binding leads to TLR activation and the initiation of inflammatory signaling pathways. The triggering of these pathways is mediated by TIR domain-containing adaptor proteins that interact with the TIR domain on the cytoplasmic side of each TLR. There are five TLR adaptors that have been identified thus far, four of which are major players in TLR downstream signaling: myeloid differentiation primary response gene 88 (MyD88), TIR-domain containing adaptor protein (TIRAP), also known as MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Lord et al., 1990;
Fitzgerald et al., 2001; Horng et al., 2002; Yamamoto et al., 2002; Jiang et al., 2004). The fifth adaptor is sterile alpha and HEAT/Armadillo motif (SARM) and is different from the other adaptors in that it inhibits TRIF (Carty et al., 2006). Each TLR uses a combination of these adaptors to start signaling. With the exception of TLR3, all the remaining TLRs use MyD88 to initiate the MyD88 dependent pathway, which induces the transcription factor nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) to produce pro-inflammatory cytokines (Akira et al., 2006). In addition to MyD88, TLR2-TLR1 and TLR2-TLR6 complexes use TIRAP also. Instead of MyD88, TLR3 uses TRIF to initiate the MyD88 independent (or TRIF-dependent) pathway which induces transcription factor IFN regulatory factor 3 (IRF3) and the production of type I interferons (Kawai and Akira, 2010). TLR4 is unique in that it uses the four adaptors MyD88, TIRAP, TRAM and TRIF and in doing so, is the only TLR that induces both downstream signaling pathways (Figure 2).

1.5 TLR4 activation and signaling

TLR4 specifically recognizes LPS from the outer membrane of Gram-negative bacteria. LPS consists of three parts: the lipid A region, an oligosaccharide core and another carbohydrate region called the O-antigen. The lipid A region, composed of phosphorylated diglucosamine and four to seven acyl chains, is the endotoxic portion recognized by TLR4 (Carpenter and O'Neill, 2009). TLR4 can detect LPS at the plasma membrane with the assistance of three other accessory proteins. LPS first must be bound by the soluble LPS binding protein (LBP) and delivered to cluster of differentiation 14 (CD14), a glycosylphosphatidylinositol (GPI)-anchored receptor. From CD14, LPS then associates with a complex composed of TLR4 and myeloid differentiation protein-2 (MD-2) (Dauphinee and Karsan, 2006). The assembly of the LPS-MD-2-TLR4 complex occurs within PIP2 rich lipid rafts and also involves dimerization with another TLR4 in
Figure 2. TLR4 signaling via the MyD88 dependent and MyD88 independent pathways. LPS from Salmonella is recognized by LBP and shuttled to CD14 where it associates with the TLR4-MD-2 complex, initiating activation of downstream TLR4 adaptors. TIRAP localizes to the plasma membrane via its PIP2 binding domain and recruits MyD88 to the TIR region of TLR4. This association activates the MyD88 dependent pathway (indicated by red arrows). IRAK4 phosphorylates IRAK1 which promotes the formation of a complex with TRAF6. The signaling cascade continues and culminates in the phosphorylation of IκB-α, facilitating the translocation of NF-κB into the nucleus which stimulates the transcription of proinflammatory cytokines like TNFα. The MyD88 independent pathway (blue arrows) is mediated by TRAM which is attached to the plasma membrane via its myristoylation domain. This pathway is triggered after LPS-induced endocytosis of TLR4 which occurs upon association of TIRAP and MyD88 with the TIR region of TLR4. During endocytosis, TIRAP detaches from TIR domain and is replaced by TRAM. Efficient TRAM signaling requires its phosphorylation by PKCε. Activation of TRAM facilitates its binding with TRIF, initiating downstream signaling from an endosomal location. Subsequent association of TRAF3 and NAP1 induces the phosphorylation and dimerization of IRF3. This initiates its translocation into the nucleus and transcription of IFNβ.
order for TLR4 to be activated (Triantafilou et al., 2002). TLR4 induces both signaling pathways in a sequential manner (Kagan et al., 2008). The TIRAP-MyD88 complex binds first to the TIR domain of TLR4 at the plasma membrane before the TRAM-TRIF complex for reasons not yet known (Kagan et al., 2008). TIRAP contains a PIP$_2$ binding domain that is essential for its recruitment to the plasma membrane and for TLR4 downstream signaling (Kagan and Medzhitov, 2006). The PIP$_2$ binding domain is located on the N-terminal end while the TIR domain, which mediates subcellular localization, is on the C-terminal side. TIRAP functions as a binding partner for MyD88, which is normally found in the cytosol, and assists in its recruitment to the plasma membrane in a TIR-dependent manner (Kagan and Medzhitov, 2006). MyD88 interacts with TLR4 via the TLR4 TIR domain and activates downstream signaling. The binding of TIRAP and MyD88 initiates the MyD88 dependent pathway and also the endocytosis of the receptor though a dynamin-dependent mechanism (Kagan et al., 2008). A crucial event during endocytosis is the decrease in the level of PIP$_2$ as the endosome forms, an event similar to that seen in macrophages during phagocytosis (Botelho et al., 2000). The reduction in PIP$_2$ is due to its hydrolysis by phospholipase C (PLC), which also produces diacylglycerol (DAG) as a byproduct. The hydrolysis of PIP2 is imperative to the actin remodeling involved in phagocytosis as was highlighted when the reduction in the availability of PIP$_2$ disrupted phagocytosis (Botelho et al., 2000). The hydrolysis of PIP$_2$ causes TIRAP to detach from the plasma membrane, thereby allowing the TRAM-TRIF complex to bind to the TIR domain of TLR4 in the newly formed endosome (Kagan et al., 2008). This initiates the MyD88 independent pathway.

1.6 TLR4 signaling: the MyD88 dependent and independent pathways

During the MyD88 dependent pathway, the recruitment of MyD88 to the plasma membrane initiates a complex with IL-1 receptor-associated kinase-4 (IRAK-4). IRAK-4 is vital for the
production of pro-inflammatory cytokines as seen in studies showing that IRAK-4 macrophages are deficient in the production of these cytokines when stimulated with LPS (Lu et al., 2008). The role of IRAK-4 is to aid in the transphosphorylation of IRAK-1. Subsequently, IRAK-1 becomes activated which facilitates the binding of TNF receptor-associated factor 6 (TRAF6). Once this occurs, the IRAK-TRAF6 complex associates with transforming growth factor-β-activated kinase 1 (TAK1). Stimulation of TAK1 causes the recruitment of both TAK1-binding protein 1 and 2 (TAB1 and TAB2) (Dauphinee and Karsan, 2006). TAB1 is responsible for the activation of TAK1 while TAB2 mediates the binding of TAK1 to TRAF6 and also aids in its ubiquitination (Dauphinee and Karsan, 2006). From TAK1, a complex of kinases are triggered in both the IkB kinase (IKK) and MAPK pathways (Lu et al., 2008). Briefly, stimulation of the IKK pathway causes the recruitment of the IkB kinase (IKK) complex made up of two catalytic subunits IKKα and IKKβ and a regulatory subunit, IKKγ, also known as NF-κB essential modulator (NEMO). Together the IKK complex phosphorylates IkB-α (inhibitor of κ light chain gene enhancer in B cells). Normally in the cytosol, IkB-α is bound to the transcription factor, NF-κB, which prevents it from migrating to the nucleus. However, once IkB-α is phosphorylated, it undergoes ubiquitin-directed proteosome-mediated degradation and releases NF-κB to translocate to the nucleus where it can trigger the transcription pro-inflammatory cytokines like TNFα, IL-6 and IL-12 (Lu et al., 2008). Also contributing to the expression of these cytokines is the MAPK pathway which is initiated by TAK1 and involves p38, extracellular signal-regulated kinase (ERK) and c-jun NH2-terminal kinase (JNK). The recruitment of these MAPK’s leads to the activation of another transcription factor, activator protein 1 (AP1) (Dauphinee and Karsan, 2006).
The TLR4 MyD88 independent pathway leads to the expression of IFN-inducible genes, like interferon-inducible protein 10 (IP10) and glucocorticoid-attenuated response gene 16 (GARG16) through activation of the transcription factor, interferon response factor 3 (IRF3) (Dauphinee and Karsan, 2006). As mentioned earlier, the induction of this signaling pathway occurs from the endosomal compartment after LPS-induced endocytosis of TLR4 (Kagan et al., 2008). Because the level of PIP$_2$ declines during endocytosis of TLR4, TIRAP detaches from membrane, leaving the TIR domain of TLR4 available for TRAM to bind. TRAM is different from TIRAP in that it contains a bipartite localization domain consisting of an amino-terminal myristate group followed by a polybasic domain (Kagan et al., 2008). Both parts of this domain are required for TRAM to localize to the plasma membrane while only the myristate region is necessary for its placement in endosomes (Kagan et al., 2008). Indeed, in resting cells TRAM is situated in both the plasma membrane and early endosomes however, endosomal localization is critical for TRAM to engage the TRAM/TRIF pathway (Rowe et al., 2006; Kagan et al., 2008). TRAM acts as a bridging adaptor and binds to TRIF directly, recruiting it to TLR4 (Oshiumi et al., 2003), triggering two downstream signaling pathways. The first branch begins with the recruitment of TRAF6, a protein that also participates in the MyD88 dependent pathway, and receptor-interacting protein 1 (RIP1) to TRIF (Carpenter and O'Neill, 2009). It is important to note that this complex triggers a late stage activation of NF-$\kappa$B and the expression of pro-inflammatory cytokines by recruiting IKK$\alpha$, IKK$\beta$ and IKK$\gamma$ and initiating the degradation of I$\kappa$B, as seen in the MyD88 dependent pathway (Jenkins and Mansell, 2010). In the other branch of the pathway, TRAF3 and NF-$\kappa$B-activating kinase (NAK)-associated protein 1 (NAP1) form a complex which then stimulates the interaction between TRAF family member-associated NF-$\kappa$B activator (TANK)-binding kinase 1 (TBK1) and IKK$\epsilon$. The formation of this complex initiates the activation of the transcription factor IFR3, leading to its phosphorylation and
dimerization. Once it has formed a homodimer, IFR3 migrates into the nucleus inducing the expression of IFNβ (Dauphinee and Karsan, 2006). It is important to note that the MyD88 independent pathway can only be triggered from endosomal compartments because TRAF3 is not present in the plasma membrane.

1.7 TLR4 distribution in the intestinal epithelium

The intestinal epithelium requires a mechanism to avoid unwarranted stimulation of TLR4 by commensal bacteria, which may lead to unnecessary immune responses. TLR stimulation is controlled in IECs by limiting the expression of TLR4 at the cell surface and by restricting TLR4 to the basolateral membrane (Fusunyan et al., 2001; Abreu et al., 2002; Otte et al., 2004). In addition, the expression MD-2, a soluble glycoprotein that assists in TLR4 recognition of LPS, is also low in colonic epithelial cells and IECs, which leads to poor LPS responsiveness as indicated by a reduced expression level of NF-κB and the downstream interleukin, IL-8 (Abreu et al., 2002). The localization of TLR4 at the basolateral surface ensures that bacteria that have breached the cell surface, elicit an immune response. TLR4 expression is not only reduced in IECs but it is also confined to specific regions in the intestine, notably in the crypts (Lotz et al., 2007). As mentioned earlier, crypts are regions of stem cell differentiation protected by the antimicrobial peptides that Paneth cells secrete. The presence of antimicrobial peptides leaves the crypt essentially germ-free; in order to prevent unnecessary stimulation by commensal bacteria, TLR4 is restricted to the lower regions (Lotz et al., 2007). TLR4 distribution ensures that a delicate balance is maintained between unwanted immune responses provoked by luminal bacteria and a warranted reaction brought on by pathogenic bacterial invasion.
1.8 TLR4 signaling during *Salmonella* invasion

It has been shown that C3H/HeJ mice, which harbour a TLR4 mutation, are more resistant to septic shock when LPS or wild type *Salmonella* is administered either by intravenous or intraperitoneal injection than wild type mice (O’Brien *et al.*, 1980). However, this difference in reactivity was not seen when these mice were orally infected with wild type *Salmonella*. In this case, the TLR4 knockout mice showed only a small difference in mortality and bacterial colonization (Weiss *et al.*, 2004). Because oral administration of *Salmonella* mimics the natural mode of bacterial uptake, it is significant that the response from the wild type mice was similar to that of the TLR4 knock out. This result signifies that infection ensues regardless of the presence of TLR4, indicating *Salmonella* may inactivate TLR4. In addition, the importance of MyD88 has been shown in mice whose MyD88 deficiency lead to their elevated susceptibility to infection by *Salmonella* (Weiss *et al.*, 2004).

1.9 The role of *Salmonella* effector SigD during invasion

*Salmonella* SPI-1 T3SS effector, SigD, is an inositol phosphate phosphatase. SigD contains two motifs that are found in mammalian inositol polyphosphate 4-phosphatases (Norris *et al.*, 1998) (Figure 3). SigD also shares homology with *Shigella flexneri* effector IpgD, a similar inositol 4-phosphatase (Norris *et al.*, 1998). *In vitro*, SigD can hydrolyze many inositol phosphates and phosphatidylinositides such as PI(3,4)P$_2$, PI(3,5)P$_2$ and PI(3,4,5)P$_3$ (Norris *et al.*, 1998; Marcus *et al.*, 2001). *In vivo*, SigD causes the disappearance of PIP$_2$ during infection (Terebiznik *et al.*, 2002). This is attributed to the dephosphorylation of PIP$_2$ at the plasma membrane. SigD 4-phosphatase activity, which causes dephosphorylation on the fourth position of the inositol ring to produce PI(5)P (Mason *et al.*, 2007), ensures efficient membrane fission and thus contributes to the formation of SCVs (Terebiznik *et al.*, 2002).
Figure 3. Schematic of the SigD gene. SigD contains two motifs that have homology with inositol polyphosphate 4-phosphatases. Within Motif 2, there is a conserved cysteine residue at 460 which is necessary for phosphatase function (Drecktrah et al., 2004). Motif 2 enables the hydrolysis of PI(4,5)P2. The ΔsigD + psigDC462S Salmonella mutant has a point mutation in Motif 2, hence its phosphatase function is inactive. In addition, SigD also has shares homology with the mammalian type II inositol 5'-phosphatase, synaptojanin. Mammalian type II 5-phosphatases hydrolyze inositol (Ins)1,4,5-triphosphate and Ins(1,3,4,5)P4, in addition to PI(4,5)P2 and PI(3,4,5)P3 (Marcus et al., 2001).
The phosphatase activity of SigD is essential for the activation of SGEF, which stimulates RhoG to induce actin modifications that cause endocytosis of bacteria (Patel and Galan, 2006). In addition, SigD is implicated in the production of PI(3)P on SCVs via an indirect mechanism; SigD first recruits Rab5 to the SCV followed by Vsp34 which then phosphorylates PI to form PI(3)P (Mallo et al., 2008). SigD also prevents the apoptosis of epithelial cells by inducing the activation of Akt (Knodler et al., 2005).

Because we know that SigD is directly causing the removal of PIP$_2$ from the plasma membrane and PIP$_2$ is necessary for TIRAP and subsequently, MyD88 recruitment, then this could imply that SigD plays a role in the disruption of the TLR4 pathway. Specifically, it is my contention that the inositol phosphatase activity of SigD and its demonstrated ability to cause the disappearance of PIP$_2$ at membrane invaginations and SCVs during *Salmonella* infection is responsible for this disruption. I suggest that the effect of SigD occurs in the initial moments of infection, preceding the effect of TIRAP.

### 1.10 Hypothesis

I hypothesize that by altering the composition and distribution of PIP$_2$ in the host cell membrane and in the SCVs, SigD will impede TIRAP and TRAM activation early in the invasion process, consequently interrupting TLR4 signaling. The disruption of TLR4 signaling will allow *Salmonella* to bypass host immune detection.

### 1.11 Objectives

For this study, my objectives were the following:

- Determine the effect of SigD on TIRAP localization and activation during *Salmonella* infection;
• Determine the effect of SigD on TLR4 MyD88 dependent pathway and;

• Determine the effect of SigD on TRAM localization and activation in the MyD88 independent pathway.
2 Materials and Methods

2.1 Salmonella strains and DNA constructs

Wild type (Hoiseth and Stocker, 1981), $\Delta$sigD (Steele-Mortimer et al., 2000), $\Delta$sigD + psigD (Steele-Mortimer et al., 2000), $\Delta$sigD + psigD$_{C462S}$ SL1344 (Steele-Mortimer et al., 2000) and RFP-expressing wild type and $\Delta$sigD SL1344 Salmonella enterica serovar Typhimurium strains used in this study were generously provided by Drs. John H. Brumell and Sergio Grinstein (Hospital for Sick Children, Toronto, ON, Canada). RFP expression was controlled by the pBR-RFP plasmid and has been previously described (Birmingham et al., 2006). The TIRAP-GFP and TRAM-GFP plasmid was from Dr. Ruslan Medzhitov (Yale University, New Haven, CT, USA). The pcDNA-CD14 plasmid was from Dr. Fabio Re (University of Tennessee Health Sciences Centre, Memphis, TN, USA). The NF-κB reporter luciferase plasmid was a gift from Dr. Stephen E. Girardin (University of Toronto, ON, Canada). The GFP-PKC$\varepsilon$ plasmid was from Dr. Michelle R. Lennartz (Albany Medical Center, Albany, NY, USA). The IRF3-GFP plasmid was from Dr. Douglas T. Golenbock (University of Massachusetts Medical School, Worcester, MA, USA). All plasmids were chemically transformed and propagated in MAX Efficiency DH5$\alpha$ Competent E. coli cells (Invitrogen, Burlington, ON, Canada). Plasmid DNA was purified using Endofree Plasmid Maxi kit as indicated by the manufacturer (Qiagen, Mississauga, ON, Canada).

2.2 Cell culture and transfection

Human epithelial (HeLa) cell line stably expressing TLR4 (pMyc-CMV-TLR4) and MD-2 (pBOS-MD-2), kindly provided by Dr. Re, was maintained in Dulbecco’s Modified Eagle’s medium (DMEM) (Wisent, St. Bruno, QC, Canada) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Wisent) at 37$^0$C in 5% CO$_2$ with 2 µg/ml blastocytin S (InvivoGen, San Diego, CA, USA). Murine RAW 264.7 macrophage and HeLa cell lines were obtained from
the American Tissue Culture Collection (ATCC) (Manassas, VA, USA) and maintained in DMEM supplemented with 10% heat inactivated FBS (Wisent) at 37°C in 5% CO₂ with no antibiotics. Human colonic HT-29 cell line was also obtained from the ATCC and maintained in McCoy’s 5a Medium Modified (Wisent) supplemented with 10% heat inactivated FBS at 37°C in 5% CO₂ with no antibiotics. Human embryonal kidney cell line stably expressing MD-2 and CD14 (293-hMD2-CD14) was acquired from InvivoGen and cultured in DMEM (Wisent) with 10% heat inactivated FBS and 50 µg/ml HygroGold (InvivoGen). Both HeLa cell lines and HT-29 cells were passaged at near confluency while RAW 264.7 and 293-hMD2-CD14 cells were passaged at 70% confluency. HeLa cell lines and HT-29 cells were used between passage numbers 4-26, RAW 264.7 cells between passage numbers 0-14 and 293-hMD2-CD14 cells between passage numbers 4-20. Approximately 36 hr before infection, cells were seeded onto 18 mm glass cover slips in 12-well tissue culture treated plates at a density of 1x10⁵ cells/ml. Approximately 18-24 hrs before Salmonella infection, transfections were performed using FuGENE HD Transfection Reagent (Roche Diagnostics, Laval, QC, Canada). Transfections were carried out when HeLa cell lines, HT-29 and 293-hMD2-CD14 cells were 50% confluent and 70% confluent for RAW 264.7 cells. Briefly, for a 12 well plate, the following was added per well: 50 µl DMEM (FBS free), 2 µl FuGENE HD, and 1 µg plasmid DNA. The transfection reaction was incubated for 35 min at RT before being added to cells (50 µl/well). Cells were returned to normal growth conditions.

2.3 Preparation of Salmonella for infection of eukaryotic cells

The bacteria used for infection were wild type, ΔsigD, ΔsigD + pfsigD and ΔsigD + pfsigD_C462S Salmonella. Bacterial cultures were grown from glycerol stocks frozen at −80°C 16-20 hr prior to infection in Luria-Bertani (LB) medium at 37°C while shaking at 200 rpm overnight. The
following day, a fresh culture was grown from the overnight culture for 3 hr in the same growth conditions as mentioned previously. An aliquot of bacteria at an optical density of 0.3 was pelleted down at 13 000 rpm for 3 min at RT. The bacterial pellet was washed twice with warm DMEM and pelleted again. Each aliquot of bacteria was suspended in 250 µl of warm DMEM. Invasions were conducted when cells had reached 95% confluency. The inoculum was added to the cells at a multiplicity of infection (MOI) of 100:1, unless otherwise indicated, at 37°C for the specified amount of time. After invasions were completed, cells were washed three times with cold phosphate buffered saline (PBS), pH 7 supplemented with MgCl₂ and CaCl₂ to a final concentration of 1 mM each.

2.4 Live cell imaging of TIRAP

HeLa TLR4/MD-2 cells were seeded onto 25 mm glass cover slips in 6-well tissue culture treated plates and transfected as detailed above except with the following volume changes: 100 µl DMEM (FBS free), 4 µl FuGENE HD and TIRAP-GFP and CD14 (0.8 µg and 0.4 µg, respectively). Wild type and ΔsigD Salmonella (including the RFP strains) were prepared as indicated above except the bacteria pellet was resuspended in Roswell Park Memorial Institute (RPMI) medium (Wisent) supplemented with 10% heat inactivated FBS (Wisent). Cover slips were placed in a temperature and CO₂ controlled chamber, at 37°C and 5% respectively. To assess the distribution of TIRAP during Salmonella invasion, the cells were infected with RFP wild type and ΔsigD Salmonella and imaged throughout a 30 min invasion.

In addition, another live cell imaging experiment was performed to assess the timing of TIRAP recruitment during SCV formation by infecting cells with wild type or ΔsigD Salmonella for 15 min. The cells were then washed with cold PBS to remove extracellular bacteria. The plasma membranes of the cells were visualized using FM4-64 (Invitrogen, Burlington, ON, Canada) that
had been diluted in PBS (MgCl$_2$ and CaCl$_2$ free) to a final concentration of 10 µg/ml. The temperature of the cover slip chamber was lowered to 4°C to stop the invasion and prevent endocytosis of FM4-64. Images were taken with a Leica DMI6000 inverted epifluorescent microscope outfitted with an ImagEM-1K EM-CCD digital camera (Hamamatsu). The images were captured in 3-D, in addition to time, and the stage automatically changed positions in order to document multiple points in the field of view. Images were acquired as 0.5 µm Z-stacks. Images were deconvolved using Volocity software (PerkinElmer) with a point spread function according to the following parameters: a refractory index of 1.5, a numerical aperture of 1.4, magnification at 63x, axial spacing in Z plane of 0.05 µm and lateral spacing in XY of 0.067 µm.

2.5 Assessing TIRAP localization by immunostaining and confocal microscopy

HeLa, HT-29 or RAW 264.7 cells were seeded onto 18 mm glass cover slips in 12-well tissue culture treated plates and transfected with TIRAP-GFP. Wild type SL 1344, ΔsigD, ΔsigD + psigD and ΔsigD + psigD$_{C462S}$ Salmonella were prepared as mentioned and used at an MOI of 100:1. After 15 min, the cells were washed twice with warm PBS to eliminate bacteria that had not been internalized and then warm DMEM was added. Invasions were carried out for 10, 20 and 30 min time points. Note that for the 10 min time point, the wash step was omitted. Cells were washed three times with cold PBS to terminate invasions. All incubations were carried out at RT unless otherwise indicated. Cells washed with PBS and then fixed with 4% paraformaldehyde (PFA) (Canemco Inc., Lakefield, QC, Canada) diluted in PBS for 30 min. After washing with PBS, cells were blocked with 5% non-fat milk (NFM) (Carnation) in PBS for 1 hr. For immunofluorescence, both primary and secondary antibodies were diluted in 5% NFM in PBS and incubations were for 1 hr. To label external bacteria, Salmonella O antisera Group B factors 1, 4, 12, 27 (BD Difco, Franklin Lakes, NJ, USA) was used at a dilution of 1:50 followed
by Alexa Fluor 647 donkey anti-mouse IgG (Molecular Probes, Eugene, OR, USA) at a dilution of 1:2000. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 30 min. Internal bacteria was labeled in the same manner as external bacteria except using Alexa Fluor 555 donkey anti-mouse IgG (Molecular Probes, Eugene, OR, Canada). Cover slips were mounted onto glass slides (0.13 – 0.17 mm) using Dako Fluorescent Mounting Medium (Dako, Mississauga, ON, Canada). Fixed samples were analysed using a Zeiss LSM 510 laser scanning confocal microscope with 63x oil immersion objective.

To quantify the percentage of TIRAP positive bacteria per cell, the number of internal bacteria enclosed in a TIRAP positive SCV 30 mpi were counted for 100 cells in three independent experiments and averaged. To quantify the percentage of invasion, the number of internalized bacteria 30 mpi, as indicated by differential immunostaining, was counted for 100 cells. The data are a representation of three independent experiments. The values were tabulated with Prism 4 (Graphpad Software Inc.) and are given as means ± standard deviations. Student’s T test was performed on the data for the number of TIRAP positive bacteria per cell and efficiency of invasion, with values of p <0.05 considered significant.

2.6 Detection of IκB-α degradation by western blot analysis

HeLa TLR4/MD-2 cells were seeded onto 3.5 cm tissue culture treated Petri dishes at a density of 1 x 10^5 cells/ml. When the plates had reached near total confluency, cells were infected with wild type SL 1344, ΔsigD and ΔsigD + psigD Salmonella. Bacteria were prepared in the same manner as described above and added to the cells at a MOI of 100:1. Invasions were carried out at 37°C, after which cells were washed three times with cold PBS, scraped and pelleted at 1000 rpm for 10 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 100-200 µl RIPA lysis buffer (150 mM NaCl, 1% Nonidet P40 (NP40), 0.5% deoxycholate,
0.9% SDS, 50 mM Tris, pH 8.0) supplemented with phosphatase inhibitor cocktail 3, phosphatase inhibitor cocktail 2 and protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA), all at a dilution of 100:1. Cells were incubated on ice for 30 min, vortexed intermittently and pelleted at 13 000 rpm for 10 min at 4\(^0\)C. Protein concentration was determined by Lowry protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada) as indicated by the manufacturer, using bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO, USA) as protein standards. Proteins were mixed with 4x Sample buffer (1M Tris-Hcl, pH 6.8, 20% glycerol, 10% SDS, 10% 2-mercaptoethanol, 1% bromophenol blue) and boiled for 10 min. Proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membrane. Equal protein loading was confirmed by Ponceau S staining. The membrane was blocked with 5% NFM in Tris-buffered saline (TBS)/Tween-20 (TBST) (1 x TBS, pH 7.4, 0.1% Tween-20) for 1 hr at RT with slight agitation. All washes consisted of three repetitions, 5 min each time with TBST. After the membrane was washed, they were incubated with rabbit I\(\kappa\)B-\(\alpha\) (Cell Signaling Technology, Pickering, ON, Canada) in 5% BSA/TBST at a dilution of 1:1000, overnight with slight agitation at 4\(^0\)C. The membrane was washed and I\(\kappa\)B-\(\alpha\) was detected with peroxidase-conjugated AffiniPure donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was added at a dilution of 1:2000 in TBST for 1 hr at RT with slight agitation. The membrane was washed again with TBST and luminescence was visualized with Supersignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) was used according to the manufacturer’s instructions.

To demonstrate the inhibition of LPS induced activation of TLR4 by polymixin B (PMB), samples were prepared as described in the detection of I\(\kappa\)B-\(\alpha\). However, the preparation of the
Salmonella involved resuspending the bacterial pellet in DMEM treated with 100 µg/ml PMB (InvivoGen, San Diego, CA, USA) prior to infection. The inoculum was added to the cells at a MOI of 100:1 at 37°C. After 10 min, cells were washed twice with warm PBS and DMEM treated with 50 µg/ml PMB was added. The invasions were allowed to proceed for the indicated amount of time.

Densitometric analysis of protein bands was performed using NIH Image J software to determine the relative fold decrease of IκB-α degradation in comparison to uninfected (control) cells.

2.7 Detection of TIRAP phosphorylation by immunoprecipitation (IP) and western blot analysis

HeLa TLR4/MD-2 cells were seeded onto 10 cm tissue culture treated Petri dishes at a density of 3.75 x 10⁶ cells/ml. When the plates had reached 50% confluency, the cells were transfected with TIRAP-GFP and CD14 using the procedure described above. However, the following volumes were used per reaction: 500 µl DMEM (serum free), 10 µg DNA (5 µg TIRAP, 5 µg CD14) and 20 µl FuGENE HD. After the incubation, 500 µl of the transfection reaction was added per plate. Wild type and ΔsigD Salmonella was prepared as previously mentioned. The inoculum was added to the cells at a MOI of 100:1 (in this case, 425 µl bacteria per plate). In addition, Ultra Pure E. coli LPS, (InvivoGen, San Diego, CA, USA) was used at 0.1µg/ml. The invasions were carried out at 37°C for 15 min and then the cells were washed three times with cold PBS. The cell lysates were collected as described above for the degradation of IκB-α however to account for a larger Petri dishes, the volume of RIPA used to resuspend the cells was approximately 500 µl. After protein quantification, each IP sample was diluted in RIPA buffer so that a minimum of 500 µg of protein was used, for a final concentration of 0.8-1 µg/µl in RIPA lysis buffer. Each sample was pre-cleared using Protein G on Sepharose 4B fast flow (Sigma-Aldrich, Saint Louis,
MO, USA) for 1 hr at 4°C with slight agitation. Samples were gently centrifuged for 5 sec so as
not to break the beads. The supernatant was transferred to a clean tube to which anti-GFP, rabbit
IgG fraction (Molecular Probes Inc., Eugene, OR, USA) was added at dilution of 1:500 over
night at 4°C with slight agitation. The following day, approximately 40 µl of beads to each 500
µl IP sample and incubated at 4°C with slight agitation for 1 hr. The beads were then washed
carefully three times with RIPA lysis buffer. The supernatant was gently removed and the beads
were resuspended in 25 µl of 2x Sample buffer in MilliQ water and boiled for 10 min. The
supernatant was collected, separated on a 10% SDS-PAGE and transferred onto nitrocellulose
membrane as previously mentioned. The membrane was blocked with 3% BSA for 1 hr at RT
and then incubated with mouse 4G10 Platinum anti-phosphotyrosine (Millipore, Temecula, CA,
USA) in 3% BSA at a dilution of 1:1000 over night at 4°C with slight agitation. The membrane
was washed four times with TBST, 3 min each time. To detect tyrosine phosphorylated proteins,
peroxidase-conjugated AffiniPure donkey anti-mouse IgG antibody was used at 1:2000 in 3%
BSA/TBST for 1 hr at RT. The membrane was washed one last time for 5 min with TBS-0.05%
Tween-20. The Supersignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL,
USA) was used as per manufacturer’s instructions.

2.8 NF-κB luciferase assay

HeLa TLR4/MD-2 cells were seeded into 24-well tissue culture treated plates (no glass cover
slips) at a cell density of 5 x 10⁴ cells/ml. The day before infection, cells were transfected with
NF-κB luciferase and CD14 plasmids according to the same volumes as indicated above for a
12-well plate with the following exceptions: per reaction, 0.6 µg NF-κB and 0.4 µg CD14 was
used and only 25 µl of the transfection reaction was added to each well. When the cells had
reached near total confluency, they were infected with wild type and ΔsigD Salmonella. The
inoculum was added to the cells at a MOI of 100:1 (25 µl bacteria per well) and 50:1 (25 µl of diluted 100:1 bacteria) in separate plates. In addition, Ultra Pure E. coli LPS was used at 0.1µg/ml. The infection proceeded for 30 min at 37°C then the cells were washed twice with warm PBS and warm DMEM was added. One hr post infection (hpi), PMB-treated DMEM was added to each well to a final PMB concentration of 50 µg/µl. Samples were taken at 2, 3 and 6 hpi by washing cells three times with cold PBS and then using the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI) according to manufacturer’s instructions. Samples were frozen at –20°C for short term storage. Luminescence of each sample was analyzed using the Biotek Synergy HT microplate reader. The data are a representation of three independent experiments. The values were tabulated with Prism 4 (Graphpad Software Inc.) and are given as means ± standard deviations. Student’s T test was performed on the data with values of p <0.05 considered significant.

2.9 Assessing TRAM localization by immunostaining and confocal microscopy

293-hMD2-CD14 cells were seeded onto glass cover slips in 12 well plates as mentioned. 293-hMD2-CD14 cells were transfected with TRAM-GFP and infected with wild type and ΔsigD Salmonella at an MOI of 100:1. After 15 min, 293-hMD2-CD14 cells were washed with cold PBS and fixed with 4% PFA as indicated above. Internal and external bacteria were immunostained as outlined for the localization of TIRAP-GFP.

2.10 Assessing PKCε and IRF3 localization by immunostaining and confocal microscopy

HeLa TLR4/MD-2 cells were seeded onto 18 mm glass cover slips in 12-well tissue culture treated plates and transfected with either GFP-PKCε or IRF3-GFP. Cells transiently expressing
GFP-PKCɛ were infected wild type and ΔsigD Salmonella for 30 min. Cells transiently expressing IRF3-GFP were infected wild type and ΔsigD Salmonella for 6 hr. Ultra Pure E. coli LPS, (InvivoGen) was included in cells transiently expressing IRF3-GFP and used at 0.1µg/ml. For both experiments, cells were washed with warm PBS 10 min after and resuspended in warm DMEM. Internal and external Salmonella was immunostained as detailed for TIRAP-GFP localization.

2.11 Detection of PKCɛ phosphorylation by western blot analysis

HeLa TLR4/MD-2 cells were seeded onto 10 cm tissue culture treated Petri dishes at a density of 3.75 x 10^6 cells/ml. Treatment of wild type and ΔsigD Salmonella and infections were performed out as described in the detection of IκB-α degradation. Cells were washed with warm PBS 10 min after infection and resuspended in warm DMEM. Invasions were carried out for 30 and 45 min after the start of infection and cell lysates were collected as indicated above. Proteins were resolved on a 10% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 5% NFM in TBS- 0.05% Tween 20 for 1 hr at RT with slight agitation. Rabbit anti-phospho PKCɛ (s379) (Millipore) was used to detect phosphorylated PKCɛ at a dilution of 1:1000 in 5% NFM/TBS-0.05% Tween-20 overnight at 4°C with slight agitation. The membrane was rinsed twice with MilliQ water and exposed to peroxidase-conjugated AffiniPure donkey anti-rabbit IgG antibody at 1:2000 in 5% NFM/TBS-0.05% Tween-20 for 1 hr at RT. The membrane was washed with TBS-0.1% Tween-20 for 15 min at RT. The Supersignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) was used as per manufacturer’s instructions.
3 Results

3.1 SigD disrupts TIRAP localization to plasma membrane invaginations and SCVs

Kagan and colleagues showed that an N-terminal PIP$_2$ binding domain mediates TIRAP localization to the plasma membrane (Kagan and Medzhitov, 2006). Terebiznik and colleagues demonstrated that PIP$_2$ had a distinct distribution during Salmonella infection; in addition to accumulating in the ruffling membrane, PIP$_2$ was also displaced from the invaginating membrane enclosing wild type Salmonella (Terebiznik et al., 2002). Although SigD was the cause of PIP$_2$ displacement from the membrane, how this affected the association of TIRAP to the membrane during infection remained unclear. Thus, I assessed if the phosphatase activity of SigD would interfere with TIRAP localization. I first followed TIRAP distribution during the initial stages of infection to assess the timing of its removal and from where along the membrane it was detaching. HeLa cells that stably express TLR4 and MD-2 were transiently transfected with CD14 and TIRAP-GFP. To visualize TIRAP during the invasion process by live cell imaging, cells were infected with wild type or ΔsigD Salmonella that express red fluorescent protein (RFP). The infection was carried out at $37^\circ$C and imaging began immediately as the bacteria were added to the cells in the heated chamber on the microscope stage. Over a period of 30 min after the onset of infection, images were acquired every 45 sec as 0.5 µm Z-stacks. At the start of the infection, TIRAP was clearly present in ruffling membrane of cells infected with either wild type or ΔsigD bacteria (Supplementary Movie 1 and 2). As infection continued, internalized wild type bacteria did not associate with TIRAP (Supplementary Movie 1). In contrast, I observed that subsequent to the internalization of ΔsigD bacteria (17 min post infection), the SCVs were TIRAP positive and remained that way as they migrated from the plasma membrane towards the nucleus (Supplementary Movie 2). The time-lapsed images
indicated that as early as 6 min after the onset of membrane ruffling, the wild type SCVs formed were not delineated by TIRAP-GFP (Figure 4, indicated by arrow in inset of top panel). This suggests that TIRAP is removed from the membrane early during the formation of SCVs. However, the SCVs enclosing ΔsigD bacteria that had pinched off by 6 min were clearly labeled by TIRAP-GFP (Figure 4, indicated by arrow in inset of bottom panel). This indicates that TIRAP associated with SCVs is derived from the plasma membrane.

I next focused on when TIRAP was being displaced from the membrane during infection. This required the membrane to be labeled with the solvatochromatic dye, FM4-64. This dye only remains associated with the outer leaflet of the plasma membrane thus it will label structures that are continuous with the membrane such as invaginations but not SCVs which have pinched off already. HeLa TLR4/MD-2 cells transiently transfected with CD14 and TIRAP-GFP were infected with wild type or ΔsigD Salmonella for 15 min. Cells were cooled to 4°C to stop the invasion. To inhibit endocytosis of the dye, FM4-64 was added to cells at 4°C. For both strains of bacteria, the ruffling membrane demonstrated the presence of TIRAP-GFP. However, in cells infected with wild type Salmonella, the clefts at the bottom of the ruffling membrane were defined only by FM4-64, showing no TIRAP-GFP (Figure 5A, indicated by arrow in inset). This was in contrast to ΔsigD-infected cells, which demonstrated membrane clefts clearly defined by both FM4-64 and TIRAP-GFP (Figure 5B, indicated by arrow in inset). Thus, this conclusively showed that TIRAP was eliminated in the membrane before pinching off of the SCVs.

To quantify TIRAP recruitment to SCVs, HeLa cells were transiently transfected with TIRAP-GFP and infected with wild type (Hoiseth and Stocker, 1981) or ΔsigD (Steele-Mortimer et al., 2000) Salmonella at a multiplicity of infection (MOI) of 100 bacteria to 1 cell (100:1). Control cells were not infected and showed TIRAP clearly in the plasma membrane (data not shown). As
Figure 4. TIRAP is removed during SCV formation in a SigD dependent manner. Live cell imaging depicting the time course distribution of TIRAP during invasion of HeLa cells with RFP wild type (top panels) or ΔsigD (bottom panels) *Salmonella*. HeLa cells are stably transfected with TLR4 and MD-2 and transiently express TIRAP-GFP and CD14. Images were acquired as 0.5 μm Z-slices taken at 45 sec intervals and deconvolved using Volocity software. Scale bar, 10 μm.
Figure 5. TIRAP is removed from membrane clefts before complete SCV fission. Live cell imaging of TIRAP distribution during SCV formation. HeLa cells stably expressing TLR4/MD-2 and transfected with CD14 and TIRAP-GFP were invaded with wild type (A) or ΔsigD (B) Salmonella for 15 min. Plasma membrane was visualized using FM4-64. Insets show membrane clefts stained with FM4-64 (red) and TIRAP localization in the clefts (green). Images were acquired as 0.5 μm Z-slices at 45 sec intervals and deconvolved using Volocity software. Scale bar, 10 μm.
seen during the initial minutes of live cell infections, the ruffling plasma membrane of cells infected with either wild type or \(\Delta\text{sig}D\) \textit{Salmonella} distinctly showed TIRAP (data not shown). Differential immunostaining revealed that at 10 min post infection (mpi), the SCVs that contained wild type \textit{Salmonella} did not show TIRAP-GFP (Figure 6A). However, at the same time, cells infected with \(\Delta\text{sig}D\) \textit{Salmonella} contained TIRAP-GFP positive SCVs (Figure 6D). The phenotype seen in \(\Delta\text{sig}D\)-infected cells is consistent throughout the 30 min invasion as seen at the 20 min (Figure 6B and E) and 30 min (Figure 6C and F) time points. After quantification, we observed that at 30 min, only 5% of SCVs containing wild type bacteria were labeled with TIRAP-GFP while 60% of SCVs from \(\Delta\text{sig}D\) bacteria showed TIRAP-GFP (\(p < 0.05\)) (Figure 6G). SigD has been shown to facilitate the formation of SCVs, such that \(\Delta\text{sig}D\) bacteria demonstrate a reduced rate of membrane fission during SCV formation (Terebiznik \textit{et al}., 2002). Thus, we needed to assess whether both strains of bacteria were being internalized in the same quantity so that the percentage of TIRAP positive SCVs per cell was independent of the efficiency of invasion. The number of total internalized bacteria was counted for the same experiment at 30 min post invasion. Considering the efficiency of invasion for wild type and \(\Delta\text{sig}D\) bacteria was approximately 20% and 25%, respectively, the difference in internalization was not significant (\(p > 0.05\)). Thus, they both demonstrated a similar level of invasion and so their percentage of TIRAP positive SCVs was comparable (Figure 6H). Therefore, we determined that the displacement of TIRAP from SCVs is PIP\(_2\) dependent. This disruption of TIRAP distribution was not due to varying levels of invasion efficiency because both wild type and \(\Delta\text{sig}D\) \textit{Salmonella} had similar success in invading cells.
Figure 6. TIRAP remains associated to ΔsigD Salmonella. HeLa cells were transfected with TIRAP-GFP and invaded with wild type (A, B and C) or ΔsigD (C, D and F) at a multiplicity of infection (MOI) of 100:1. After 10 min, bacteria was removed, fresh media was added and the invasion continued until the specified time (see figure). After cells were fixed, external bacteria was immunostained with Alexa Fluor 647 (blue) and total bacteria was labelled with Alexa Fluor 555 (red). Top insets for A-F show the co-localization of TIRAP with bacteria in the SCVs. The bottom insets show TIRAP recruitment in the SCVs alone. Images are representative of three independent experiments. The percentage of internalized bacteria enclosed in vacuoles expressing TIRAP-GFP after 30 min with wild type or ΔsigD Salmonella (G) and the efficiency of invasion from the same assay (H) are representative of 100 cells from three independent experiments. Scale bar, 10µm.
3.2 The displacement of TIRAP from the plasma membrane and SCVs is due to the phosphatase activity of SigD

To investigate if SigD phosphatase activity removes TIRAP from the SCVs, we incorporated two additional SigD mutant Salmonella strains. The ΔsigD + psigD strain (Steele-Mortimer et al., 2000) is complemented with a plasmid that expresses the wild type SigD gene. The ΔsigD + psigD<sub>C462S</sub> strain (Steele-Mortimer et al., 2000) is similar to ΔsigD + psigD bacteria however, it is complemented with a SigD gene containing a cysteine to serine point mutation (C462S) in the 4-phosphatase domain which inactivates the phosphatase function (Figure 3). Infection of cells with wild type bacteria once again showed SCVs lacking TIRAP (Figure 7A, see arrowhead in inset). As shown before, cells infected with ΔsigD bacteria had TIRAP positive SCVs (Figure 7C, see arrowhead in inset). Interestingly, our results show that cells infected with the ΔsigD + psigD mutant showed SCVs devoid of TIRAP mirroring the phenotype induced by wild type bacteria (Figure 7B, see arrowhead in inset). In contrast, infection with ΔsigD + psigD<sub>C462S</sub> bacteria showed the presence of TIRAP positive SCVs, as seen with ΔsigD Salmonella (Figure 7D, see arrowhead in inset). Taken together, this proves that not only is SigD responsible for the removal of TIRAP from the membrane, but it is strictly the PIP<sub>2</sub> hydrolyzing activity of SigD that is interfering with the association of TIRAP to the membrane.

3.3 The SigD dependent removal of TIRAP from the plasma membrane during Salmonella infection is confirmed in macrophage and intestinal cell lines

Macrophages form part of the innate immune system and are required in the clearance of pathogenic bacteria that have passed from the intestinal lumen into IECs (Hisamatsu et al., 2008). We reasoned that because TLR4 is essential for Salmonella recognition, then we should expect SigD to cause a disruption in TIRAP localization in macrophages. When RAW 264.7
Figure 7. The displacement of TIRAP in SCVs is dependent on the phosphatase activity of SigD. HeLa cells were invaded with wild type (A), ΔsigD + psigD (B), ΔsigD (C) or ΔsigD + psigDc462s (D) Salmonella at an MOI of 100:1. At 10 minutes post invasion, bacteria was removed, fresh media was added and the invasion continued until 30 min. Cells were fixed and external bacteria was fluorescently labeled with Alexa Fluor 647 (blue). Cells were then permeabilized with 0.1% Triton X-100 and total bacteria was fluorescently labelled with Alexa Fluor 555 (red). The top insets for C and D show the co-localization of TIRAP with bacteria in the SCVs. The bottom insets for C and D show TIRAP recruitment in the SCVs alone. Images are representative of three independent experiments. Scale bar, 10 μm.
cells that transiently expressed TIRAP-GFP were infected with wild type bacteria, SCVs were devoid of TIRAP-GFP. However, in \( \Delta sigD \) Salmonella infected cells, the distribution of TIRAP mirrored that observed in HeLa cells such that SCVs were positive for TIRAP (Figure 8A, arrowhead in inset). Thus, in macrophages as in HeLa cells, SigD phosphatase activity disrupted the recruitment of TIRAP to SCVs. In addition, we used a human colonic cell line (HT-29) instead of epithelial cells as a better representation of the intestinal environment. HT-29 cells are polarized cells, demonstrating distinct apical and basolateral domains. Once again as in HeLa cells, HT-29 cells exhibited bacteria enclosed in TIRAP-positive SCVs when infected with \( \Delta sigD \) Salmonella (Figure 8B, arrowhead in inset). This data is significant because it validates my previous TIRAP results that were carried in epithelial cells.

In addition, I have preliminary data showing that TIRAP phosphorylation may be disrupted during Salmonella invasion, indicating another possible way in which SigD interrupts the MyD88 dependent pathway (Figure 9). TIRAP is tyrosine phosphorylated by BTK during TLR4 signaling (Gray et al., 2006). The kinase activity of BTK is stimulated by Src kinases once it translocates to the membrane (Jefferies et al., 2003). Western blot analysis shows that during the initial 15 min of invasion, cells infected with \( \Delta sigD \) Salmonella had an increased level of TIRAP phosphorylation than did cells infected with wild type Salmonella as indicated by the intense band at approximately 59 kDa (Figure 9, top blot). In addition, I quantified the level of TIRAP protein in the immunoprecipitated samples and found that although there was a substantially lower amount of total TIRAP in the cells infected with \( \Delta sigD \) Salmonella, these cells still exhibited a dramatically greater level of activated TIRAP than wild type-infected cells (Figure 9, lower blot). This indicates that SigD not only disrupts TIRAP localization, but it may also interfere with its activation as well.
Figure 8. SigD dependent removal of TIRAP from SCVs also occurs in RAW 264.7 and HT-29 cell lines. RAW 264.7 (A) and HT-29 (B) cells were transfected with TIRAP-GFP and infected with wild type or ΔsigD Salmonella (MOI 100:1) for 15 min. After cells were fixed, external bacteria was immunostained with Alexa Fluor 647 (blue) and total bacteria was labelled with Alexa Fluor 555 (red). In cells infected with ΔsigD Salmonella, top insets show the co-localization of TIRAP with bacteria in the SCVs; the bottom insets show TIRAP recruitment in the SCVs alone. Scale bar, 10 μm.
Figure 9. SigD may attenuate TIRAP activation during *Salmonella* invasion. HeLa TLR4/MD-2 cells were transfected with CD14 and TIRAP-GFP and infected with wild type or ΔsigD *Salmonella* (MOI 100:1) for 15 min. As a positive control, 0.1 μg/ml Ultra Pure *E. coli* LPS was used. Control cells were uninfected. TIRAP-GFP was immunoprecipitated using rabbit anti-GFP antibody. Phosphorylated TIRAP (59 kDa) was detected by immunoblotting with phospho-tyrosine antibody. Using the same blot, TIRAP-GFP was detected using mouse anti-GFP antibody. The molecular weight markers are indicated by MW (the 62 kDa standard is shown).
3.4 SigD interferes with the MyD88 dependent pathway

Because SigD caused the disappearance of TIRAP from membrane clefts and SCVs in addition to reducing TIRAP phosphorylation, I reasoned that SigD would ultimately inhibit downstream TIRAP-TLR4 signaling. I investigated this by following the degradation of IκB-α during Salmonella invasion. IκB-α is an inhibitor of NF-κB by binding to it in the cytosol. Upon activation of TLR4, IκB-α is degraded thereby releasing NF-κB, allowing it to translocate to the nucleus and initiate the transcription of pro-inflammatory genes. HeLa TLR4/MD2 cells were infected with wild type or ΔsigD Salmonella for 6 hr and sampled at 30 min post infection and again at 1, 3 and 6 hrs. Although wild type infected cells showed minimal IκB-α degradation 1 hr after the onset of invasion (indicated by the 59 kDa band), the amount of IκB-α was consistent thereafter (Figure 10A, left blot). However, 30 min after infection with ΔsigD Salmonella, IκB-α levels begin to diminish and by 3 hrs, it had completely degraded (Figure 10A, right blot). Densitometric analysis reveals that IκB-α levels in ΔsigD infected cells had diminished by 60% compared to uninfected (control) cells while wild type infected cells only demonstrated a 40% decrease (Figure 10B). This indicates that by interfering with TIRAP localization and phosphorylation, SigD is consequently attenuating TLR4 signaling. In a similar experiment, I infected cells with the ΔsigD + pσD mutant in addition to wild type and ΔsigD Salmonella. Previous immunofluorescent imaging revealed that the wild type phenotype could be rescued by the expression of exogenous SigD, proving that SigD disrupts recruitment of TIRAP to SCVs (Figure 7). Cells infected with the ΔsigD + pσD mutant did not exhibit IκB-α degradation at 1 and 2 hrs post invasion as seen with wild type-infected cells (Figure 10C). Densitometric analysis reveals that IκB-α levels in ΔsigD infected cells had diminished by 62% compared to uninfected (control) cells by the first hour while wild type infected cells only
Figure 10. **SigD interrupts the degradation of NF-κB inhibitor, IκB-α.** HeLa TLR4/MD-2 cells were cultured on 10 mm Petri dishes and invaded with wild type or ΔsigD Salmonella (A) or ΔsigD + psigD Salmonella (C). Control cells, C on blot, were uninfected. After 10 min, bacteria were removed, fresh media was added and the invasion continued until the specified amount of time. Whole cell lysates were collected and immunoblotting was performed using anti-IκB-α antibody (59 kDa). Equal sample loading was verified by Ponceau staining. Densitometric analysis of IκB-α degradation for wild type and ΔsigD Salmonella (B) or ΔsigD + psigD Salmonella (D). Results are representative of three independent experiments.
demonstrated a 39% decrease (Figure 10D). In addition, as I showed in the immunofluorescent images, densitometric analysis confirmed that the wild type phenotype could be rescued by incorporating the $\Delta\text{sig}D + p\text{sig}D$ mutant; infected cells only showed a 36% reduction IκB-α levels (Figure 10D). This confirms that SigD disrupts the MyD88 dependent pathway, specifically at the level of IκB-α degradation.

As mentioned previously, LPS recognition occurs through a series of protein-protein interactions. LBP binds LPS and shuttles it to CD14, which can be membrane bound or occur in a soluble form. CD14 then transfers LPS to the TLR4/MD-2 complex for LPS recognition and activation of TLR4 (Wright et al., 1990). Thus to confirm the results of the IκB-α degradation assay, I reconstructed the TLR4 receptor complex by transfecting cells with CD14. As in the previous experiment, IκB-α shows degradation with $\Delta\text{sig}D$ but not wild type Salmonella (Figure 11). In addition, I wanted to specifically attribute the observed IκB-α degradation to LPS recognition occurring through TLR4 only. I achieved this by incorporating PMB, an inhibitor of LPS-induced activation of TLR4 during invasion. PMB is an antibiotic that binds and neutralizes the effect of LPS (Okamura et al., 2001). Thus, by sequestering LPS, PMB prevents it from activating TLR4. In cells infected with either wild type or $\Delta\text{sig}D$ Salmonella and treated with 50 µg/ml PMB, degradation of IκB-α was completely blocked (Figure 11, PMB ‘+’ lanes). Thus, SigD was specifically interfering with downstream TLR4 signaling pathway.

3.4.1 SigD activity interferes with downstream TLR4 signaling by attenuating NF-κB activation

Although I demonstrated that SigD was disrupting the MyD88 dependent pathway at the level of IκB-α degradation, I wanted to further explore how it ultimately affected the activation of NFκB, a process that induces the expression of pro-inflammatory genes. To investigate this, I
Figure 11. SigD disrupts TLR4 downstream signaling in the MyD88 dependent pathway. HeLa TLR4/MD-2 cells were cultured on 10 mm petri dishes and invaded with wild type or ΔsigD Salmonella. After 10 min, bacteria were removed and fresh media was added that either contained no Polymixin B (PMB) or 50 µg/ml PMB. The invasion was allowed to proceed for the specified amount of time. The PMB treated cells were infected for 2 hr. Control cells were uninfected. Whole cell lysates were collected and immunoblotting was done using an anti-IκB-α antibody (59 kDa) (A). Equal sample loading was verified by Ponceau staining. Densitometric analysis of corresponding blot shown in B. Results are representative of three independent experiments.
measured the transcriptional activity of NF-κB utilizing a luciferase reporter assay. In this assay, the luciferase reporter gene is under the control of NF-κB; changes in the signaling activity of luciferase, measured as luminescence (relative luciferase units, RLU), will be indicative of changes in the activity of NF-κB (Ghim et al., 2010). HeLa TLR4/MD-2 cells transfected with CD14 and a luciferase-reporter construct of NF-κB (Tattoli et al., 2008) were infected with wild type or ΔsigD Salmonella for 6 and 8 hrs (Figure 12). Transfected cells that were neither stimulated with LPS or bacteria were measured but showed negligible luminescence indicating that RLU values from bacteria infected samples were not the result of an immune response induced by the transfection procedure alone. As a control, transfected cells were stimulated with 0.1 µg/ml LPS. When measured, the LPS-stimulated cells had a significant 40-fold increase in NF-κB activity in comparison to control cells, p <0.05 (data not shown). This result verified the luciferase assay as a valid method for detecting NF-κB activity. At 6 hrs post invasion, cells infected with ΔsigD Salmonella had an elevated level of NF-κB activity, showing a 47-fold difference in NF-κB activity in contrast to wild type-infected cells which only demonstrated a 32-fold difference (Figure 12). Although the luminescence decreased by 8 hrs post infection, cells infected with ΔsigD bacteria still showed a greater level of NF-κB activity than in infections carried out with wild type bacteria (a 30- and 21-fold difference, respectively). Thus, these data conclusively showed that the SigD mediated disruption of TIRAP recruitment in SCVs interferes with TLR4 downstream signaling by attenuating the activity of NF-κB.

3.5 SigD and TLR4 MyD88 independent pathway

3.5.1 TRAM localization is unaffected by SigD activity

TRAM contains a myristoylated region which enables it associate with the plasma membrane and therefore, unlike TIRAP, its localization to the membrane is not dependent on PIP₂
Figure 12. SigD interferes with the MyD88 independent pathway by decreasing NF-κB activity. HeLa cells stably expressing TLR4 and MD-2 were transfected with CD14 and a luciferase-reporter construct of NF-κB. Cells were infected with wild type or ΔsigD Salmonella at an MOI of 100:1 for 6 and 8 hr. As a positive control, LPS was added to cells at 0.1 µg/ml. Transfected control cells were uninfected. Values represent fold increase of luciferase activity in treated cells over values from untreated cells. At 6 hr, LPS-stimulated cells had a 40 fold increase in luciferase activity in comparison to control (data not shown). Data shown are the means ± standard deviations of triplicates and are representative of three independent experiments.
(Kagan et al., 2008). In agreement with this, 293-hMD2-CD14 cells transfected with TRAM-GFP and infected with either wild type or ΔsigD bacteria for 15 min demonstrated TRAM localized in the membrane (Figure 13, inset). Thus, the phosphatase activity of SigD did not displace TRAM from the plasma membrane as was seen with TIRAP-GFP (Figure 6).

I then assessed TRAM activation by focusing on the localization and activation of PKCε. PKCε is necessary for the phosphorylation of TRAM (McGettrick et al., 2006). We explored PKCε recruitment to the membrane during infection by confocal microscopy by incorporating a GFP-PKCε construct (Larsen et al., 2002). In cells infected with wild type Salmonella, PKCε did not localize to SCVs (Figure 14A). However, PKCε showed recruitment to the SCVs when HeLa TLR4/MD-2 cells were infected with ΔsigD Salmonella (Figure 14B). These data are consistent with the fact that PKCε recruitment to the membrane should be impeded due to the lack of DAG present in the membrane. DAG production would be interrupted due to SigD mediated hydrolysis of PIP2. If PIP2 is not available, PLC cannot hydrolyze it to produce DAG.

Considering SigD activity was affecting PKCε localization, I assessed its activation via western blot. TRAM activation is dependent on phosphorylation of serine residue 16 by PKCε (McGettrick et al., 2006). In turn, PKCε activation needs to be phosphorylated by glycogen synthase kinase (GSK) and PKC (Durgan et al., 2008). HeLa TLR4/MD-2 cells infected with ΔsigD Salmonella showed a greater induction of phosphorylated PKCε than cells infected with wild type Salmonella at 45 min after the onset of infection (Figure 14C). Put together, this indicated that SigD interrupts the localization as well as, the phosphorylation of PKCε. As a result, this would suggest that TRAM activation may also be disrupted, leading to inefficient signaling of the MyD88 independent pathway.
**Figure 13. SigD has no affect on TRAM-GFP localization.** 293-hMD2-CD14 were transfected with TRAM-GFP and infected with wild type or ΔsigD Salmonella (MOI 100:1) for 15 min. Cells were fixed and external bacteria was fluorescently labelled with Alexa Fluor 647 (blue). Cells were then permeabilized with 0.1% Triton X-100 and total bacteria was fluorescently labelled with Alexa Fluor 555 (red). Top insets show the co-localization of TRAM with bacteria in SCVs. Bottom insets show TRAM recruitment in the SCVs alone. Images are representative of two independent experiments. Scale bar, 10 μm.
Figure 14. SigD impedes GFP-PKCε recruitment and attenuates its activation. HeLa TLR4/MD-2 cells were transfected with GFP-PKCε and invaded with wild type (A) or Δ sigD Salmonella (B) (MOI 100:1). After cells were fixed, external bacteria was immunostained with Alexa Fluor 647 (blue) and total bacteria was labelled with Alexa Fluor 555 (red). The top insets for A and B show the co-localization of PKCε with bacteria in the SCVs. The bottom insets show PKCε recruitment in the SCVs alone. Images are representative of two independent experiments. Detection of phospho-rylated PKCε (C) was determined by collecting lysates from HeLa TLR4/MD-2 cells invaded with wild type or Δ sigD Salmonella and immunoblotting with anti-phospho-PKCε (ser729) (95 kDa). Equal sample loading was verified by Ponceau staining. Data representative of three independent experiments. Scale bar, 10 μm.
3.5.2 Localization of IRF3 during Salmonella infection

To investigate the disruption of signaling downstream of TRAM, IFR3 localization was assessed. Once the MyD88 independent pathway is activated, IRF3 becomes phosphorylated and forms a dimer in the cytosol. This leads to its translocation into the nucleus, where it initiates the transcription of INFβ (Fitzgerald et al., 2003). However, both cells infected with wild type or ΔsigD Salmonella did not show nuclear translocation of IRF3 as expected (Figure 15A and B, respectively). However, these observations are inconclusive because cells stimulated with 0.1 µg/ml LPS did not induce IRF3 nuclear translocation as expected (Figure 15D).
Figure 15. Analysis of IRF3-GFP localization during *Salmonella* infection. HeLa cells stably expressing TLR4 and MD-2 were transfected with IRF3-GFP and infected with wild type (A) and ∆*sigD* (B) *Salmonella* (MOI 100:1). At 10 min, bacteria was removed, fresh media was added and the invasion continued for 6 hrs. Transfected control cells (C) were not exposed to bacteria. As a positive control, LPS was added at 0.1 µg/ml (D). Cells were fixed and external bacteria was fluorescently labelled with Alexa Fluor 647 (blue). Cells were then permeabilized with 0.1% Triton X-100 and total bacteria was fluorescently labelled with Alexa Fluor 555 (red). Images are representative of two independent experiments. Scale bar, 10 µm.
4 Discussion

Non-typhoidal Salmonella serotypes, such as S. enterica serotypes Typhimurium (S. Typhimurium) and (S. Enteritidis), are currently the most significant food borne pathogens, leading to millions of cases of diarrheal disease and thousands of hospitalizations and deaths worldwide each year (Galanis et al., 2006). The prevalence of Salmonella is indicative of its success as an invasive pathogen. Salmonella uses the T3SS and its effector proteins to forcibly enter into host epithelial cells, spreading infection as it proliferates. Part of this clever invasive strategy involves disabling the host immune system in order to interfere with Salmonella detection. However, it is unclear as to how Salmonella mediates this. Previous work has indicated that Salmonella may cause a disruption at the level of TLR4 recognition (Weiss et al., 2004). Work by Tobar and colleagues supports this notion by showing that antigen presentation and stimulation of T-cells in DCs, a TLR4 regulated process (Blander and Medzhitov, 2004), is suppressed in Salmonella SPI-2 mutants (Tobar et al., 2006). In addition, Salmonella effector SigD eliminates PIP$_2$ from the plasma membrane during infection (Terebiznik et al., 2002). PIP$_2$ is necessary for the function of TLR4 adaptor proteins and thus, its elimination would suggest a disabling of the TLR4 pathway. Thus, I reasoned that disruption of TLR4 signaling was key to Salmonella pathogenicity. The main objective of this study was to elucidate the role of Salmonella effector SigD on the TLR4 signaling pathway by assessing its effect on TLR4 adaptors, TIRAP and TRAM whose recruitment and/or activation is dependent on PIP$_2$. With regards to the TLR4 MyD88 dependent pathway, I demonstrated that during Salmonella infection, the phosphatase activity of SigD causes TIRAP to be displaced from membrane invaginations before complete closure of SCVs. I also showed that TIRAP co-localizes with SCVs from ΔsigD Salmonella infected cells while there is no association of TIRAP with wild type Salmonella SCVs. I showed that SigD disrupts the MyD88 dependent pathway by
decreasing NF-κB activation. In addition, I demonstrated that SigD also affects the MyD88 independent pathway by interfering with PKCε recruitment to the SCVs and by attenuating its activation. Our proposed model of SigD mediated disruption of the TLR4 signaling pathway is depicted in Figure 16.

As mentioned earlier, *Shigella flexneri* effector, IpgD, demonstrates homology with SigD (Norris *et al.*, 1998). Like SigD, IpgD dephosphorylates PIP2 during infection of epithelial cells (Niebuhr *et al.*, 2002). An *ipgD* mutant was shown to induce less membrane ruffling during invasion, signifying the importance of IpgD in bacterial internalization (Niebuhr *et al.*, 2000). The fact that both *Salmonella* and *Shigella* have an effector protein with homology to a 4-phosphatase indicates that this activity is key during the invasion process and implicates that it may be a common trait amongst enteropathogenic bacteria. In addition, my findings on the impairment of TIRAP recruitment and the disruption of TLR4 signaling suggest that enteropathogenic bacteria may employ similar methods to disable TLR recognition in order to avoid detection by the host.

### 4.1 SigD phosphatase activity disrupts TIRAP localization: a novel *Salmonella* virulence mechanism for the evasion of TLR4 detection

Prior to my study, TIRAP recruitment to the membrane was shown to be disrupted in chinese hamster ovary (CHO) cells when a SigD plasmid was expressed (Kagan and Medzhitov, 2006). Although this gave an indication that 4’-phosphoinositides were crucial for TIRAP localization, TIRAP recruitment to the plasma membrane during *Salmonella* infection had not yet been explored. Using confocal and live cell microscopy, I demonstrated that during *Salmonella* infection of cultured human epithelial cells, SigD mediates the disruption of TIRAP to plasma membrane invaginations and SCVs. Of particular importance, I also showed that TIRAP was excluded from the membrane prior to complete SCV fission. In addition, my analysis of
**Figure 16. SigD mediated disruption of TLR4 signaling.** *Salmonella* injects SPI-1 T3SS effector, SigD, into the host cell during infection. SigD phosphatase activity hydrolyzes PIP2 into PI(5)P. Consequently, PIP2 mediated recruitment of TIRAP to the TIR domain of TLR4 is inhibited. TIRAP can not associate with downstream TLR4 adaptor, MyD88, and therefore, the MyD88 dependent pathway is blocked. Similarly, SigD hydrolysis of PIP2 interferes with PLC activity. PLC cleaves PIP2 into DAG, which activates PKCε. In turn, PKCε phosphorylates TRAM. However, the lack of DAG production inhibits PKCε activity and TRAM activation. This leads to the interruption of the MyD88 independent pathway.
Salmonella invasion using a mutant strain defective in 4-phosphatase activity confirmed that SigD mediated depletion of PIP₂ directly caused the displacement of TIRAP from the membrane. The fact that I not only demonstrated these trends in human epithelial cells but also in human colonic (HT-29) cells, lends greater credibility to our findings because HT-29 cells mimic the natural environment of Salmonella invasion, the intestinal lumen. Lastly, the effect of SigD on TIRAP localization implies that the TLR4 dependent pathway is impaired early in the invasion process.

The observed interruption of TIRAP localization to the membrane has implications on the efficient signaling of the MyD88 dependent pathway. This is due to the fact that PIP₂ mediated association to the membrane is essential for TIRAP function (Kagan and Medzhitov, 2006). If TIRAP is not associated to the membrane, it can not bind to the TIR domain of TLR4 which is necessary for interaction with MyD88 (Ohnishi et al., 2009). Previous work with a mutant form of TIRAP (Mal D96N), which is unable to bind to MyD88, demonstrated an inability to stimulate downstream TLR4 signaling like NF-κB activation (Nagpal et al., 2009). In agreement with these findings, I demonstrated that cells infected with wild type Salmonella exhibited a lack of IκB-α degradation and a corresponding reduction in NF-κB activity as compared in cells infected with ΔsigD Salmonella.

In addition, I have shown preliminary data showing that TIRAP phosphorylation may be attenuated during Salmonella invasion. I reason that the disruption in TIRAP localizing to the plasma membrane interferes with its ability to become phosphorylated by BTK. Recent work has shown three key tyrosine residues (86, 106 and 159) on TIRAP whose phosphorylation are involved in TIRAP activity (Piao et al., 2008). Mutations at these residues impaired tyrosine phosphorylation of TIRAP and interfered with its ability to activate p38 phosphorylation and
stimulate IκB-α degradation resulting in reduced NF-κB activity (Piao et al., 2008). Piao and colleagues speculated that TIRAP phosphorylation might induce conformational changes in TIRAP and aid in the creation of docking platforms for the recruitment of downstream factors such as other adapter proteins (Piao et al., 2008). Another possibility is that phosphorylated TIRAP acts as a shuttle for MyD88, recruiting it the plasma membrane. Furthermore, a recent study has shown that not only does TIRAP act as a bridging adapter for MyD88, it is essential for binding directly to TRAF6, an important player in the MyD88 dependent signaling cascade (Verstak et al., 2009). Thus, the observed lack of TIRAP phosphorylation may cause a distortion in the TRAF-6 binding motif and therefore, inhibit proper binding with TRAF6. Taken together, the disruption of TIRAP phosphorylation is sufficient in impairing the MyD88 pathway by hindering the NF-κB pathway. This provides a mechanism by which SigD blocks this branch of the TLR4 pathway.

Because TIRAP recruitment to the membrane and/or lack of TIRAP phosphorylation hinders normal TIRAP function, it is apparent that TIRAP is an essential player in host cell immune responses. In agreement with this, recent work has highlighted the association of TIRAP and disease. For example, a single nucleotide polymorphism (SNP) in the TIRAP protein, S180L, has been shown to increase susceptibility to contracting invasive pneumococcal disease, bacteremia, malaria and tuberculosis in homozygous individuals (Khor et al., 2007). Further analysis revealed that heterozygotes carrying this mutation also had an enhanced inflammatory response and a reduced chance of acquiring septic shock (Ferwerda et al., 2009). The relevance of these data supports our reasoning that the phosphatase activity of SigD and its impairment of TIRAP function are pivotal in disabling immune responses such as those mediated by TLR4 signaling.
During our assessment of the effects of SigD on the downstream TLR4 pathway, I showed there was a disruption of IκB-α degradation in wild type-infected cells (Figure 10A). I had expected this result to translate into an equivalent level of reduction in NF-κB activation as well. Instead however, there was some residual activation of NF-κB (Figure 12). This slight induction of NF-κB activity in wild type-infected cells may be due to late stage activation of NF-κB triggered by the MyD88 independent pathway (Oshiumi et al., 2003). I can also attribute the residual activation of NF-κB to LPS contamination in the media. Although I used PMB to inhibit TLR4 activation by LPS, it could be that the inhibitory effect of PMB was slowly waning by the end of the luciferase assay, at 8 hrs. In addition, although the possibility is small, the residual NF-κB activity may be due to some aberrant activity of TIRAP. A recent study showed that a TIRAP mutant (Mal 4KK), lacking the ability to localize to the membrane, is still able to stimulate MAP kinases and NF-κB transactivation (Verstak et al., 2009).

4.2 Inhibition of PKCε phosphorylation by SigD implicates a possible disruption in the TLR4 MyD88 independent pathway

To explore the effect of SigD on TRAM localization during Salmonella infection, I assessed TRAM-GFP localization in the plasma membrane via confocal microscopy. I demonstrated that upon infection with either wild type or ΔsigD Salmonella, the localization of TRAM in the plasma membrane was unaffected by SigD. My data is in agreement with the observations by Kagan and colleagues that TRAM is associated with the plasma membrane (Kagan et al., 2008). This is because TRAM localization is dependent on its myristoylate domain and not on association with PIP2. Therefore, SigD phosphatase activity does not alter TRAM localization. Because LPS induces TLR4 endocytosis and efficient TRAM signaling occurs from endosomes (Kagan et al., 2008), I reason that during Salmonella invasion, TRAM stays associated with the
plasma membrane. However, a transient disassociation from the membrane during TRAM activation is plausible. This may be the case because myristoylated proteins like TRAM detach from the membrane when they become phosphorylated (Walker et al., 1993). Indeed, McGettrick and colleagues demonstrated via membrane fractionation that upon LPS stimulation of HEK293-TLR4 (human embryonic kidney) cells, TRAM is depleted from the membrane as it becomes phosphorylated by PKCε (McGettrick et al., 2006). In addition, TRAM detachment was observed 30 min after infection whereas, my results were based on 15 min infections. Thus, it may be possible for ΔsigD Salmonella infected cells to exhibit SCVs devoid of TRAM-GFP indicating that TRAM had been phosphorylated and detached from the plasma membrane. Also, the activation of the MyD88 independent pathway occurs after the initiation of the MyD88 dependent pathway (Kagan et al., 2008). Thus, localization of TRAM during Salmonella invasion still requires further study.

Because activation of TRAM involves phosphorylation of serine 16 by PKCε (McGettrick et al., 2006), I investigated the localization and phosphorylation of PKCε. I observed that the recruitment of PKCε to the plasma membrane was impaired in cells infected with wild type Salmonella in contrast to ΔsigD infected cells which showed PKCε in the membrane of SCVs. PKCε activation would also be affected as a result of its impaired localization because PKCε requires DAG, which is produced at plasma membrane by PLC, for activation. Indeed, when I assessed the phosphorylation of PKCε as an indicator of its activity, I observed that there was a decreased induction of PKCε phosphorylation in cells infected with wild type bacteria in contrast to ΔsigD infected cells. This implicates a definitive role for SigD in the impairment of PKCε activity during Salmonella infection. Ultimately, a lack of PKCε activation would hinder TRAM
phosphorylation and the ability of TRAM to associate with TRIF, thereby interrupting TLR4 signaling from endosomes.

As I demonstrated with TIRAP and the MyD88 dependent pathway via the NF-κB luciferase assay, I also wanted to show the effect of SigD downstream of TRAM and PKCε. To this end, I chose to follow the translocation of IRF3 from the cytoplasm to the nucleus during invasion. To initiate MyD88 independent signaling, IRF3 is transcribed in the cytosol and phosphorylated, inducing its dimerization. Once in its homodimer form, it shuttles to the nucleus where it stimulates the expression of IFN-β (Sakaguchi et al., 2003). Thus, I anticipated that because PKCε phosphorylation, and in turn its activation, is interrupted by SigD, downstream signaling to IRF3 would be blocked and cells infected with wild type bacteria would demonstrate nuclei devoid of IRF3 whereas ΔsigD-infected cells would show a clear translocation of IFR3 from the cytoplasm to the nucleus. However, when I infected cells with wild type or ΔsigD Salmonella, I did not observe any difference in IRF3 localization. Considering that the LPS control did not stimulate IRF3 translocation, these results are inconclusive. The IRF3-GFP construct I used was confirmed as a valid probe in HEK293 cells, a human embryonic kidney cell line (Fitzgerald et al., 2003). However, in that study, IRF3-GFP translocation was demonstrated by over-expressing a flag-tagged TRAM construct. Over-expression of TRAM may have artificially amplified MyD88 independent signaling, therefore making IRF3 translocation very apparent.

Although, the experimental conditions I used did not favor the visualization of TRAM movement during infection, I hypothesize that SigD mediated hydrolysis of PIP₂ does not affect TRAM localization from the plasma membrane however, SigD may interrupt some transient detachment that occurs when TRAM becomes phosphorylated. Dissociation from the plasma membrane may allow TRAM to bind with other downstream factors that contribute to MyD88 independent
signaling. But TRAM phosphorylation is most likely disrupted by SigD mediated inhibition of PKC\(\varepsilon\) recruitment to the plasma membrane. PKC\(\varepsilon\) recruitment to the plasma membrane is dependent on DAG (Shirai et al., 2000), which is a product of PLC mediated cleavage of PIP\(_2\) (Nishizuka, 1995). However, if SigD depletes PIP\(_2\), it can no longer act as a substrate for PLC. The absence of DAG production by PLC would inhibit PKC\(\varepsilon\) activation. Therefore, TRAM phosphorylation and activation would be impeded leading to the inability of TRAM to mediate signaling in the MyD88 independent pathway.

4.3 Future directions and implications

Initial attempts at investigating TRAM localization during infection were inconclusive. One way to remedy the confocal microscopy results would be to allow for longer time points than 15 min. However, taking into consideration the dynamics of TRAM localization and signaling during TLR4 activation, I might try to follow TRAM during infection via live cell imaging. Live cell imaging will enable a better way to monitor events occurring throughout the cell and thus, it could indicate any transient localization of TRAM in the membrane and SCVs that may have been missed with fixed cells. During live cell imaging, in addition to observing the plasma membrane, I would focus on the formation of SCVs to follow TRAM as TLR4 is being endocytosed. Also, I may not have observed TRAM movement because implementing a TRAM-GFP construct instead of observing endogenous TRAM via confocal microscopy may have induced an over abundance of TRAM in the membrane. This could have limited phosphorylation by PKC\(\varepsilon\) and/or made any small amount of TRAM detachment hard to visualize. To remedy this issue, we can track endogenous TRAM during invasion by using an anti-TRAM antibody and immunofluorescent imaging. Timing is also an important factor to consider when monitoring transient events such as the activation of TRAM. Phosphorylation of endogenous TRAM has
been shown to be a transient event, occurring 15 min after LPS stimulation and showing the greatest induction by 45 min (McGettrick et al., 2006). Thus, I would use this period of TRAM activity as a framework for our in vivo studies. Lastly, after working with epithelial we would reconfirm these live cell imaging results in human colonic cells in order give greater relevance to our model of Salmonella invasion.

To remedy the inconclusive results of the IRF3 localization experiment, it is possible to use a specific anti-IRF3 antibody and immunostain for total endogenous IRF3 as it translocates into the nucleus (Husebye et al., 2010). The use of an antibody against endogenous IRF3 would be favored over exogenous IRF3 because we will be able to assess IRF3 localization in an experimental condition that more closely resembles in vivo conditions. Alternatively, instead of assessing IRF3 activation by confocal microscopy, I might assess IRF3 dimerization via native PAGE (Clement et al., 2008). I would expect that in cells infected with wild type Salmonella, SigD would disrupt the MyD88 independent pathway at TRAM activation and/or PKCε activation and thus, IRF3 dimerization would not take place. In contrast, samples taken from ΔsigD-infected cells would contain IRF3 dimers, indicating that MyD88 independent signaling has not been interrupted. Lastly, to verify if the SigD can completely abolish MyD88 independent signaling, I would test the induction of IFN-β promoter by using the p-125 luc reporter plasmid which contains the human IFN-β promoter region (Matsumoto et al., 2002). I expect that cells infected with wild type Salmonella will show a reduced level of IFN-β expression than ΔsigD-infected cells.

4.4 Conclusion

I have shown that the phosphatase activity of SigD impairs the recruitment of TLR4 adapter, TIRAP, to the plasma membrane invaginations and SCVs during Salmonella infection. This
impairment in TIRAP recruitment has downstream effects on TLR4 signaling. I demonstrated that not only was the degradation of IκB-α interrupted but also the activity of NF-κB was ultimately reduced. Because the induction of pro-inflammatory cytokines occurs as a result of NF-κB activity, I have shown that by SigD disrupting the TLR4 MyD88 dependent pathway, SigD can attenuate the host immune response. In addition, I also investigated the role of SigD on the MyD88 independent pathway. Although it is still unclear as to how SigD affects the localization of TRAM during infection, I demonstrated that PKCε recruitment to the plasma membrane is blocked and also that its phosphorylation is significantly reduced. This indicates SigD most likely interferes with this branch of the TLR4 pathway as well, culminating in the disruption of the type 1 interferon expression. This study has provided a model of Salmonella pathogenicity which involves disabling TLR4 signaling in order to evade host recognition. This contributes to an attenuation or inhibition of the host immune response that allows Salmonella to proliferate.
References


