

**TLR-AGONIST TREATMENT AND SALMONELLA INFECTION**

THE ROLE OF TOLL-LIKE RECEPTOR AGONIST TREATMENT  
ON  
SALMONELLA INFECTION IN MACROPHAGES

By

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**TITLE:** The Role of Toll-Like Receptor agonist Treatment on *Salmonella* Infection in Macrophages

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## ABSTRACT

*Salmonella* is a Gram-negative intracellular pathogen that causes gastroenteritis and typhoid fever in humans. *Salmonella* can survive and replicate within host cells and has adapted several mechanisms to evade host immune defenses. The innate immune system plays an important role as a first-line of defense against pathogens such as *Salmonella*, and is mediated in part by toll-like receptors (TLRs). TLRs recognize fundamental components of pathogenic microorganisms and activation of TLRs leads to downstream signaling cascades eventually resulting in the expression of pro-inflammatory cytokines (4) and also has a role in activating adaptive immunity through presentation of antigens to lymphocytes (86). There are several lines of evidence that suggest that TLR activation may have therapeutic potential in therapies against infectious disease and several TLR agonists have been shown to protect against both bacterial and viral infection in mice (7; 8; 38; 66; 75; 84; 89; 121). To understand how TLR-agonist treatment of host cells affects *Salmonella* pathogenesis, RAW 264.7 murine macrophages were treated with the TLR agonists liposaccharide (LPS), poly(I:C), peptidoglycan, and CpG-ODN. Treatment of macrophages with all TLR-agonists results in increased phagocytosis of *Salmonella* compared to control-treated macrophages. These increases in phagocytic activity, however, do not enhance macrophage anti-microbial activity, since *Salmonella* infection of TLR-treated macrophages results in increased intracellular replication compared to control-treated cells. Infection with *Salmonella* mutants indicates that increased intracellular replication of *Salmonella* in TLR-treated macrophages is

dependent on a functional SPI-2 type III secretion system. This also indicates that there was not a generalized defect in macrophage anti-bacterial function. These data exemplify how interactions between macrophage defense mechanisms and bacterial virulence factors can result in evasion of the innate immune response. Studying how TLR-agonist treatment affects *Salmonella* pathogenesis will give us a better understanding of the host-pathogen relationship and may provide insight into novel strategies to fight intracellular microorganisms.

**Key words:** *Salmonella*, bacterial pathogenesis, macrophage, Toll-like receptor, innate immunity

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## LIST OF ABBREVIATIONS

AP-1	activator protein-1
Arp2/3	actin-related protein 2/3
CD	clusters of differentiation
DMEM	Dulbecco's Modified Eagle's Medium
EEA1	early endosome antigen-1
ERK	extracellular signal-regulated kinase
GPI	glycosylphosphatidylinositol
IFN	interferon
I $\kappa$ B	inhibitor of NF- $\kappa$ B
IKK	inhibitor of $\kappa$ B kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor-associated kinase
IRF3	IFN regulatory factor-3
JAK	Janus kinase
Lamp	lysosomal-associated membrane protein
LBA	lysobisphosphatidic acid
LBP	LPS binding protein
LPS	lipopolysaccharide
LRR	leucine rich repeat
M6PR	mannose-6-phosphate receptor
MAL	MyD88 adaptor-like
MAPK	mitogen-activated protein kinase
MDR	multi-drug resistance
MEK	MAPK and ERK kinase
MyD88	myeloid differentiation protein-88
NADPH	nicotinamide adenine dinucleotide phosphate
NF- $\kappa$ B	nuclear factor $\kappa$ B
NO	nitric oxide
Nramp1	natural resistance-associated macrophage protein 1
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PG	peptidoglycan
Poly(I:C)	polyinosine-polycytidylic acid
PRR	pattern recognition receptor
RIP1	receptor-interacting protein-1
ROS	reactive oxygen species
SCV	<i>Salmonella</i> -containing vacuole
SEM	standard error of the mean

Sif	<i>Salmonella</i> -induced filament
SNARF	seminaphtharhodafuor
SPI	<i>Salmonella</i> pathogenicity island
SR	scavenger receptor
TAB	TAK1-binding
TAK	TGF- $\beta$ activated kinase
TANK	TRAF family member-associated NF- $\kappa$ B activator
TBK1	TANK-binding kinase-1
TF	transferrin receptor
TGF $\beta$	transforming growth factor- $\beta$
TIR	Toll/IL-1R homology
TIRAP	TIR-associated protein
TLR	toll-like receptor
TNF	tumour necrosis factor
TRAF6	tumour necrosis factor receptor-associated factor 6
TRIF	TIR-domain-containing adapter-inducing IFN- $\beta$ /TLR-associated activator of IFN
TRAM	TRIF-related adaptor molecule
V-ATPase	vacuolar adenosine triphosphatase
WASP	Wiskott-Aldrich Syndrome protein

## 1. INTRODUCTION

### 1.1 *Salmonella enterica*

*Salmonella enterica* is an intracellular Gram-negative bacterium that infects multiple hosts and causes a variety of diseases (141). There are over 2500 known serovars of *Salmonella*. Serovars such as *Salmonella* Typhi or *Salmonella* Paratyphi are human-restricted and are the infective agents of typhoid fever (65). Other serovars such as *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) have a broad host range, infecting a wide variety of species including humans, mice, reptiles, and birds (65). *Salmonella* is usually acquired following ingestion of contaminated food or water. In humans, *S. Typhimurium* typically causes self-limiting gastroenteritis and enteric fever (141), whereas in mice it causes a systemic infection which resembles the human-specific typhoid fever (65). Therefore murine infection with *S. Typhimurium* serves as an excellent animal model for typhoid fever.

An important part of *Salmonella*'s virulence strategy is its ability to survive and replicate within eukaryotic host cells. Once inside host cells, the bacteria reside within a specialized membrane-enclosed compartment, termed the *Salmonella*-containing vacuole or SCV, which provides a niche where *Salmonella* is able to survive and replicate. Type III secretion systems (T3SSs) play an important role in the ability of *Salmonella* to infect host cells and cause disease. These systems consist of over 20 proteins that form a needle-like structure that spans both the inner and outer bacterial membrane and extends to the host cell membrane, allowing for the translocation of bacterial effectors into the host cell cytosol (78). While several Gram-negative bacteria possess similar T3SSs, the

effector proteins secreted vary. *Salmonella* has two distinct T3SSs encoded within two different horizontally acquired *Salmonella* pathogenicity islands (SPI), termed SPI-1 and SPI-2.

### 1.1.1 Bacterial invasion

The SPI-1 T3SS plays an important role in the bacteria's ability to gain entry into the host and cause disease. *Salmonella* is able to enter its host through invasion of the epithelial cells of the small intestine. Mutations that prevent the formation of the SPI-1 T3SS result in a 10 to 100-fold decrease in virulence (11; 49; 83). Following contact with the host cell the SPI-1 T3SS injects effectors such as SopB, SopE, SopE2 and SopD which activate the Rho GTPases Cdc42, Rac-1, and RhoG in the host cell (10; 45; 67; 112; 115; 139; 160). Once activated, Rho GTPases activate Wiskott-Aldrich Syndrome protein (WASP) family members N-WASP and WAVE2, which in turn recruit the actin-related protein 2/3 (Arp2/3) complex to the site of entry (33; 134; 139; 143). This leads to membrane ruffling and ultimately causes the host cell to engulf the bacterium (50). Actin cytoskeleton rearrangement is critical to *Salmonella* entry since treatment with drugs that disrupt actin dynamics prevent bacterial uptake into epithelial cells (42). Interestingly, recent studies have shown that treatment with TLR-agonists can also lead to activation of the Rho GTPases Cdc42 and Rac, resulting in an increase in phagocytosis (88).

*Salmonella* entry also activates mitogen activated protein kinases (MAPKs) such as Erk, Jnk and p38, which in turn activate the transcription factors nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1), leading to the production of pro-inflammatory

cytokines (50). These signaling events are significant because they contribute to inflammatory diarrhea, which is a characteristic symptom of *Salmonella* infection (50).

### **1.1.2 *Salmonella* pathogenicity island-2 (SPI-2)**

The SPI-2 T3SS is prominently induced after the bacterium has entered host cells and plays an important role in intracellular survival and replication. The SPI-2 locus encodes for a T3SS apparatus and SPI-2 genes are regulated by SsrAB, a two-component regulatory system (113; 133). SPI-2 is required for prevention of fusion between the SCV and the lysosomes (142), for avoidance of macrophage killing through NADPH oxidase (51; 146), and for prevention of delivery of inducible nitric oxide synthase (iNOS) to the SCV (27). All of these processes are carried out by secreted bacterial effector proteins that are encoded both within and outside of SPI-2 and are important for the survival of the bacterium within the host cell (153).

Previous studies have suggested that bacteria can upregulate expression of genes required for intracellular survival when infecting cells that are better equipped to kill bacteria (159). Natural resistance-associated macrophage protein-1 (Nramp1, also known as Slc11a1) is a divalent cation transporter that localizes to the late endocytic/lysosomal vacuole (60). Nramp1 can restrict the growth of *Salmonella* by removing ions from the SCV (148). Nramp1 has an important role in host defense and infection of mice lacking Nramp1 with various intracellular bacteria such as *S. Typhimurium* and *Mycobacterium bovis* bacillus Calmette-Guérin results in high levels of bacterial replication and fatality (16). Zaharik *et al.* examined potential crosstalk between host Nramp1 status and *Salmonella* virulence effectors (159). They found that when *Salmonella* is infecting an



Nramp1<sup>+/+</sup> host there was an increase in expression of SPI-2 genes such as *sseA* and *ssrA* compared to infections of Nramp1<sup>-/-</sup> hosts (159). This suggests that bacteria can sense the immune status of its host, and upregulate SPI-2 gene expression accordingly.

### 1.1.3 Intracellular survival within the *Salmonella*-containing vacuole (SCV)

Within the SCV, bacteria are able to redirect the normal phagosomal maturation process to allow for survival (22) (Figure 1). The SCV maturation process involves interaction with the endosomal system in a highly orderly and selective manner (1). The SCV interacts briefly with early endosomes (138), acquiring marker proteins such as early endosome antigen-1 (EEA1), transferrin receptor (TF), and the GTPases Rab5 and Rab11 (123; 136; 138). However, the interaction with these proteins is only transient, and after EEA1 and Rab5 dissociate, the SCV then acquires select markers of the late endosome/lysosome such as Rab7 (101), which in turn attracts lysosomal-associated membrane proteins (Lamps) Lamp1, Lamp2, and Lamp3 and vacuolar ATPase, which acidifies the SCV (37; 52; 138). Despite the presence of these late endosome/lysosome markers, the SCV does not merge with lysosomes. This is confirmed by the absence of mannose-6-phosphate receptors (M6PR), cathepsin D, and lysobisphosphatidic acid (LBA) (27; 50; 52; 54; 68). Therefore, *Salmonella* is able to redirect the normal phagosome maturation process to its advantage, creating an environment that is conducive to its survival and growth. Interestingly, some studies have suggested that TLR signaling may also have an effect on phagosome maturation and proteolytic capacity in macrophages (17; 155).

Approximately 4-6 hours after invasion in epithelial cells, the SCV extends elongated tubular structures from its surface called *Salmonella*-induced filaments (Sifs) (53). This structural change corresponds with the beginning of bacterial replication and like the SCV, Sifs are associated with Lamps and vacuolar ATPase (23; 53). Sifs were originally thought to be restricted to epithelial cells, however studies using interferon gamma (IFN- $\gamma$ )-primed macrophages demonstrated that *Salmonella* forms Sifs in macrophages as well (87). Though their exact physiological purpose is not known, it is possible that they function to increase the size of the SCV in a specific direction that is more conducive for bacterial replication. Additionally, it is thought that they may deliver nutrient-containing vacuoles and organelles to the SCV (65).

One method that macrophages have developed to combat infection by intracellular pathogens is the generation of reactive oxygen species (ROS) and nitrogen species such as nitric oxide (NO), superoxide and hydrogen peroxide. Superoxide is created by the membrane-associated NADPH oxidase complex. This process of generating reactive oxygen species is called the respiratory burst and is usually effective at killing microbes within the phagosome (79; 106). SPI-2 effectors enable *Salmonella* to avoid the respiratory burst and survive within the macrophage (51; 146). Thus, *Salmonella* has evolved to survive within several cell types and has acquired multiple mechanisms to resist host immune defenses.

#### **1.1.4 SPI-1 and SPI-2: Complex interactions and coordination within the host**

It was previously believed that SPI-1 and SPI-2 were expressed at distinct points of infection with SPI-1 being responsible for induction of an inflammatory response in

the gut, invasion of epithelial cells, and colonization of the intestine and with SPI-2 being responsible for establishing systemic infection and for intracellular survival in macrophages. However, an increasing number of studies are concluding that this view is too simplistic and that there is likely more interplay between these pathogenicity islands. For example, it was shown that SPI-1 is also required for *S. Typhimurium* survival and replication within epithelial cells (137), which expands the role of SPI-1 from beyond the initial invasion of the gut. Also, SPI-2 has been shown to be expressed in the intestine, prior to intestinal colonization (21). Lack of functional SPI-2 T3SS can reduce the expression of some SPI-1 T3SS genes and be detrimental to colonization, which provides further evidence for the importance of SPI-1 and SPI-2 T3SS interplay (34). In addition, several groups have shown that some SPI-1 effectors are present in host cells long after the initial invasion (65). Therefore, there is much more spatio- and temporal-overlap between these two pathogenicity islands than previously thought and this is further evidence for the complex interactions that have evolved between *Salmonella* and its host.

## **1.2 Innate immunity**

The mammalian immune system has evolved to combat numerous pathogens that pose a daily threat. The immune system in mammals consists of two main branches: innate immunity and adaptive immunity. The innate immune system plays an important role in early host defense against pathogens (9; 13-15). It involves the recognition of microorganisms through germline-encoded pattern-recognition receptors (PRRs) (4). PRRs recognize pathogen-associated molecular patterns (PAMPs) that are conserved components of microorganisms such as lipopolysaccharide (LPS), flagellin,

peptidoglycan, bacterial DNA, and viral RNA (4). PRRs recognize specific PAMPs and this interaction results in the activation of downstream signaling cascades and distinct anti-microbial responses by the host cell (4). Unlike the adaptive immune system, this form of recognition is nonclonal and is not associated with immunological memory (4). These basic recognition mechanisms are found in all classes of species, in both plants and mammals (4).

### 1.3 Toll-like receptors

Toll-like receptors (TLRs) are a family of PRRs that are conserved in many species (4). Toll was first discovered in *Drosophila* as a critical component involved in establishing embryonic dorsal-ventral patterning (6; 107) and was later found to play an important role in resisting fungal infections (94). One year later, the first human homologue of the *Drosophila* Toll protein (hToll) was identified by Janeway and colleagues and was shown to induce both innate and adaptive immune responses through activation of the NF- $\kappa$ B pathway (99). This receptor was later renamed TLR4 and mice with defective TLR4 were found to be hyporesponsive to LPS, further supporting a role for TLRs in mammalian immunity (76; 118). To date, 13 mammalian TLRs have been identified along with numerous TLR ligands, signaling molecules, and regulatory molecules (55).

TLRs are type I integral membrane glycoproteins that contain leucine rich repeat (LRR) motifs in the extracellular domains and a Toll/IL-1R homology (TIR) signaling domain in the intracellular domain (18). TLRs are expressed by both immune cells such as macrophages, dendritic cells and B cells, as well as on non-immune cells such as

epithelial cells and fibroblasts (4). Some TLRs such as TLRs 1, 2, 4, 5, and 6 are expressed on the surface of cells where they can sample extracellular ligands such as lipopeptides, peptidoglycan, LPS, and flagellin. Other TLRs such as TLRs 3, 7, 8, and 9 are localized to an intracellular compartment for recognition of internalized bacterial and viral DNA (4).

#### **1.4 Toll-like receptor signal transduction**

The molecules involved in TLR signaling are the same as those that are used for IL-1R signaling (3). The TLR signaling cascade begins with the recruitment of TIR-containing adaptor molecules such as Myeloid differentiation factor-88 (MyD88), MyD88 adaptor-like (MAL, also known as TIR-associated protein, TIRAP), TIR domain containing adaptor inducing IFN $\beta$  (TRIF), and TRIF-related adaptor molecule (TRAM) (86). The different TLRs have specificity for different signaling pathways by recruiting different adaptor proteins (36).

MyD88 is a shared adaptor molecule in all TLR signaling pathways with the exception of TLR3 (5). MyD88 contains a C-terminal TIR domain, which allows it to interact with the TIR domain in the cytoplasmic tail of TLRs (36). MyD88 also has N-terminal death domains, which interact with the death domains of the interleukin-1 receptor-associated kinase (IRAK) family members (36). There are two main TLR signaling pathways, the MyD88-dependent pathway and the MyD88-independent pathway.

In the MyD88-dependent pathway, MyD88 interacts with the cytoplasmic tail of TLRs, recruits IRAK4 and together they mediate IRAK1 phosphorylation (97). Once

phosphorylated, IRAK1 interacts with and activates tumour necrosis factor receptor-associated factor-6 (TRAF6) and together they interact with a membrane complex consisting of TGF- $\beta$  activated kinase (TAK1), and the TAK1-binding (TAB) family members, TAB1, and TAB2 (36). The complex is activated following TRAF6 and TAK1 ubiquitination, resulting in the phosphorylation of TAK1 and TAB2 and the dissociation of the complex from the membrane (81; 122; 151). In the cytosol IRAK1 is degraded and activated TAK1 can phosphorylate the inhibitor of  $\kappa$ B kinase (IKK) complex, leading to the phosphorylation and degradation of I $\kappa$ B and the release of NF- $\kappa$ B (100). Free NF- $\kappa$ B translocates to the nucleus where it promotes the expression of pro-inflammatory genes.

TLR3 and TLR4 activate the MyD88-independent pathway which is also known as the TRIF-dependent pathway (86). TLR4 recruits TRAM, which subsequently recruits TRIF, whereas TLR3 can recruit TRIF directly (43). TRIF can bind to receptor-interacting protein-1 (RIP1), which recruits TRAF6, resulting in the activation of the IKK complex (103). Direct binding of TRIF and TRAF6 can result in IRAK1/4-independent activation of TAK1 (80; 82). TRIF can bind TANK-binding kinase-1 (TBK1) and IKK $\epsilon$ , which activates the transcription factor Interferon regulatory factor-3 (IRF3) which plays an important role in the production of type 1 interferons and defense against viral infections (129) (Figure 2).

TLR4 is unique in its ability to participate in both the MyD88-dependent and TRIF-dependent signaling pathways. It was recently shown that MyD88-dependent and TRIF-dependent signaling are not initiated simultaneously in TLR4 (85). The MyD88-dependent signaling is initiated after TLR4 activation by LPS on the plasma membrane

whereas the TRIF-dependent signaling pathway is initiated in the early endosome after TLR4 has been endocytosed (85). Therefore, it can be generalized that TRIF-dependent signal transduction is initiated intracellularly for both TLR3 and TLR4.

### **1.5 Toll-like receptor agonists**

Several TLR-agonists have been reported and studied, however for the purpose of this study I focused on lipopolysaccharide (LPS), poly(I:C), peptidoglycan, and CpG oligodinucleotides. These ligands are all commercially available and their effects on the innate immune response have been studied previously, but not in the context of *Salmonella* infection of macrophages.

One of the strongest immunostimulants identified to date is LPS from the Gram-negative cell wall. LPS is made up of three main components – lipid A (endotoxin), a core oligosaccharide, and an O-antigen (26). Lipid A is the active component of LPS and is responsible for many Gram-negative pathologies such as endotoxin shock. The initial clue that TLR4 was the receptor for LPS was through the observation that C3H/HeJ mice were hyporesponsive to LPS. It was later discovered that this was due to a mutation in the TLR4 gene (118). The TLR4 signaling pathway requires the accessory proteins CD14 and MD-2 (4). Free LPS binds to the LPS binding protein (LBP), which is an acute-phase protein that is found in the bloodstream. LPS-LBP then binds to CD14, which is a glycosylphosphatidylinositol (GPI) linked protein that is expressed on the cell surface of phagocytic cells. Next, LPS is transferred to MD-2, which associates with the extracellular portion of TLR4. This leads to TLR4 oligomerization and downstream signaling.

Many viruses produce double stranded RNA as part of their life cycle. Double stranded RNA and its synthetic analog, polyinosine-polycytidylic acid, (poly(I:C)), are both recognized by TLR3 (5). TLR3 is expressed intracellularly in several types of cells and its expression is upregulated significantly when cells are treated with poly(I:C) (4).

Another strong stimulator of the innate immune system is peptidoglycan (PG, also known as murein). Peptidoglycan is a polymer of  $\beta$ -(1,4) linked N-acetylglucosamine and N-acetylmuramic acid. A small peptide chain consisting of 3-5 amino acids is attached to the N-acetylmuramic acid. Peptidoglycan makes up approximately 10% of the dry weight of the Gram-negative cell wall and 90% of the Gram-positive cell wall. TLR2 is involved in the recognition of peptidoglycan (132; 157). TLR2 can recognize a wide variety of ligands due to its ability to form heterodimers with TLR1 and TLR6 (26).

Bacterial DNA is also highly immunostimulatory. In mammalian DNA, CpG dinucleotides are highly methylated (4). In contrast, bacterial DNA contains unmethylated CpG dinucleotides, which is referred to as CpG-DNA (4). Unmethylated CpG-DNA is found approximately twenty times more frequently in bacterial DNA than in mammalian DNA (150) and is recognized by TLR9, which is an intracellular receptor located in endosomes (72; 90). In order to stimulate a response, the bacterial DNA must be delivered to the endosome where it is degraded into smaller single-stranded segments which are then able to interact with TLR9 (2; 93). Synthetic oligonucleotides that contain the CpG motif, such as CpG-oligodeoxynucleotide (CpG-ODN), are also able to stimulate an immune response (4).



## 1.6 Potential of Toll-like receptor agonist treatment against infection

There are several lines of evidence that suggest that TLR activation may have therapeutic potential. In fact, development of synthetic TLR ligands in cancer, vaccine improvement, infectious disease and allergies are already in development, with some already in the early clinical trial phase (91). For example, treatment with oligonucleotides that contain the CpG motif has been shown to provide protection against several pathogens in mice including *Listeria monocytogenes* (38; 89), *Francisella tularensis* (38), Herpes Simplex Virus-2 (HSV-2) (7; 66; 121) and *Mycobacterium tuberculosis* (84). Treatment with the TLR3 ligand, poly(I:C), can protect against infection with HSV-1 and HSV-2 (8; 75). The TLR7 agonist imiquimod has been approved for use as a topical treatment against viral infections such as human papillomavirus (41). One advantage of enhancing innate immunity through TLR stimulation is that it will not result in direct selective pressure on the pathogens (41). The effect of TLR stimulation on *Salmonella* infection is an area that requires further study.

## 1.7 Rationale and objectives

Worldwide, *Salmonella* is one of the most widespread sources of food-borne illness and disease. Every year, millions of people are infected with *Salmonella*, resulting in thousands of fatalities (59). In developing countries, *Salmonella* infection continues to be a significant public health concern (77), especially in children, the elderly, and patients with HIV (59). In addition, the emergence of multidrug-resistant strains of *Salmonella* are threatening to become a serious public health problem, not just in developing countries, but in developed nations such as Canada as well. For example, the *Salmonella* Typhimurium strain DT104 is resistant to several antibiotics including ampicillin, chloramphenicol, florfenicol, streptomycin, spectinomycin, sulfonamides, and tetracycline (71). It has been hypothesized by many groups that drug-resistant strains of *Salmonella* are more virulent than drug-susceptible strains since infection with multi-drug resistant (MDR) *Salmonella* is associated with increased morbidity and mortality compared to the drug-susceptible strains (57; 108; 145). The multidrug-resistance region of DT104 is located on the chromosome allowing for more stable maintenance in the absence of selective-pressures (19).

Based on these observations, there is a strong need to examine alternative therapies to antibiotics. Scientists have proposed a variety of strategies as novel alternatives to antibiotic therapy, some of which include enhancing immunity, the creation of novel anti-bacterials, prebiotics and probiotics, as well as bacteriophage therapy. For my graduate studies, I have focused on investigating the potential to

enhance or modulate the innate immune system through TLRs as a strategy to combat bacterial infection.

Modulating the innate immune response through TLR-agonist treatment is an area of research that has received increased attention. Several reviews have examined the effects of TLR agonists and signaling on animal models of infectious disease (58; 91; 114). However, few have examined the specific role of TLRs on macrophages, which are important since macrophages play a dual role in immunity by acting as both a first line of defense through destruction of pathogens as well as antigen-presenting cells to initiate the adaptive immune response (98). Therefore macrophages are truly at the interface between the innate and adaptive branches of the immune system. In addition, few have examined the role of innate immune modulation on *Salmonella* pathogenesis. Studying *Salmonella* infection of macrophages is of particular importance since mutants that are unable to survive within macrophages are also unable to cause systemic disease in mice (40).

For my graduate research, I have focused on the role of TLR-agonist treatment on the host response to *Salmonella* infection. **My objectives were: (i) To understand how treatment with TLR-agonists affects phagocytosis, phagosome maturation, and bactericidal ability of host macrophages (ii) To understand the host-pathogen relationship in TLR-agonist primed macrophages.**

Studying how TLR-agonist treatment affects *Salmonella* pathogenesis will provide us with a better understanding of this complex host-pathogen relationship and may provide insight into novel strategies to fight intracellular microorganisms.

## **2. MATERIALS AND METHODS**

### **2.1 Materials**

Please refer to Table 1 for a list of all chemicals and reagents used.

### **2.2 Methods**

#### **2.2.1 Bacterial strains and growth conditions**

*Salmonella enterica* serovar Typhimurium strain SL1344 (154) was used throughout. Bacteria were cultured in Luria-Bertani (LB) broth and on LB agar plates containing streptomycin (50 µg/ml), ampicillin (100 µg/ml), or chloramphenicol (15 µg/ml) where appropriate. The *ssaR* deletion mutant ( $\Delta$ *ssaR*) was derived from SL1344 (23). The recombinase-based *in vivo* expression technology (RIVET) reporter strains to measure SPI-2 promoter activity were derived from SL1344 (21).

#### **2.2.2 Mammalian cell lines and growth conditions**

RAW 264.7 murine macrophages (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (HyClone). Cells were maintained in 75-cm<sup>2</sup> culture flasks at 37°C in 5% CO<sub>2</sub> without antibiotics.

#### **2.2.3 Toll-like receptor agonist treatments**

RAW 264.7 cells were treated with TLR agonists approximately 20 hours before infection with 100 ng/ml lipopolysaccharide from *Escherichia coli* (Sigma), 10 µg/ml poly(I:C) (Sigma), 10 µg/ml peptidoglycan from *Staphylococcus aureus* (Sigma), or 10

$\mu\text{g/ml}$  CpG-ODN 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (Mobix Labs, McMaster University, Canada). Cells treated with phosphate buffered saline (PBS) were used as a control.

#### **2.2.4 Gentamicin protection assay**

RAW 264.7 cells were seeded 24 hours before infection at a density of  $2.0 \times 10^5$  cell per well. *S. Typhimurium* SL1344 was grown overnight in 3 ml of LB broth in culture tubes, with shaking at 225 rpm. RAW cells were infected in triplicate with human-serum opsonized stationary phase bacteria at a multiplicity of infection (MOI) of approximately 50 bacteria per cell. After a 30 minute incubation period to allow for bacterial uptake, cells were washed 3 times with PBS and incubated in DMEM containing 100  $\mu\text{g/ml}$  gentamicin, which kills extracellular bacteria. After 2 hours, the gentamicin concentration was reduced to 10  $\mu\text{g/ml}$ . At 2 and 20 hours post-infection, cells were washed 2 times with PBS and then lysed with 250  $\mu\text{l}$  of 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS) in PBS. Lysates were diluted in PBS and plated onto LB agar containing the appropriate drug-selection and incubated overnight at 37°C. Colonies were counted and expressed as colony forming units (cfu). Two hour uptake levels were normalized by dividing the 2 h cfu values of each treatment group by the average 2 h cfu value for the PBS control. The fold increase in the number of intracellular bacteria was calculated by dividing the cfu values at 20 h by the cfu values at 2 h post infection for each treatment group. Fold increase was normalized by dividing by the fold increase for each treatment group by the average fold increase of the PBS control. All experiments

were performed in triplicate, on at least three separate occasions. Error bars represent the standard error of the mean,  $p \leq 0.5$ .

### **2.2.5 Fluorescent microscopy**

RAW 264.7 cells were seeded onto 1.2 cm coverslips at a density of  $2.0 \times 10^5$  cells/well in 24-well culture plates. *S. Typhimurium* SL1344 was grown overnight in 3 ml of LB broth in culture tubes, with shaking at 225 rpm. RAW cells were infected in triplicate with human-serum opsonized stationary phase bacteria at a multiplicity of infection of approximately 50 bacteria per cell. Samples were fixed with 2.5% paraformaldehyde in PBS for a minimum of 30 min at 37°C. Samples were washed twice with PBS and blocked and permeabilized with 10% goat serum and 0.2% saponin in PBS (SS-PBS) for 30 minutes. Primary and secondary antibodies were spread over the surface of the coverslips in SS-PBS for 1 hour, followed by three washes with PBS. Coverslips were mounted onto glass slides using fluorescent mounting medium (Dako). Widefield microscopy was performed using a Leica DMI 600B (100x 1.4NA objective) and Velocity 4 software. Images were taken with a Hamamatsu Orca ER-AG camera, imported into Adobe Photoshop 7.0 and assembled in Adobe Illustrator CS for labeling.

### **2.2.6 Antibodies and reagents**

Rat anti-Lamp1 (1D4B) antibody developed by August, J.T. was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA 52242). 1D4B

was used at 1:100. Mouse Anti-Mono- and Polyubiquitinated Conjugates, Monoclonal (clone FK2) (Biomol International) was used at 1:500. Rabbit anti-Salmonella O Antiserum Group B Factors 1, 4, 12, 27 (Difco) was used at 1:100. Alexa-488-conjugated donkey anti-rat and goat anti-mouse antibodies and Alexa-568-conjugated donkey anti-rabbit and goat anti-rabbit antibodies were used at 1:200 and were purchased from Molecular Probes. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used at 1:2500 and was purchased from Molecular Probes.

### **2.2.7 Phagocytosis of fluorescent microspheres**

RAW 264.7 cells were seeded onto 2.5 cm coverslips at a density of  $1.0 \times 10^5$  cells/ml in 35 x 10 mm petri dishes. 1.0  $\mu\text{m}$  diameter green-yellow fluorescent microspheres (Molecular Probes) were opsonized in 20% human serum for 30-60 minutes. RAW 264.7 cells were washed with PBS and incubated with  $1.0 \times 10^7$  beads/ml for 3.5 hours and then washed 3 times with PBS and transferred to Attofluor cell chambers (Invitrogen) in HEPES buffer for live cell imaging. Samples were treated with 3  $\mu\text{M}$  5- (and 6)-carboxy SNARF-1 acetoxymethyl ester, acetate (Molecular Probes) to label the cytoplasm. Confocal microscopy was performed using a Leica DMI 600B microscope with the Leica TCS SP5 AOBS scanner (63x 1.3NA objective) and Leica Application Suite Advanced Fluorescence Software. The 100 mW 488 nm Argon ion and 10 mW 561 nm DPSS lasers were used. Internalized beads were identified using ImageJ software and through enumeration of at least 200 cells per treatment group. Images were imported into Adobe Photoshop 7.0 and assembled in Adobe Illustrator CS for labeling.

### **2.2.8 Recombinase-based *in vivo* expression technology**

A recombinase-based *in vivo* expression technology (RIVET) reporter was used to measure the transcriptional activity of the *sseA* promoter (21). RAW 264.7 cells were cultured and seeded as described previously and infected with human serum opsonized stationary phase RIVET strains for 30 minutes and then washed and incubated in DMEM containing 100 µg/ml gentamicin. Macrophages were lysed at 15 minute intervals post-infection with 1% Triton X-100 and 0.1% SDS. Resolution was determined by plating bacteria on streptomycin-containing LB-agar and then replica stamping onto chloramphenicol-containing LB-agar.

### **2.2.9 Transmission electron microscopy**

RAW 264.7 cells were seeded in 24-well plates at a density of  $2 \times 10^5$  cells/well and treated with TLR agonists approximately 20 hours before infection. *S. Typhimurium* SL1344 was grown overnight in 3 ml of LB broth in culture tubes, with shaking at 225 rpm. RAW cells were infected in triplicate with human-serum opsonized stationary phase bacteria at a multiplicity of infection of approximately 50 bacteria per cell. After a 30 minute incubation period to allow for bacterial uptake, cells were washed 3 times with PBS and incubated in DMEM containing 100 µg/ml. Two hours post-infection, cells were fixed with 2% glutaraldehyde (Canemco) in 0.1 M sodium cacodylate (NaCac) (Canemco) pH 7.4 for one hour at room temperature. Sample dehydration, embedding, sectioning, and staining were performed by the Integrated Microscopy Facility in the Health Science Centre at McMaster University. Following fixation, samples were incubated in 0.2 M NaCac pH 7.4 two times for five minutes and post fixed with 1%



osmium tetroxide ( $\text{OsO}_4$ ) (Canemco) in 0.1 M NaCac buffer for 30 minutes. Samples were dehydrated in an ethanol series (50%, 70%, 95%, and 100%) and infiltrated with TAAB 812 (Canemco) in 1:1 (TAAB 812 : 100% ethanol) for one hour and 3:1 (TAAB 812 : 100% ethanol) for one hour. Samples were then incubated in TAAB 812 twice for one hour. Excess TAAB 812 was aspirated off the samples and small BEEM capsules were placed into the surface of the wells and polymerized at approximately  $65^\circ\text{C}$  overnight. BEEM capsules (Canemco) were topped off with TAAB 812 and polymerized at approximately  $65^\circ\text{C}$  overnight. Capsules were snapped off and ultra-thin sections (approximately 70 nm thick) were cut with an Ultracut T (Leica) and placed onto 200 mesh Cu/Pd grids. Sections were stained with saturated aqueous uranyl acetate (Canemco) for 5 minutes and rinsed with distilled water followed by lead citrate (Canemco) for 2 minutes. Sections were viewed and representative micrographs were taken using a 1200 EX TEMSCAN (JEOL, Tokyo).

**Table 1: Chemicals and Reagents**

Reagent	Catalogue #	Source
1D4B-s (rat anti-LAMP-1 antibody)	1D4B	Hybridoma Bank
4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	D1306	Molecular Probes
5- (and 6)-carboxy SNARF-1 acetoxymethyl ester, acetate	C1272	Molecular Probes
Agar	AGR001.1	Bioshop
Alexa Fluor 488 donkey anti—rat IgG	A21208	Molecular Probes
Alexa Fluor 488 goat anti—mouse IgG	A11029	Molecular Probes
Alexa Fluor 568 donkey anti - rabbit IgG	A10042	Molecular Probes
Alexa Fluor 568 goat anti—rabbit IgG	A11036	Molecular Probes
Ampicillin Sodium Salt	A0166	Sigma
Chloramphenicol	CLR201	Bioshop
CpG Oligodinucleotide 5'TCGTCGTTTTGTCGTTTTGTCGTT-3'	-	Mobix Labs
Dimethylsulfoxide	DMS666	Bioshop
Dulbecco's modified Eagle's medium	11995	Gibco
Fetal bovine serum	SH30397.03	HyClone
Fluorescence mounting media	S3023	Dako
Fluospheres polystyrene microspheres	F13081	Molecular Probes
Gentamicin Sulfate	GTA202	Bioshop
Glutaraldehyde	111-30-8	Canemco
Goat serum	053-150	Wisent
HEPES Sodium Salt	HEP002	Bioshop
HyQ Trypan Blue Solution	SV30084.01	HyClone

Lead Citrate	512-26-5	Canemco
Lipopolysaccharide from <i>Escherichia coli</i>	L2654	Sigma
Mouse Anti-Mono- and Polyubiquitinylated Conjugates, Monoclonal (clone FK2)	PW-8810	Biomol International
Osmium tetroxide	20816-12-0	Canemco
Paraformaldehyde (16%)	15710	Electron Microscopy Sciences
Peptidoglycan from <i>Staphylococcus aureus</i>	77140	Sigma
Peptone	PEP403.1	Bioshop
Phosphate buffered saline (+Mg, +Ca)	14080-055	Invitrogen
Phosphate buffered saline	405.4	Bioshop
Pluronic F-127	P3000MP	Molecular Probes
Poly(I:C)	P1530	Sigma
Rabbit anti-Salmonella O Antiserum Group B Factors 1, 4, 12, 27	229731	Difco
Saponin	CA80058-630	VWR
Sodium cacodylate	124-65-2	Canemco
Sodium chloride	SOD001.205	Bioshop
Sodium dodecyl sulfate	SDS001	Bioshop
Streptomycin sulfate	STP101	Bioshop
TAAB 812	CE001-3	Canemco
Triton X-100	TRX777	Bioshop
Uranyl acetate	541-09-3	Canemco
Yeast extract	YES401.500	Bioshop

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### 3. RESULTS

#### 3.1 Treatment with TLR-agonists leads to increased uptake of *S. Typhimurium*

To understand how treatment of host cells with TLR-agonists affects the host cell response to *Salmonella* infection I performed gentamicin protection assays using RAW 264.7 murine macrophages. Macrophages were treated approximately 20 hours prior to infection with the TLR-agonists LPS, poly(I:C), peptidoglycan, CpG or with PBS as a control as a similar time-point was defined previously to be effective against HSV-2 infection (7). Serum-opsonized *S. Typhimurium* cells were incubated with the macrophages for 30 minutes to allow for phagocytic uptake, and then extracellular bacteria were washed off and killed with a 1.5 hour gentamicin treatment. The number of intracellular bacteria were then enumerated by colony counting following lysis of the macrophages. Treatment with all TLR-agonists significantly increases the bacterial uptake after 2 hours compared to the control treatments (Figure 3). Specifically, treatment with LPS, poly(I:C), peptidoglycan, or CpG increased bacterial uptake at 2 hours post-infection by 3.0, 2.3, 4.6, 5.0-fold, respectively, as compared to control cells treated with PBS (Figure 3). Similar results were observed using immunofluorescence (Figure 4) and transmission electron microscopy (Appendix 1). Therefore, TLR-agonist treatment results in an increased uptake of *S. Typhimurium* by RAW 264.7 cells.

### **3.2 Treatment with TLR-agonists does not affect phagocytosis of fluorescent microspheres**

Treatment with all TLR-agonists tested results in an increase of *S. Typhimurium* uptake two hours post-infection (Figure 3, Figure 4). To see if this increased uptake was specific to *Salmonella* or a more general increase in phagocytic capacity, I examined the effect of TLR-agonist treatment on the phagocytosis of fluorescent microspheres. Human serum-opsonized 1.0  $\mu\text{m}$  green-yellow fluorescent microspheres were incubated with TLR-agonist treated macrophages for 3.5 hours to allow for phagocytic uptake. The cytoplasm was labeled with SNARF-1 dye and live cells were viewed under a confocal microscope. Following z-stack acquisition, ImageJ software was used to distinguish internalized beads from external beads (Appendix 2, Appendix 3). There was not a significant increase in the number of cells phagocytosing beads in macrophages treated with LPS, peptidoglycan, or CpG compared to the PBS-control (Figure 5). This demonstrates that while TLR-agonists treatment results an increase in *S. Typhimurium* uptake, it does not lead to an increased uptake of inert microspheres.

### **3.3 Treatment with TLR-agonists leads to increased intracellular replication of *S. Typhimurium***

Since TLR-agonist treatment results in increased phagocytic capacity, I next examined whether it also leads to increased intracellular killing of the bacteria. TLR-agonist treated macrophages were infected with *Salmonella* and the macrophages were lysed at 2 and 20 hours post infection. The number of intracellular bacteria was determined by colony counting and the fold increase in *Salmonella* colony forming units was calculated (20 hour CFU/2 hour CFU) and normalized to the PBS control. Under all

TLR-agonist treatment conditions there was a significant increase in intracellular replication compared to the PBS control (Figure 6). Specifically, treatment with LPS, Poly(I:C), peptidoglycan, and CpG increased bacterial replication by 3.0, 2.0, 3.7, 6.1-fold, respectively, as compared to the PBS treated control (Figure 6). Thus, while treatment with TLR-agonists increases bacterial uptake, it does not result in increased bacterial killing ability since *S. Typhimurium* replicates better in TLR-agonist treated cells.

#### **3.4 Increased bacterial replication is observed even with reduced multiplicity of infection**

To ensure that the increase in replication is not due to the burden of an initially high bacterial load, infections were performed with the MOI reduced from 50:1 to 25:1, 10:1, or 5:1 so that the number of bacteria at the 2 hour time point was similar to the PBS-control (Figure 7A). Despite the reduced bacterial load, there was still a significant increase in bacterial replication for all of the TLR-agonist treated macrophages (Figure 7B). This suggests that the increase in bacterial replication following TLR-agonist treatment is not due to the burden of an initially high bacterial load.

#### **3.5 Stimulation with interferon-gamma results in net killing of *Salmonella* within TLR-agonist treated cells**

Interferon-gamma (IFN- $\gamma$ ) is a dimeric cytokine that plays an important role in the immune response by participating in antigen presentation and in the Th1 response (109). IFN- $\gamma$  is the prototypic cytokine for activating macrophages (61). It was long thought that IFN- $\gamma$  production and secretion was restricted to natural killer (NK) cells and T

lymphocytes and was not produced by macrophages themselves (131). However several groups have shown that upon stimulation with certain cytokines and immune-stimulatory ligands, IFN- $\gamma$  can be produced by some murine and human macrophages (39; 48; 109; 119; 120; 127). In macrophages, stimulation with IFN- $\gamma$  leads to increased expression of major histocompatibility complex (MHC) class II molecules, nitric oxide (NO) production, cytokine production, and cytolytic activity (131). IFN- $\gamma$  signaling can prime macrophages to respond faster and more efficiently against bacteria (125) and *in vitro* treatment of macrophages with IFN- $\gamma$  allows them to control intracellular replication of *S. Typhimurium* (124).

Since IFN- $\gamma$  is a key activator of macrophages both *in vivo* and *in vitro* and plays an important role in resistance against intracellular pathogens such as *Salmonella* (124) and *Mycobacterium tuberculosis* (31; 44), I examined whether the addition of IFN- $\gamma$  to the TLR-agonist treatments would increase macrophage bactericidal ability. RAW 264.7 macrophages were treated with TLR-agonist, IFN- $\gamma$ , or TLR-agonist in conjunction with IFN- $\gamma$  approximately 20 hours before infection with *S. Typhimurium*. Macrophages were lysed at 2 and 20 hours post-infection and intracellular bacteria were enumerated by colony counting to calculate the fold-increase. Unlike treatment with PBS or TLR-agonists alone (Figure 6), treatment with IFN- $\gamma$  and IFN- $\gamma$  in conjunction with TLR-agonist resulted in a net-killing of internalized bacteria (Figure 8). Therefore, addition of IFN- $\gamma$  can activate macrophages to effectively kill *Salmonella*.

### **3.6 The increase in *S. Typhimurium* replication in TLR-agonist treated macrophages is dependent on a functional SPI-2 type 3 secretion system**

SPI-2 is known to be important for intracellular survival of *S. Typhimurium* within host macrophages. To determine whether SPI-2 effectors played a role in the increased bacterial replication within TLR-agonist treated macrophages, the gentamicin protection assay was performed using a strain with a deletion of the *ssaR* gene ( $\Delta$ *ssaR*). The *ssaR* gene is within SPI-2 and has homology to the *yscR* gene in *Yersinia enterocolitica*, which encodes for membrane-bound component of the T3SS (73; 117). The  $\Delta$ *ssaR* strain does not have a functional SPI-2 T3SS and SPI-2 effectors cannot be translocated into the host cell. RAW 264.7 cells were treated with PBS, LPS, poly(I:C), peptidoglycan, or CpG-ODN approximately 20 hours prior to infection with wild-type SL1344 or SL1344  $\Delta$ *ssaR*. Cells were lysed at 2 and 20 hours post-infection and fold increase was determined by colony counting (2 hour CFU/20 hour CFU). TLR-agonist treatment resulted in a significant increase in uptake of both the wildtype and  $\Delta$ *ssaR* strains (Figure 9A). However, unlike the wild-type strain, there was a net killing of mutants lacking the SPI-2 T3SS (Figure 9B), suggesting that SPI-2 type 3 secretion is required for the increase in bacterial replication following treatment with TLR-agonists.

### **3.7 SPI-2 promoter activity is not enhanced in TLR-agonist treated cells early in infection**

As mentioned earlier, previous studies have suggested that bacteria can sense the immune status of the host, and regulate gene expression accordingly (159). Since SPI-2 T3SS is required for increased bacterial replication following TLR-agonist treatment



(Figure 9), I wanted to examine whether SPI-2 gene expression was increased to improve bacterial survival within these activated macrophages. To do this, the recombinase-based *in vivo* expression technology (RIVET) system was used to examine the promoter activity of the SPI-2 gene, *sseA*. *SseA* is the first gene in one of the SPI-2 operons and its expression is required for the secretion of other SPI-2 effectors (30). *SseA* is a chaperone for two other SPI-2 proteins, *SseB* and *SseD* (126; 161) and the *sseA* mutant strain is attenuated for virulence and has a severely impaired ability to replicate within cells (74).

In the RIVET system, activation of the *sseA* promoter leads to transcription and translation of *tnpR*, which encodes for a site-specific recombinase. TnpR directs recombination between two *res* sites that flank a chloramphenicol resistance cassette on the chromosome, resulting in the permanent loss of the chloramphenicol resistance (Figure 10). Thus, *sseA* promoter activation can be measured by determining the percentage of bacteria that gain chloramphenicol sensitivity. This can be achieved by replica plating onto media with or without chloramphenicol. RAW 264.7 cells were treated with PBS, LPS, or CpG approximately 20 hours before infection with wild-type SL1344. Cells were lysed at 15-minute intervals post-infection and replica plating was used to determine SPI-2 promoter activation by calculating the percentage of bacteria that had resolved the chloramphenicol resistance cassette and thereby gained chloramphenicol sensitivity. The LPS- and CpG-treated macrophages shared a similar trend to the PBS control over the 90-minute time course (Figure 11). Therefore SPI-2 promoter activity is not enhanced in TLR-agonist treated cells early in infection.

### **3.8 Bacteria are not in the cytosol but are found within Lamp1-positive vacuoles**

Since TLR-stimulation appears to have an effect on phagocytosis, I chose to investigate its role in phagosome maturation. Loss of SCV integrity can result in increased bacterial replication in the cytosol of epithelial cells (24). Therefore, it is possible that the increase in *S. Typhimurium* replication observed in TLR-agonist treated macrophages could be due to disruption of the SCV, bacterial escape, and replication in the cytosol. Alternatively, since *Salmonella* is able to redirect phagosome maturation (22), it is possible that *Salmonella* is altering the SCV maturation process in TLR-agonist treated cells.

To test this hypothesis, macrophages treated with TLR-agonists and infected with wild-type *S. Typhimurium* were fixed at two hours post infection and co-stained with antibodies against *S. Typhimurium*, FK2, and DNA. The FK2 monoclonal antibody recognizes mono- and polyubiquitinated proteins in the cytosol, but not free ubiquitin (47). Therefore, the FK2 antibody labels ubiquitinated bacteria found within the cytosol. Immunofluorescence experiments revealed that approximately 10% of bacteria in the TLR-agonist treated cells and the control cells are found in the cytoplasm and not within the SCV (Figure 12). This was confirmed by immunofluorescence with antibodies against Lamp1, a lysosomal glycoprotein that is a marker of SCV (Figure 13). In untreated cells, 93.5% of bacteria were found with Lamp1-positive vacuoles. This was comparable to the LPS, poly(I:C), peptidoglycan, or CpG-treated cells, in which had 92.4, 93.3, 94.0, and 93.4% of bacteria were in Lamp1-positive vacuoles, respectively (Figure 14). Therefore, at 2 hours post-infection, approximately 90% of *S. Typhimurium* are

within the SCV in both the TLR-agonist treated cells and the PBS control. Thus, treatment with TLR-agonist does not affect the percentage of bacteria within SCVs or the percentage of bacteria in the cytosol.

## 4. DISCUSSION

### 4.1 TLR-agonist treatment and phagocytosis

This work has shown that treatment with a range of TLR-agonists such as LPS, poly(I:C), CpG oligonucleotides, and peptidoglycan leads to an increase in phagocytosis of *Salmonella enterica* serovar Typhimurium using the gentamicin protection assay (Figure 3), immunofluorescence (Figure 4) and transmission electron microscopy (Appendix 1). Treatment with TLR-agonists LPS, peptidoglycan, and CpG, however, does not lead to a significant increase in phagocytosis of fluorescent microspheres (Figure 5). This suggests that enhanced phagocytosis following TLR-stimulation is restricted to *Salmonella*. Since both the bacteria and the beads were opsonized with human serum, this also suggests that the increase in phagocytosis is not due to an increase in Fc $\gamma$ -receptors or complement receptor 3 (CR3), since IgG and complement are major components of serum.

Phagocytosis is a characteristic function of macrophages and other immune cells such as neutrophils and dendritic cells. It plays an important role in host defense because it ultimately results in the destruction of phagocytosed pathogens and presentation of antigens in the context of MHC to activate an adaptive immune response (149). Phagocytosis is a complex and highly regulated process, involving a variety of molecules including receptors, protein kinases and Rho GTPases. It begins with recognition and binding of pathogens to cell surface receptors on the phagocyte. Ligand-receptor interaction activates a series of downstream signaling events, leading to actin cytoskeleton rearrangements and phagocytic uptake (149). Macrophages express a

variety of cell-surface receptors, and the underlying molecular mechanisms behind complement receptor 3 (CR3) and the Fc $\gamma$  receptor ligand binding and phagocytosis, have been well characterized (32; 62). However, macrophages also express TLRs on their cell surface and there is a growing body of evidence suggesting a possible link between TLR-stimulation and phagocytosis (17; 28; 35; 88; 96; 111; 144).

Several other studies have also found that TLR signaling has an effect on phagocytosis. Impaired phagocytosis of *Escherichia coli* has been observed in macrophages lacking the adapter molecule MyD88, suggesting a connection between TLR-signaling and phagocytosis (17). Treatment with TLR-agonists has also been shown to affect phagocytic capacity. Flow cytometric analysis of murine macrophages treated with CpG oligodinucleotides showed increased phagocytosis of *Burkholderia pseudomallei*, *Salmonella enterica* serovar Typhi, and *E. coli* in a dose-dependent manner (144). Doyle *et al.* expanded on these studies by showing that treatment of macrophages with TLR agonists CpG, lipid A, peptidoglycan and poly(I:C) increases the phagocytosis of *E. coli* and *Staphylococcus aureus*, but not latex beads (35). Recently, Kong and Ge have also found that stimulation of RAW 264.7 cells with LPS, peptidoglycan, CpG and poly(I:C) leads to an increase in the phagocytosis of GFP-*E. coli* and that LPS-stimulation increases uptake of FITC-dextran, but not latex beads (88). However, they did not find that the degree of phagocytosis enhancement differed with the various TLRs as we (Figure 3) and Doyle *et al.* (35) have observed, with TLR9 providing the strongest increase in phagocytosis and TLR3 providing the weakest. This is possibly due to the fact that Kong and Ge treated with TLR-agonists for 2 hours (88), compared to the 20-24

hour treatment times performed by us and others (35). Other recent studies have also linked TLR2 to phagocytosis of amyloid  $\beta$  peptide by microglia cells (28) and fungi by macrophages (96), and TLR4 to bacterial phagocytosis by enterocytes (111). Two mechanisms for this increase in phagocytic capacity following TLR-agonist treatment have been proposed, one that is dependent on Cdc42, Rac and actin (88) and another that is dependent on scavenger receptors (SR) and MyD88-IRAK4-p38 signal transduction (35).

Kong and Ge found that stimulation with LPS resulted in an increase in phagocytosis of *E. coli* in a manner that is dependent on the small GTPases Rac and Cdc42 (88). Rac and Cdc42 have long been known to play an important role in phagocytosis (25). When Rac and Cdc42 are inhibited either by chemical inhibitors or through RNAi knockdown, LPS-stimulated *E. coli* phagocytosis is reduced (88). This pathway is independent of MyD88 but requires actin (88). It is interesting to note that while enhanced phagocytosis of *E. coli* occurs 12-24 hours following LPS stimulation, Rac and Cdc42 are activated less than one hour following TLR stimulation (88). This suggests that signaling events downstream of these Rho GTPases play a role in TLR-stimulated phagocytosis.

Doyle *et al.* found that TLR-agonist treatment induced the upregulation of scavenger receptors in a manner that depended on the MyD88, IRAK4, and p38 signaling pathway (35). This suggests overlap between TLR and SR intracellular events. Due to the high number of molecules involved in the signal transduction and regulation

of these pathways, this demonstrates that intracellular events are more intricately connected than previously thought.

#### **4.2 TLR-agonist treatment increases intracellular bacterial replication**

The increase in bacterial uptake following TLR-agonist treatment does not result in enhanced bactericidal activity of the macrophages. Treatment with LPS, poly(I:C), peptidoglycan, and CpG all lead to increased bacterial replication within macrophages compared to the PBS control (Figure 6). This increase in bacterial replication is not due to an initially high bacterial load because there was still a significant increase when the multiplicity of infection was reduced to establish similar levels of bacteria at two hours (Figure 7). Wang *et al.* also observed an increase in *Salmonella enterica* serovar Dublin and *Listeria* replication following treatment with CpG (152). CpG treatment, however, was able to partially inhibit the growth of *M. tuberculosis* (152), suggesting that TLR-agonist stimulation may have different effects on uptake and survival of different bacteria.

*Salmonella*'s ability to survive within macrophages activated through TLR-stimulation may be due to the fact that *Salmonella* has multiple effectors that are able to subdue a host inflammatory response (65). For example, the SPI-1 effector SptP has a role in antagonizing the pro-inflammatory signaling cascade that is activated following invasion (110). SptP can downregulate activation of the MAPK Erk through its tyrosine phosphatase activity (110). SspH1 has also been implicated in downregulating the innate immune response. SspH1 contains leucine rich repeat motifs and has been shown to localize to the nucleus and inhibit NF- $\kappa$ B gene expression (64; 105). AvrA, another SPI-

I effector, has also been shown to inhibit NF- $\kappa$ B-dependent cytokine secretion (29). The presence of multiple mechanisms for antagonizing the host immune response may explain why *Salmonella* is able to survive and replicate within macrophages activated through TLR-agonist treatment.

Where treatment with TLR-agonist alone results in an increase in *Salmonella* replication (Figure 6), treatment with TLR-agonist in combination with IFN- $\gamma$  results in a net killing of intracellular bacteria (Figure 8). TLR-agonists are able to activate macrophages, resulting in the production of cytokines such as TNF- $\alpha$ , IFN- $\beta$ , IL-6, IL-5 and IL-12. IFN- $\gamma$  is one of the most well characterized activators of macrophages, so much so that it was previously known as “macrophage activating factor” (131). While production of IFN- $\gamma$  has been reported in some macrophages (39; 48; 109; 119; 120; 127), it is not produced by RAW 264.7 cells treated with these TLR-agonists. IFN- $\gamma$  treatment inhibits *Salmonella* replication through activation of MEK/Erk kinase signal transduction and phagocyte NADPH oxidase (124). Since addition of IFN- $\gamma$  results in a net killing of bacteria in all treatment groups (Figure 8), this suggests that the production of the inflammatory cytokines TNF- $\alpha$ , IFN- $\beta$ , IL-6, IL-5 and IL-12 is not sufficient to launch an aggressive defense against *Salmonella* infection.

Another reason for the increase in bacterial replication following TLR-agonist treatment could be due to limitations of the cell culture model. *In vivo*, macrophage activation by TLR-agonist treatment would also result in the recruitment of other immune cells and the initiation of an adaptive immune response (4). It is likely that the production



of inflammatory cytokines is insufficient to combat *Salmonella* infection in the absence of other immune cells.

#### **4.3 TLR agonist-dependent increase in *Salmonella* replication through Cdc42, Rac and actin remodeling**

Recent studies have shown that treatment with TLR-agonists results in an increase in phagocytosis in a manner that is dependent on actin and the Rho GTPases Cdc42 and Rac (88). Interestingly, *Salmonella* has many effectors that activate Cdc42 and Rac within the host cell as well as effectors that interact with actin.

*Salmonella* effectors SopB, SopE, SopE2 and SopD have been shown to activate the Rho GTPases Cdc42 and Rac-1 in the host cell (10; 45; 67; 115; 139; 160). While they have mainly been characterized for their role in invasion, a recent study has shown that SopB, SopD and SopE2 are all synthesized at later stages of infection in mice (56). If both TLR-agonist treatment and *Salmonella* are able to activate Cdc42 and Rac signal transduction, then a combination of TLR-agonist treatment followed by *Salmonella* infection may result in conditions that are favourable for *Salmonella* survival. Alternatively, the *Salmonella* effector SptP has been shown to antagonize Cdc42 and Rac-1 (46; 92). Therefore it is possible that any beneficial effects of Rac-1 and Cdc42 activation following TLR-stimulation could be dampened, instead leading to an environment that is advantageous for the bacteria.

Actin cytoskeletal rearrangements are important for bacterial invasion in the gut, however, they also play a role in later stages of infection since treatment of infected cells with actin polymerization inhibitors leads to a decrease in bacterial replication (102). The SPI-1 T3SS effectors SipA and SipC (also known as SspA and SspC, respectively) are

actin-binding proteins that are able to directly modulate actin remodeling (65). SipA has a role in bacterial entry by reducing the critical concentration required for the initiation of actin polymerization and SipC can nucleate and bundle actin and interact with intermediate filaments (70; 130; 160). Even though they are SPI-1 effectors, SipA has been shown to persist after entry and to have a role in phagosome maturation and bacterial replication (20; 56). SPI-2 T3SS can induce actin polymerization around the SCV, though this may not be due to the action of effectors but rather may represent a host reaction to the translocon insertion into the vacuole since a similar response has been observed with *Yersinia* T3SSs (147). In fact, there is evidence to suggest that some SPI-2 effectors may function to inhibit vacuole-associated actin polymerization (VAP) (65). SpvB and SspH2 have been reported to have roles in promoting actin depolymerization and reducing actin polymerization, respectively (95; 104). Therefore, since both *Salmonella* and TLR signaling can affect actin dynamics, perhaps *Salmonella* is able to benefit from actin rearrangements induced by TLR-agonist treatment. Alternatively, *Salmonella* may be able to tolerate and modify any changes in actin dynamics through the use of multiple effectors that result from TLR-agonist treatment if they are unfavourable for its survival.

#### **4.4 TLR-agonist treatment and SCV maturation**

The results from these experiments show that approximately 90% of bacteria were within Lamp1-positive vacuoles, in all of the TLR-agonist treated cells as well as the PBS control (Figure 13). These results are comparable to those observed by others (12; 24). While there was no significant difference in Lamp1-association between the TLR-

agonists and the PBS control, this does not exclude the possibility that TLR-agonist treatment modulates SCV maturation in a manner that allows for increased bacterial replication.

The downstream consequences of TLR signaling on phagosome maturation is an area of some controversy. Medzhitov and Blander found a role for TLR signaling in phagosome maturation since MyD88-deficient and TLR2/4-deficient macrophages display delayed acidification and phagosome-lysosome fusion following uptake of bacteria (17). They concluded that innate sensing and the ability to initiate TLR signaling can induce phagosome maturation. A later study by Yates and Russell disputed these results and instead concluded that phagosome maturation is in fact unaffected by TLR stimulation (156). They argue that there is no difference in the kinetics of phagosome maturation in MyD88-deficient macrophages stimulated with the TLR ligands LPS or Pam<sub>3</sub>Cys (156). Further studies are necessary to clarify the effect of TLR signaling on phagosome maturation and the role this may have on *Salmonella* infection. Also, phagosome-lysosome fusion and acidification kinetics are not the only markers of phagosome maturation. It would be interesting to see if acquisition of other SCV markers other than Lamp1 is altered following TLR-agonist treatment. Since *Salmonella* is already known to modulate phagosome maturation on its own (22), any additional alterations to phagosome maturation due to treatment with TLR-agonists may result in an additive effect that is more conducive for *Salmonella* growth.

Additionally, a study by Yates *et al.* in 2007 found that treatment with LPS and/or IFN- $\gamma$  resulted in reduced proteolytic capacity of the early phagosome (155). Reduced

lipase activity was also observed in cells treated with LPS or LPS with IFN- $\gamma$  (155). The authors hypothesize that the activated macrophages have a general decrease in degradative capacity. This may allow for epitope preservation so the macrophages are better able to present antigen through MHC class II receptors and subsequently initiate the adaptive immune response (156). It is possible that this is also the case for the TLR-agonist treated cells in this study. Activation through TLR-agonist treatment may result in cells that favour antigen presenting over degradation. This may create conditions that are more favourable for *Salmonella* survival. Examining the effect of LPS, poly(I:C), peptidoglycan, and CpG treatment on protease and lipase activity could confirm this hypothesis and may provide insight into macrophage function.

#### **4.5 Host-pathogen interaction in TLR-agonist treated macrophages**

In this study I used the RIVET system to examine SPI-2 promoter activity in TLR-agonist treated cells and found that there was no noticeable difference between the LPS- and CpG- treated macrophages and the PBS controls over the 90-minute time course (Figure 11). However, this result does not exclude the possibility that SPI-2 gene expression is not increased, particularly at later time-points in infection. This reporter system is extremely sensitive, and ultimately perhaps too sensitive for this experiment. One of the drawbacks of this system is that activation of the promoter of interest results in the permanent loss of chloramphenicol resistance. Therefore, any subsequent fluctuations in gene expression cannot be measured. Other reporter systems such as  $\beta$ -galactosidase, green fluorescent protein (GFP), or lux could be used to measure changes in SPI-2 gene

expression in TLR-agonist treated macrophages. The implementation of these other systems will allow for promoter activation to be measured over a longer period of time.

#### **4.6 Model for TLR agonist-dependent increase in *Salmonella* replication through scavenger receptor-A, Hook3 and SPI-2 effector SsaB**

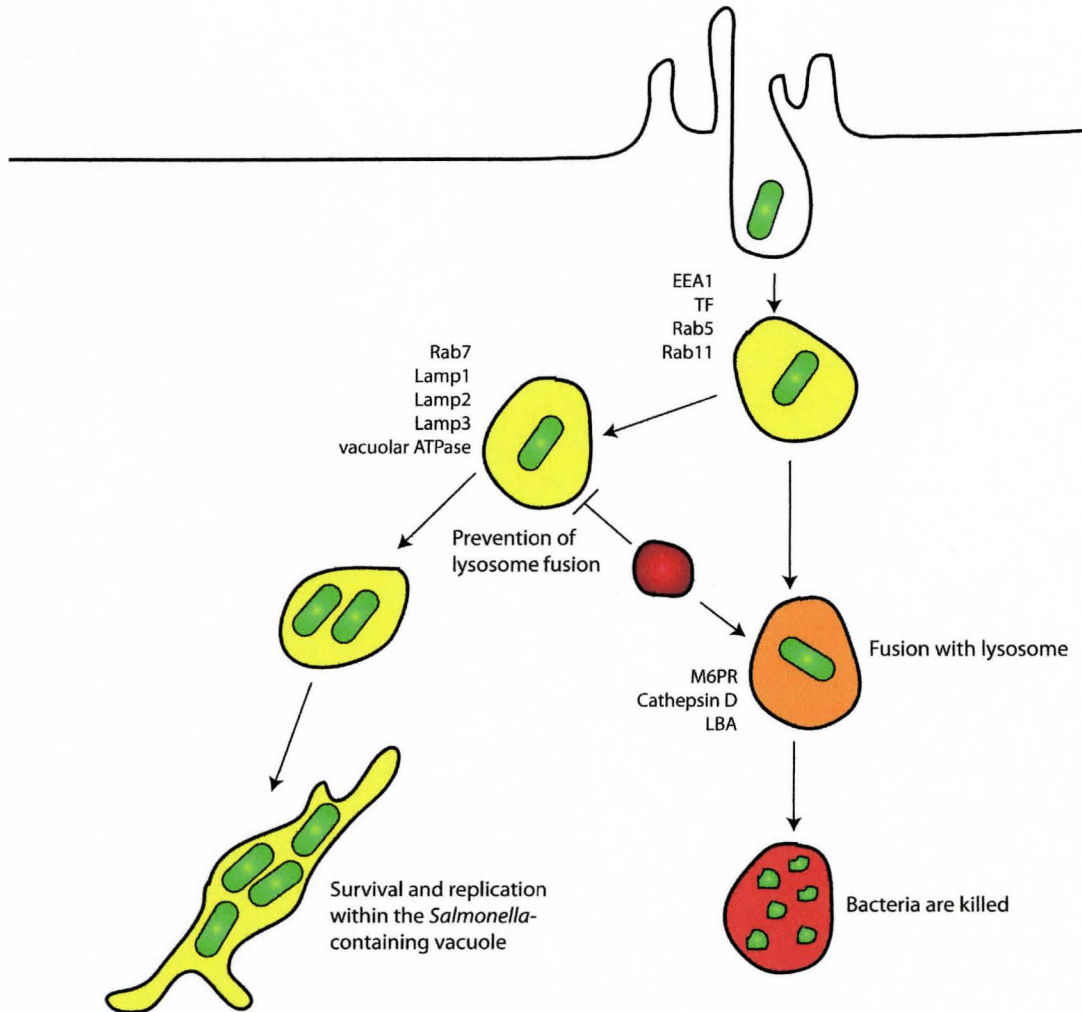
Macrophage scavenger receptor-A (SR-A) plays a role in innate immunity through recognition and phagocytosis of both Gram-positive and Gram-negative bacteria (116; 140) as well as in inactivating toxic microbial products (63; 69). Recently Sano *et al.*, found that the cytoplasmic tail of SR-A interacts with the microtubule-binding protein Hook3 and that cells transfected with Hook3 siRNA have increased surface and total SR-A expression (128). They suggest that Hook3 may play a role in intracellular trafficking and clearance of SR-A following endocytosis (128). Hook3 has also been implicated in endocytic trafficking through interaction with the *Salmonella* effector SsaB (135).

SsaB (also known as SpiC) is encoded within SPI-2 (113) and is required for virulence in mice and for replication within macrophages (142). SsaB is thought to be part of the SPI-2 type 3 secretion apparatus. Some groups have demonstrated that SsaB is also translocated into the host cytosol (135), however other groups using less sensitive methods have claimed that SsaB is not translocated (158). Hook3 is found in infected and uninfected macrophages and Shotland *et al* demonstrated that full length Hook3 from macrophages binds to SsaB (135). Since expression of SsaB leads to Golgi and late-endosome/lysosome disruption in a manner that resembles a Hook3 dominant negative mutant, it is likely that SsaB interacts with Hook3, inactivating it, and thereby resulting in the inhibition of phagosome-lysosome fusion (135).

Taken together, there is a possible explanation for why TLR-agonist treatment leads to an increase in bacterial uptake and intracellular replication (Figure 15). Treatment with TLR-agonists leads to an increase in SR-A expression and an increase in phagocytosis of bacteria (35). Though the bacteria used in that study were *E. coli* and *S. aureus*, it is likely that *Salmonella* would share a similar pathway. The cytoplasmic tail of SR-A interacts with the microtubule-binding protein Hook3 (128), a protein that has also been shown to bind to the translocated *Salmonella* effector, SsaB (135). Hook3 is involved in SR-A trafficking (128) and the effect on Hook3 availability and activity following an increase in SR-A expression due to TLR-agonist treatment has not yet been examined. It is possible that an increase in SR-A expression results in a decrease in active and available Hook3, which is also observed following interaction with SsaB (135). It has previously been hypothesized that Hook3 inactivation by SsaB results in decreased phagosome-lysosome fusion and therefore enables *Salmonella* replication within the phagosome (135). I hypothesize that this effect is exacerbated following TLR-agonist treatment since additional Hook3 would be needed for SR-A recycling. Therefore a combination of TLR-agonist treatment and *Salmonella* infection leads to less active and available Hook3 than either would alone. This may explain why TLR-agonist treatment results in increased bacterial uptake, and also increased bacterial replication.

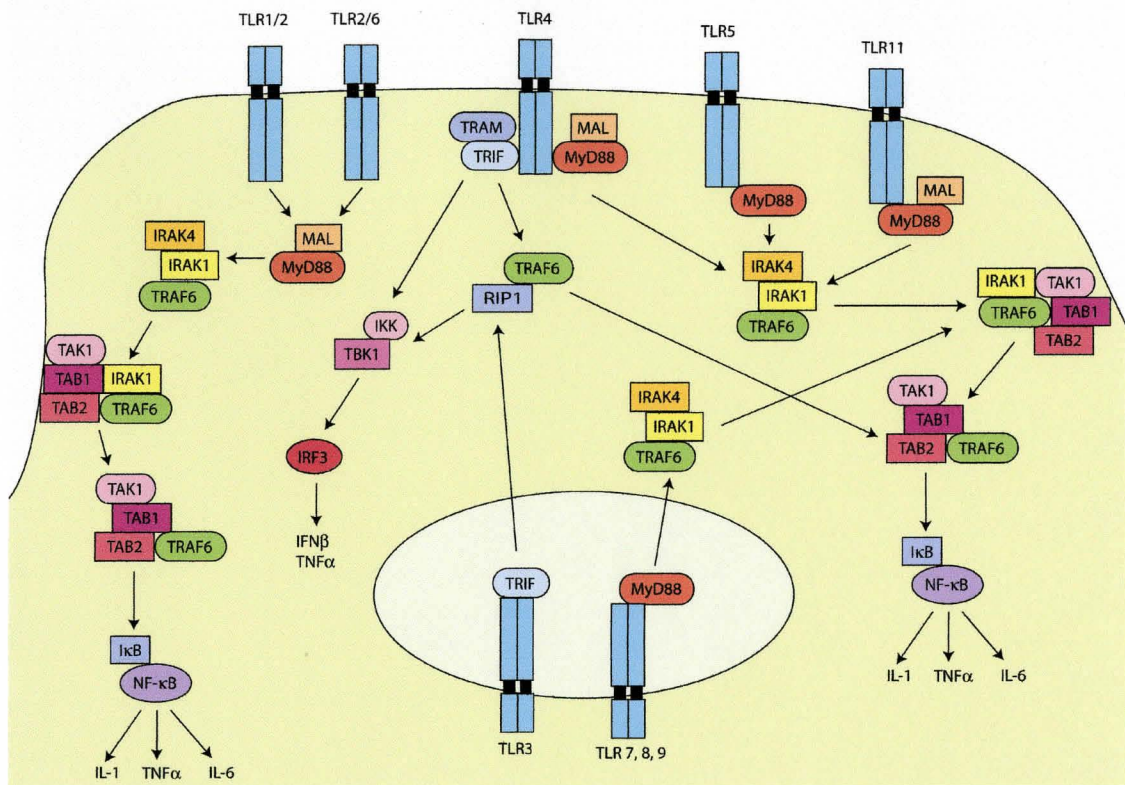
#### 4.7 Summary

This study has shown that treatment of RAW 264.7 cells with LPS, poly(I:C), peptidoglycan, and CpG-ODN results in increased phagocytosis of *S. Typhimurium*. This increase in bacterial uptake, however, does not result in increased bactericidal ability since bacterial replication is higher in TLR-agonist treated macrophages compared to the PBS-control. The increase in bacterial replication following TLR-agonist treatment is dependent on functional SPI-2 type 3 secretion, but can be abolished when macrophages are also activated with IFN- $\gamma$ . A recent study has suggested that TLR stimulation can lead to an increase in phagocytosis in a Rac, Cdc42, and actin-dependent manner (88). Interestingly, *Salmonella* has several effectors that have been shown to interact with Rac, Cdc42, and actin (65), and this may represent a connection between TLR signaling, phagocytosis, and *Salmonella* pathogenesis. TLR signaling has also been shown to lead to an increase in phagocytosis through scavenger receptors (35). I hypothesize that the observed increase in bacterial replication may be due to interactions between the SPI-2 effector SsaB and the host protein Hook3, which has also been implicated in scavenger receptor recycling (128). The more we learn about TLR signaling, the more apparent it becomes that there is extensive interplay between intracellular signaling pathways involved in immunity and in macrophage function. Understanding these pathways, how they interact with one another, and the role they have on *Salmonella* pathogenesis may present novel opportunities to combat infection and disease.

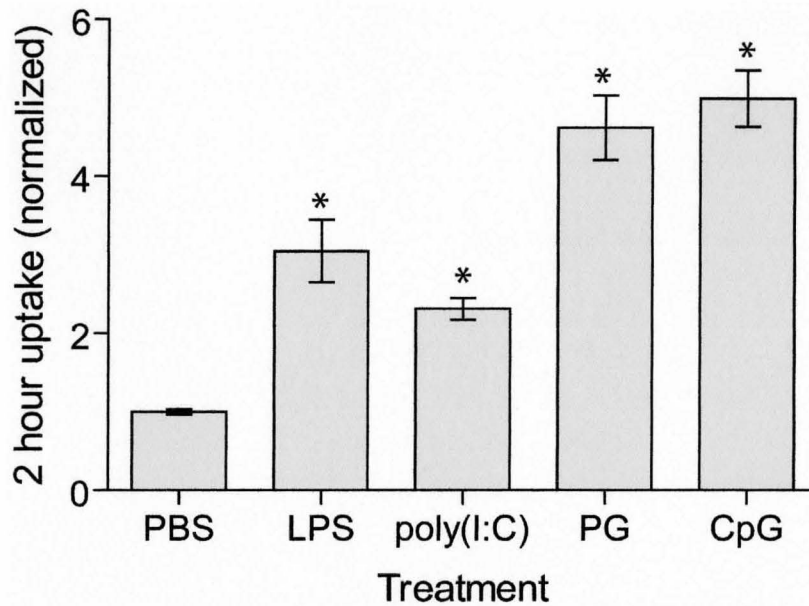


**Figure 1: *Salmonella* redirects phagosome maturation.** The SCV maturation process involves interaction with the endosomal system in a highly orderly and selective manner (1). The SCV interacts briefly with early endosomes (138), acquiring marker proteins such as early endosome antigen-1 (EEA1), transferrin receptor (TF), and the GTPases Rab5 and Rab11 (123; 136; 138). However, the interaction with these proteins is only transient, and after EEA1 and Rab5 dissociate, the SCV then acquires select markers of the late endosome/lysosome such as Rab7 (101), which in turn attracts lysosomal membrane glycoproteins (Lamps) Lamp1, Lamp2, and Lamp3 and vacuolar ATPase, which acidifies the SCV (37; 52; 138). Despite the presence of these late endosome/lysosome markers, the SCV does not merge with the lysosomes. This is confirmed by the absence of mannose-6-phosphate receptors (M6PR), cathepsin D, and lysobisphosphatidic acid (LBA) (27; 50; 52; 54; 68). Adapted from (149).

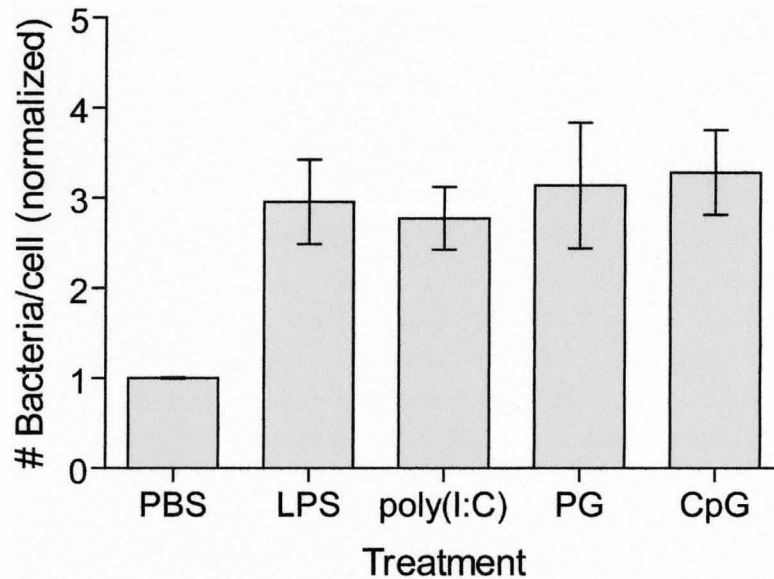




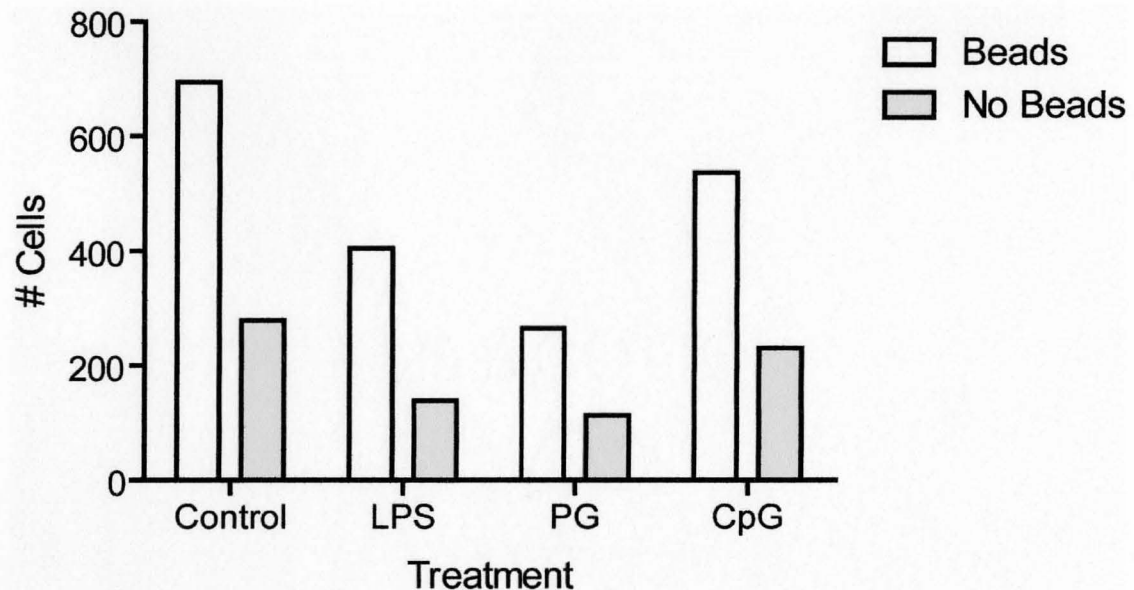
**Figure 2: TLR signaling pathway.** Myeloid differentiation factor-88 (MyD88) interacts with the cytoplasmic tail of TLRs and recruits interleukin-1 receptor-associated kinase-4 (IRAK4) and together they mediate IRAK1 phosphorylation (97). Once phosphorylated, IRAK1 interacts with and activates tumour necrosis factor receptor-associated factor-6 (TRAF6) and together they interact with a membrane complex consisting of TGF- $\beta$  activated kinase (TAK1), and the TAK1-binding (TAB) family members, TAB1, and TAB2 (36). The complex is activated following TRAF6 and TAK1 ubiquitination, which leads to the phosphorylation of TAK1 and TAB2 and the dissociation of the complex from the membrane (81; 122; 151). In the cytosol IRAK1 is degraded and activated TAK1 can phosphorylate the IKK complex, leading to the phosphorylation and degradation of I $\kappa$ B and the release of NF- $\kappa$ B (100). NF- $\kappa$ B translocates to the nucleus where it promotes the expression of pro-inflammatory genes. TLR3 and TLR4 activate the TRIF-dependent pathway (86). TLR4 recruits TRIF-related adaptor molecule (TRAM), which subsequently recruits TRIF, whereas TLR3 can recruit TRIF directly (43). TRIF can bind to receptor-interacting protein-1 (RIP1), which recruits TRAF6, resulting in the activation of the inhibitor of  $\kappa$ B kinase (IKK) complex (103). Direct binding of TRIF and TRAF6 can result in IRAK1/4-independent activation of TAK1 (80; 82). TRIF can bind TANK-binding kinase-1 (TBK1) and IKK $\epsilon$ , which activate the transcription factor IRF3 (129). Adapted from (26) and (36).



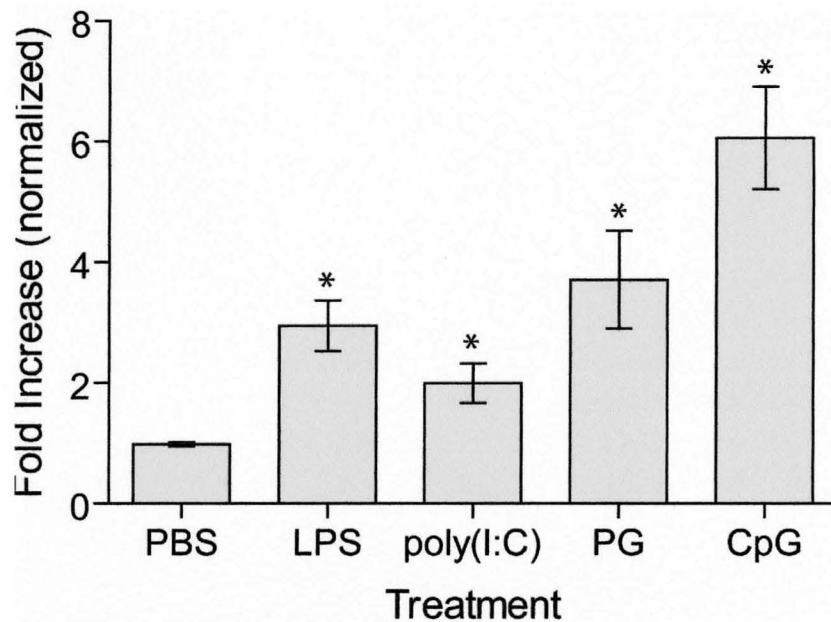
**Figure 3: Treatment with TLR agonists results in an increase in *Salmonella* uptake at 2 hours.** RAW 264.7 cells were treated with PBS, LPS, poly(I:C), peptidoglycan or CpG-ODN approximately 20 hours before infection with wild-type SL1344. Two hour uptake was calculated as the intracellular cfu count, normalized to the PBS control. Bars represent mean  $\pm$  SEM normalized to the PBS control. All experiments were performed in triplicate on at least 3 separate occasions. Asterisks represent significant difference from the PBS control ( $p \leq 0.05$ ) using a one-way ANOVA.



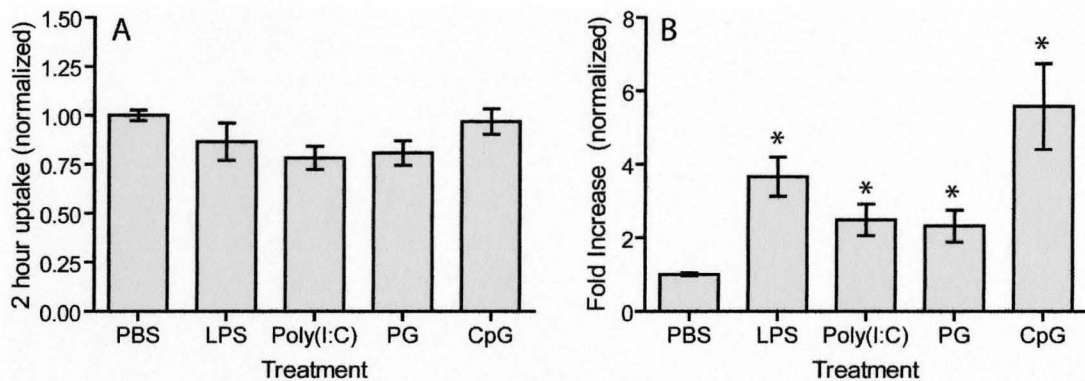
**Figure 4: Immunofluorescence of TLR-agonist treated cells shows an increase in *Salmonella* uptake at 2 hours.** RAW 264.7 cells were treated with PBS, LPS, poly(I:C), peptidoglycan or CpG-ODN approximately 20 hours before infection with wild-type SL1344. Cells were fixed with 2% paraformaldehyde 2 hours post infection and stained with antibodies against *Salmonella*. The number of bacteria per cell was counted for at least 100 cells and normalized to the PBS control. Bars represent mean  $\pm$  SEM normalized to the PBS control. All experiments were performed in duplicate, on at least 3 separate occasions.



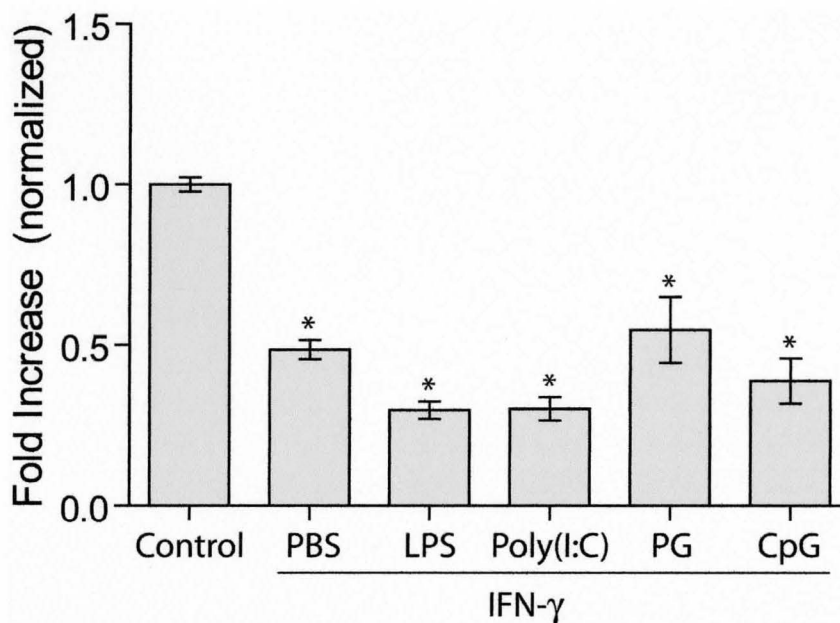
**Figure 5: Treatment with TLR-agonists does not affect the phagocytosis of fluorescent microspheres.** 1.0  $\mu\text{m}$  fluorescent microspheres were opsonized in 20% human serum and incubated with RAW 264.7 macrophages to allow for phagocytosis. Cells were treated with SNARF-1 dye for 30 minutes to stain the cytoplasm and then viewed under a confocal microscope. Software was used to determine if beads were internalized or on the surface of the cell and internalized beads were counted and plotted. Experiments were performed on at least 3 separate occasions. No significant difference between treatment groups as determined by Chi-squared test.



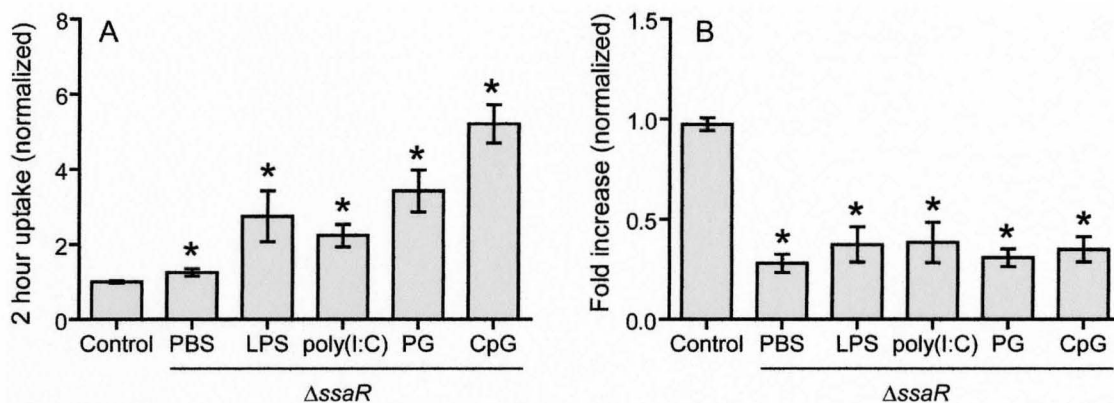
**Figure 6: Treatment with TLR agonists results in increased intracellular replication of *Salmonella*.** RAW 264.7 cells were treated with PBS, LPS, poly(I:C), peptidoglycan or CpG-ODN approximately 20 hours before infection with wild-type SL1344. Fold increase was calculated as cfu count at 20 hours divided by cfu count at 2 hours. Bars represent mean  $\pm$  SEM normalized to the PBS control. All experiments were performed in triplicate on at least 3 separate occasions. Asterisks represent significant difference from the PBS control ( $p \leq 0.05$ ) using a one-way ANOVA.



**Figure 7: Reducing the multiplicity of infection still results in increased intracellular replication of *Salmonella*.** RAW 264.7 cells were treated with PBS, LPS, poly(I:C), peptidoglycan, or CpG-ODN approximately 20 hours before infection with wild-type SL1344 with MOI of 50:1, 25:1, 10:1, or 5:1. (A) MOI was modified so that macrophages treated with TLR-agonists had similar number of bacteria at 2 hours post infection (B) Fold increase (20 hour/2 hour CFU), normalized to PBS-control that had similar number of bacteria at 2 hours. All experiments were performed in triplicate, on at least 3 separate occasions. Bars represent mean  $\pm$  SEM. Asterisks represent significant difference from PBS control ( $p \leq 0.05$ ) using a one-way ANOVA.

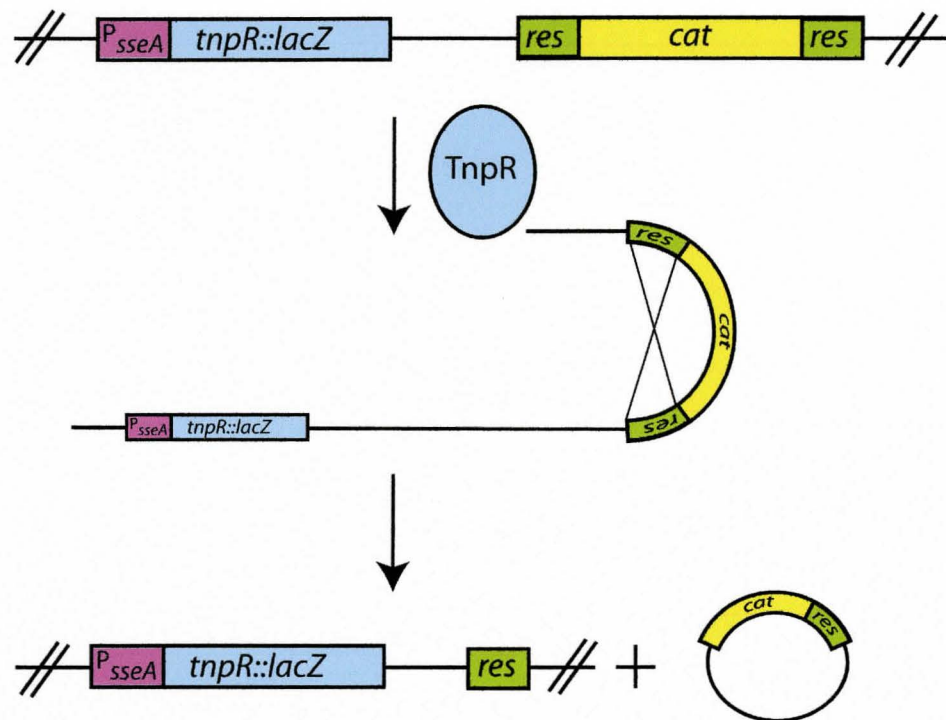


**Figure 8: Net killing of bacteria in cells treated with TLR-agonist plus interferon-gamma.** RAW 264.7 cells were treated with PBS alone or with IFN- $\gamma$  in addition to PBS, LPS, poly(I:C), peptidoglycan or CpG-ODN approximately 20 hours before infection with wild-type SL1344. Fold increase was calculated as cfu count at 20 hours divided by cfu count at 2 hours. Bars represent mean  $\pm$  SEM normalized to the PBS control with no IFN- $\gamma$  treatment. All experiments were performed in triplicate on at least 3 separate occasions. Asterisks represent significant difference from the control ( $p \leq 0.05$ ) using one-way ANOVA.

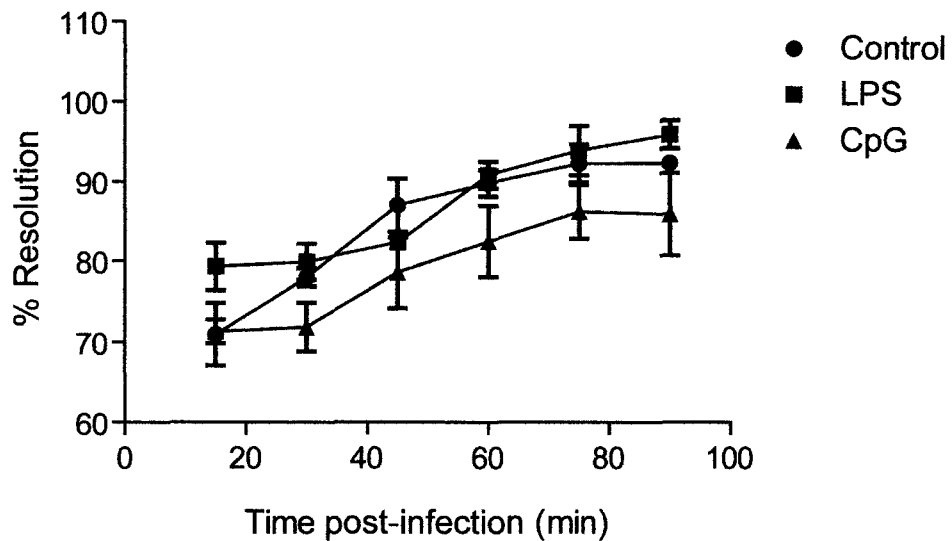


**Figure 9: Increase in *Salmonella* intracellular replication RAW 264.7 cells is dependent on functional SPI-2 type 3 secretion.** RAW 264.7 cells were treated with PBS, LPS, poly(I:C), peptidoglycan, or CpG-ODN approximately 20 hours before infection with wildtype SL1344 or mutant strain SL1344  $\Delta ssaR$ . (A) Two hour uptake normalized to wildtype PBS control. (B) Fold increase normalized to wildtype PBS control. All experiments were performed in triplicate, on at least 3 separate occasions. Bars represent mean  $\pm$  SEM. Asterisks represent significant difference from the wildtype PBS control ( $p \leq 0.05$ ) using a one-way ANOVA.

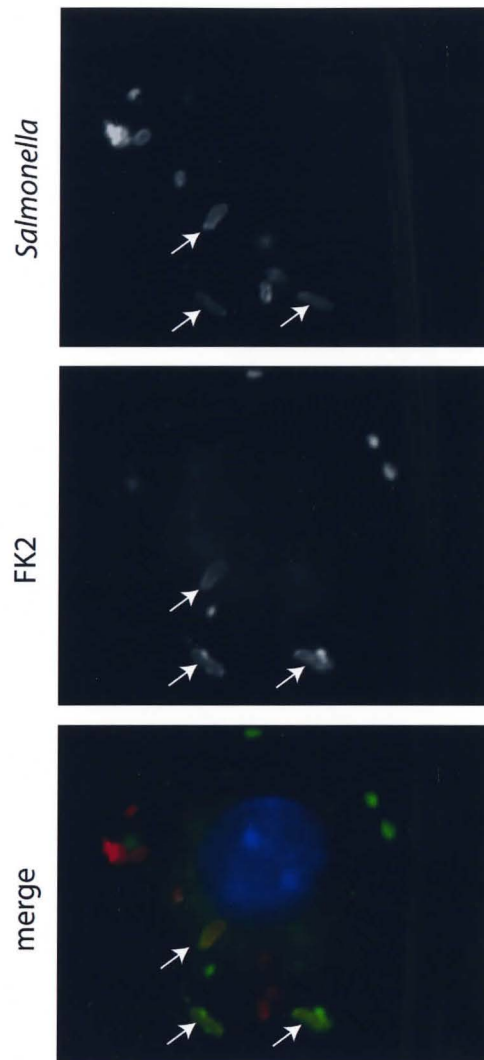




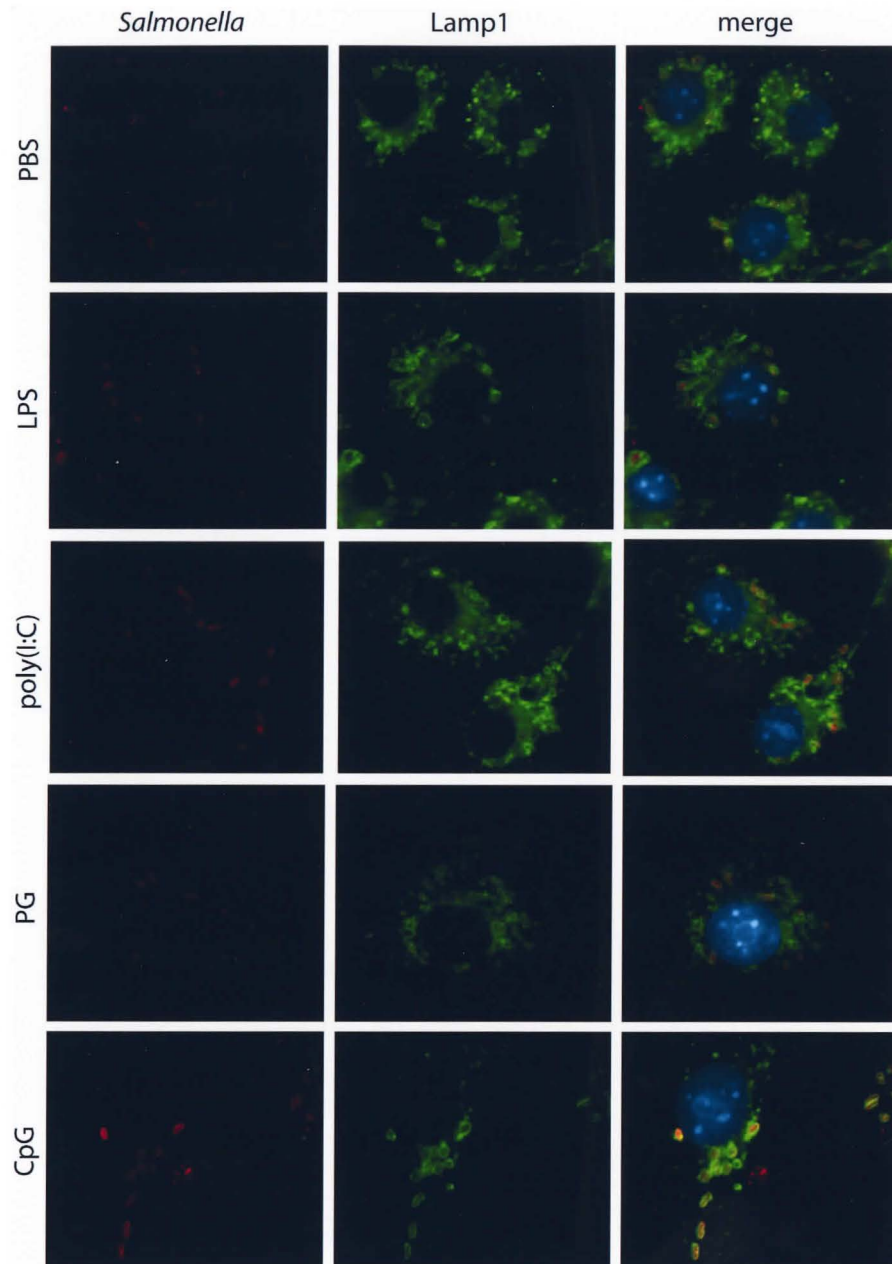
**Figure 10: Schematic representation of recombinase-based *in vivo* expression technology (RIVET).** Activation of the *sseA* promoter results in the transcription and translation of the site-specific recombinase gene, *tnpR*. TnpR will direct recombination between the two *res* sites flanking the chloramphenicol resistance cassette, resulting in the permanent loss of the chloramphenicol resistance. Percent resolution can then be calculated by replica stamping onto plates with or without chloramphenicol.



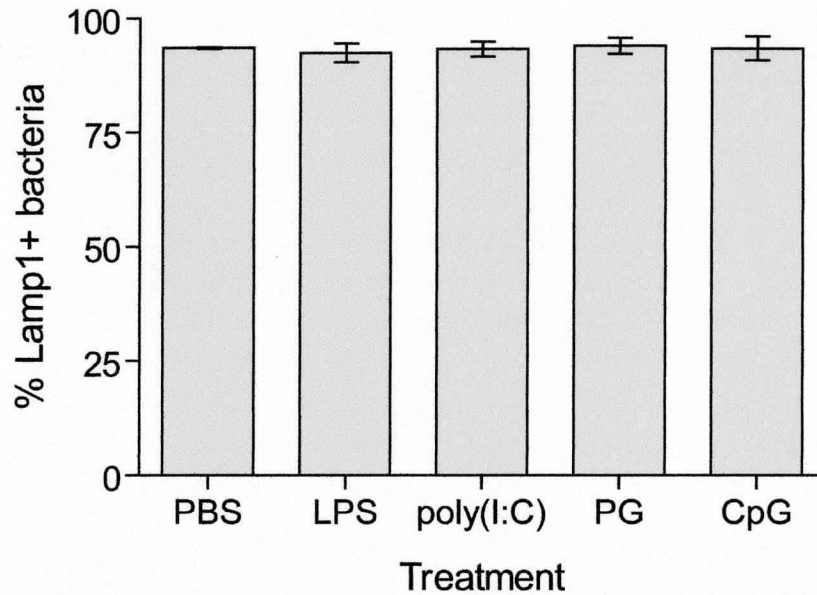
**Figure 11: SPI-2 promoter activity is not enhanced in TLR-agonist treated cells early in infection.** SPI-2 promoter activation in early time-points post-infection was measured using recombinase-based in vivo expression technology. Replica plating was used to determine the percentage of bacteria in which SPI-2 promoter activation resulted in permanent resolution of the chloramphenicol resistance cassette. Experiments were performed in triplicate, on two separate occasions and shown as mean  $\pm$  SEM.



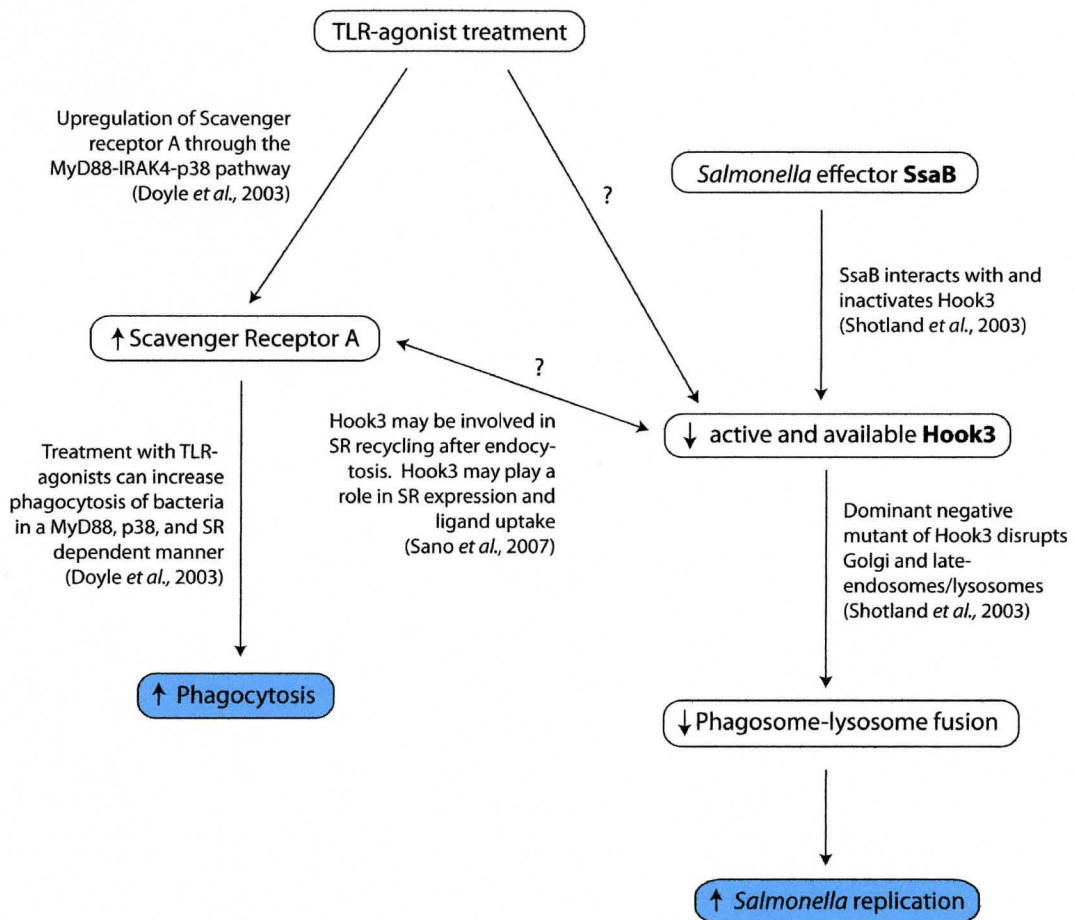
**Figure 12: Immunofluorescent staining for cytosolic bacteria in LPS-treated cells.** RAW 264.7 cells were treated with TLR-agonists approximately 20 hours before infection with wild-type *S. Typhimurium*. Cells were fixed with 2% paraformaldehyde 2 hours post-infection and co-stained with antibodies against *Salmonella* (top panel, red in merge) or FK2 (middle panel, green in merge). Bottom panel represents merged colorized image with red representing *Salmonella*, green representing FK2 and blue representing DNA. Arrows suggest possible colocalization of *Salmonella* with FK2.



**Figure 13: Immunofluorescent staining for bacteria within Lamp1-positive vacuoles.** RAW 264.7 cells were treated with TLR-agonists approximately 20 hours before infection with wild-type *S. Typhimurium*. Cells were fixed with 2% paraformaldehyde 2 hours post-infection and co-stained with antibodies against *Salmonella* (left column, red in merge), Lamp1 (middle column, green in merge), and DNA (blue in merge). Experiment was performed in triplicate, on at least three separate occasions and representative images are shown.



**Figure 14: Most bacteria reside within Lamp1-positive vacuoles.** RAW 264.7 cells were treated with TLR-agonists approximately 20 hours before infection with wild-type *S. Typhimurium*. Cells were fixed with 2% glutaraldehyde 2 hours post-infection and co-stained with antibodies against *Salmonella*, Lamp1, and DNA. Bacteria within Lamp1+ vacuoles were enumerated and expressed as a percentage of total intracellular bacteria. At least 100 bacteria were counted on each occasion. Bars represent mean  $\pm$  SEM. Experiment was performed in triplicate, on at least three separate occasions.



**Figure 15: Hypothesized interaction map between TLRs, SRs, SsaB, and Hook3.** Treatment of macrophages with TLR-agonists leads to an increase in phagocytosis, in a manner that is dependent on MyD88, p38, and SR (35). Hook3 interacts with the cytoplasmic tail of SR and may play a role in SR expression and trafficking (128). The *Salmonella* protein SsaB also interacts with Hook3 and inactivates it, resulting in a decrease in phagosome-lysosome fusion (135) and a subsequent increase in *Salmonella* replication.

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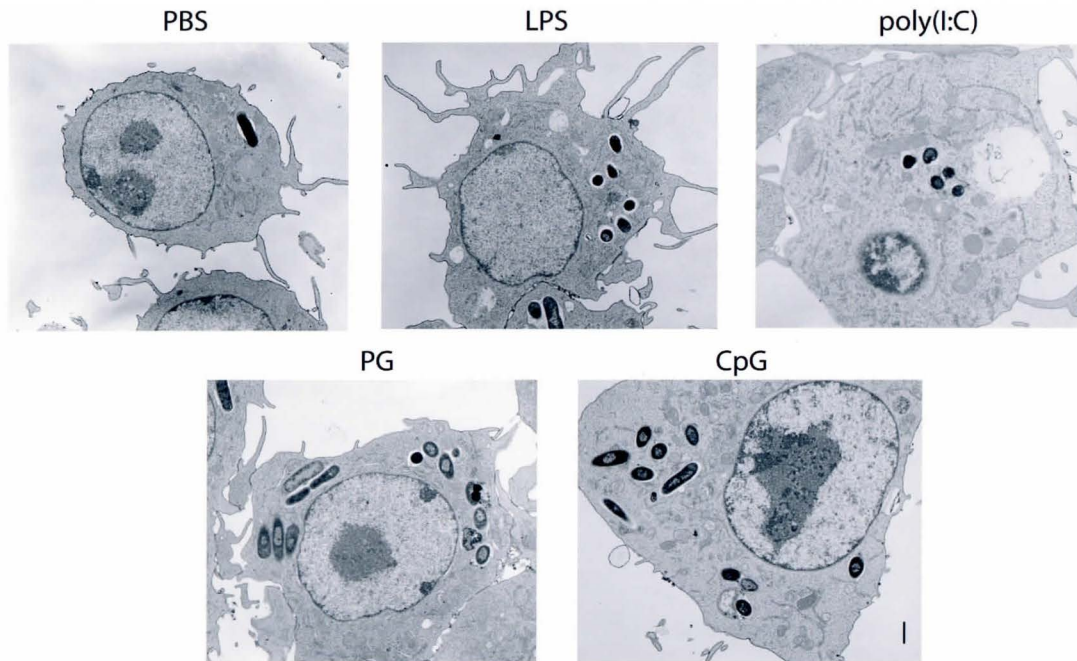
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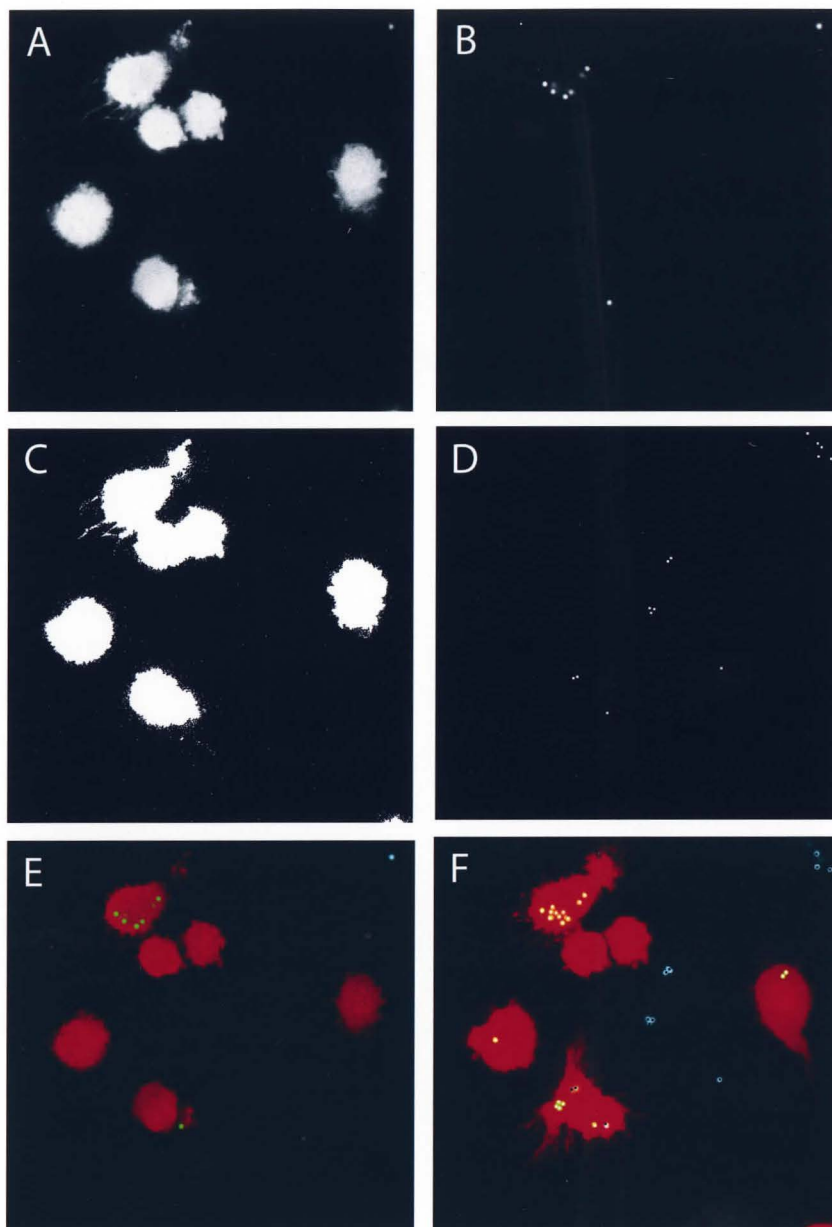
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**6. APPENDIX**

**Appendix 1: Transmission electron micrographs of TLR-agonist treated macrophages 2 hours following infection with *Salmonella*.** RAW 264.7 cells were treated with TLR-agonists approximately 20 hours before infection with *Salmonella*. Cells were fixed with 2% glutaraldehyde 2 hours post-infection and prepared for viewing under the transmission electron microscope at 5000x magnification. Bars represent 1  $\mu\text{m}$ .



**Appendix 2: Sample images from ImageJ software program.** Raw images from the original z-stacks using the red channel (A) and green channel (B). (C) Program identifies maximum intensities for the red channel and fills in gaps to create a mask to represent the cytoplasm. (D) Program identifies maximum intensities for the green channel and marks location of beads. (E) Combining the results from C and D, the program labels the beads that colocalize within the mask as internal, and those that do not as external. (F) Compressed z-stack where internal beads are marked with a white dot and external beads with a black dot.

**Appendix 3: Macro for phagocytosis of fluorescent beads assay**

Created by: Dr. Tony Collins, McMaster Biophotonics Facility Manager  
For use in ImageJ software

```
originalTitle=getTitle();
origID=getImageID();

//setBatchMode(true);
steps = nSlices/2;
width = getWidth();
height = getHeight();

newImage("red", "8-bit", width, height, steps);
redID = getImageID();

for (i=1; i<=(steps); i++)
    {selectImage(origID);
      setSlice((i*2)-1);
      run("Copy");
      selectImage(redID);
      setSlice(i);
      run("Paste");
    }

newImage("green", "8-bit", width, height, steps);
greenID= getImageID();

for (i=1; i<=(steps); i++)
    {selectImage(origID);
      setSlice((i*2));
      run("Copy");
      selectImage(greenID);
      setSlice(i);
      run("Paste");
    }
//setBatchMode(false);

//get unique image ID #s
//selectImage(nImages-1);
//redID=getImageID();
//selectImage(nImages);
//greenID=getImageID();
```



```
//subtract green from red to correct bleedthru
imageCalculator("Subtract stack",redID,greenID);
redProcessID=getImageID();
rename("red proc");
run("Duplicate...", "title=red-copy duplicate");
steps=nSlices;

//make mask cells using SNARF channel then fill in bead holes
setThreshold(15, 255);
//run("Otsu Thresholding 8Bit");
run("Convert to Mask", " black");

run("Fill Holes", "stack");
maskID=getImageID();
rename("mask");

//subtract cell outlines from green bead image to get 'outside beads'
imageCalculator("Subtract create stack", greenID,maskID);
outsideID=getImageID();
rename("outside");

//make a projection of the 'outside bead' stack
run("Z Project...", "start=1 stop="+steps+" projection=[Max Intensity]");
maxProjID=getImageID();
rename("max");

//subtract outside bead image from green beads stack = all beads
imageCalculator("Subtract create stack", greenID,maxProjID);
insideID=getImageID();
rename("inside");

//analyse
//count all beads

selectImage(greenID);
run("Z Project...", "start=1 stop="+steps+" projection=[Max Intensity]");
greenMax = getImageID();
rename("greenMax");

setThreshold(60, 255);
//run("Otsu Thresholding 8Bit");
run("Find Maxima...", "noise=10 output=Count exclude above");
run("Find Maxima...", "noise=10 output=[Single Points] exclude above");
run("Dilate");
```

```
allPointsID=getImageID();

selectImage(greenMax);
close();

selectImage(outsideID);
run("Z Project...", "start=1 stop="+steps+" projection=[Max Intensity]");
outsideMax = getImageID();
rename("outsideMax");
setThreshold(60, 255);
//run("OtsuThresholding 8Bit");

run("Find Maxima...", "noise=10 output=Count exclude above");
run("Find Maxima...", "noise=10 output=[Single Points] exclude above");
outsidePointsID=getImageID();
run("Dilate");

selectImage(outsideMax);
close();

//merge images for colour overlay
selectImage(redProcessID);
redTitle=getTitle();
selectImage(greenID);
greenTitle=getTitle();
selectImage(outsideID);
blueTitle=getTitle();
run("RGB Merge...", "red=["+redTitle+"] green=["+greenTitle+"]
blue=["+blueTitle+"]");
rename("RGB - "+ originalTitle);
run("Z Project...", "start=1 stop="+steps+" projection=[Max Intensity]");
rgbID=getImageID();

imageCalculator("Add stack", rgbID,allPointsID);
imageCalculator("Subtract stack", rgbID,outsidePointsID);

rename("RGB proj - "+ originalTitle);

//tidy up
selectImage(maskID);
close();
selectImage(maxProjID);
close();
selectImage(insideID);
```

```
close();  
selectImage(outsidePointsID);  
close();  
selectImage(allPointsID);  
close();
```