Salmonella Typhimurium promotes cholesterol accumulation and bacterial survival in host macrophages through the cholesterol acyltransferase activity of the SPI-2 effector protein SseJ

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Abstract

Upon infection of host cells, *Salmonella enterica* serovar Typhimurium resides in a modified endosomal compartment referred to as the *Salmonella*-containing vacuole (SCV). Following invasion of host cells, *Salmonella* uses multiple effector proteins translocated through two type III secretion systems (T3SS-1 and T3SS-2) to alter the host environment and manipulate intracellular signaling. While many host proteins targeted by effector proteins have been characterized, the role of host lipids in *Salmonella* pathogenesis remains poorly understood. Previous studies have shown that *S.* Typhimurium infection in macrophages leads to accumulation of intracellular cholesterol, much of which concentrates in and around SCVs; however, the underlying mechanisms for cholesterol accumulation and the role of cholesterol in *S.* Typhimurium virulence remains unknown. Here, we show that *S.* Typhimurium utilizes the T3SS-2 effector SseJ to downregulate expression of the host cholesterol transporter ABCA1 and increase cellular free cholesterol during infection of macrophages. We identify a novel role for SseJ in the activation of the host kinases FAK and Akt and the enrichment of host intracellular cholesterol concentrations through the suppression of *Abca1*. Pharmacological induction of *Abca1* prevents cholesterol accumulation and attenuates *S.* Typhimurium survival, while the depletion of *Abca1* restores *Salmonella* survival in FAK-deficient macrophages, suggesting that the inhibition of cholesterol efflux and increased intracellular cholesterol plays an important role in *Salmonella* pathogenesis. Collectively, these findings highlight the importance of manipulating host lipid homeostasis and suggests a critical role for cholesterol in promoting *Salmonella* survival in macrophages.

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List of Abbreviations

ABCA1	ATP-binding cassette subfamily A member 1
ABCG1	ATP-binding cassette subfamily G member 1
ACAT	Acyl-CoA:cholesterol acyltransferase
Akt	Protein kinase B (also referred to as PKB)
ApoA-1	Apolipoprotein A-1
ApoB-100	Apolipoprotein B-100
ANOVA	Analysis of variance
APOE1	Apolipoprotein E-1 (Gene)
ATP	Adenosine triphosphate
BMDM	Bone marrow derived macrophage
CE	Cholesteryl esters
CFU	Colony-forming unit
CHOP	C/EBP homologous protein
Co-IP	Co-immunoprecipitation
DMEM	Dulbecco's modified Eagle medium
EE	Early endosome
EEA1	Early endosome antigen 1
ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FBS	Fetal bovine serum

FERM	4.1 protein, ezrin, radixin, moesin
FOXO3	Forkhead box O3
GAP	GTPase activating protein
GCAT	Glycerophospholipid:cholesterol acyltransferase
GDI	glycosylphosphatidylinositol
GDP	Guanosine diphosphate
GDSL	Family of lipolytic enzymes with a signature GDSL motif
GEF	Guanosine nucleotide exchange factors
GFP	Green fluorescent protein
GRAMD1	GRAM domain-containing protein 1A
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate hydrolase
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
HMGCR	HMG-CoA reductase
HMGCS1	HMG-CoA synthase
iBMDM	immortalized bone marrow-derived macrophage
IL	Interleukin
INSIG1	Insulin induced gene 1
INSIG2	Insulin induced gene 2
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase

LAMP1	Lysosomal-associated membrane protein 1
LB	Luria-Bertani
LCAT	Lecithin:cholesterol acyltransferase
LC3	Microtubule-associated protein 1A/1B-light chain 3
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MCS	Membrane contact site
MEF	Murine embryonic fibroblast
MFI	Mean fluorescence intensity
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MyD88	Myeloid differentiation primary response factor 88
M6PR	Mannose 6-phosphate receptor (also referred to as MPR)
NAPs	Nucleoid-associated proteins
NF-κB	Nuclear factor-kappa B
NOD1	Nucleotide binding oligomerization domain containing 1

NPC1	Niemann-Pick type C protein 1
NPC2	Niemann-Pick type C protein 2
NTS	Non-typhoidal *Salmonella*
nSREBP	N-terminal fragment of SREBP
ORP	OSBP-related protein
ORP1L	OSBP-related protein 1, variant L
ORP5	OSBP-related protein 5
ORP6	OSBP-related protein 6
OSBP1	Oxysterol binding protein 1
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PDK	3-phosphoinositide-dependent protein kinase 1
PFA	Paraformaldehyde
PIP_3	Phosphatidylinositol (3,4,5) trisphosphate
PI3K	Phosphoinositide 3-kinase
PI(3)P	Phosphatidylinositol 3-phosphate
PI(4)P	Phosphatidylinositol 4-phosphate
PI5K	Phosphoinositide 4 phosphate 5-kinase
$PI(3,4)P_2$	Phosphatidylinositol (3,4) bisphosphate
$PI(4,5)P_2$	Phosphatidylinositol (4,5) bisphosphate
PKC	Protein kinase C
PM	Plasma membrane
PRR	Pattern recognition receptor

qRT-PCR	Quantitative real-time polymerase chain reaction
RCT	Reverse cholesterol transport
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
SCAP	SREBP cleavage-activating protein
SCV	*Salmonella*-containing vacuole
SEM	Standard error of the mean
SIF	*Salmonella*-induced filament
siRNA	Small interfering RNA
SKIP	SifA and kinesin-interacting protein
SNARE	SNAP receptor
SPI-1	*Salmonella* pathogenicity island-1
SPI-2	*Salmonella* pathogenicity island-2
SR-B1	Scavenger receptor B1
SREBP	Sterol regulatory element-binding protein
SREBP2	Sterol regulatory element-binding protein 2
STAT3	Signal transducer and activator of transcription 3
S1P	Site 1 protease
S2P	Site 2 protease
TAL1	T-cell acute lymphocytic leukemia protein 1
TGN	Trans-Golgi network

TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TSC-1	Tuberous sclerosis 1 protein
TSC-2	Tuberous sclerosis 2 protein
T1SS	Type one-secretion system
T3SS-1	Type three-secretion system (encoded by *Salmonella* SPI-1)
T3SS-2	Type three-secretion system (encoded by *Salmonella* SPI-2)
T6SS	Type six-secretion system
V-ATPase	Vacuolar adenosine triphosphate hydrolases
VAP-A	Vesicle-Associated Membrane Protein-Associated Protein A
VAP-B	Vesicle-Associated Membrane Protein-Associated Protein B
WT	Wild type
25HC	25-hydroxycholesterol

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Chapter 1:

Introduction

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Salmonella enterica: A Global Threat to Public Health

Salmonellae are a genus of Gram-negative facultative anaerobic bacteria belonging to the family Enterobacteriaceae. The bacteria were first isolated in 1880 by Karl Eberth as the causative agent of typhoid fever, although the organism remained unofficially named until 1900 [1]. In 1885, Theobald Smith identified *Salmonella enterica* as a secondary bacterial invader in swine cholera and the genus was later named after Daniel E. Salmon, Smith's chief at the Bureau of Animal Industry in the Department of Agriculture who claimed the discovery [2]. Following the advent of genome sequencing, differences in the 16S RNA sequences have divided the genus into two species, *Salmonella bongori* and *Salmonella enterica*. *Salmonella enterica* has further been divided into six subspecies and over 2,600 serovars, including Typhi and Typhimurium. While Salmonellae infect a broad range of animal hosts, the host specificity and the severity of disease caused by infection varies greatly among serovars [3]. The typhoidal serovars (specifically *S. enterica* serovar Typhi) are human-adapted *Salmonella* which cause typhoid fever - a severe systemic disease characterized by fever, abdominal rash, lethargy, and diarrhea. Dissemination of *S.* Typhi through the bloodstream to secondary sites of infection may lead to life-threatening complications including splenomegaly, hepatomegaly, and intestinal hemorrhaging. Non-typhoidal *Salmonella* (NTS) serovars, including *S. enterica* ser. Typhimurium, are zoonotic bacteria predominantly found in animal reservoirs, but nearly all are pathogenic and can infect human cells. NTS are the etiological agents of salmonellosis, leading to acute gastroenteritis of ranging severity accompanied by diarrhea, vomiting, and/or cramping. As with *S.* Typhi, severe NTS infections can infect resident and circulating macrophages

and disseminate to distal sites of infection, including the liver and spleen [4]. However, moderate salmonellosis is typically self-limiting and cleared within three to seven days in immunocompetent individuals.

While about 90% of genes identified in *S*. Typhi and *S*. Typhimurium are identical, the remaining differences may contribute towards variations in host specificity and pathology [5; 6]. One of the major virulence factors differentiating *S*. Typhi from other *S. enterica* serovars is the presence of a bacterial capsule composed of the polysaccharide Vi antigen. The Vi antigen is thought to prevent Toll-like Receptor 4 (TLR4)-mediated detection of bacterial lipopolysaccharide (LPS), reducing the inflammatory response and facilitating the systemic dissemination of *S*. Typhi out of the intestines [7]. Conversely, *S*. Typhimurium expresses ~200 genes which are inactive pseudogenes in *S*. Typhi, as well as five unique fimbrial sequences. The presence of these genes may account for the expanded host range of NTS serovars and the enhanced affinity for the intestinal epithelium [8; 9]. Furthermore, multiple epidemic strains of *S*. Typhimurium harbor a prophage encoding a virulence factor which acts as a guanine exchange factor (GEF) for Cdc42 and Rac1, inducing actin cytoskeleton rearrangements that enhance invasion of epithelial cells [10; 11]. These examples reveal how both typhoidal and non-typhoidal Salmonellae have acquired a vast array of virulence factors which may account for the diverse clinical presentations between typhoid fever and salmonellosis.

Although the incidence and mortality associated with typhoidal and NTS infections is relatively low in the United States, both pose a significant risk to public health in the endemic regions of South Asia and Sub-Saharan Africa. Two vaccines against *Salmonella* Typhi have been developed: a live attenuated oral vaccine and an injected vaccine against

the Vi polysaccharide in the bacterial capsule [12]. While these vaccines help curtail the prevalence of typhoid fever in developed regions, the lack of availability in endemic regions is reflected by the global incidence of disease. In 2015, the CDC reported only 309 cases of typhoid fever occurred in the United States, however, most cases were associated with travel to endemic regions and no deaths occurred [13]. In contrast, typhoid fever caused nearly 11 million cases and 116,000 deaths globally in 2017, with peak incidence and mortality occurring in children under 5 years old [14. Without antibiotic intervention, individuals can display intermittent symptoms for weeks or months after infection. However, an estimated 2-5% of infected individuals fail to clear the infection and become chronic carriers of the disease, with the majority of chronic infections being asymptomatic [15]. As such, management of typhoid fever will require a concerted effort of antibacterial use, vaccine employment, and sanitation strategies worldwide.

As with Typhoidal serovars, non-typhoidal *Salmonella* also represents a massive burden on global public health. Diarrheal diseases are a leading cause of global morbidity, responsible for almost 4.5 billion cases and 1.6 million deaths in 2016 alone. Of those, NTS infections were estimated to cause 200 million episodes and 85,000 deaths, making it the fourth-most common cause of disease and the fifth-leading cause of death from diarrheal diseases [16]. In contrast to *S.* Typhi, NTS serovars colonize a broad range of hosts and are typically acquired from environmental sources, such as contaminated food and water. As such, salmonellosis presents a more universal challenge to public health and infrastructure. The importance of food, water, and healthcare infrastructure is underscored by the majority of morbidity localized to underdeveloped regions lacking access to proper healthcare and clean water [16]. Most NTS infections result in self-limiting gastroenteritis which resolves

within 7 days of exposure. Although NTS infects individuals of all ages, severe complications are more prevalent in children and adults over 70. As such, the dehydration and chronic malnutrition due to NTS infections result in a disproportionate morbidity in children under 5 years old, with the death rate of children increasing five-fold over the mean [16]. Compounding the global public health threat imposed by NTS infections is the widespread emergence of multiple antibiotic resistance strains. From 2004-2012, over 3000 clinical isolates of *S.* Typhimurium were reported in the United States, with 29% of those demonstrating resistance to three common antibiotic strategies [17]. Due to the growing threat of antibiotic resistance, antibiotic therapy is discouraged for all but the most severe cases and the primary treatment for NTS infections instead recommends oral hydration to replace lost fluids and nutrients. Global initiatives aiming to reduce the prevalence of NTS and all diarrheal diseases are already underway, promoting improved sanitation, food safety practices, and access to proper healthcare and treatments [18; 19]. However, gaining a better understanding of the molecular mechanisms behind NTS disease is required to overcome the rising antibiotic resistance and develop new therapies to combat severe *S.* Typhimurium infections.

Invasion of host cells by *S.* Typhimurium

Like other NTS serovars, *Salmonella* Typhimurium colonizes the intestines of animals and is transmitted through the fecal-oral route of infection. Following ingestion of contaminated food or water, *Salmonella* utilizes an acid tolerance response to combat the low pH of the stomach and pass into the intestinal tract [20]. Upon reaching the ileum of the small intestines, *S.* Typhimurium invades intestinal epithelial cells through three major routes of infection: translocation through the microfold cells (M cells) of the Peyer's

Patches, dendritic cell sampling of the intestinal lumen, and direct penetration of intestinal epithelial cells.

Due to the ease of infecting cultured monolayers *in vitro,* the invasion of epithelial cells by non-typhoidal *Salmonella* has been extensively studied over the past few decades [**Figure 1-1**]. Studies have found that the cell surface cellulose, fimbriae, and flagella of *Salmonella* are important for the attachment to both animal and plant cells [21; 22; 23]. While numerous bacterial factors responsible for attachment have been identified, the host cell receptor(s) involved in *S.* Typhimurium attachment remains unknown. Following attachment, *S.* Typhimurium induces extensive rearrangements of target host cell plasma membrane to generate macropinosomes and promote the uptake of attached bacteria into non-phagocytic epithelial cells [24; 25]. To this end, multiple virulence factors encoded within *Salmonella* pathogenicity island-1 (SPI-1) induce the dramatic actin cytoskeleton rearrangements and membrane ruffling events characteristic of *Salmonella* invasion (discussed below). *In vivo*, *Salmonella* invasion of murine epithelial cells may also occur without extensive actin rearrangements in a process termed 'discreet-invasion'. Although not as dramatic as the hallmark membrane ruffling seen during epithelial cell infection in vitro, discreet-invasion induces mild elongation of the microvilli to internalize attached bacteria near cell-cell junctions in a SPI-1-dependent manner [26]. Following invasion of polarized epithelial monolayers, *Salmonella* is largely retained within the cells. However, a small portion of these bacteria transcytose through the host cells and gain access to the basolateral space, where they can encounter the underlying resident immune cells [27]. Furthermore, the attachment of *S.* Typhimurium to the apical membranes of cultured human intestinal epithelial cells induces the recruitment of neutrophils to sites of infection,

indicating that *Salmonella* induces a robust, localized inflammatory response at initial sites of invasion [28]. These findings suggest that *Salmonella* is highly adapted to infect intestinal epithelial cells and resist the innate immune response initiated upon infection. This inflammatory microenvironment may allow for pathogenic *Salmonella* to outcompete the non-pathogenic gut microbiota and disseminate into host macrophages.

Although the widespread use of *in vitro* cell culture has resulted in a detailed understanding of epithelial cell invasion, several lines of evidence suggest that the translocation of *Salmonella* through M cells may constitute a primary method of invasion during *in vivo* mouse infections. M cells are specialized antigen-sampling intestinal epithelial cells which reside in the Peyer's patches of the intestinal epithelium and transport antigens to the underlying lymphoid follicles to initiate immune responses. Histological surveys have suggested that *S.* Typhi causes ulceration of the Peyer's Patches, leading to intestinal perforation and hemorrhaging [29]. Similarly, invasive *S.* Typhimurium selectively infects the M cells in both murine and calf ligated ileal loops, leading to the destruction of the M cells and invasion of the underlying lymphoid follicle [30]. While the role of M cells in *S.* Typhimurium infection of human tissues is poorly understood, infection of a simplified *in vitro* human M cell model generated by co-culture of human colonic Caco cells with Raji B lymphocytes has suggested that *S.* Typhimurium can breach the intestinal epithelium by translocating through human M cells [31].

Multiple physiological characteristics of M cells may explain the selective targeting of these cells for invasion by *Salmonella*. The surface of M cells contains less mucous and IgA compared to neighboring cells, reducing the physical barrier against infection. Furthermore, M cells generate shorter, irregular microvilli, contrasting the long, uniform

microvilli of normal intestinal epithelial cells [32]. The exposed, flexible apical membrane of M cells may provide a more hospitable surface necessary for the dramatic membrane and actin cytoskeleton rearrangements induced by *Salmonella* invasion. As opposed to the regularly-shaped neighboring columnar epithelial cells, the basolateral surface of M cells is warped into an intraepithelial pocket which allows for the intimate association with lymphoid and macrophage cells, facilitating efficient presentation of luminal antigens to underlying immune cells [33; 34]. Along with this unique morphology, M cells have a high endosomal trafficking potential due to a dense network of actin filaments, tubules, vesicles, and multivesicular bodies (MVBs) [32; 35]. This close association between M cells and immune cells may benefit both the host and pathogen. Antigen presentation plays an important role in the induction of innate and adaptive immune functions. By having this close proximity between M cells and antigen processing cells, the host is able to initiate a rapid immune response to contain the source of infection. However, invasive *Salmonella* exploits the induced proinflammatory state to survive and replicate within host macrophages. Therefore, *Salmonella* may target M cells in order to penetrate the intestinal epithelial layer and subsequently infect the resident phagocytic cells for further dissemination.

Regardless of whether *Salmonella* directly penetrates the intestinal epithelial cells or invades through M cells, both result in the exposure of pathogenic *Salmonella* to the underlying immune cells. Following colonization and transcytosis through the intestinal epithelial layer, *Salmonella* encounters resident and circulating macrophages in the lamina propria. These macrophages phagocytose *S.* Typhimurium, and in cases of acute salmonellosis, contain the infection. As professional phagocytes, macrophages utilize a

number of mechanisms to kill pathogens, including reactive oxygen species (ROS), lysosomal degradation, production of antimicrobial peptides, and the autophagic killing of pathogens [36]. These innate immune functions are upregulated when pattern recognition receptors (PRRs) in and on macrophages recognize conserved structural components, or pathogen-associated molecular patterns (PAMPs), commonly found on bacteria or other pathogens. This localized immune response allows for the rapid containment and clearance of potential pathogens from the body. Along with the passive initiation of inflammation through PAMPs such as LPS, *Salmonella* actively induces inflammatory cell death in macrophages in a SPI-1-dependent manner [37; 38]. This programmed cell death upon *S.* Typhimurium invasion may lead to the localized inflammation and tissue damage seen during acute salmonellosis and the recruitment of additional immune cells to contain the infection within the lamina propria. By eliciting a robust, localized inflammatory response, the immune cells of the lamina propria prevent the spread of bacteria and contribute to the gastroenteritis commonly associated with *S.* Typhimurium infection.

However, as a facultative intracellular pathogen, *Salmonella* employs numerous strategies to adapt to and/or avoid macrophage killing mechanisms, such as encoding superoxide dismutase to avoid host ROS, resisting damage from host antimicrobial peptides, and manipulating host cell signaling to avoid autophagic clearance from macrophages [39; 40; 41]. Remarkably, *S.* Typhimurium utilizes colonization-induced inflammation to outcompete the normal microbiota and enhance the secondary invasion of macrophages [42]. Similar to *S.* Typhi, highly virulent *S.* Typhimurium infections are capable of overcoming the innate immune response and survival within macrophages, leading to the dissemination to distal sites of infection. Furthermore, chronic infection by

S. Typhimurium has been attributed to the persistence of bacteria within macrophages of the lymph nodes [43]. The induction of and resistance to inflammation confers invasive *Salmonella* a competitive advantage over commensal microbiota in the intestines. By causing the inflammation-mediated dysbiosis of the commensal microbiota, *Salmonella* eliminates competition for nutrients and the colonization of intestinal epithelial cells and promotes the recruitment of additional immune cells for further infection, dissemination, and possibly even chronic persistence.

The Intravacuolar Lifestyle of *Salmonella*

Following internalization by host cells, *Salmonella* remains within the vacuolar compartment named the *Salmonella*-containing vacuole (SCV). Within the SCV, *Salmonella* translocates multiple effector proteins into the host cell cytoplasm to manipulate various host processes, including cell signaling and lipid homeostasis (see **Chapter 2**). In addition to promoting virulence, these translocated bacterial proteins facilitate the continual remodeling of the SCV membrane, converting it to a modified late endosome-like compartment where the bacteria typically remain throughout the course of infection [**Figure 1-l**].

Rab-family proteins are critical in the maturation and function of both host endosomes and *Salmonella* SCVs [44]. Rab proteins are a group of over 70 small GTPases which serve as key mediators of endosomal trafficking and fusion [45]. During the initial stages of infection, *Salmonella* actively recruits early endosome markers EEA1, Rab4, and Rab5 to the SCV [46; 47]. Notably, many of these endosomal markers are consistent between SCVs formed in epithelial cells or macrophages [46], suggesting that SCV development is fairly similar during infection of different cell types. In addition to

conventional markers of endosomal development, sorting-nexin 1 (SNX1) is also recruited to the SCV to prevent the delivery of lysosomal hydrolases to the SCV. TGN-associated cargo, including lysosomal degradative enzymes, is removed from the SCV at sites of SNX1-induced tubular membrane extensions [48]. Additionally, *S.* Typhimurium inhibits Rab14-dependent fusion with lysosomes [49], further protecting the SCV against harmful lysosomal enzymes. Rab5 is an important regulator of endosomal trafficking and the maturation of early endosomes into late endosomes and plays a crucial role in the maturation of SCVs. During the maturation of the early SCV, Rab5 recruits the late endosome marker Rab7, which is necessary for the subsequent acquisition of LAMP1 [50; 51]. During the early stages of infection, Rab7 recruits host RILP and the microtubule dynein motor complex to the SCV, promoting localization of the SCV towards the periphery of host cells [52]. Furthermore, the host Vps34 PI3-kinase (PI3K) is recruited to the SCV in a Rab5-dependent manner where it generates PI(3)P on the SCV membrane [47]. While the SCV contains several late endosome markers and lysosomal glycoproteins, other late endosomal proteins and lysosomal enzymes are notably absent from the SCV [46; 53; 54]. Through these mechanisms, the SCV remains intimately associated with the host endocytic network, allowing *Salmonella* to reside and replicate within a protected intracellular niche while avoiding lysosomal fusion.

Acquisition of host endosomal trafficking proteins involves the extensive interaction between SCVs and the host endocytic compartments. Endosomal markers are gradually acquired by the SCV, suggesting that endocytic proteins are delivered through reoccurring contacts between the SCV and host endosome instead of a single fusion event [55]. Immediately following internalization, the nascent SCV actively promotes fusion

with host early endosomes in a Rab5-dependent manner [56]. Within one hour of infection, the SCV begins to fuse with host late endosomes, acquiring late endosomal markers LAMP1, Rab7, and Rab9, while selectively avoiding fusion with or depleting the SCV membrane of other endosomal markers and lysosomal proteins such as mannose 6-phosphate receptor (M6PR) and Rab14 [46; 57; 58]. These SCV-endosome fusion events may result in the sequential acquisition of host endosomal proteins to enhance the maturation and positioning of SCVs within the endocytic network. During the later stages of infection, a population of mature SCVs form elongated tubular structures called *Salmonella*-induced filaments (SIFs) in order to acquire nutrients and membrane proteins while diluting out harmful lysosomal enzymes [59; 60; 61]. SIF-forming *Salmonella* are more metabolically active than those lacking SIFs [60], demonstrating the importance of SCV-endosome contact in nutrient and membrane acquisition. Together, the presence of SIFs and the selective acquisition or avoidance of endosomal transport and regulatory proteins demonstrate the importance of SCV-endosome interactions in the biogenesis and maturation of SCVs.

The original model of SCV maturation suggested that SCVs inhibit fusion with host lysosomes [54; 57], however, more recent work has contradicted this model by demonstrating SCVs follow canonical endosomal trafficking pathways and fuse with host lysosomes. Live cell imaging has revealed that SCVs directly acquire luminal cargo of lysosomes as soon as 1 hour post-infection and persist throughout the course of infection in both epithelial cells and primary macrophages [62; 63]. In uninfected cells, Rab7 controls the fusion of late endosomes and lysosomes [64]. Rab7 also resides on early SCVs, where it promotes the perinuclear localization of SCVs towards the lysosome-rich

microtubule organizing center before mature SCVs actively uncouple Rab7 from microtubule motors during the later stages of infection [52]. Therefore, the bacterial manipulation of Rab7 may be important for regulating SCV contacts with lysosomes, allowing for selective lysosomal contact to occur while preventing long-term association. As SCV lysosomes interactions require Rab7 and SCV acidification [62], the maturation of the SCV parallels that of host endosomes, where endosomal proteins are transiently acquired and lost in a sequential manner as nascent endosomes mature and sort through the endocytic network.

The establishment and maintenance of the SCV is highly important to *Salmonella* virulence following infection. Although the majority of *Salmonella* remains within the SCV throughout the course of infection, a fraction of bacteria escapes the SCV and access the host cytosol. Importantly, the cytosolic escape of *Salmonella* leads to dramatically different outcomes dependent on the host cell type. While *S.* Typhimurium replication typically occurs between 6-12 hours post-infection, the cytosolic escape of *Salmonella* results in rapid bacterial proliferation prior to the onset of intravacuolar replication [65; 66]. However, vacuolar escape of *S.* Typhimurium is not limited to mutant bacteria, as a portion of wild-type *Salmonella* also escape the SCV and undergo early-onset hyper-replication within HeLa cells [67]. Escape from the SCV in epithelial cells dramatically shifts the transcriptomic profile of *S.* Typhimurium, upregulating invasion and motility genes and undergoing replication independent of the conventional regulatory genes [67; 68]. To date, no studies have demonstrated sustainable hyper-replication of *Salmonella* within macrophages. Although a small population of *S.* Typhimurium continues to escape the SCV in macrophages, cytosolic bacteria fail to replicate and the majority of wild-type

Salmonella remain within the SCV [69; 70]. Furthermore, cytosolic *S.* Typhimurium induces macrophage pyroptosis through the detection of bacteria by pattern recognition receptors inside macrophages [71]. Therefore, vacuolar escape and cytosolic hyper-replication may play an important role in *Salmonella* virulence and dissemination in host epithelial cells. Conversely, macrophages readily detect cytosolic *S.* Typhimurium, restricting bacterial growth and dissemination through the induction of inflammatory cell death. Thus, establishing and maintaining an intact SCV plays an important role in *S.* Typhimurium survival and dissemination within infected hosts. While the role of host proteins in SCV integrity have been briefly discussed above, future studies will reveal the contributions of host cholesterol and other lipids to SCV stability.

Function and Regulation of *Salmonella* Pathogenicity Islands

Salmonella encodes numerous virulence factors encoded within gene clusters named *Salmonella* pathogenicity islands (SPIs). These virulence genes are critical for promoting *Salmonella* invasion and the manipulation of host cell signaling and cholesterol homeostasis. Whereas 21 SPIs have been identified throughout the *Salmonella* genus, *S.* Typhimurium contains 14, only one of which is specific to the serovar [8]. Proteins encoded by SPIs serve diverse functions towards *Salmonella* virulence. *S.* Typhimurium SPIs encode at least four secretion systems: two type three secretion systems (T3SS), one T1SS, and one T6SS [72; 73; 74], while additional secretion systems have been identified in other *S. enterica* serovars. Each of these secretion systems translocate a specific set of bacterial effector proteins into target host cells to promote host cell invasion and survival, and the manipulation of a diverse array of host cell signaling pathways. Additional virulence factors encoded within SPIs regulate the expression of these secretion systems

and exert temporal control over the translocation of effectors. Along with these regulatory virulence factors, SPIs encode numerous other proteins that elicit *Salmonella* virulence by acting on or within the bacterium itself. Although all SPIs play an important role in imparting host specificity, invasion, and *Salmonella* virulence, SPI-1, SPI-2, and their associated effectors have been the most extensively characterized and will be further discussed here.

SPI-1

The SPI-1 pathogenicity island plays a crucial role in *S.* Typhimurium entry into non-phagocytic epithelial cells. This 40 kb genetic cluster contains 39 genes encoding the structural and regulatory components of a T3SS and the 13 effector proteins translocated through the SPI-1 T3SS (T3SS-1; **Table 1-1**). Type three secretion systems act as molecular syringes which inject bacterial effector proteins into the host cell cytosol. Generally speaking, the T3SS is comprised of several substructures: a sorting platform and export apparatus on the inner membrane of the bacteria, an inner rod which spans the inner and outer membranes of Gram-negative bacteria, a needle complex which extends into the extracellular space, and a translocon complex at the tip of the needle which forms a pore in the host plasma membrane to facilitate effector protein translocation [75]. Although T3SSs are commonly found in pathogenic Gram-negative bacteria, the structural, regulatory, and effector proteins vary to facilitate the specific needs of the bacteria.

The sorting complex of the *S.* Typhimurium T3SS-1 assembles on the inner bacterial membrane and is composed of five SPI-1 proteins, SpaO, OrgA, OrgB, InvI, and InvC [76; 77; 78]. These proteins serve to control the delivery of structural and effector proteins through the needle complex to form and translocate through the mature T3SS. In

order to control the translocation of effector proteins, the SicA and InvE chaperone proteins recruit and load effector proteins onto the T3SS-1 sorting complex [77]. The inner ring proteins PrgH and PrgK and the outer ring protein InvG span the inner and outer bacterial membranes, respectively [79]. Importantly, deletion of either OrgA or InvG results in the inability for T3SS-1 to translocate the external components of the needle apparatus or any of the effectors. As such, Δ*orgA* and Δ*invG* strains serve as a functional ΔT3SS1 mutant and provide an established model of infection for macrophages to avoid potential T3SS-1-induced cytotoxicity. Together, these proteins form the export apparatus of the *S.* Typhimurium T3SS-1 [**Figure 1-2**], regulating the secretion of bacterial effector proteins into the host cell cytosol.

Following the formation of the export apparatus on the bacterial membranes, several additional proteins begin assembling the needle complex of the *S.* Typhimurium T3SS-1. The inner rod protein PrgJ forms a cylindrical structure between the inner and outer bacterial membranes, where it connects to the main needle protein, PrgI, at the outer ring of the export apparatus [78; 80]. InvJ regulates needle length, preventing the continued elongation of the needle [80]. SipD interacts with PrgI at the tip of the T3SS-1 needle, where it provides a base for the assembly of the SipB/SipC pore-forming translocon [81]. SipB and SipC have dual roles in *Salmonella* virulence, facilitating intimate contact with the host cell membrane and generating a pore through which soluble effector proteins are translocated into the host cell. SipB binds cholesterol on the host cell plasma membrane, promoting the intimate attachment of *S.* Typhimurium to the host [82]. As a result, depletion of cholesterol from the host cell membrane reduces *Salmonella* attachment and invasion. The SipB/SipC complex is necessary for the insertion of both proteins into the

host cell membrane, where they form approximately 2nm-wide pores [83]. Together, the concerted actions of structural, sorting, and chaperone proteins facilitate the direct secretion of *S*. Typhimurium effector proteins through the T3SS-1 into the host cytosol. Although many of the SPI-1 genes are conserved across multiple *Salmonella* serovars, evolutionary differences in expression and the translocation machinery of the T3SS-1 may account for host range specificity [84; 85].

The expression of SPI-1 genes and *S.* Typhimurium invasion is regulated by a complex network of environmental and genetic factors, centering around the master transcriptional regulator, HilA. Upon passing through the stomach, the environmental conditions of the intestines (anaerobic, high osmolarity, slightly above neutral pH) induce the expression of SPI-1 and prime *Salmonella* for invasion [86]. Stimulation of HilA environmental factors induces expression of the T3SS-1 components, as well as the expression of the InvF and SicA regulators. These in turn promote expression of the HilC/HilD/RtsA transcriptional regulators, which act as a feed-forward complex, driving the further activation of HilA and SPI-1 expression [87]. Most regulatory pathways controlling SPI-1 expression converge at HilD. This includes environmental sensing by the two-component regulatory systems OmpR/EnvZ and PhoP/PhoQ [87]. HilE serves as the most important negative regulator of SPI-1 expression and *Salmonella* invasion by binding to and preventing the activity of HilD [88]. Following invasion into epithelial cells, SPI-1 expression is downregulated due to the lon protease-dependent degradation of HilA, HilC, and HilD [89]. As such, the expression of the majority of SPI-1-associated genes is effectively silenced within 4 hours of infection [90]. Together, this complex network of

regulatory pathways moderates the invasiveness of *S.* Typhimurium by sensing environmental cues and regulating the expression of the T3SS-1 and effector proteins.

The 13 SPI-1 effector proteins translocated through the T3SS-1 enable the efficient penetration of host intestinal epithelial cells and begin modifying the intracellular vacuolar environment during the initial stages of infection. *Salmonella* invasion of epithelial cells induces membrane ruffling events through the rearrangement of the host actin cytoskeleton and plasma membrane. As membrane ruffling events are necessary for the efficient internalization of bacteria, many SPI-1 effectors regulate actin nucleation and membrane delivery to sites of infection. In addition to its role as a translocon protein, SipC is secreted into host cells as a soluble effector where the SipA/SipC complex cooperatively binds and nucleates F-actin polymerization independent of host cell factors [91; 92]. Further remodeling of the actin cytoskeleton occurs through SopB, SopE, and SopE2. SopE and SopE2 serve as potent GEFs for Rac1 and Cdc42 [11]. As a result, these proteins promote the extensive actin cytoskeleton rearrangements which generate the characteristic membrane ruffling event necessary for *Salmonella* internalization by an otherwise non-phagocytic host cell. Although SopB, SopE, and SopE2 are encoded outside of the SPI-1 locus, all translocate through the T3SS-1 where they activate the Rho family GTPases, Rac1 and Cdc42 [93; 94]. By promoting the GTP-binding of Rac1 and Cdc42, SopB/E/E2 induce the downstream activation of the Arp2/3 complex, resulting in the nucleation and branching of actin filaments. The functional redundancy of SopB/E/E2 reflects the importance of Rac1 and Arp2/3-mediated actin remodeling during invasion of host epithelial cells [94; 95; 96]. Furthermore, both SopB and SopE manipulate host lipid homeostasis during invasion. SopB plays a central role in the early stages of *Salmonella*

infection. As an inositol phosphate phosphatase, SopB hydrolyses multiple phosphoinositide species to generate PI(3)P on the SCV membrane and enrich PI(3,4)P_2 and PIP_3 in membrane ruffles at the initial sites of epithelial cell invasion [47]. These lipid modifications lead to the recruitment of Rab5 and SNX1, which promote SCV maturation and prevent the delivery of lysosomal enzymes, respectively [48; 50]. Similarly, SopE rapidly depletes the host inositol phosphate InsP5 and induces membrane ruffling [97]. In addition to actin dynamics, many SPI-1 effectors, including SipB and SopA, B, D, E, and E2, also regulate host inflammation [37; 98; 99; 100]. To antagonize the effects of these virulence effectors, SPI-1 encodes two negative regulators of inflammation and actin polymerization. AvrA restricts *S.* Typhimurium infection of cultured polarized epithelial cells by stabilizing the tight junctions between adjacent cells. Additionally, AvrA enhances *Salmonella* invasion and virulence *in vivo* by downregulating JNK signaling and inhibiting proinflammatory cytokine release [101; 102]. SptP is a tyrosine phosphatase responsible for reversing the actin cytoskeleton rearrangements induced during *Salmonella* invasion and promotes intracellular replication [103; 104].

While the concerted actions of the SPI-1 effectors serve to enhance *Salmonella* invasion and survival within host epithelial cells, they can cause significant cytotoxicity during infection of macrophages. In contrast to non-invasive *Salmonella*, the active invasion of macrophages under SPI-1-inducing conditions leads to a significant increase in macrophage cell death [105]. Cell death occurs early during *Salmonella* infection, where SipB and SipD activate caspase-1 and caspase-3, leading to the subsequent lysis of macrophages via pyroptosis [37; 38; 106]. Pyroptosis is a form of programmed cell death which is accompanied by the production of inflammatory cytokines. However, non-

pyroptotic cell death has also been attributed to SPI-1 expression [107], suggesting that *Salmonella*-induced macrophage cytotoxicity occurs via multiple mechanisms. Indeed, late-stage macrophage cell death also occurs dependent upon LPS and the SipB-mediated signaling through TLR4 [108]. To avoid potential cell death, *S.* Typhimurium lacking structural components of the SPI-1 T3SS, such as Δ*invG* mutant strains, are frequently used to avoid macrophage cytotoxicity. As SPI-1 is dispensable for *Salmonella* dissemination from epithelial cells and the invasion of phagocytic cells [109], Δ*invG S.* Typhimurium serves as an effective model of infection for macrophages.

SPI-2

While SPI-1 is generally responsible for invasion of epithelial cells, the SPI-2 pathogenicity island is largely responsible for facilitating the various intracellular signaling events necessary for the survival and replication of *Salmonella* within host cells, including the accumulation of cholesterol within host macrophages (see **Chapter 2**). Similar to the SPI-1 pathogenicity island, SPI-2 is a 40 kb genetic region that harbors 31 proteins encoding two operons for a second T3SS: one operon for chaperone and effector proteins, and one operon for a two-component regulatory system [110]. While the majority of the T3SS-1-associated effectors are encoded within the SPI-1 locus, most of the 30+ T3SS-2-associated effector genes are located outside of the SPI-2 locus on the bacterial chromosome or within virulence plasmids [111; **Table 1-2**]. As with SPI-1, the SPI-2 locus is largely conserved between *Salmonella* Typhi and Typhimurium. However, multiple T3SS-2 effectors encoded outside of the *S.* Typhimurium SPI-2 locus are either pseudogenes or missing altogether in *S.* Typhi, suggesting that T3SS-2 effector differences

could contribute towards the host range and pathology specificity of *Salmonella* serovars [8].

As with all type 3 secretion systems, the SPI-2 T3SS is composed of the bacterial membrane-associated export apparatus, the extracellular needle complex, and the pore-forming translocon complex. On the inner bacterial membrane, SsaK, SsaN, SsaO, and SsaQ assemble to form the sorting complex for the T3SS-2 [110; 112]. The Ssc operon encodes two putative chaperone proteins, SsaA and SsaB. Additionally, the chaperones SsaE and SsaH have been demonstrated to regulate secretion and stability of other T3SS-2 components [110; 113]. Within the inner bacterial membrane, SsaV, SsaU, and SsaL serve as "gatekeeper" proteins, controlling the translocation of T3SS-2 components in response to environmental conditions [112]. Importantly, the loss of SsaV results in the loss protein translocation through T3SS-2. As such, the deletion of SsaV fails to assemble the T3SS-2 and serves as a model for ΔSPI-2 infections. The inner ring proteins SsaD and SsaJ, and the outer ring proteins SsaC (also known as SpiC) and SpiA reside on the inner and outer bacterial membranes, where they interact with the sorting proteins to form the export apparatus of the SPI-2 T3SS. SpiA shares considerable homology to InvG in the T3SS-1 and SpiA-deficient bacteria are incapable of forming a functional T3SS-2 [114]. As such, Δ*spiA* mutants also serve as a useful model for ΔT3SS infection. The inner rod protein SsaI spans the bacterial membranes and connects to the needle protein SsaG, which extends out from the bacterial outer membrane. The molecular "ruler" SsaP regulates SsaG filament length, homologous to InvJ regulating the PrgI filament length in the T3SS-1 [112; 115]. SseB serves as the base for the translocon complex, linking the SsaG needle filament

to the SseC/SseD pore-forming translocon. Together, these proteins assemble to form the functional T3SS-2 apparatus [**Figure 1-2**].

Similar to other type 3 secretion systems, SPI-2 and the T3SS-2 are under tight regulation by a number of systems [116]. While the regulatory cascade of SPI-2 expression is only partially understood, the SPI-2-encoded regulatory proteins SsrA and SsrB play a central role in the expression of SPI-2 and its associated virulence genes. Following internalization into host cells, *Salmonella* remains within the vacuolar compartment where the SPI-1 and -2 effectors promote the maturation of the endosome into the *Salmonella*-containing vacuole (SCV). Shortly following colonization of the intestinal epithelia, SsrA and SsrB sense the acidic pH and nutrient-limited conditions within the nascent SCV, binding to the promoters of SPI-2 genes and driving their expression [117; 118; 119]. Interestingly, the SPI-1 regulatory protein HilD binds the promoter regions and drives the expression of SsrA and SsrB following the expression of SPI-1 genes [120], revealing the sequential expression of SPI-1 and -2. Further demonstrating the inverse relationship between SPI-1 and SPI-2 expression, upregulation of SPI-2 generally occurs between 1-5 hours post-invasion while SPI-1 is downregulated over the same period [121].

As mentioned above, the SPI-1 pathogenicity island is regulated in part by the two-component regulatory systems PhoP/PhoQ and OmpR/EnvZ. Similar to the involvement of HilD in both SPI-1 and SPI-2 expression, PhoP/Q and OmpR/EnvZ also regulate the expression of SPI-2 genes. During macrophage infection, PhoP binds the SsrB promoter, driving the expression of SPI-2 genes [122; 123]. Similarly, OmpR binds the promoters of SsrA and SsrB in order to upregulate SPI-2 expression [123; 124; 125]. How PhoP/PhoQ and OmpR/EnvZ differentiate SPI-1 from SPI-2 genes remains unclear. Along with two-

component regulatory systems, *Salmonella* utilizes multiple nucleoid-associated proteins (NAPs) to regulate the expression of SPI-2 genes. IHF promotes SPI-2 expression by bending and altering the accessibility of the *Salmonella* genome [116], while Fis promotes the expression of both SPI-1 and SPI-2 [126; 127]. NAPs also serve a critical role in the negative regulation of SPI-2 expression. H-NS binds and silences various SPI-2 genes, antagonizing the activity of SsrB [119]. Additionally, YdgT and its homolog Hha are both necessary for the suppression of SPI-2 [128; 129]. Together, the two-component regulatory systems and NAPs serve as part of the regulatory cascade controlling the expression of SPI-2 and associated T3SS-2 effector genes in response to various environmental stimuli. The proper regulation of these SPI-2 genes is necessary for *Salmonella* virulence, allowing for the survival and replication of *Salmonella* within host cells and the subsequent dissemination to distal sites of infection.

Following internalization into host cells, the SPI-2 T3SS translocates over 30 effector proteins across the SCV membrane into the target host cytoplasm. SPI-2 effector proteins interact with numerous host proteins and manipulate many host signaling pathways. These interactions facilitate a diverse array of bio-enzymatic functions necessary for *Salmonella* virulence, including regulating host inflammatory signaling, maintaining proper SCV positioning within the host cell, regulating SCV membrane dynamics and interactions with the endosomal network, and manipulating host lipid homeostasis [**Table 1-2**]. While multiple effectors may converge on a specific host process, each effector displays considerable specificity in their biochemical activities and target host proteins.

As discussed above, manipulating host inflammation plays a large role in the virulence of *Salmonella*. Similar to SPI-1, many T3SS-2 effector functions manipulate

innate immune responses by positively or negatively regulating host inflammatory signaling pathways during infection of host cells. GogA, GogB, GtgA, PipA, SspH1, and SpvD all inhibit inflammatory NF-κB signaling [110; 111; 130]. Additionally, the SseK1, SseK2, SseK3 are part of a large family of bacterial proteins responsible for the inhibition of TNF-α-induced NF-κB signaling [131]. Previous studies came to conflicting conclusions on the role of SseL in NF-κB signaling [132]. However, recent data suggest that SseL suppresses activation of NF-κB, enhancing *Salmonella enterica* ser. Pollorum virulence in chickens [133]. NF-κB is a critical regulator of host inflammation and innate immune responses, producing a number of pro-inflammatory cytokines and chemokines while inducing activation and differentiation of host immune cells. Therefore, the inhibition of NF-κB signaling prevents *Salmonella*-induced cell death and allows for bacterial survival within host cells. Additional anti-inflammatory signaling results from the T3SS-2 effector proteins SlrP, SpvC, SteD, and SteE [111]. Along with its role in activating anti-inflammatory STAT3 signaling, SteE further suppresses host inflammation by inducing anti-inflammatory M2 polarization of macrophages [134; 135]. While many of the T3SS-2 effectors inhibit inflammatory signaling pathways, SspH2 activates NOD1 signaling, resulting in inflammatory IL-8 production [136]. Together, these T3SS-2 effectors work in concert to regulate host cell inflammation during *Salmonella* infection.

Along with inflammatory signaling, T3SS-2 effectors regulate the position and interaction of SCVs with the host endocytic network. The only effector proteins located within the SPI-2 genetic locus, SseF and SseG, are integral membrane proteins which tether the SCV to the Golgi network during the course of infection [137; 138]. This close association with the Golgi network is necessary for *S.* Typhimurium survival [138],

underscoring the importance of SCV trafficking within host cells. As mentioned above, Rab GTPases are important regulators of SCV positioning and fusion with host endosomes during infection [52; 57; 139]. The T3SS-2 effectors GtgE and SopD2 both directly bind specific Rab proteins to regulate their localization and activity. SopD2 binds Rab7, disrupting endosomal trafficking by preventing the Rab-dependent recruitment of microtubule motor proteins [140]. Similar to SPI-1, multiple T3SS-2 effectors inhibit SCV-lysosome fusion and promote mistrafficking of lysosomal enzymes. The manipulation of both Rab7 and Rab32 by SopD2 prevents lysosomal fusion to SCVs [140; 141]. Additionally, GtgE binds Rab32 as well as Rab29, which regulates the trafficking of M6PR and the subsequent targeting of lysosomal enzymes to endolysosomal compartments [141; 142; 143]. Endosome-endosome fusion is also inhibited by purified SsaB, which is translocated as an effector protein as well as functioning as part of the pore-forming translocon [144]. Together these effectors act in concert with T3SS-1 effectors to manipulate the host endocytic network, regulating SCV-endosome contacts to allow for nutrient and membrane acquisition while preventing fusion with lysosomes and exposure to antibacterial enzymes.

The maintenance and stability of the SCV is critical to *Salmonella* virulence, as the destabilization of the SCV results in the cytosolic detection of *Salmonella* and the induction of macrophage pyroptosis [71]. Unsurprisingly, many T3SS-2 effector proteins serve to maintain the integrity of the SCV. Among these effector proteins, SifA directly anchors into the SCV membrane where it serves as a key regulator of SCV stability. The majority of mutant *ΔsifA Salmonella* strains gradually lose their SCV membrane within the first few hours of infection, leading to hyper-replication in epithelial cells and causing pyroptosis in

macrophages [69; 145; 146]. Conversely, the overexpression of SifA is sufficient to drive tubulation of host LAMP1$^+$ endosomes in uninfected cells [52]. PipB2, SopD2, and SseJ all have antagonistic roles towards SifA, contributing to the loss of SCV membrane integrity. PipB2 recruits the microtubule motor kinesin-1 to the SCV while SifA downregulates kinesin-1 recruitment [147]. Although a *ΔsifAΔpipB2* double mutant still results in the destabilization of the SCV, inhibition of kinesin-1 prevents SCV rupture during *ΔsifA* infection [147; 148], suggesting a role for PipB2 in SCV membrane destabilization. In the absence of SifA, both SopD2 and SseJ result in the destabilization of the SCV and the cytosolic release of intracellular bacteria. Conversely, *ΔsifAΔsopD2* and *ΔsifAΔsseJ* double-mutants remain within an intact, SIF-less SCV [145; 149; 150]. Notably, the co-deletion of SifA and SopD2 is sufficient to reduce the replication defect of a *ΔsifA* mutant and restore *S.* Typhimurium replication to near wild-type levels; however, *ΔsifAΔsseJ* double-mutants are still attenuated for replication in macrophages [149], suggesting that both SifA and SseJ play important, independent roles in *Salmonella* virulence. While the mechanism for SopD2-mediated SCV destabilization remains uncertain, the deacylation of glycerophospholipids and the esterification of cholesterol by SseJ (described in further detail below) suggests that manipulations of the SCV lipid content regulates the fluidity and stability of the SCV membrane.

Under specific infection and host cell environmental conditions, a select population of mature SCVs will generate SIFs [59]. SIFs are highly dynamic double-membraned tubular extensions from the SCV which are enriched in endosomal and lysosomal markers as well as cholesterol [151; 152]. The membranes and lumen of SIFs are a continuation of the main SCV body, dramatically increasing the absorptive area of the SCV and allowing

for the rapid diffusion of membrane and luminal content from SIFs to the SCV [60; 151]. While the specific functions and environmental regulation of SIF formation remains unclear, numerous bacterial effector proteins important for regulating SIF dynamics have been identified.

The T3SS-2 effector proteins SifA, PipB2, and SteA all play important roles in the regulation of SIF formation. Following infection or ectopic expression, SifA stimulates the tubulation of the SCV or endosomal membrane into elongated protrusions [152; 153]. Upon insertion into the SCV membrane, SifA interacts with and recruits the host kinesin-interacting protein, SKIP, to the SCV for the subsequent generation of SIFs and fission of vesicles from the SCV body [154; 155]. Additionally, PipB2 localizes to the SCV and SIF membrane where it plays an important, yet undefined role in proper SIF formation and dynamics [156; 157]. SIFs derived from *ΔpipB2 Salmonella* are bulky and static compared to wild-type bacteria [157]. Therefore, PipB2 and SifA may balance the membrane-destabilizing presence of kinesin-1 on the SCV to ensure proper development of SIFs. SteA binds PI(4)P on the SCV membrane and is important for partitioning the SCV membrane around dividing bacteria [158]. In the absence of SteA, multiple bacteria are clustered into enlarged, irregular SCVs which lack SIFs [159]. Together, the concerted actions of these T3SS-2 effectors regulate the biogenesis and dynamics of SIFs on permissible SCVs, promoting *Salmonella* virulence within host epithelial cells and macrophages.

Finally, T3SS-2 effectors have also been shown to manipulate membrane lipids and cholesterol content. SrfJ is a T3SS-2 effector with unknown biochemical activity, however, it contains a C-terminal domain similar to host glucosylceramidases which generate ceramide from glucosylceramide [160]. This raises the possibility that SrfJ mediated

ceramide synthesis may influence a diverse array of signaling pathways, including autophagy, sphingolipid metabolism, and cell death [161]. Additionally, the T3SS-2 effectors SseJ and SseL both play important roles in cholesterol homeostasis and trafficking and will be discussed in detail below. As cholesterol plays an important role in the regulation of membrane stability and intracellular signaling (see below), T3SS-2-mediated cholesterol manipulation likely impacts a diverse array of cellular processes.

Together, the combined arsenal SPI-1 and SPI-2 effectors are critical for *Salmonella* invasion of host cells. Although many secreted effector proteins display unique biochemical activity, the diverse, yet interconnected functions play important roles in facilitating *Salmonella* invasion, survival, and manipulation of host lipid homeostasis and inflammatory signaling responses during infection.

Cholesterol: An Integral Component of Eukaryotic Membranes

On a fundamental level, biological membranes partition interior components of cells and organelles from the external milieu. Cellular membranes are selectively permeable, regulating the diffusion of water and molecules across the membrane to maintain intracellular homeostasis regardless of variable extracellular conditions. The majority of biological membranes form as bilayers comprised of lipids, proteins, and carbohydrates; however, the composition of membranes varies dramatically between cells and organelles. Three major membrane lipid species form the basis of the majority of membranes: phospholipids, glycolipids, and sterols [162]. Sterols are a group of ringed lipids composed of a polar hydroxyl head, a rigid sterol body, and short hydrophobic tail. Although many species of sterols have been found in prokaryotic and eukaryotic membranes, cholesterol is the most abundant species found in animal membranes [163].

Importantly, cholesterol is not just an inert structural component of lipid membranes; rather, it is a multifaceted regulator of numerous cellular processes. Therefore, the manipulation of host cholesterol homeostasis by *Salmonella* suggests that one or more functions of cholesterol may play an important role in *Salmonella* virulence.

The Multiple Functions of Cholesterol

Cholesterol is a vital component of eukaryotic cells, constituting 20-30 mol% of membrane lipid bilayers [164]. In contrast, the vast majority of bacterial membranes are entirely devoid of cholesterol. Cholesterol is not homogeneously distributed within individual cells; instead, cholesterol concentrations vary dramatically between membranes of different organelles and generally increase along organelles of the secretory pathway. Within a membrane lipid bilayer, the distribution of cholesterol is asymmetrical, altering membrane curvature and local lipid environment [165]. Although the ER is the primary site of cholesterol biosynthesis, cholesterol concentrations in the ER and Golgi are significantly lower than phospholipid concentrations. Conversely, endosomes and the plasma membrane are enriched for cholesterol, with the majority of total cellular cholesterol content found in the plasma membrane [166; 167]. The varied abundance of cholesterol in the membranes of different organelles can lead to significant diversity in the properties and functions of membranes.

In eukaryotic cells, cholesterol serves many purposes, including steroid hormone signaling, bile salt production, and regulation of cell adhesion [168; 169]. Despite these numerous roles, on a molecular level, the most basic function of cholesterol is to modulate the biophysical properties of lipid bilayers. As an integral component of membranes, cholesterol provides structural rigidity, decreases membrane permeability, and buffers the

fluidity of lipid bilayers [170; 171; 172]. These functional impacts can be attributed to the ability of cholesterol to increase ordering of lipids in a membrane. The ordering effect of cholesterol refers to the effect cholesterol has on regulating the phase changes of lipid bilayers from a highly fluid liquid-disordered phase, to a stable liquid-ordered phase, to a rigid solid-ordered phase. Lipid ordering affects the rotational mobility and lateral diffusion of phospholipids, with a more disordered state having more motion and diffusion, while highly-ordered states restricted mobility and diffusion. Physiological plasma membrane conditions typically occur in a liquid-ordered state, allowing for a level of rotational mobility while reducing the extent of lateral diffusion throughout the membrane [163; 172]. How cholesterol impacts membrane ordering depends on the cholesterol concentration, phospholipid composition, and temperature of a membrane [164; 172]. At warmer physiological temperatures, the bulky, rigid sterol rings of cholesterol insert themselves between the long hydrophobic tails of phospholipids, reducing their mobility and increasing the rigidity of the membrane. However, at lower temperatures, cholesterol prevents the clustering and freezing of phospholipid tails, maintaining membrane fluidity [173]. Therefore, cholesterol acts as a buffer for membrane integrity against changes in temperature and membrane lipid composition.

As briefly mentioned above, the plasma membrane cholesterol content is highly asymmetrical, both between the inner and outer leaflets, as well as within a single leaflet. The concept of segregated lipid domains has existed since the first observations of cellular membrane extractions existing in detergent-soluble and detergent-insoluble fractions. However, advances in microscopy and membrane modeling have produced a significant body of evidence suggesting that lipid monolayers are a laterally heterogeneous assortment

of lipid microdomains [174; 175]. The lipid raft model proposes that membrane asymmetry arises from the preferential aggregation of cholesterol and sphingolipids into mobile lipid microdomains (lipid rafts) which are enriched for specific proteins and serve as hubs for the induction of intracellular signaling events [176]. Cholesterol has a higher affinity for sphingolipids compared to other phospholipids, allowing for cholesterol to insert between the hydrophobic tails of sphingolipids and act as a sort of molecular glue holding lipid rafts together following their generation in the Golgi [177; 178]. The cholesterol-rich lipid rafts of the plasma membrane vary in size and are linked between inner and outer leaflets [179]. This coupling of lipid rafts across the inner and outer leaflets of the PM is thought to potentiate intracellular signaling events by concentrating external-facing receptor proteins and increasing the local density of intracellular adaptor proteins.

Lipid rafts are thought to play a significant role in the induction of many intracellular signaling events. Since lipid rafts selectively enrich certain proteins, including glycosylphosphatidylinositol (GDI)–anchored proteins, while excluding others, signaling proteins localized to lipid rafts may be protected from phosphatases and other enzymes excluded from lipid rafts [177]. Along with numerous other signaling pathways, multiple immunogenic pathways, including TLR4 signaling, involve lipid raft-dependent signaling [177; 180]. Disruption of lipid rafts through the expression of the cholesterol export protein ABCA1 attenuates TLR4 signaling, significantly reducing macrophage sensitivity to LPS [181; 182]. These findings demonstrate that plasma membrane cholesterol concentrations regulate inflammation by altering cellular TLR responses. Thus, cholesterol is not simply a structural component regulating the biophysical properties of membranes; rather,

cholesterol plays a large role in numerous intracellular signaling events through the selective recruitment of signaling proteins to cholesterol-rich lipid rafts.

Intracellular Trafficking of Cellular Cholesterol

While import, export, synthesis, and storage play crucial roles in regulating cholesterol concentrations on a whole-cell level (see below), the trafficking of cholesterol between various compartments is important for intracellular homeostasis [**Figure 1-5**; **Figure 1-6**]. Cholesterol transport within host cells is a highly coordinated process utilizing both vesicular and non-vesicular transport mechanisms. Vesicular transport refers to the ATP-dependent shuttling of cholesterol within a vesicular carrier where it is transferred between lipid compartments as a consequence of membrane fusion events [183].

Endosomes contain two proteins, Niemann-Pick type C proteins NPC1 and NPC2, that are responsible for mediating non-vesicular transport of cholesterol out of endosomes onto other compartments. NPC2 acts in the endosomal lumen to load cholesterol onto membrane-associated NPC1, which in turn transports cholesterol across the endosomal membrane [184]. NPC1 tethers late endosomes/lysosomes to the ER by directly interacting with the ER tether protein VAP-A and promotes cholesterol efflux through the recruitment of various lipid transport proteins. The absence of functional NPC1 results in the accumulation of unesterified cholesterol in the endocytic network and the dysregulation of cholesterol synthesis in the ER [185].

The exact pathway of cholesterol trafficking following egress from the endocytic pathway remains a matter of current debate; however, endosomal cholesterol is transported to the PM and ER using a series of protein transport proteins [**Figure 1-6**]. Delivery of cholesterol to the PM requires NPC1, although the exact protein carriers remain unknown

[186]. ER-PM membrane contact sites facilitate phospholipid and cholesterol transport to the ER through OSBP-related protein 5 (ORP5) and GRAMD1 proteins [187; 188]. Notably, NPC1 colocalizes with ORP5 and GRAMD1b in endosomes [189; 190], suggesting that cholesterol may directly transfer from endosomes to the ER. In support of direct endosome-ER cholesterol trafficking, multiple ORP proteins mediate the transport of cholesterol from endosomes to the ER [191]. Conversely, OSBP1 typically localizes to the Golgi to facilitate cholesterol transport from the ER to the Golgi in exchange for PI(4)P [192]. Paradoxically, the cholesterol efflux protein ABCA1 may also contribute to retrograde cholesterol transport from endosomes to the ER. Depletion of ABCA1 results in the accumulation of cholesterol within the endocytic network and the loss of cholesterol sensing by the ER [193]. Whether ABCA1 plays a direct role in endosome to ER transport, or whether the loss of cholesterol efflux prevents ER sensing by generating a bottleneck of cholesterol in the endosomal network remains unclear. This complex network of transport proteins allows for redundant mechanisms to ensure proper trafficking of host cholesterol, allowing for newly acquired extracellular cholesterol to sort through the endocytic network towards the ER. This pathway places the ER at the center of cholesterol sensing and processing, where it regulates cholesterol biosynthesis, storage, and signaling in order to maintain cholesterol homeostasis.

Regulation of Cholesterol Homeostasis

Maintaining cholesterol homeostasis is crucial for eukaryotic cells, as elevated concentrations of free cholesterol induce ER stress and cellular cytotoxicity, while the depletion of cholesterol leads to the destabilization of membranes and increased cellular permeability. Given the importance of cholesterol homeostasis, it is unsurprising that

pathogens such as *Salmonella* disrupt the intracellular balance of host cholesterol to promote virulence. Due to the crucial roles of cholesterol in maintaining membrane integrity, modulating the biophysical properties of membranes, and facilitating numerous intracellular signaling pathways, cholesterol homeostasis is highly regulated within eukaryotic cells. To achieve cholesterol homeostasis, cells must balance cholesterol biosynthesis and uptake from extracellular sources with the storage of free cholesterol and cholesterol export out of the cell.

Regulation of Cholesterol Homeostasis - Cholesterol uptake

The majority of circulating cholesterol accessible to peripheral tissues is transported in the form of LDL. LDL consists of an apolipoprotein (predominantly ApoB-100) packaged around a hydrophobic core of phospholipids, cholesterol, and other fatty acids. Cholesterol makes up approximately half of the total molecular composition of mature LDL particles, with the majority being in the form of cholesteryl esters [194]. As such, the uptake of circulating LDL contributes towards a large fraction of cholesterol within peripheral cells. Cellular uptake of LDL occurs through receptor-mediated endocytosis via the LDL receptor (LDLR) and scavenger receptors (SR-B1) [195]. LDLR is found on both liver cells, as well as peripheral tissues. Under low intracellular cholesterol conditions, LDLR expression is upregulated, while elevated intracellular cholesterol suppresses LDLR expression.

LDL binding to LDLR drives endocytosis of the complex and enters LDL into the host endocytic pathway [**Figure 1-4**]. Following endosomal maturation, the acidic pH of late endosomes promotes the dissociation of LDL-LDLR complexes, liberating LDLR where it is either recycled to the cell surface or degraded in lysosomes [196; 197].

Subsequently, the cholesteryl esters packaged in LDL are hydrolyzed within late endosomes/lysosomes by lysosomal acid lipase, while the other phospholipids and triglycerides are hydrolyzed by additional lipases [195]. Newly hydrolyzed free cholesterol is then transported out of the endocytic network to the cell surface and/or ER. Importantly, once integrated into the ER membrane, increased local concentrations of free cholesterol negatively regulate cholesterol biosynthesis.

Regulation of Cholesterol Homeostasis - Cholesterol biosynthesis

Paradoxically, ER is the primary site of cholesterol biosynthesis, yet it contains minimal concentrations of cholesterol [166]. This cholesterol limitation in the ER may allow for the detection of slight changes in total cellular cholesterol reflected by the amount of cholesterol delivered to the ER from extracellular and endosomal sources [**Figure 1-5**], increasing ER sensitivity and allowing for rapid responses to maintain cellular homeostasis. Cholesterol biosynthesis is controlled by the master regulator sterol regulatory element-binding protein 2 (SREBP2), whereas SREBP1 regulates fatty acid synthesis [198]. SREBP proteins are synthesized as inactive precursors and insert into the ER membrane in a hairpin conformation, with the N-terminal transactivation domain and C-terminal regulatory domain exposed to the cytoplasm. Following translation, SREBP proteins are retained in the ER membrane as a complex with SREBP cleavage-activating protein (SCAP) through the interaction of SCAP with the cholesterol-sensing proteins INSIG1 and INSIG2. In cholesterol-replete conditions, oxysterol binding stabilizes INSIG binding to SCAP, while cholesterol directly binds the sterol-sensing domain of SCAP, causing the retention of SREBP-SCAP in the ER [199]. Conversely, cholesterol starvation destabilizes the INSIG-SCAP interaction and promotes SREBP-SCAP translocation to the

Golgi. Upon reaching the Golgi membrane, SREBP undergoes sequential proteolytic cleavage by the S1P and S2P proteases. These cleavage events free the N-terminal fragment of SREBP (nSREBP) from SCAP and releases nSREBP into the cytoplasm [200]. nSREBP homodimers translocate into the nucleus where they bind SRE promoter regions to induce the transcription of biosynthetic proteins [201]. Although SREBPs demonstrate some overlap in downstream targets, SREBP2 preferentially targets genes involved in cholesterol biosynthesis, including *HMGCS1* (HMG-CoA synthase) and *HMGCR* (HMG-CoA) reductase [202].

Cholesterol biosynthesis is a multi-enzyme, multi-intermediate process [**Figure 1-3**]. Although all nucleated mammalian cells can synthesize cholesterol, the bulk of cholesterol production is thought to occur in the liver and intestines [203]. The first step in the synthesis of cholesterol is conversion of acetyl-CoA into 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). This initial stage of cholesterol synthesis is catalyzed by HMG-CoA synthase in the cytoplasm, while all subsequent steps occur in the ER [203]. The rate-limiting step in cholesterol biosynthesis is the irreversible conversion of HMG-CoA into mevalonate by the ER integral membrane protein HMG-CoA reductase [204]. Statins are potent competitive inhibitors of HMG-CoA reductase. As such, pharmacological treatment with these drugs reduces cholesterol synthesis and lowers circulating and intracellular cholesterol content [205]. Subsequent enzymatic reactions convert mevalonate through a series of intermediates, including isoprenoids, farnesol, squalene, lanosterol, and desmosterol. Many of these intermediates have important biological functions of their own [206], underscoring the importance of cholesterol biosynthesis in regulating numerous host processes.

Regulation of Cholesterol Homeostasis - Cholesterol storage

In order to maintain cholesterol homeostasis and avoid toxicity, irregular increases in intracellular cholesterol concentrations are combated by the storage and export of excess cholesterol. As mentioned above, extracellular and biosynthetic cholesterol converges in the ER membrane where it serves to regulate synthesis. Importantly, surplus cholesterol in the ER is esterified and stored in the form of lipid droplets or exported from hepatocytes as vLDL [207]. Esterification of cholesterol depends on host cholesterol acyltransferases, mainly ACAT. ACAT is primarily located in the ER membrane where it functions as a homotetramer [208]. Interestingly, the primary regulation of ACAT appears to be post-translational, as cholesterol feeding increases ACAT activity without altering mRNA levels [209]. By utilizing post-translational regulation, ACAT is able to rapidly respond to the dynamic variation in intracellular cholesterol content. Elevated cholesterol concentrations promote ACAT activity, catalyzing the transfer of acyl chains from Acyl-CoA onto cholesterol, generating CoA and cholesteryl esters [210]. Remarkably, ACAT has been demonstrated to utilize a variety of long-chain fatty acids as a substrate to esterify multiple oxidized cholesterol derivatives [211]. Esterified cholesterol is subsequently deposited with phospholipids and specific proteins into cytosolic lipid droplets. Although conventionally considered as storage compartments, lipid droplets play important roles in regulating cell and ER stress, cholesterol turnover in membranes, and starvation-induced autophagy [212; 213]. Thus, the storage of cholesterol is not just important for the buffering of intracellular cholesterol content, but also for regulating numerous cellular processes.

Regulation of Cholesterol Homeostasis - Cholesterol export

In addition to storing surplus cholesterol in the form of lipid droplets, excess cholesterol can be removed from the cell in a process called reverse cholesterol transport (RCT). RCT is a highly regulated set of cellular functions which facilitate cholesterol transport within the cell, efflux of cholesterol out of peripheral cells, and removal of cholesterol from the body. Within cells, excess cholesterol is transported out of endosomal compartments and the ER and positioned for removal by one of three efflux pathways: passive diffusion, facilitated diffusion by SR-B1, or efflux facilitated by the ATP-binding cassette (ABC) family of proteins [214]. Although all three pathways contribute to the efflux of cholesterol out of peripheral cells, active export of cholesterol via the ABC transporters has been the most extensively studied. Efflux through ABCA1 results in the direct lipidation of apoA-1 in the serum. Notably, ABCA1 utilizes cholesterol and other membrane phospholipids as substrates for export [215], suggesting that ABCA1 may be involved in the remodeling of entire membranes. Mechanistically, ABCA1 localizes to membrane surfaces where it acts as a 'floppase', transferring cholesterol and other phospholipids across the inner leaflet onto the outer leaflet [216]. The current dogma for ABCA1 activity proposes that the vast majority of ABCA1-mediated efflux occurs at the plasma membrane; however, a small pool of ABCA1 resides on a subset of early endosomes [217], presumably to load cholesterol onto endocytosed apoA-1. Nevertheless, the localized concentration of cholesterol on the plasma membrane recruits circulating apoA-1, facilitating cholesterol efflux and generating nascent HDL. The removal of lipid molecules from the exofacial membrane generates positive membrane curvature, which further enhances apoA-1 recruitment and cholesterol efflux [218]. In contrast, ABCG1

facilitates cholesterol export onto complete HDL, not nascent apoA-1 [219]. Through these mechanisms, peripheral cells maintain cellular cholesterol homeostasis through the export of excess cholesterol through ABC transport proteins, increasing circulating HDL levels.

As professional phagocytes, macrophages internalize a large amount of cholesterol through both receptor mediated endocytosis of LDL and the phagocytosis and degradation of apoptotic cells. To prevent an overabundance of cholesterol leading to cellular toxicity and the conversion of macrophages into arterial plaque-inducing foam cells, macrophages heavily rely on RCT to remove excess cholesterol. In macrophages, more than 50% of total cellular cholesterol efflux occurred through ABCA1, while ABCG1 was responsible for ~20% [220]. In the absence of ABCA1/ABCG1, foam cell formation and development of atherosclerotic lesions is enhanced [221]. Furthermore, individual knockout of *ABCA1* in macrophages results in reduced HDL production and the increase in intracellular free cholesterol [222]. In humans, the inherited mutation of *ABCA1* (Tangier's disease) leads to the dramatic reduction or complete absence of circulating HDL and the concurrent accumulation of cholesteryl esters in peripheral tissues [223]. Left untreated, Tangier's disease significantly increases the risk of coronary artery disease, underscoring the importance of ABCA1 and the proper regulation of cholesterol export.

Regulation of ABCA1 in Response to Intracellular Cholesterol Concentration

As a cholesterol efflux protein, ABCA1 plays an important role in maintaining intracellular cholesterol homeostasis. Disruptions in ABCA1 regulation have severe impacts on intracellular cholesterol concentrations and contribute to *Salmonella* pathogenesis [223; **Chapter 2**]. To prevent erroneous perturbation of cholesterol homeostasis, the expression and function of ABCA1 is under tight regulatory control on

both a transcriptional and post-translational level. *ABCA1* expression is positively correlated with intracellular cholesterol levels, with elevated intracellular cholesterol leading to enhanced *ABCA1* expression and depletion of cholesterol downregulating *ABCA1.* In healthy cells, the regulatory control of *ABCA1* by cholesterol is manifested in the form of liver X receptors (LXRs). LXRs are a family of nuclear hormone receptors which serve as the master regulator of *ABCA1* expression in hepatocytes and peripheral tissues. Elevated cholesterol due to uptake or biosynthesis leads to the generation of oxidized cholesterol derivatives, known as oxysterols, which serve as the major ligand for LXR activation [224; 225]. Subsequently, activated LXR translocates into the nucleus where it binds promoter LXR response elements to enhance the expression of target genes. Many LXR-induced genes are involved in cholesterol efflux, including *APOE1*, *ABCG1*, and *ABCA1* [226]. As such, pharmacological induction of LXR upregulates *ABCA1* and drives cholesterol efflux [227], highlighting the importance of LXR signaling in regulating cholesterol efflux and lipid homeostasis.

Intriguingly, LXR-independent pathways of ABCA1 regulation have also been identified. One such mechanism is mediated by focal adhesion kinase (FAK) and Akt in the context of cell density. Sparsely-grown fibroblasts contained increased amounts of active FAK, resulting in the downstream signaling through Akt and the Akt-dependent phosphorylation of two transcription factors, FOXO3 and TAL1. Phosphorylation of FOXO3 inhibits translocation to the nucleus while TAL1 phosphorylation leads to its degradation, preventing transcription of *ABCA1* and increased cellular cholesterol content. In contrast, highly crowded cells with reduced FAK activation, as well as FAK-knockout cells, demonstrated higher levels of ABCA1 and a concurrent reduction in cellular

cholesterol [228]. Additionally, exposure of macrophages to bacterial LPS upregulates *ABCA1* expression in a p38 MAP kinase-dependent manner [229], suggesting that host cells innately respond to bacteria by stimulating ABCA1-mediated cholesterol efflux. These alternative mechanisms for ABCA1 regulation suggest that cholesterol efflux may not be solely for preventing the toxic accumulation of excess cholesterol, but it may serve as a means to modulate cholesterol homeostasis in order to respond to specific cellular stimuli.

Given the importance of cholesterol export in maintaining cellular cholesterol homeostasis, it is unsurprising that ABCA1 is regulated by multiple mechanisms. Loading macrophages with LDL stimulates a disproportionate increase in ABCA1 protein levels compared to mRNA [230], suggesting post-transcriptional modifications and protein stability play a significant role in regulating ABCA1 activity. ABCA1 protein is turned over rapidly, with a half-life of 1-2 hours [231]. ABCA1 protein stability is regulated by multiple cellular factors. The direct interaction of ABCA1 with the scaffolding protein α1-syntropin dramatically increases ABCA1 stability [231]. Furthermore, the binding of ABCA1 to apoA-1 and other apolipoproteins during cholesterol efflux delays proteolytic degradation of ABCA1 [232; 233], revealing a positive feedback loop where ABCA1-mediated cholesterol efflux stabilizes ABCA1. In contrast, abnormally high concentrations of fatty acids enhance ABCA1 degradation and reduce cholesterol efflux [234]. This rapid turnover of proteins allows cells to quickly respond to depletion of cholesterol on a transcriptional level, preventing excessive efflux and allowing for the restoration of intracellular cholesterol concentrations.

The manipulation of host cholesterol homeostasis by *S.* Typhimurium

The disruption of host cell cholesterol homeostasis is a common theme among disease-causing microorganisms. One frequently occurring commonality is the targeting of cholesterol-rich domains for pathogen invasion into target host cells. Intriguingly, both viruses and bacteria both share an affinity for cholesterol in host membranes. Numerous viruses, including HIV, Ebola, and influenza, specifically target cholesterol-rich membranes for viral attachment and entry [235; 236; 237]. Cholesterol-rich host membranes also play important roles in the attachment and/or invasion by bacteria, including *E. c*oli, *Chlamydia*, and *Salmonella* [82; 238; 239]. *Salmonella* invasion of epithelial cells begins with the T3SS-1 pore forming translocon protein, SipB, targeting cholesterol in the host PM. Importantly, depletion of host cholesterol prevents the delivery of bacterial effectors into the host, preventing the invasion of non-phagocytic cells [82]. Furthermore, cholesterol can be seen in close proximity to *Salmonella* immediately following attachment and invasion, suggesting that cholesterol is enriched within the membrane ruffles at sites of *S.* Typhimurium invasion [240]. These findings demonstrate the importance for cholesterol in the initial stages of *Salmonella* invasion of host epithelial cells.

Salmonella infection of macrophages has previously been demonstrated to increase intracellular cholesterol concentrations. In a study by Catron, *et al*., *S.* Typhimurium increased both cholesterol and the cholesterol precursor lanosterol by 16 hours post-infection in immortalized macrophages. Furthermore, relocalization of cholesterol within close proximity of SCVs occurred within 8 hours of infection in a T3SS-2-dependent manner. Notably, inhibition of lanosterol synthesis (a cholesterol precursor immediately

upstream of cholesterol) had no effect on cholesterol recruitment towards SCVs [241], indicating that the main source of cholesterol accumulation seen during *Salmonella* infection is due to the uptake of extracellular cholesterol. In a follow up study, inhibition of the initial stages of cholesterol biosynthesis by statins significantly attenuated *S.* Typhimurium growth in macrophages [242]. As statins inhibit HMG-CoA reductase, these studies suggest that *de novo* cholesterol biosynthesis *per se* is not essential for *Salmonella* survival [**Figure 1-3**]; rather, cholesterol biosynthetic signaling and/or synthesis of cholesterol precursors between mevalonate and squalene oxide are important for *Salmonella* survival.

As previously mentioned, *Salmonella* contains multiple T3SS-2 effectors involved in manipulating host cholesterol homeostasis. SseJ is a cholesterol acyltransferase containing both deacylase and glycerophospholipid:cholesterol acyltransferase (GCAT) activity. Following translocation into host cells, SseJ is activated by the host Rho-family GTPase RhoA. SseJ competes with traditional eukaryotic RhoA effector proteins for binding to the regulatory switch region of RhoA [243]. Mutations within the RhoA interacting surface of SseJ prevents RhoA binding and significantly reduces the phospholipase activity of SseJ. Although SseJ interacts with RhoA independent its GDP- or GTP-bound state, SseJ activity is specifically enhanced by RhoA-GTP [244], suggesting that RhoA activity may be important for the downstream activation of SseJ. The interaction between SseJ-RhoA also plays a critical role in the recruitment of both proteins to endosomal compartments, as binding-deficient SseJ mutants remain inactive and dispersed throughout the cell [243].

During infection, SseJ localizes to the surface of SCVs and SIFs; however, transfected recombinant SseJ colocalizes with RhoA on LAMP-1$^+$ endosomes in the absence of bacteria [150; 245; 246]. Ectopic expression of SseJ in uninfected cells leads to the formation of large SCV-like LAMP1$^+$ aggregates which are enriched for cholesterol [150], suggesting that SseJ plays an important role in modifying the SCV. Structurally, the N-terminus of SseJ contains the T3SS translocation signal, while the C-terminus shares considerable homology with glycerophospholipid:cholesterol acyltransferase (GCAT) proteins found in other bacterial species [247; 248; 249].

As with other GCAT proteins, SseJ catalyzes the transfer of acyl chains from phospholipids onto cholesterol, generating both lysophospholipids and cholesteryl esters [250; 251]. This function is homologous to eukaryotic cholesterol acyl transferase proteins, including ACAT. Therefore, SseJ may serve as a central hub for cholesterol homeostasis, redirecting cholesterol towards SseJ-containing compartments, including SCVs, where it is subsequently esterified. In support of this hypothesis, SseJ activity has been demonstrated to increase cholesteryl ester synthesis and lipid droplet formation during *S.* Typhimurium infection of both HeLa cells and RAW 264.7 macrophages [251]. In contrast, significantly more lipid droplets are generated in gallbladder cells during infection with an SseL-deficient mutant [252], suggesting that SseL inhibits lipid droplet formation. SseL is a deubiquitinase which preferentially hydrolyses lysine-63 ubiquitin chains, preventing the accumulation of *Salmonella*-induced ubiquitinated protein aggregates and inhibiting autophagic flux [253; 254]. How this function inhibits lipid droplet formation is unknown. Although infection has been demonstrated to increase cholesteryl ester formation, the specific function of cholesteryl esters in *Salmonella* virulence remains unclear.

GDSL lipases (which include GCAT proteins) contain a conserved triad of Ser, Asp, and His residues for catalytic function [255]. Remarkably, this Ser-His-Asp catalytic triad is a common feature of many enzymes, including serine proteases [256]. In SseJ, the catalytic triad of Ser141, Asp247, and His384 are all critical for deacylase activity. Although catalytically inactive SseJ remains on the SCV and other endosomal compartments, these mutants fail to produce cholesteryl esters within host cells. Importantly, mutating any one of these three catalytic residues abolishes SseJ activity, reduces survival within macrophages, and attenuates *Salmonella* virulence in mice comparable to a complete *ΔsseJ S.* Typhimurium strain [250; **Figure 1-4**]. The presence of homologous GCAT proteins in other pathogenic bacteria suggests an intriguing possibility that the manipulation of host cholesterol storage may be a conserved mechanism in promoting the virulence of numerous pathogens.

In addition to modulating cholesterol storage, SseJ has been implicated in SIF formation and SCV integrity. Recombinant SseJ reduces SIF formation and/or endosomal tubulation in both infected cells and uninfected cells transfected with SifA [150]. Furthermore, *ΔsseJ S.* Typhimurium generates significantly more, albeit morphologically similar to wild-type, SIFs during infection [59; 157]. This suggests that SseJ may not be directly involved in the generation of SIFs, but rather it may indirectly regulate SIF formation by altering the permissiveness of the SCV environment for SIF production. Additionally, the unopposed acylation of free cholesterol due to SseJ activity in *ΔsifA Salmonella* strains may significantly impair SCV membrane integrity, exposing bacteria to the host cytoplasm and potentially eliciting an inflammatory host response. Conversely, SseJ activity in wild-type bacteria enhances *S.* Typhimurium survival by maintaining SCV

integrity. Both SseJ and SseL interact with the cholesterol transporter oxysterol-binding protein 1 (OSBP1), recruiting OSBP1 to the SCV surface. The loss of OSBP1 on SCVs correlates with the loss of SCV integrity and cytosolic release of *S.* Typhimurium [257; 258]. This suggests that the SseJ-dependent acquisition of free cholesterol through OSBP1 may increase SCV stability, while the unopposed SseJ-mediated conversion of free cholesterol into cholesteryl esters destabilizes SCV membranes. Together, these findings reveal the delicate balance of SseJ activity and cholesterol homeostasis in SCV integrity and *S.* Typhimurium survival.

The Diverse Functions of Focal Adhesion Kinase

The cell surface is a highly dynamic region of the cell, continuously extending and retracting as it adheres to and disassembles from extracellular substrates at localized sites called focal adhesions. Focal adhesions are a highly complex network of integrins, scaffolding, and signaling proteins that link the extracellular matrix to the cellular cytoskeleton. Within focal adhesions, focal adhesion kinase (FAK) acts as a mechanosensor, integrating mechanical signals and biochemical signaling pathways throughout the cell. Remarkably, previous studies and the work shown in Chapter 2 reveal FAK has a significant role in regulating cholesterol independent of focal adhesions [41; 228; **Chapter 2**], demonstrating the wide-ranging signaling pathways regulated by FAK.

FAK is a non-receptor tyrosine kinase typically associated with focal adhesion and integrin-mediated signaling. FAK contains a central tyrosine kinase domain flanked by an N-terminal FERM and a C-terminal focal adhesion targeting (FAT) domain. Unstimulated FAK resides in an autoinhibited conformation, with the FERM domain directly binding to the catalytic domain and blocking the activation loop [259]. Mechanical forces within focal

adhesions alter the conformation of the FERM domain, releasing it from the catalytic domain and allowing for Tyr397 autophosphorylation and additional phosphorylation steps necessary for the optimal activation of FAK [260; 261]. Additionally, RhoA-mediated cytoskeletal rearrangements induce FAK activation [262], indicating that FAK directly responds to cytoskeletal dynamics independent of focal adhesions. Furthermore, increased $PI(4,5)P_2$ in membranes is sufficient to induce conformational changes and autophosphorylation of FAK [263], suggesting that lipid composition of target membranes also plays a significant role in the regulation of FAK signaling. Together, active FAK/Src complexes signal through downstream substrates to regulate focal adhesion and cytoskeletal dynamics.

Although conventional FAK signaling centers around cell adhesion and migration, FAK-induced signaling pathways impact numerous cellular functions, including cholesterol efflux, Akt signaling, and autophagic clearance of bacteria [228; 264; 265]. Mechanistically, FAK activation of Akt occurs through PI3K binding to the phosphorylated Tyr397 residue of FAK, resulting in phosphorylation of the p85 subunit of PI3K. PI3K catalyzes the conversion of $PI(4,5)P_2$ on membranes to PIP_3, resulting in the recruitment of PDK1 and the subsequent phosphorylation and activation of Akt [266]. Downstream signaling of Akt impacts many cellular processes, including autophagy through the indirect activation of mTOR [267]. Interestingly, FAK/Akt and Akt/mTOR demonstrate reciprocal activation [267; 268], resulting in a considerable positive feedback loop to enhance signaling through the FAK/Akt/mTOR signaling axis.

Mammalian target of rapamycin (mTOR) is the primary regulatory protein of autophagy in cells. Autophagy is an evolutionarily conserved process for regulating the

degradation and recycling of cellular components and organelles due to cellular stress and nutrient starvation. In nutrient-replete conditions, activated mTOR suppresses autophagy, while starvation decreases mTOR activity and enhances autophagy [269]. It is becoming increasingly evident that manipulating autophagy plays an important role in the virulence of numerous pathogens [270]. Previous findings from our lab revealed that *S*. Typhimurium infection of macrophages results in the T3SS-2-dependent recruitment FAK and Akt to the cytosolic surface of the SCV membrane. Signaling through the FAK/Akt/mTOR axis inhibited the autophagic killing of *Salmonella*, enhancing bacterial survival. In the absence of a functional T3SS-2, SCVs became highly decorated with the autophagic scaffolding protein LC3 and bacterial survival was significantly attenuated [41]. This indicates that the SCV may serve as a central signaling hub for the manipulation of the FAK/Akt/mTOR signaling axis and the subsequent inhibition of autophagy.

Summary and Thesis Rationale

Cholesterol homeostasis in peripheral tissues depends on the balancing of cholesterol uptake and synthesis with cholesterol storage and efflux. Previous studies have shown that *S*. Typhimurium infection leads to the accumulation of cholesterol in infected macrophages and the redirection of some of that cholesterol towards SCVs [241]. In the absence of T3SS-2 activity, cholesterol fails to relocalize towards the SCV [164], suggesting that *S*. Typhimurium actively disrupts host cholesterol trafficking during infection. Furthermore, the SPI-2 effector proteins SseJ and SseL interact with the host cholesterol transport protein OSBP1 to enhance SCV stability [257; 258]. As the loss of either SseJ or SseL reduces *Salmonella* pathogenesis, these findings suggest that *Salmonella* directly manipulates host cell cholesterol homeostasis during infection to promote bacterial survival. Prior to the research presented here, the mechanism(s) of *Salmonella*-mediated cholesterol accumulation and how elevated cholesterol enhances *S*. Typhimurium survival remained unknown. In **Chapter 2**, we will detail the mechanism of *S*. Typhimurium-induced cholesterol accumulation, demonstrating that the T3SS-2 effector and functional homolog of eukaryotic ACAT, SseJ downregulates *Abca1* in a FAK- and Akt-dependent manner. In **Chapter 3**, we will discuss the potential functions for cholesterol in mediating *Salmonella* survival and other outstanding questions generated by this research.

Discovery of this novel, SseJ-dependent mechanism of cholesterol accumulation during *Salmonella* infection provides a better understanding of potential roles for the manipulation of host cholesterol in facilitating intracellular survival. Further clarification of the relationship between cholesterol and *Salmonella* virulence may provide insight into

potential targeted approaches to indirectly treat *Salmonella* infection through the regulation of host cholesterol homeostasis.

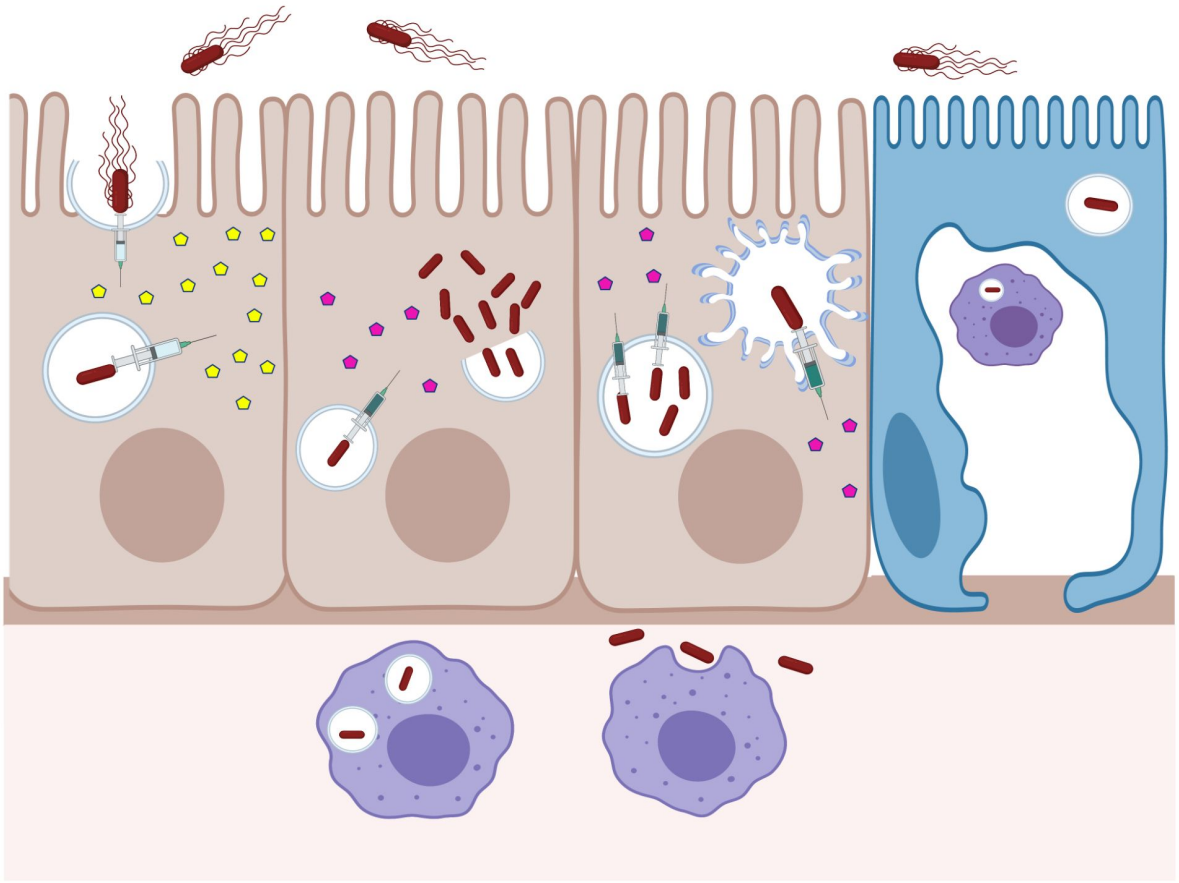


Figure 1-1. *Salmonella* Typhimurium Invasion of Host Organisms.

A simplified model of *Salmonella* invasion of host intestinal epithelial cells and macrophages. Following ingestion of contaminated food or water, *Salmonella* passes through the stomach and reaches the intestinal tract. Here, *Salmonella* contacts the intestinal epithelium (brown) and targets host cell cholesterol for intimate attachment by the T3SS-1 translocon complex. *Salmonella* then induces characteristic actin polymerization and membrane ruffling to promote the endocytic uptake of attached bacteria, where the bacteria remain and establish an intracellular replicative niche called the *Salmonella*-containing vacuole (SCV). Immediately following internalization, *Salmonella* translocates 13 effector proteins (yellow pentagons; a complete list is presented in **Table 1-1**) through T3SS-1 (see **Figure 1-2**) into the cytoplasm to promote the internalization and establishment of the SCV. In the absence of a functional SCV, *Salmonella* undergoes hyper-replication in the epithelial cell cytoplasm. Within 1-4 hours of invasion, the T3SS-1 and associated effectors are downregulated, while the T3SS-2 is upregulated with its vast panel of effector proteins. More than 30 T3SS-2 effectors (pink pentagons; a complete list is presented in **Table 1-2**) are involved in maintaining SCV integrity, regulating endosomal positioning, and manipulating host cell signaling to promote the survival and replication of *Salmonella* within the host cell. During the later stages of infection, certain SCVs may produce elongated tubular structures from the SCV called Salmonella-induced filaments (SIFs). Following invasion of the intestinal epithelium, a small population of bacteria may breach the basal membrane of the intestinal epithelium where they are phagocytosed by the resident macrophages (purple) of the lamina propria. Alternatively, *Salmonella* can be endocytosed by the M cells (blue) within

the Peyer's patches and be directly delivered to resident macrophages. Following internalization by macrophages, SPI-2 remains upregulated and facilitates *Salmonella* survival and dissemination to distal sites of infection [Model based on 271].

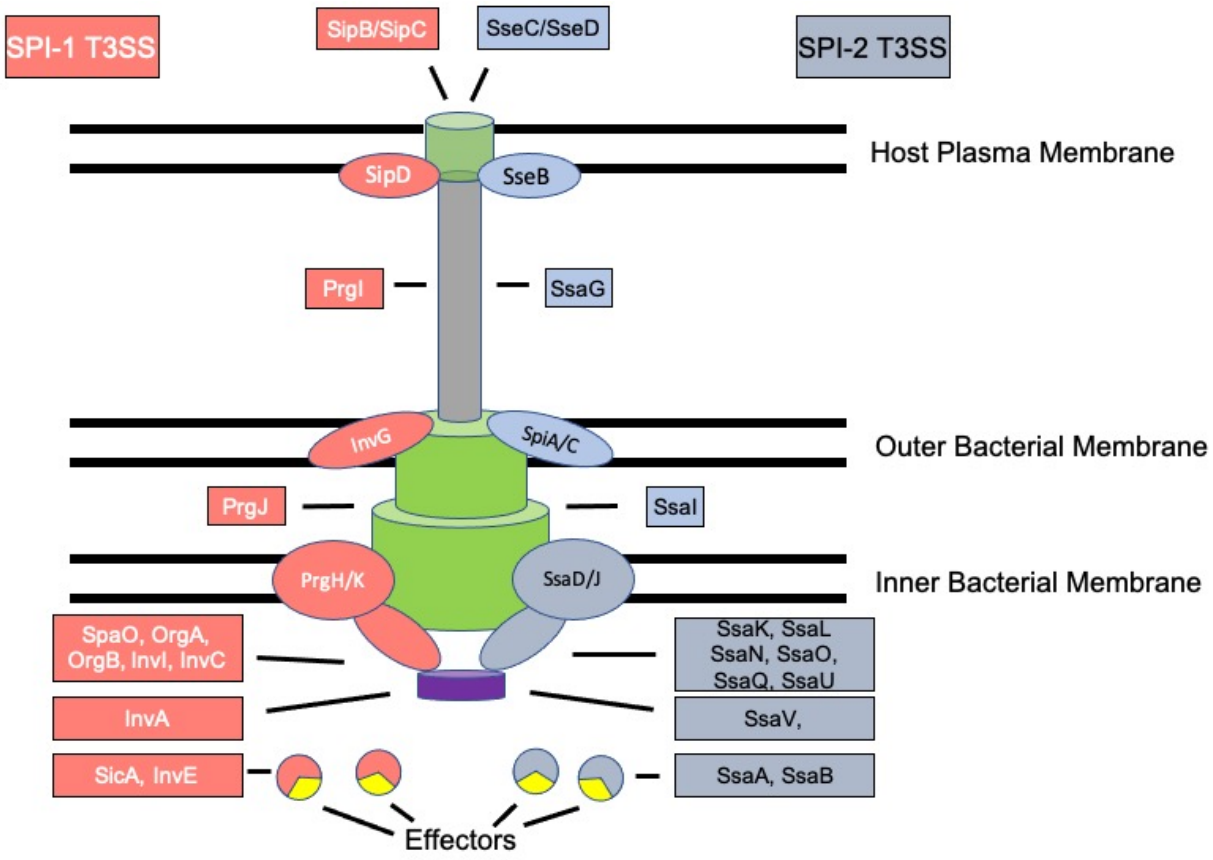

SPI-1 T3SS
SipB/SipC
SseC/SseD
SPI-2 T3SS
Host Plasma Membrane
SipD
SseB
PrgI
SsaG
InvG
SpiA/C
Outer Bacterial Membrane
PrgJ
SsaI
PrgH/K
SsaD/J
Inner Bacterial Membrane
SpaO, OrgA, OrgB, InvI, InvC
SsaK, SsaL SsaN, SsaO, SsaQ, SsaU
InvA
SsaV,
SicA, InvE
SsaA, SsaB
Effectors

Figure 1-2. Schematic of *Salmonella* Type-Three Secretion Systems T3SS-1 and T3SS-2.

Salmonella Typhimurium encodes two different type III secretion systems (T3SS-1 and T3SS-2) through which bacterial effector proteins are translocated through into the host cytosol. The T3SS is a multi-protein molecular syringe that forms a continuous transport channel through the two bacterial membranes and extends into extracellular space by a needle structure [75]. T3SS-1 and T3SS-2 are two of the best characterized T3SSs and are critical for *S.* Typhimurium invasion of host epithelial cells and survival within the SCV [272]. While the general structural assembly of the T3SS remains similar between T3SS-1 and T3SS-2, the underlying proteins of each component vary. Here, we show several key components that make up the SPI-1 (red) and SPI-2 (blue) T3SS. Mutation of the outer ring protein InvG prevents the formation of the T3SS-1 and serves as a ΔT3SS-1 mutant to avoid macrophage cytotoxicity during our research presented in **Chapters 2** and **3**. Furthermore, the deletion of sorting platform component OrgA also prevents T3SS-1 formation and function and serves as the background for the SPI-2 effector mutants detailed in **Chapter 2**. The T3SS-2 component SpiA shares considerable homology to the T3SS-1 InvG. Therefore, the deletion of both OrgA and SpiA forms the ΔT3SS1/ΔT3SS2 mutant used in **Chapter 2** (Adapted from 75; 273)

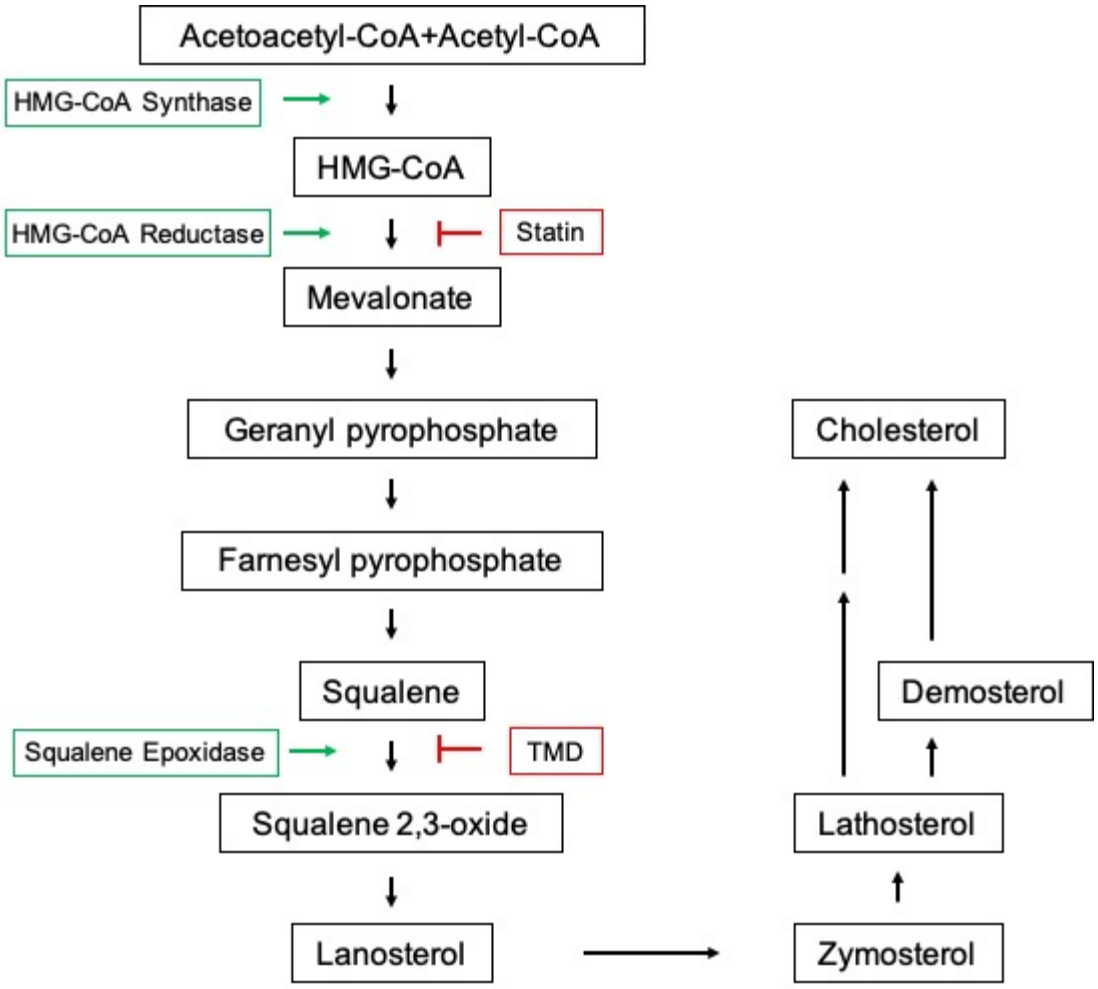

Acetoacetyl-CoA+Acetyl-CoA
HMG-CoA Synthase
HMG-CoA
HMG-CoA Reductase
Statin
Mevalonate
Geranyl pyrophosphate
Farnesyl pyrophosphate
Squalene
Squalene Epoxidase
TMD
Squalene 2,3-oxide
Lanosterol
Zymosterol
Lathosterol
Demosterol
Cholesterol

Figure 1-3. Cholesterol Biosynthetic Pathway.

A schematic overview of SREBP2-mediated cholesterol biosynthesis. Notable enzymes are in green, while inhibitors are in red. In cholesterol-depleted conditions, SREBP2 translocates to the nucleus and stimulates the transcription of a number of genes responsible for cholesterol synthesis. de novo cholesterol biosynthesis converts acetoacetyl-CoA and acetyl-CoA to cholesterol through a number of cholesterol precursors. HMG-CoA Synthase is the first step in cholesterol synthesis, generating HMG-CoA. Subsequently, HMG-CoA reductase converts HMG-CoA into mevalonate and downstream isoprenoids. Statins are potent pharmacological inhibitors of HMG-CoA reductase, preventing cholesterol biosynthesis by inhibiting mevalonate generation. Geranyl pyrophosphate and farnesyl pyrophosphate are two important cholesterol precursors responsible for prenylating cytosolic proteins to promote association with target membranes. TMD is an inhibitor of squalene epoxidase, which generates the terminal cholesterol precursor lanosterol. Subsequently, lanosterol is converted through a series of enzymatic interactions into mature cholesterol. Notably, statin treatment reduced *Salmonella* survival, while TMD treatment did not, suggesting maintaining cholesterol biosynthesis is important for *Salmonella* survival through the production of cholesterol precursors between mevalonate and squalene [242]. How these cholesterol precursors impact *Salmonella* survival remains unknown and will be discussed in **Chapter 3**. [Model based on 241; 274].

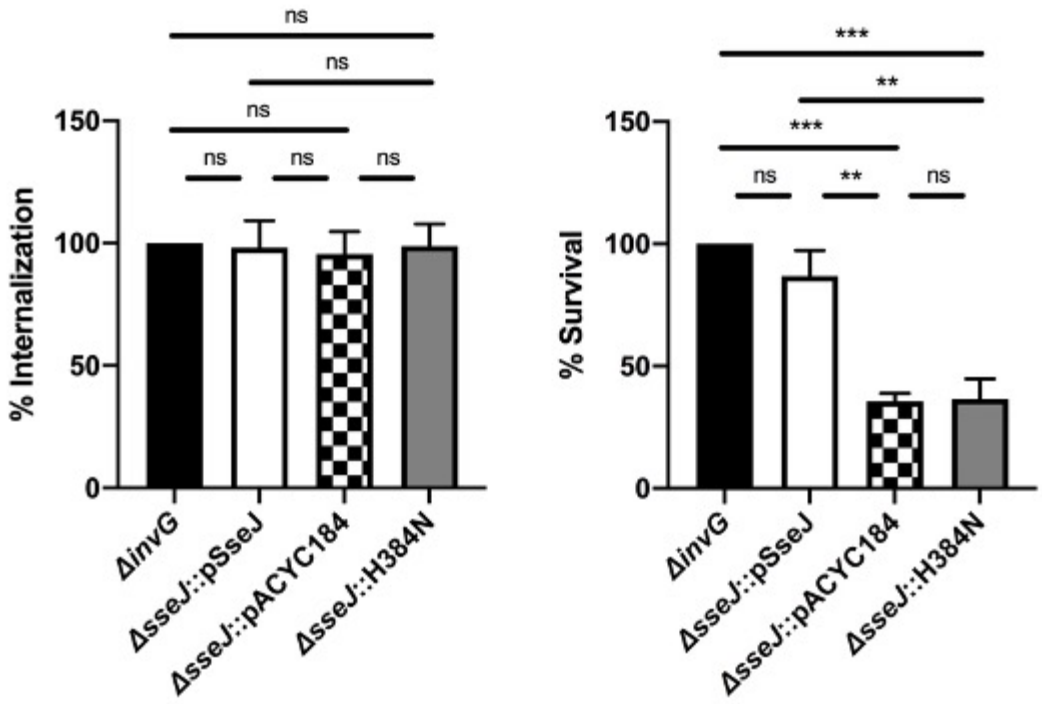

% Internalization
150
100
50
0
ns
ns
ns
ns
ns
ns
ΔinvG
ΔsseJ::pSseJ
ΔsseJ::pACYC184
ΔsseJ::H384N
% Survival
150
100
50
0
***
**
***
ns
**
ns
ΔinvG
ΔsseJ::pSseJ
ΔsseJ::pACYC184
ΔsseJ::H384N

Figure 1-4. Catalytically-inactive SseJ Mutants are Attenuated for Survival in Macrophages.

Internalization and survival rates were determined using a standard gentamicin protection assay as described in the materials and methods section of **Chapter 2**. For all conditions, cells were infected with Δ*invG,* Δ*sseJ*::H384N (catalytically inactive SseJ mutant; see **Chapter 2**), Δ*sseJ*::pSseJ (complementation vector), or Δ*sseJ*::pACYC184 (empty vector) *S.* Typhimurium at an MOI of 75 for 0.5, 1, or 18 hours prior to lysing cells and plating to enumerate CFUs. Internalization rate = CFU at 1 hour/CFU at 0.5 hour. Survival rate = CFU at 18 hour/CFU at 1 hour. Rates normalized to Δ*invG* control infection. All values are mean ± SD and n=3. **p<0.005, ***p<0.0005, ns=not significant. SseJ H384N mutants are significantly attenuated for deacylase activity and have a competitive disadvantage during in vivo mouse infection compared to wild-type *S.* Typhimurium [250]. Restoration of SseJ using a complementation vector with wild-type SseJ under its endogenous promoter restores survival compared to empty vector or catalytically inactive complementation. Unpublished data.

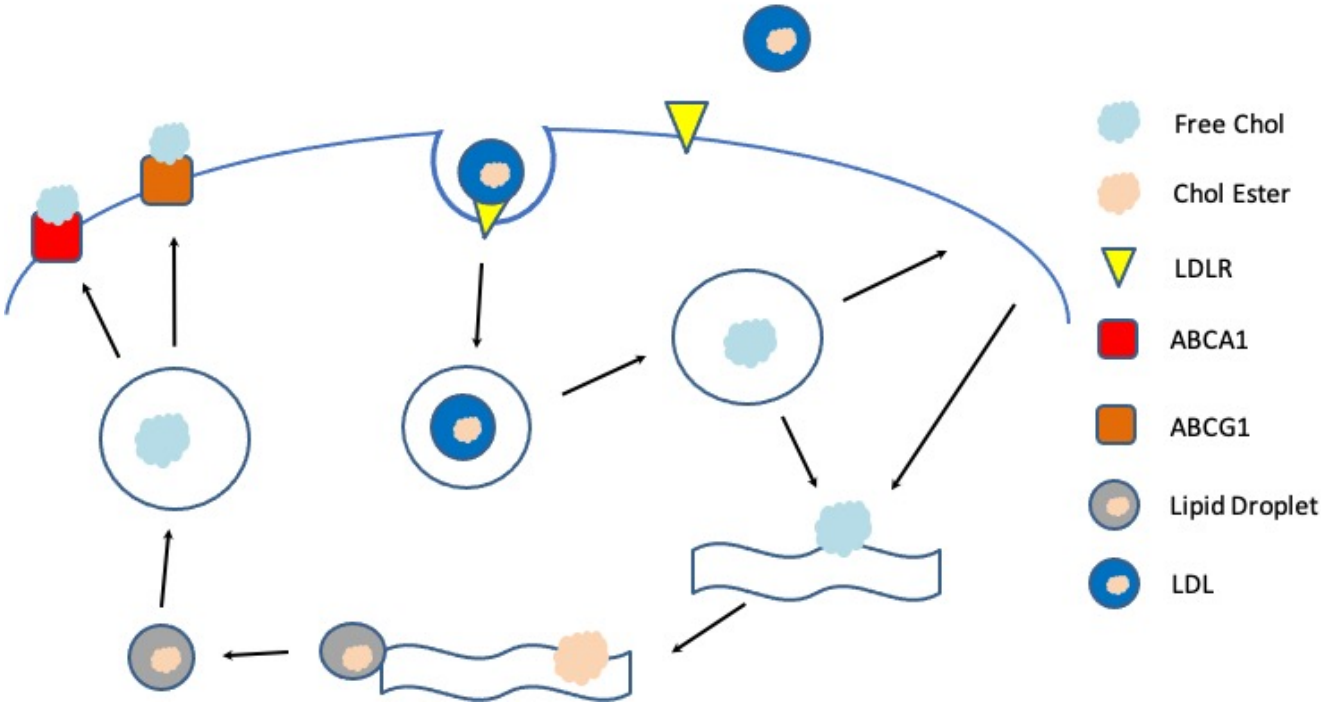

Free Chol
Chol Ester
LDLR
ABCA1
ABCG1
Lipid Droplet
LDL

Figure 1-5. Uptake and Transport of Extracellular Cholesterol.

Peripheral cells bind circulating LDL through both LDL and scavenger receptors. Subsequently, LDL is internalized via receptor-mediated endocytosis. Within the early endosome, receptors dissociate from LDL and are transported back to the PM or targeted to lysosomes for degradation. LDL is hydrolyzed in late endosomal compartments, dissociating the free cholesterol, fatty acids, and apolipoproteins. Free cholesterol is then shuttled across the endosomal membrane and trafficked out of the endocytic network directly to the ER, or indirectly through the PM as an intermediate (see **Figure 1-6**). In the ER, free cholesterol is esterified into cholesteryl esters. ER-resident proteins package cholesteryl esters along with triglycerides and additional proteins to form lipid droplets. Cholesteryl esters within lipid droplets can then be hydrolyzed back into free cholesterol and transported between cellular organelles. Excess free cholesterol is exported from the cell, mainly through ABCA1 and ABCG1. ABCA1 actively transports cholesterol across the PM where it is bound by apoA1 to form nascent HDL particles in the serum. Alternatively, ABCG1 facilitates cholesterol export onto circulating HDL particles.

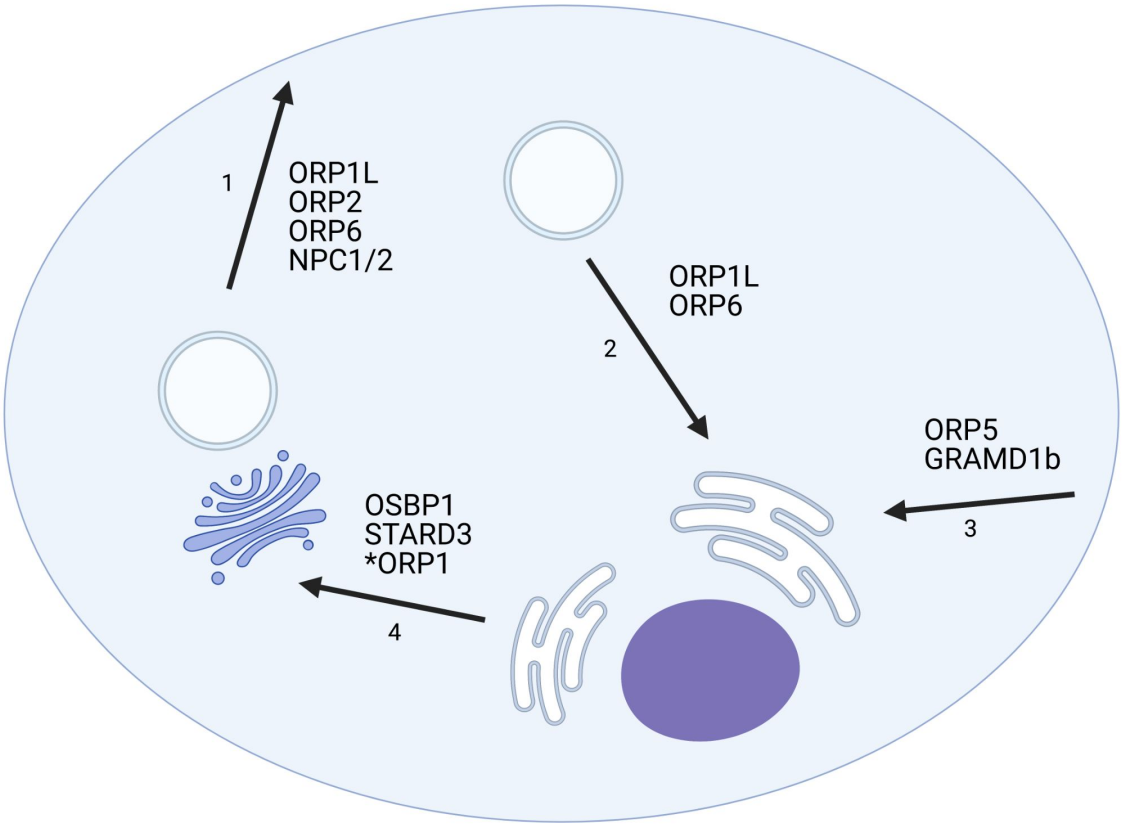

1
ORP1L
ORP2
ORP6
NPC1/2
ORP1L
ORP6
2
ORP5
GRAMD1b
3
OSBP1
STARD3
*ORP1
4

Figure 1-6. Intracellular Cholesterol Transport Proteins.

Intracellular trafficking of cholesterol is a highly coordinated effort requiring numerous lipid transport proteins. Here, we present a simplified view of a few major lipid transport proteins and where they function. 1) Following internalization, LDL cholesterol is hydrolyzed to free cholesterol in the endosomal network. Recent findings have suggested that cholesterol efflux out of the endosomes transports cholesterol to the PM prior to cholesterol transfer to the ER. NPC1/NPC2 are endosomal proteins that are required for cholesterol transport out of the endosomal lumen and towards the PM/ER. In the absence of NPC1 or NPC2, cholesterol accumulates in the endocytic network, leading to Niemann-Pick disease. While ORP1L and ORP6 are both important for cholesterol transport from the endosome to the ER, their exact location of transport is unclear. One possibility is that both proteins exchange endosomal cholesterol for PI(4)P on the PM, where it is subsequently transported to the ER. ORP2 has also been shown to promote cholesterol efflux from endosomes to the PM in exchange for $PI(4,5)P_2$. 2) Alternatively, endosomal free cholesterol has been suspected to directly transport from the endosome to the ER through ORP1L and ORP6. However, since the ER is deficient in PI(4)P, ORP1/6-mediated cholesterol transport would occur with an unidentified counterlipid or through unidirectional cholesterol transport without a counterlipid. 3) PM to ER cholesterol transport is facilitated by the GRAMD1 proteins, specifically GRAMD1b. ORP5 has also been implicated in PM to ER cholesterol transport; however, the potential counterlipids for these proteins remains unknown. 4) While a large portion of cholesterol is transported out of the ER following esterification and storage in lipid droplets, free cholesterol can directly be transported through OSBP1 and STARD3. OSBP1 is a cytosolic lipid transport protein

responsible for the exchange of ER cholesterol for PI(4)P in the Golgi. Additionally, STARD3 has been shown to promote endosome/ER MCS and promote the transport of protein out of the ER to the endosomal network. Paradoxically, ORP1L has been implicated in cholesterol transport out of the ER to endosomes and multivesicular bodies during sterol depletion, acting counter to its conventional role as an endosome to ER cholesterol transport protein. Through these mechanisms (and more), cells are able to transport cholesterol to various organelles to maintain cellular cholesterol homeostasis.

Effector	Location	Function	Citation
AvrA	SPI-1	Anti-inflammatory; anti-apoptotic	101; 102; 275
SipA	SPI-1	Actin Remodeling; induction of neutrophil transmigration	276; 277
SipB	SPI-1	Translocon; macrophage pyroptosis	37; 38; 83; 106
SipC	SPI-1	Translocon; actin remodeling	83; 91
SipD	SPI-1	Epithelial cell adhesion; apoptosis	38; 278
SopA	Chromosome	Proinflammatory response	98
SopB	SPI-5	Actin remodeling; PI phosphatase; proinflammatory response	47; 94; 99
SopD	Chromosome	Macropinosome formation; membrane fission; inflammatory response regulation	100; 279
SopE	Prophage	Actin remodeling; proinflammatory response; inositol phosphate phosphatase	11; 94; 97; 99
SopE2	Chromosome	Actin remodeling; proinflammatory response	11; 94; 99
SptP	SPI-1	Disruption of actin remodeling; Inhibition of NF-κB signaling	103; 104; 280
SteA*	Chromosome	SIF regulation, SCV positioning; Inhibition of NF-κB signaling	158; 159; 281
SteB*	Chromosome	Putative dipicolinate reductase	282

Table 1-1. List of *Salmonella enterica* T3SS-1 Effector Proteins.

Salmonella enterica effector proteins translocated through the SPI-1-encoded T3SS-1, where they are encoded within the *Salmonella* genome, and function. *SteA and SteB are primarily translocated through T3SS-2, with some translocation through T3SS-1. Both are found in *S*. Typhi, not *S*. Typhimurium. Gene localization acquired from [8; 283; 284; 285].

Effector	Location	Function	Citation
CigR	SPI-3	Unknown	
GogA	Prophage	Inhibition of NF-κB signaling	130
GogB	Prophage	Inhibition of NF-κB signaling	286
GtgA	Prophage	Inhibition of NF-κB signaling	130
GtgE	Prophage	Prevents localization of Rab proteins to SCV	141; 142
PipA	SPI-5	Inhibition of NF-κB signaling	130
PipB	SPI-5	Unknown	
PipB2	Chromosome	Recruitment of kinesin to SCV; SIF extension	142; 157; 287
SifA	Chromosome	SIF formation; maintenance of SCV membrane integrity	69; 145
SifB	Chromosome	Unknown	
SopD2	Chromosome	SCV membrane dynamics; prevents SCV-lysosome fusion	140; 145; 149; 150
SlrP	Chromosome	Inhibition of IL-1β	288
SrfJ	Chromosome	Putative glucosylceramidase	160
SsaB	SPI-2	Chaperone; inhibition of endosomal fusion	144
SseF	SPI-2	SCV positioning; microtubule transport	137; 289
SseG	SPI-2	SCV positioning; microtubule transport	137; 138; 289
SseI	Prophage	Actin cytoskeleton dynamics; inhibition of macrophage migration	247; 290
SseJ	Chromosome	Deacylase; Glycerophospholipid-cholesterol acyltransferase; membrane stability	150; 250; 251
SseK1	Chromosome	Inhibition of TNFα and NF-κB signaling	131
SseK2	Chromosome	Inhibition of TNFα and NF-κB signaling	291
SseK3	Prophage	Inhibition of TNFα and NF-κB signaling	131; 292
SseL	Chromosome	Lipid droplet suppression; inhibition of autophagy; inhibition of NF-κB signaling	133; 252; 258
SspH1	Prophage	Inhibition of NF-κB signaling	280

Effector	Location	Function	Citation
SspH2	SPI-12	Inhibition of actin polymerization; activation of NOD1 signaling	136; 293
SteA*	Chromosome	SIF regulation, SCV positioning; Inhibition of NF-κB signaling	158; 159; 281
SteB*	Chromosome	Putative dipicolinate reductase	282
SteC	Chromosome	SCV actin polymerization	294
SteD	Chromosome	Inhibition of MHC2 antigen presentation	295
SteE	Prophage	Promotes macrophage M2 polarization; anti-inflammatory signaling	135; 291; 296
SpvB	Plasmid	Inhibition of actin polymerization	293
SpvC	Plasmid	Dephosphorylation of MAP kinases	297
SpvD	Plasmid	Inhibition of NF-κB signaling	298

Table 1-2. List of *Salmonella* Typhimurium T3SS-2 Effector Proteins

A partial list of *Salmonella enterica* effector proteins translocated through the SPI-2-encoded T3SS-2, where they are encoded within the *Salmonella* genome, and function. *SteA and SteB are primarily translocated through T3SS-2, with some translocation through T3SS-1. Both are found in *S.* Typhi, not *S.* Typhimurium. Gene localization acquired from [8; 111; 283; 284; 285]

Chapter 2:

Salmonella Typhimurium manipulates macrophage cholesterol homeostasis through the SseJ-mediated suppression of the host cholesterol transport protein ABCA1

The following work has been published:
Greene AR, Owen KA, Casanova JE. Salmonella Typhimurium manipulates macrophage cholesterol homeostasis through the SseJ-mediated suppression of the host cholesterol transport protein ABCA1. *Cell Microbiol*. 2021 Mar 20:e13329. doi: 10.1111/cmi.13329. Epub ahead of print. PMID: 33742761.

Greene AG. performed most experiments and all analysis shown in Chapter 2 and wrote and revised the publication. Owen KA performed qPCR experiments in the published works of Chapter 2. Casanova JE provided intellectual guidance and reviewed and revised the manuscript.
*Minor wording changes have been made from the published manuscript to improve sentence clarity

Chapter 2: *Salmonella* Typhimurium manipulates macrophage cholesterol homeostasis through the SseJ-mediated suppression of the host cholesterol transport protein ABCA1

Abstract

Upon infection of host cells, *Salmonella enterica* serovar Typhimurium resides in a modified endosomal compartment referred to as the *Salmonella*-containing vacuole (SCV). SCV biogenesis is driven by multiple effector proteins translocated through two type III secretion systems (T3SS-1 and T3SS-2). While many host proteins targeted by these effector proteins have been characterized, the role of host lipids in SCV dynamics remains poorly understood. Previous studies have shown that *S.* Typhimurium infection in macrophages leads to accumulation of intracellular cholesterol, some of which concentrates in and around SCVs; however, the underlying mechanisms remain unknown. Here, we show that *S.* Typhimurium utilizes the T3SS-2 effector SseJ to downregulate expression of the host cholesterol transporter ABCA1 in macrophages, leading to a ~45% increase in cellular cholesterol. Mechanistically, SseJ activates a signaling cascade involving the host kinases FAK and Akt to suppress *Abca1* expression. Mutational inactivation of SseJ acyltransferase activity, silencing FAK, or inhibiting Akt prevents *Abca1* downregulation and the corresponding accumulation of cholesterol during infection. Importantly, RNAi-mediated silencing of ABCA1 rescued bacterial survival in FAK-deficient macrophages, suggesting that *Abca1* downregulation and cholesterol accumulation are important for intracellular survival.

Introduction

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that colonizes the intestinal epithelium and circulating phagocytes, causing disease ranging from mild gastroenteritis to severe septicemia. Upon internalization by host cells, *Salmonella* resides and proliferates within membrane-bound inclusions called *Salmonella*-containing vacuoles (SCVs). Pathogenic *Salmonella* strains express two type III secretion systems (T3SS-1 and T3SS-2), each of which translocate distinct arrays of effector proteins into the host cytosol. Although there is some functional overlap between T3SS-1 and T3SS-2, T3SS-1 effectors generally facilitate bacterial penetration of the intestinal epithelium, while T3SS-2 effectors promote the biogenesis of and bacterial survival within the SCV [132; 299].

Many host proteins targeted by these secreted effectors have been intensively studied, and in many cases their respective roles in invasion and/or intracellular survival are well characterized. In contrast, the role of host lipids and their manipulation by secreted effectors is less understood. Early studies reported that infection of both epithelial cells and macrophages by *S.* Typhimurium results in a significant increase in intracellular cholesterol and its precursors, much of which accumulates in and around SCVs [241]. The observed increase in cholesterol content requires a functional T3SS-2, indicating that it is actively induced by the pathogen, although specific effectors that may drive cholesterol accumulation have not been identified. Remarkably, although cholesterol accumulation occurs independently of *de novo* synthesis, later studies reported that the synthesis of one or more cholesterol precursors is necessary for optimal *Salmonella* survival [242]. Importantly, cholesterol has been reported on both the limiting membrane of SCVs and on

Salmonella-induced filaments (SIFs) extending from them [152], suggesting an important role for cholesterol in SCV dynamics and intracellular survival.

While these early studies did not detail the mechanisms through which *Salmonella* perturbs cholesterol homeostasis, it is important to note that two T3SS-2 effectors, SseJ and SseL, have reported roles in cholesterol metabolism and transport. SseJ is a RhoA-dependent cholesterol acyltransferase containing both deacylase and glycerophospholipid:cholesterol acyltransferase (GCAT) activity. Key to SseJ activity is the catalytic triad of, Ser141, Asp247, and His384. Importantly, mutating any one of these catalytic residues abolishes SseJ activity and attenuates *Salmonella* virulence in mice [250]. SseJ localizes to the cytosolic surface of the SCV where it catalyzes the transfer of acyl chains from phospholipids onto free cholesterol, generating cholesteryl esters [245; 250; 251; 300]. This function is homologous to eukaryotic ACAT, which esterifies cholesterol to generate cholesteryl esters within the endoplasmic reticulum (ER) and promotes the storage of excess cholesteryl esters in lipid droplets [207]. Additionally, both SseJ and SseL have been shown to interact with the host cholesterol transport protein oxysterol binding protein 1 (OSBP1) and recruit it to SCVs [257, 258]. OSBP1 is known to mediate the non-vesicular transfer of cholesterol from the ER to the Trans-Golgi Network (TGN) and endosomes, suggesting that SseJ and SseL play a role in redirecting cholesterol transport from the ER to the SCV. In agreement with this hypothesis, infection with an SseL-deficient strain of *S.* Typhimurium leads to an accumulation of large lipid droplets within the host cell [178; 257]. Importantly, bacterial strains deficient in either SseJ or SseL are attenuated in virulence in both macrophage and animal models [150; 253],

demonstrating that modulation of host cell lipid homeostasis plays an important role during *Salmonella* pathogenesis.

Cholesterol is a critical component of eukaryotic membranes, whereas prokaryotic membranes are devoid of cholesterol. The distribution of lipids within eukaryotic membranes is heterogenous; plasma membrane (PM) and late endosomal lipid bilayers are enriched with cholesterol, while the ER contains low amounts of cholesterol at steady state [166]. Due to the importance of cholesterol in maintaining membrane stability and function, cellular cholesterol concentrations are normally under tight regulatory control.

Cholesterol homeostasis is maintained by balancing uptake (via low-density lipoprotein; LDL) and biosynthesis in the ER with the storage and export of excess cholesterol [301; 302]. Excess cholesterol is handled in two ways; it can be esterified and stored within ER-derived lipid droplets and/or exported out of the cell by a family of ATP-binding cassette (ABC) transporters, predominantly ABCA1 and ABCG1 [207; 303]. These proteins shuttle intracellular pools of cholesterol to the PM where it is transferred to apolipoprotein A-1 (via ABCA1), generating nascent high-density lipoprotein (HDL), or onto mature HDL (via ABCG1) [304; 305; 306]. It is becoming increasingly evident that many pathogenic microorganisms, including *Salmonella* Typhi and *Salmonella* Typhimurium, target cholesterol-enriched lipid domains as well as intracellular free cholesterol in order to facilitate microbial entry, intracellular localization, and other pathogenic functions [82; 240; 307; 308; 309; 310; 311].

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase typically associated with integrin-mediated signaling. We recently reported the surprising observation that *S.* Typhimurium recruits FAK to the cytoplasmic face of SCVs in infected macrophages in a

T3SS-2-dependent manner. FAK activation at this site stimulates the Akt/mTOR signaling axis, thereby suppressing the autophagic capture and killing of intracellular *Salmonella* [41]. Remarkably, FAK and its downstream effector Akt have been shown to control cholesterol levels in fibroblasts by inversely regulating the expression of ABCA1 as a function of cell density [228]. In that study, the absence of FAK led to elevated *Abca1* expression independent of cell density and correlated with a decrease in cellular cholesterol and sterol esters, demonstrating a novel role for FAK in the regulation of cholesterol homeostasis.

Here, we show that activation of the FAK/Akt signaling axis by intracellular *S.* Typhimurium induces cholesterol accumulation in macrophages by suppressing *Abca1* expression. Bacterial survival is significantly attenuated in FAK-deficient macrophages as well as in wild-type (WT) macrophages treated with the pharmacological LXR agonist, T0901317, to induce *Abca1* expression. Importantly, survival within FAK-depleted macrophages can be restored to wild-type levels by siRNA-mediated depletion of ABCA1. Mechanistically, we found that FAK/Akt activation is mediated by the T3SS-2 effector SseJ. In contrast to WT *S.* Typhimurium, an SseJ-deficient strain fails to activate FAK or Akt, does not downregulate *Abca1* expression, and fails to accumulate cholesterol, resulting in reduced survival. Importantly, the catalytic activity of SseJ is necessary for cholesterol accumulation, as a loss-of-function mutation of the catalytic residue His384 failed to activate FAK or increase intracellular cholesterol. Together, these results define a novel mechanism through which *Salmonella* alters intracellular cholesterol homeostasis in a FAK- and ABCA1-dependent manner to promote survival in macrophages.

Results

Salmonella infection promotes cholesterol accumulation in primary mouse macrophages

Previously, *S.* Typhimurium had been shown to induce accumulation of cholesterol and its precursors in cultured immortalized macrophages through an unknown mechanism [241]. Here, we first confirmed that cholesterol accumulation occurs in primary bone marrow-derived macrophages (BMDMs) harvested from wild-type C57BL/6 mice. C57Bl/6 mice were chosen because 1) they are susceptible to *Salmonella* infection due to the lack of functional Nramp1 and are widely used for this purpose [312] and 2) to maintain consistency with our earlier work in which we generated mice conditionally lacking FAK in the myeloid lineage on the C57Bl/6 background [41]. Since invasive *Salmonella* rapidly kills macrophages in a T3SS-1-dependent manner [107], we used a mutant of *S.* Typhimurium strain SL1344 that lacks a functional T3SS-1 (Δ*invG*) [313]. As T3SS-1 and associated effectors are dispensable for *Salmonella* dissemination from the gastrointestinal tract and subsequent invasion of phagocytic cells [109], this Δ*invG* strain provides an established model of infection for macrophages which avoids potential T3SS-1-induced cytotoxicity.

To visualize cholesterol abundance and distribution in primary macrophages, BMDMs were incubated with BODIPY-cholesterol, a stable fluorescent cholesterol analog that is widely used to study the trafficking of cholesterol in live cells [314]. For this purpose, cells were infected with RFP-expressing Δ*invG Salmonella* for a total of 18 hours prior to live-cell imaging. 90 minutes prior to imaging, cells were incubated for 45 minutes in serum-free culture media containing 0.5 μM BODIPY-cholesterol, followed by a 45 minute chase in the absence of labeled cholesterol. Images were then acquired of

uninfected and infected cells in the same culture. As shown in **Figure 2-1, A-B**, infected BMDMs exhibited markedly increased BODIPY-cholesterol fluorescence compared to uninfected cells. Interestingly, in addition to BODIPY-cholesterol being found associated with SCVs, it was also highly enriched in other intracellular compartments, presumably elements of the endocytic pathway. Quantification of the mean fluorescence intensity (MFI) of BODIPY-cholesterol in individual cells revealed a 2.6-fold increase in MFI in infected BMDMs compared to uninfected cells (**Figure 2-1, C**), in agreement with previous findings in immortalized cells [152; 241].

Next, we tested whether *Salmonella* alters the levels of endogenous cholesterol in BMDMs. Cells were infected or mock-infected for 18 hours before total cellular lipids were harvested using the Bligh and Dyer method [315]. Free cholesterol was then measured using a fluorometric cholesterol assay as previously described [316]. In agreement with the findings above, *S.* Typhimurium infection increased the content of free cholesterol by approximately 45% relative to uninfected BMDMs (**Figure 2-1, D**). As not all cells in the population become infected, cholesterol accumulation in infected cells likely exceeds 45% on a per-cell basis. Both the visual and biochemical cholesterol quantification results were recapitulated using immortalized BMDMs (iBMDMs) (**Figure 2-S1, A-E**) [317]. Together, these results demonstrate that *Salmonella* Typhimurium induces the accumulation of cholesterol during the later stages of infection in both primary and immortalized bone marrow-derived macrophages.

Activation of FAK/Akt signaling downregulates expression of the cholesterol exporter Abca1

We recently demonstrated that the non-receptor tyrosine kinase FAK is recruited to the

SCV by *S.* Typhimurium in a T3SS-2-dependent manner, and that this leads to activation of the serine/threonine kinase Akt on the surface of the vacuole [41]. Remarkably, FAK/Akt signaling has been shown to stimulate cholesterol accumulation in fibroblasts by downregulating transcription of the cholesterol export protein, ABCA1 [228]. As ABCA1 is the predominant cholesterol exporter in macrophages, we hypothesized that *Salmonella*-induced FAK/Akt signaling may similarly downregulate ABCA1 expression, leading to the cholesterol accumulation seen during macrophage infection. To test this hypothesis, iBMDMs were depleted of endogenous FAK using siFAK oligonucleotides or mock-depleted with non-coding siRNA, then infected with *S.* Typhimurium for either 5h or 24h. Cells were then lysed and immunoblotted to detect total and phosphorylated (active) FAK (pY397) or Akt (pS473) at each time point. In agreement with our previous findings, infection of untreated control macrophages resulted in robust phosphorylation of both FAK and Akt at 5h and 24h post-infection (**Figure 2-2, A-C**). As expected, depletion of FAK reduced overall FAK expression and substantially inhibited the downstream phosphorylation of Akt (**Figure 2-S2, A**).

Next, we sought to assess whether *S.* Typhimurium regulates *Abca1* expression during infection of control or FAK-depleted iBMDMs by measuring mRNA levels using qPCR. As shown in **Figure 2-2, D**, infection of control iBMDMs led to a 50% reduction in *Abca1* mRNA at 5h and 72% at 18h post-infection, relative to uninfected cells (dashed line). In contrast, infection of FAK-depleted cells failed to reduce *Abca1* mRNA below uninfected levels, and instead transiently increased *Abca1* expression (**Figure 2-2, D**). These findings suggest that *Salmonella* may interfere with cholesterol efflux through the FAK-dependent downregulation of *Abca1*. To determine whether FAK-dependent

downregulation was specific to ABCA1, we measured the expression of another ABC-family cholesterol transport protein, ABCG1. As shown in **Figure 2-2, E**, *Abcg1* mRNA levels were suppressed during infection of both control and FAK-depleted cells, suggesting that *S.* Typhimurium also downregulates expression of *Abcg1*, but independently of FAK. To determine whether the suppression of *Abca1* was specific to *Salmonella* invasion, iBMDMs were infected with non-pathogenic *Escherichia coli* (DH5α) and mRNA expression was measured by qPCR as above. Remarkably, DH5α failed to downregulate *Abca1*, instead inducing a significant increase in expression (**Figure 2-S2, B**), indicating that the suppression of *Abca1* is actively mediated by *Salmonella* and is not an innate host response to Gram-negative bacteria.

Since Akt signaling downstream of FAK is important for *Abca1* suppression in fibroblasts, we sought to determine whether Akt signaling is also necessary for the downregulation of *Abca1* during *Salmonella* infection. iBMDMs were treated with the pharmacological Akt inhibitor triciribine for 1 hour prior to and throughout a 5 hour infection with *S.* Typhimurium. As anticipated, triciribine did not affect FAK^{Y397} phosphorylation or levels of total Akt during infection, but severely attenuated Akt^{S473} phosphorylation (**Figure 2-S2, C**). Similar to FAK depletion, Akt inhibition not only prevented *Salmonella*-mediated downregulation of *Abca1*, but actually increased mRNA levels by approximately 40% relative to uninfected controls, while *Abcg1* expression was downregulated independently of Akt (**Figure 2-2, F-G**). Together, these results demonstrate that *Salmonella* specifically downregulates the expression of *Abca1* in a FAK- and Akt-dependent manner.

Failure to downregulate Abca1 prevents cholesterol accumulation in infected macrophages

ABCA1 plays a major role in maintaining lipid homeostasis in macrophages and other cell types by facilitating cholesterol efflux [303; 318]. Since the findings above reveal that FAK/Akt signaling is required for *Salmonella*-induced downregulation of *Abca1* expression, we reasoned that cholesterol retention would be reduced during infection of FAK-deficient macrophages. To test this hypothesis, we used BMDMs derived from mice conditionally lacking FAK in myeloid lineage cells, which we previously showed completely lack endogenous FAK [41]. BMDMs derived from these mice ($FAK^{-/-}$ BMDMs) or their WT littermates were infected with RFP-expressing *S.* Typhimurium and incubated with BODIPY-cholesterol as described in **Figure 2-1**. As observed above, infection of WT BMDMs resulted in a dramatic increase in BODIPY-cholesterol content by 18 hours post-infection relative to uninfected controls, with much of the fluorescence enriched in intracellular compartments (**Figure 2-3, A-B**). In contrast, BODIPY-cholesterol fluorescence remained similar between infected and uninfected $FAK^{-/-}$ BMDMs (**Figure 2-3, C-D**). These morphological observations are reflected by the MFI analysis, where infection caused a 4.8-fold increase in BODIPY-cholesterol fluorescence in infected WT BMDMs relative to uninfected cells and no significant difference in FAK-deficient macrophages (**Figure 2-3, E**). In agreement with these fluorescence data, the fluorometric cholesterol assay indicated that infected WT BMDMs exhibited a ~45% increase in endogenous free cholesterol, while no change was observed in $FAK^{-/-}$ BMDMs relative to uninfected cells (**Figure 2-3, F**).

Previous studies have shown that activation of the transcription factor liver X receptor (LXR) positively regulates ABCA1 expression and stimulates cholesterol efflux [319]. Among the pharmacological LXR agonists that have been developed, T0901317 has been shown to induce ABCA1 expression in murine macrophages [227]. We hypothesized that pharmacological induction of *Abca1* expression might counteract the suppression of *Abca1* triggered by *Salmonella* infection. To test this hypothesis, macrophages were either mock-treated or pretreated with 10 μM T0901317 18 hours prior to infection and maintained in the drug throughout the course of infection. *Abca1* mRNA was measured by qPCR at 5h and 24h post-infection. As shown in **Figure 2-3, G**, treatment of cells with T0901317 increased *Abca1* expression 6-fold at 5h and 8-fold at 24h post-infection, indicating that pharmacological activation of LXR can successfully bypass *Salmonella*-mediated *Abca1* suppression. In contrast to control BMDMs, treatment of cells with T0901317 completely abrogated the accumulation of BODIPY-cholesterol in infected cells at 18h (**Figure 2-3, H-L**). As expected, these findings were mirrored by the fluorometric cholesterol assay, where T0901317 treatment caused the concentration of free cholesterol in infected BMDMs to remain near uninfected levels (**Figure 2-3, M**). As expected, these findings were recapitulated during T0901317 treatment of iBMDMs (**Figure 2-S3, A-F**). Together, these observations strongly suggest that the *Salmonella*-mediated suppression of *Abca1* promotes cholesterol accumulation in infected macrophages.

Suppression of Abca1 enhances Salmonella survival in macrophages

The findings described above suggest that the FAK-dependent accumulation of cholesterol is important for virulence of *Salmonella* in macrophages. To test whether *Abca1* suppression and the subsequent increase in intracellular cholesterol enhanced *S.*

Typhimurium survival in macrophages, we utilized a standard gentamicin protection assay to measure bacterial internalization and survival in immortalized and primary BMDMs. As shown in **Figure 2-4, A**, internalization of *S.* Typhimurium in iBMDMs pretreated with either 10 mM triciribine or 10 μM T0901317 was similar to control cells treated with vehicle alone. In contrast, inhibition of Akt or the induction *Abca1* expression reduced bacterial survival by ~50% at 18h post-infection (**Figure 2-4, A**). To ensure that the reduced bacterial survival upon T0901317 treatment was due to *Abca1* induction and not-off target toxicity, we pretreated WT and FAK-deficient BMDMs with T0901317 and measured bacterial survival. As above, T0901317 treatment had no effect on *S.* Typhimurium internalization in either WT or $FAK^{-/-}$ BMDMs, but survival was significantly reduced in WT cells (**Figure 2-S4, A**). In agreement with our previous findings that *Salmonella*-mediated FAK activation suppresses killing of intracellular bacteria [41], bacterial survival in untreated $FAK^{-/-}$ BMDMs was significantly reduced. Importantly, pretreatment of FAK-deficient BMDMs with T0901317 did not further reduce *Salmonella* survival in these cells (**Figure 2-S4, A**), suggesting that reduced bacterial survival was not a result of off-target toxicity, but rather the inability of *S.* Typhimurium to downregulate *Abca1*.

To directly confirm that the decreased survival of *S.* Typhimurium in FAK-deficient macrophages is due to the failure to suppress *Abca1*, WT and FAK-deficient BMDMs were depleted of endogenous *Abca1* using siRNA (**Figure 2-S4, B**). As anticipated, bacterial internalization was unaffected by *Abca1* depletion in either WT or $FAK^{-/-}$ BMDMs compared to siLuciferase-treated control cells (**Figure 2-4, B**). Interestingly, *Abca1* knockdown had no effect on intracellular survival in WT BMDMs,

suggesting that any further reduction in *Abca1* beyond that caused by the bacteria had no additional impact on survival. In contrast, depletion of *Abca1* in FAK-deficient cells restored *S*. Typhimurium survival to levels found in WT cells, indicating that the observed decrease in bacterial survival is directly associated with the inability to downregulate *Abca1* expression. These data demonstrate that the FAK-dependent suppression of *Abca1* is important for *Salmonella* survival in macrophages.

The T3SS-2 effector SseJ is necessary for FAK activation and subsequent cholesterol accumulation

Within 2-6 hours of infection, conditions within the SCV trigger the expression of T3SS-2 and more than 30 effector proteins that are translocated through it into the host cytosol [124; 132]. We have previously shown that activation of FAK by *S.* Typhimurium requires T3SS-2 [41], but the specific effectors involved remained unidentified. Among the effectors translocated by T3SS-2, SseJ and SseL have been previously implicated in cholesterol regulation. To investigate whether these or other T3SS-2 effectors were involved in FAK activation, we infected iBMDMs with a panel of SL1344 T3SS-2 effector mutants. These mutants were all on a Δ*orgA* background which lacks a component of the sorting system for effector delivery through the T3SS-1. A Δ*orgA* mutant fails to produce the T3SS-1 "needle" apparatus, resulting in the loss of a functional SPI-1 T3SS, similar to a Δ*invG* mutant [77; 78]. At 24h post-infection, cells were then lysed and immunoblotted for $pFAK^{Y397}$ and $pAkt^{S473.}$ As shown in **Figure 2-5, A**, infection with Δ*sifA,* Δ*sseF,* Δ*sseI,* and Δ*sseL* strains induced similar levels of FAK^{Y397} phosphorylation relative to Δ*invG.* In contrast, infection with a strain lacking both T3SS-1 and T3SS-2 or a specific Δ*sseJ* mutant failed to increase FAK phosphorylation above uninfected levels. In agreement with our

previous observation that FAK activity is required for downstream phosphorylation of Akt, both the ΔT3SS-1/ΔT3SS-2 and Δ*sseJ* strains also failed to induce Akt^{S473} phosphorylation during infection (**Figure 2-5, B**). These observations clearly demonstrate that the activation of both FAK and Akt during *S.* Typhimurium infection requires the specific T3SS-2 effector, SseJ.

Because SseJ is essential for the activation of both FAK and Akt, we next sought to determine whether SseJ is also necessary for suppression of *Abca1* transcription. For this purpose, we infected control or FAK-depleted iBMDMs with either Δ*invG* or Δ*sseJ* mutant strains and measured *Abca1* mRNA by qPCR. As shown above (**Figure 2-2, B**), infection with the Δ*invG* strain resulted in significantly reduced *Abca1* expression in a FAK-dependent manner relative to uninfected controls (**Figure 2-5, C**). In contrast, infection with a Δ*sseJ* strain not only failed to downregulate *Abca1,* but rather increased its expression by two-fold, independent of FAK (**Figure 2-5, C**). Interestingly, this increase is similar to the response to non-pathogenic *E. coli* (**Figure 2-S2**), suggesting that in the absence of active suppression, the innate host response to Gram-negative infection increases *Abca1* expression in macrophages.

Subsequently, we confirmed that SseJ is essential for cholesterol accumulation in *S.* Typhimurium-infected macrophages. As observed above (**Figure 2-1, A-C**), BMDMs infected with the T3SS-1-deficient Δ*invG* strain contain significantly more BODIPY-cholesterol than uninfected cells (**Figure 2-5, D**). Conversely, infection with the Δ*sseJ* mutant did not detectably increase the level of BODIPY-cholesterol fluorescence relative to uninfected cells (**Figure 2-5, D, Figure 2-S5, A-B**), phenocopying the loss of FAK or Akt activity as well as T0901317 treatment in which *Abca1* is not downregulated.

Similarly, biochemical analysis indicated that while infection with the Δ*invG* strain increased endogenous free cholesterol by 50% at 18 hours post-infection, BMDMs infected with the Δ*sseJ* mutant exhibited no increase in cholesterol content relative to uninfected cells (**Figure 2-5, E**). Complementation of the Δ*sseJ* strain with a plasmid encoding SseJ under control of its endogenous promoter (pSseJ) restored accumulation of both BODIPY-cholesterol (**Figure 2-5, F, Figure 2-S5, C**) and endogenous cholesterol (**Figure 2-5, G**) in infected cells, relative to the empty vector (pACYC184) (**Figure 2-S5, D**). Together, these data suggest that SseJ-mediated activation of FAK is critical for the downregulation of *Abca1* and the subsequent accumulation of cholesterol in infected macrophages.

Finally, and consistent with previous reports [150], survival of the Δ*sseJ* mutant was reduced by ~60% in BMDMs compared to Δ*invG* (**Figure 2-S5, E**), and largely restored by complementation with the pSseJ expression vector compared to the empty vector alone (**Figure 2-S5, F**). As standard gentamicin protection assays are only able to assess bacterial survival in a population of cells, we used an immunofluorescence-based assay to determine *S.* Typhimurium survival within individual cells. Similar to the gentamicin protection assay, we found that both the Δ*invG* and Δ*sseJ* strains infected a similar percentage of BMDMs at 1h and 5h. However, by 18h post-infection, a significantly smaller percentage of cells contained the Δ*sseJ* strain compared to Δ*invG* (**Figure 2-S5, G**). The same results were found by pretreating BMDMs with T0901317 prior to Δ*invG* infection (**Figure 2-S5, G**), suggesting that the failure to suppress *Abca1* and the subsequent inability to accumulate cholesterol reduces *S.* Typhimurium survival by enhancing bacterial clearance from infected macrophages.

SseJ catalytic activity is important for S. Typhimurium-induced cholesterol accumulation

SseJ is a cholesterol acyltransferase, transferring acyl chains from glycerophospholipids onto free cholesterol, generating cholesteryl esters [250; 251]. This activity allows SseJ to modify the lipid content and potentially alter the biophysical properties of host membranes. As detailed in previous reports, mutating any of three specific catalytic residues (S141, D247, H384) eliminates both SseJ deacylase and acyltransferase activity and reduces *Salmonella* survival [250]. Therefore, to assess whether the catalytic activity of SseJ was necessary for FAK activation and cholesterol accumulation, we generated a mutation of His384N within the pSseJ rescue vector detailed above. Subsequently, iBMDMs were infected with Δ*invG,* Δ*sseJ*::pSseJ, Δ*sseJ*::pACYC184*,* or Δ*sseJ*::H384N strains of *S.* Typhimurium for 18h prior to lysing cells for immunoblotting. As described above, activation of both FAK and Akt occurs in the presence, but not absence of, SseJ (compare Δ*sseJ*::pSseJ to Δ*sseJ*::pACYC184). Importantly, catalytically inactive SseJ (Δ*sseJ*::H384N) failed to induce either FAK or Akt phosphorylation (**Figure 2-6, A-B**), suggesting that the activation of FAK/Akt signaling depends on the catalytic activity of SseJ.

Finally, we determined the importance of SseJ catalytic activity in inducing cholesterol accumulation by infecting BMDMs with the strains outlined above and extracting lipids to measure endogenous cholesterol. As expected, infection with either Δ*invG* or Δ*sseJ*::pSseJ resulted in a significant increase in cellular cholesterol levels, while the Δ*sseJ*::pACYC184 empty vector control failed to increase cholesterol above uninfected levels. Similarly, infection with the strain expressing catalytically inactive SseJ (Δ*sseJ*::H384N) completely failed to increase levels of free cholesterol in BMDMs relative

to the empty vector Δ*sseJ* strain (**Figure 2-6, C**). Taken together, these results demonstrate that the catalytic activity of SseJ is critical to induce cholesterol accumulation in infected cells, through the FAK- and Akt-dependent suppression of *Abca1* transcription.

Discussion

Cholesterol is an indispensable constituent of eukaryotic membranes and is widely acknowledged as one of the most important regulators of lipid organization. As such, cholesterol homeostasis is tightly regulated by balancing uptake and synthesis with storage and export. Unsurprisingly, numerous intracellular pathogens have been shown to manipulate cholesterol homeostasis as part of their virulence mechanisms [82; 157; 307; 308; 309; 310; 311]. In the case of *S.* Typhimurium, it is well established that infection of host cells results in a significant increase in cholesterol content, but how this is achieved has remained unknown. Here we show that the T3SS-2 effector SseJ induces cholesterol accumulation in infected macrophages by downregulating expression of the cholesterol export protein ABCA1. Mechanistically, we show that the catalytic activity of SseJ initiates signaling through the host tyrosine kinase FAK and the subsequent FAK-dependent activation of Akt. Remarkably, FAK/Akt signaling has been shown to suppress expression of *Abca1* in fibroblasts through inhibitory phosphorylation of two transcription factors that drive transcription of *Abca1*, FOXO3 and TAL1 [228]. Here, we find that *S.* Typhimurium increases cellular cholesterol concentrations by downregulating *Abca1* expression in a FAK- and Akt-dependent manner. Conversely, pharmacological induction of *Abca1* during infection circumvents bacterial suppression and suppresses cholesterol accumulation in infected cells. An *S.* Typhimurium strain lacking SseJ fails to activate FAK or Akt, fails to downregulate *Abca1*, and fails to induce cholesterol accumulation. The inability of the

ΔsseJ strain to suppress *Abca1* and accumulate cholesterol significantly attenuates bacterial survival and is phenocopied by pharmacological induction of *Abca1* during wild-type infection. As we previously reported [41], survival of *S.* Typhimurium in FAK-deficient macrophages is significantly attenuated. Here we show that depletion of endogenous *Abca1* in FAK-deficient macrophages restores survival to control levels. Together, these results indicate that bacterial manipulation of cholesterol homeostasis through the SseJ/FAK/Akt-dependent suppression of *Abca1* contributes significantly to intracellular survival.

Activation of FAK by SseJ

At present, it remains unclear how SseJ activates FAK during infection. SseJ localizes to the cytosolic surface of SCVs, both during infection and when ectopically expressed [245; 251]. We previously demonstrated that FAK is recruited to SCVs during infection, where it is activated in a T3SS-2-dependent manner [41]. FAK exists in the cytosol in an autoinhibited conformation in which an N-terminal FERM domain folds against the kinase domain [259]. Activation typically occurs by interaction of the FERM domain with integrins, other receptors, or by direct interaction with membrane phosphoinositides, thereby relieving autoinhibition [259; 263; 320]. Although it is possible that direct binding of SseJ to the FAK FERM domain could promote FAK activation, our observation that the catalytic activity of SseJ is essential for FAK activation suggests that modification of the local lipid environment, either through the generation of lysophospholipids via its deacylase activity or the extraction of cholesterol from host membranes through esterification, has an important role. Whether this leads to FAK activation directly or indirectly remains unknown and will be the subject of future investigation.

Cholesterol metabolism in Salmonella-infected cells

In uninfected cells, depletion of cholesterol induces *de novo* sterol synthesis, while increased cholesterol content leads to activation of nuclear LXR receptors. LXRs target genes that promote cholesterol clearance by coordinately downregulating expression of LDL receptors and upregulating cholesterol efflux genes, including *ABCA1* [227; 321]. Current models propose a mechanism by which ABCA1 moves cholesterol from the cytoplasmic leaflet to the extracellular leaflet of the plasma membrane where it is transferred to circulating ApoA1, generating HDL in the plasma [303; 306; 321]. Loss of ABCA1, which occurs in Tangier's disease, results in intracellular retention of cholesterol and prevents ER cholesterol sensing by impairing retrograde cholesterol transport from the PM to the ER [193; 304; 305]. This is notable, as previous studies using metabolic labeling have shown that *de novo* cholesterol synthesis is actually increased in response to *S.* Typhimurium infection despite elevated cholesterol levels [241; 242; 322]. Therefore, the downregulation of ABCA1 may also inhibit cholesterol sensing by the ER, allowing for the continuation of sterol synthesis during infection.

Although *de novo* synthesis accounted for a relatively small fraction of total cellular cholesterol in infected macrophages, these studies suggest that production of non-sterol cholesterol precursors, rather than mature cholesterol, contributes to intracellular survival. Statins, which inhibit an early stage of cholesterol biosynthesis, were found to reduce *Salmonella* survival in macrophages, while the addition of mevalonate restored survival to control levels [242]. However, inhibition of either squalene oxidase or Δ24 sterol oxidase, later enzymes in the sterol synthesis pathway, had no effect on survival [242; 322]. While these studies suggest that cholesterol biosynthesis is unnecessary for *Salmonella* survival,

our findings suggest that a large fraction of the accumulated cholesterol is derived from the uptake of and inability to export excess cholesterol.

Role of cholesterol accumulation in Salmonella survival

How cholesterol accumulation in the endocytic pathway benefits *S.* Typhimurium remains uncertain; however, there are several possibilities that are not mutually exclusive. First, high cholesterol has been shown to inhibit autophagic flux by sequestering the SNARE proteins required for autophagosome/lysosome fusion [323; 324]. Flux can be restored by depletion of cholesterol with methyl-β-cyclodextrin or T0901317 treatment [325; 326], suggesting that cholesterol accumulation may protect the bacteria from autophagic clearance. Furthermore, cholesterol delivery from the ER to the cytoplasmic leaflet of endolysosomes via OSBP promotes the recruitment and activation of mTORC1, leading to suppression of autophagy [327]. Thus, an important role of *Salmonella*-dependent cholesterol accumulation may be to inhibit autophagic clearance of the bacteria.

Second, recent evidence indicates that OSBP is specifically recruited to the SCV in epithelial cells through its interaction with both SseJ and SseL, where it is required for vacuolar stability [258]. Depletion or inhibition of OSBP leads to an increase in cytosolic bacteria that have escaped the vacuole. While this suggests that cholesterol delivery to the vacuole from the ER may be essential for SCV stability, the requirement for lipid transfer activity was not tested directly.

Third, elevated cholesterol content inhibits lysosomal maturation and the acidification of late endosomes/lysosomes by inhibiting vacuolar ATPase pump activity [328]. High cholesterol has been reported to block recycling of mannose-6-phosphate receptors (MPRs) from endosomes to the TGN, resulting in extracellular secretion of

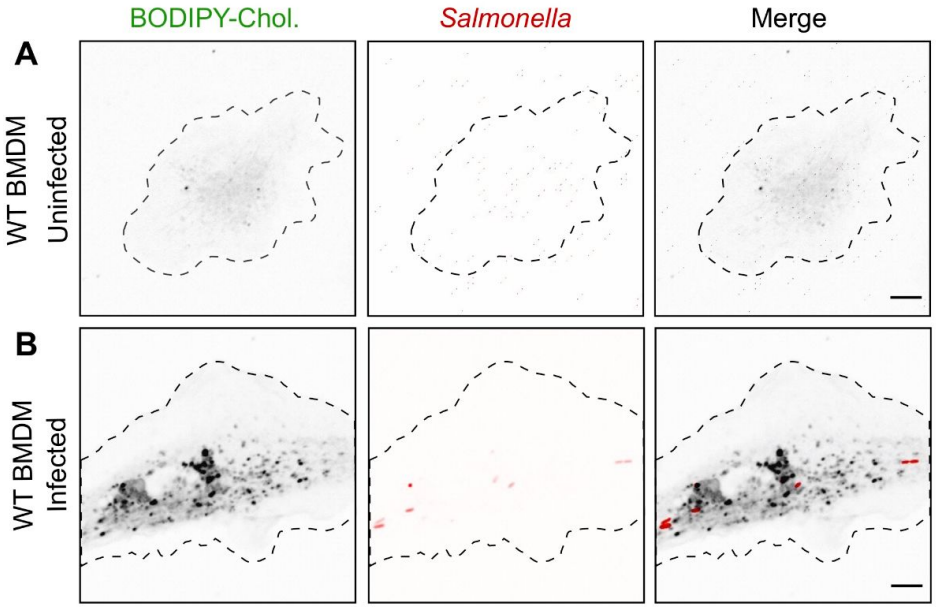
lysosomal hydrolases and their depletion from lysosomes [329; 330]. It is well established that SCVs contain low levels of lysosomal hydrolases despite the presence of lysosomal membrane proteins in their limiting membrane [54]. This is due, at least in part, to the formation of a stable complex between the T3SS-2 effector SifA and host Rab9, which inhibits the Rab9-dependent recycling of MPR to the TGN [58]. However, accumulation of endosomal cholesterol in cells also inhibits MPR recycling by sequestering Rab9 in the absence of infection [329], and it is possible these two mechanisms act in tandem to deplete lysosomal hydrolases from SCVs. Therefore, cholesterol accumulation may prevent the acidification of endosomes and allow for *S.* Typhimurium to avoid exposure to antimicrobial hydrolases, ensuring bacterial survival.

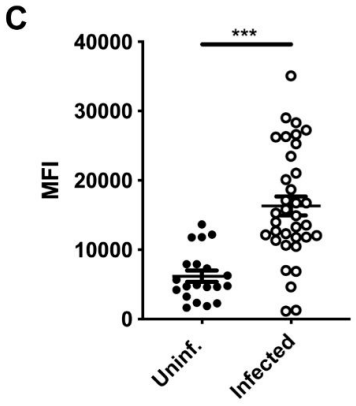
Fourth, recent evidence suggests that SIFs are important for *Salmonella* growth and survival within host cells by facilitating access of intravacuolar bacteria to host nutrients and enhancing bacterial metabolism [60; 151]. SIFs are rich in cholesterol [152], but whether cholesterol is essential for their formation or function remains unknown. Although the role of SseJ in SIF formation has been previously investigated, the data are conflicting [150; 157; 331]. However, ectopic co-expression of SseJ and SifA is sufficient to induce the formation of SIF-like tubules from endolysosomal compartments in a manner that requires SseJ catalytic activity [246], suggesting that remodeling of the endolysosomal membrane by SseJ is essential for tubulation. These findings highlight the importance of cholesterol in regulating endolysosomal dynamics and suggest that *Salmonella*-induced cholesterol accumulation may promote SCV integrity and manipulate endolysosomal trafficking. Because cholesterol plays a major role in the regulation of membrane sorting and the partitioning of proteins between membrane domains [332; 333], it is reasonable to

expect that the level of cholesterol accumulation seen during *S.* Typhimurium infection significantly impacts many host processes by altering endosomal trafficking. Future studies will be necessary to determine the exact role of cholesterol accumulation in endosomal transport.

As noted above, SseJ is a glycerophospholipid:cholesterol acyltransferase, catalyzing the transfer of *sn*-1 acyl chains from glycerophospholipids onto free cholesterol, in the process generating both lysophospholipids and cholesteryl esters [245; 250; 251; 300]. At present it is not clear which of these two activities is important for intracellular survival. Lysophospholipids regulate many cellular processes including epithelial permeability and phagocytosis [334; 335], however, the role of lysophospholipids during *Salmonella* infection remains unknown. It is possible that the generation of lysophospholipids in the SCV membrane is critical for regulating SIF formation and SCV membrane dynamics, and that the elevated local cholesterol content serves to sequester the free acyl chains removed from glycerophospholipids.

In summary, we elucidate a novel role for the *Salmonella* T3SS-2 effector SseJ in manipulating host cell cholesterol homeostasis by promoting cholesterol accumulation in macrophages. The catalytic activity of SseJ prevents bacterial clearance from BMDMs through the FAK- and Akt-dependent downregulation of *Abca1* and the subsequent increase in intracellular free cholesterol. While we show that suppression of *Abca1* enhances *Salmonella* Typhimurium survival, the specific role of cholesterol accumulation in bacterial pathogenesis remains uncertain. Our future studies will focus on determining how elevated cholesterol affects SCV membrane integrity and regulates endolysosomal trafficking.


BODIPY-Chol.
Salmonella
Merge
A
WT BMDM
Uninfected
B
WT BMDM
Infected


C
***
40000
30000
20000
10000
0
MFI
Uninf.
Infected

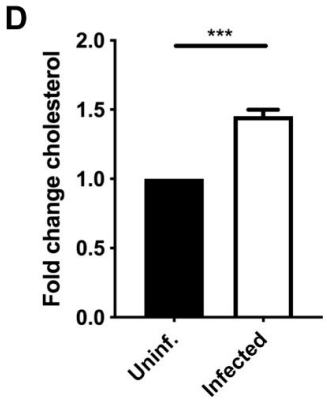

D
***
2.0
1.5
1.0
0.5
0.0
Fold change cholesterol
Uninf.
Infected

Figure 2-1 - *Salmonella* Typhimurium induces cholesterol accumulation in murine BMDMs.

Unless otherwise stated, n refers to the number of individual experimental replicates. (A-B) Bone marrow-derived macrophages (BMDMs) from WT mice were infected with RFP-expressing Δ*invG S.* Typhimurium (red) at an MOI of 50. At 16.5 hours post-infection, cells were incubated with 0.5 μM BODIPY-cholesterol (pseudo- colored black) for 45 minutes and imaged after a 45 minute chase. Uninfected cells (A) were imaged from the same dish as infected cells (B). Cells are outlined by a dashed line. Scale bar represents 5 μm. (C) Mean fluorescence intensity (MFI) of BODIPY-cholesterol was measured from uninfected (filled circles) or infected BMDMs (open circles). Each data point represents a single cell from a representative experimental replicate, mean ± SD. Uninf. n=20 cells; Infected n=35 cells. (D) BMDMs were infected (open bars) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3 experimental replicates. ***$p<0.0005$.

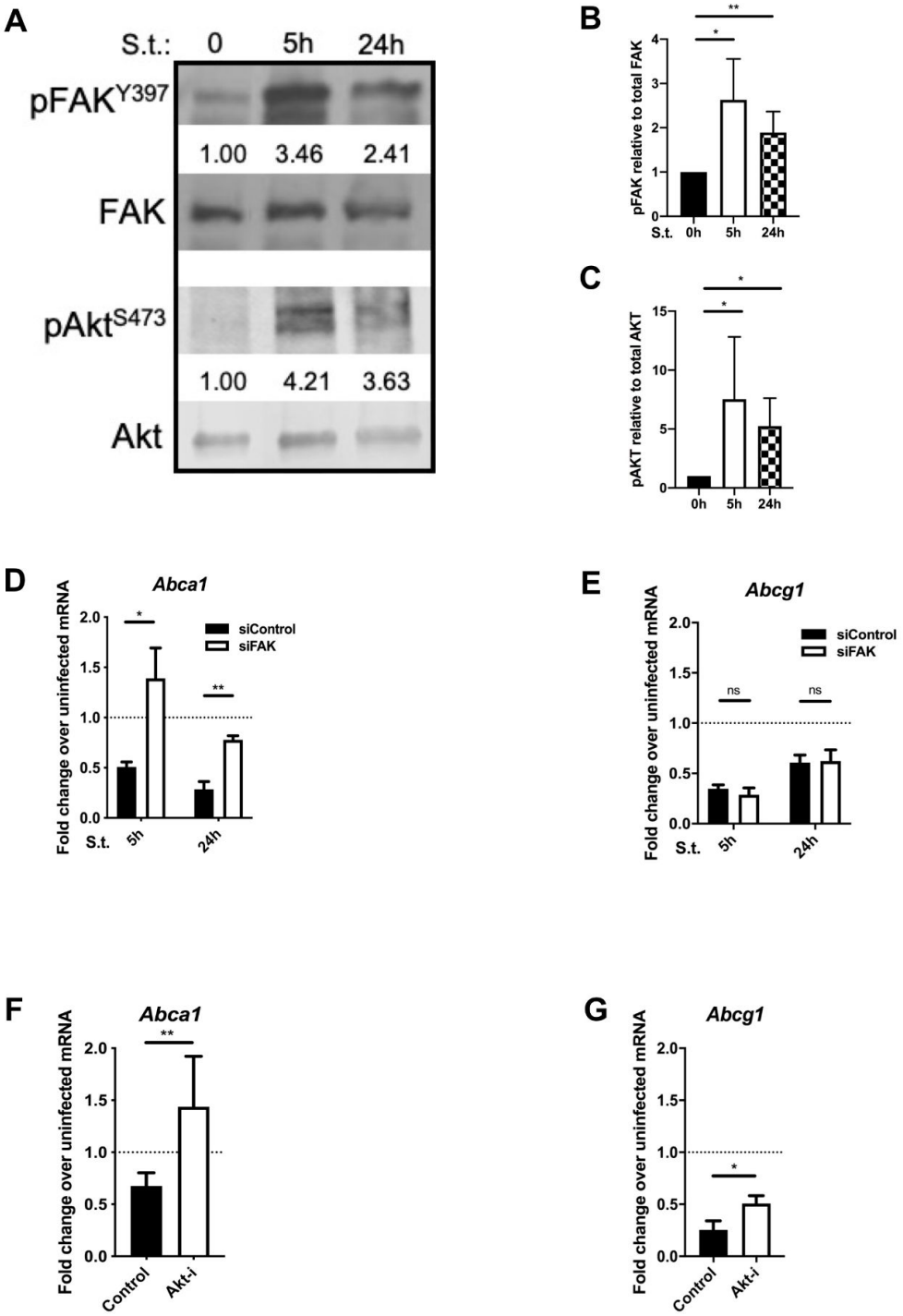

A
S.t.: 0 5h 24h
pFAK^Y397
1.00 3.46 2.41
FAK
pAkt^S473
1.00 4.21 3.63
Akt
B
pFAK relative to total FAK
S.t. 0h 5h 24h
C
pAKT relative to total AKT
0h 5h 24h
D
Abca1
siControl
siFAK
Fold change over uninfected mRNA
S.t. 5h 24h
E
Abcg1
siControl
siFAK
ns
ns
Fold change over uninfected mRNA
S.t. 5h 24h
F
Abca1
Fold change over uninfected mRNA
Control Akt-i
G
Abcg1
Fold change over uninfected mRNA
Control Akt-i

Figure 2-2 - *Salmonella* suppresses *Abca1* expression in a FAK- and Akt-dependent manner.

(A-C) Immortalized BMDMs (iBMDMs) were infected with Δ*invG S.* Typhimurium (S.t.) at an MOI of 100 for 0h (uninfected; filled bars), 5h (open bars), or 24 hours (checked bars). (A) Representative western blot of cell lysates immunoblotted to detect phospho-FAK^{Y397}, total FAK, phospho-Akt^{S473}, and total Akt. (B, C) Quantification of pFAK:total FAK (B) and pAkt:total Akt (C) normalized to uninfected control lysates averaged across multiple replicates, mean ± SD. n=4. Values represent the ratio of phospho/total protein for each condition and are normalized to uninfected controls. (D, E) iBMDMs were transfected with non-targeting siRNA (siControl, filled bars) or siFAK oligonucleotides (open bars) 48 hours prior to infection with Δ*invG* (S.t.) at an MOI of 100 for 5 or 24 hours. *Abca1* (D) or *Abcg1* (E) mRNA was measured by qPCR. Values are normalized to uninfected levels (dotted line), mean ± SD. (D) n=4; (E) n=5. (F, G) iBMDMs were infected as described above for 5h in the presence (open bars) or absence (filled bars) of the Akt inhibitor triciribine (10 mM; +Akt-i). Cells were infected for 5h and *Abca1* (F) or *Abcg1* (G) mRNA was measured by qPCR. Values are normalized to uninfected levels (dotted line), mean ± SD. (F) n=5; (G) n=3. *p<0.05, **p<0.005, ns=not significant.

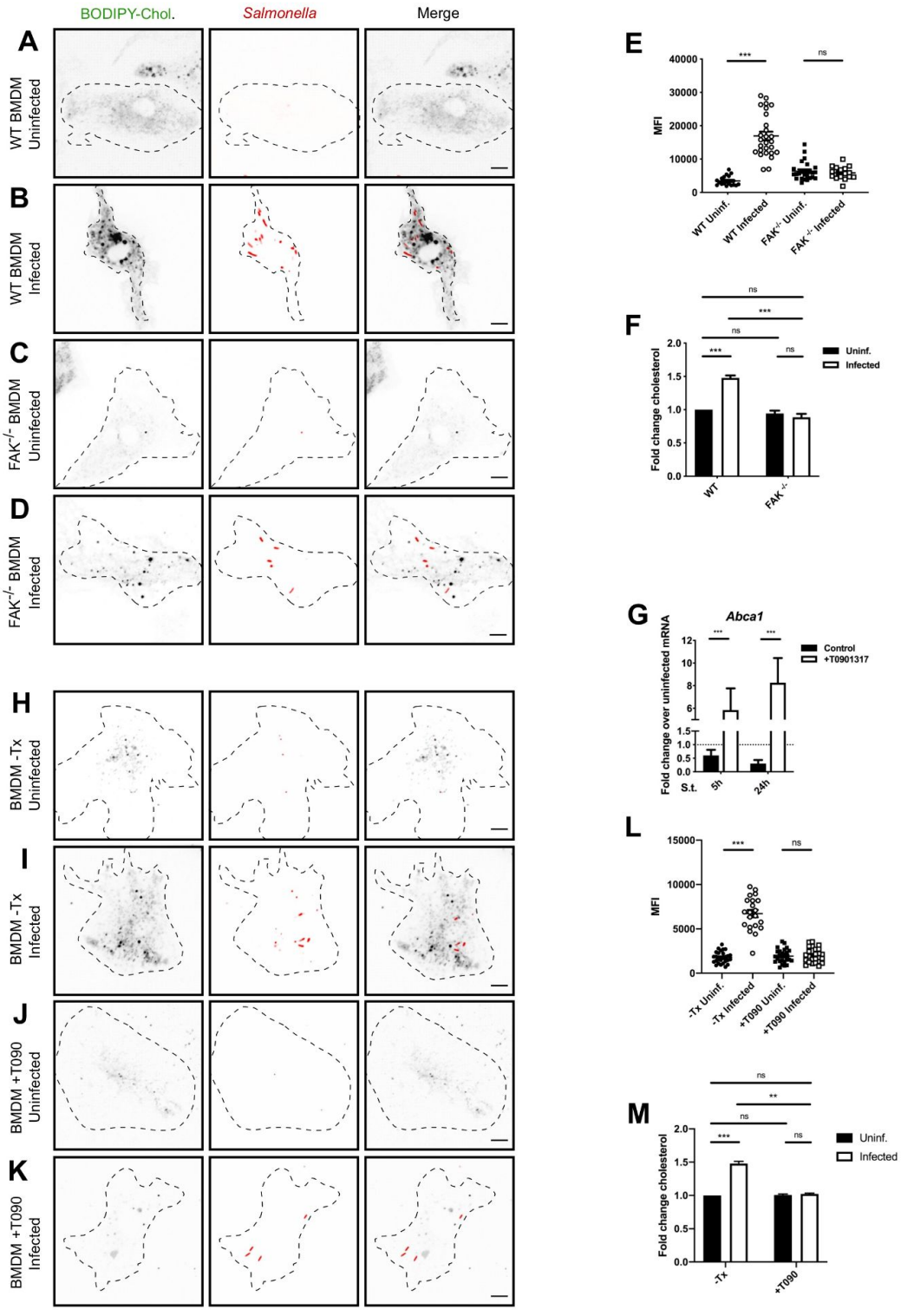

BODIPY-Chol.
Salmonella
Merge
A
WT BMDM Uninfected
B
WT BMDM Infected
C
FAK-/- BMDM Uninfected
D
FAK-/- BMDM Infected
H
BMDM -Tx Uninfected
I
BMDM -Tx Infected
J
BMDM +T090 Uninfected
K
BMDM +T090 Infected
E
MFI
WT Uninf.
WT Infected
FAK-/- Uninf.
FAK-/- Infected
F
Fold change cholesterol
Uninf.
Infected
WT
FAK-/-
G
Abca1
Fold change over uninfected mRNA
Control
+T0901317
S.t.
5h
24h
L
MFI
-Tx Uninf.
-Tx Infected
+T090 Uninf.
+T090 Infected
M
Fold change cholesterol
Uninf.
Infected
-Tx
+T090

Figure 2-3 - Failure to downregulate *Abca1* prevents cholesterol accumulation in infected macrophages.

(A-D) WT BMDMs (A-B) or FAK$^{-/-}$ BMDMs (C-D) were uninfected (A, C) or infected with RFP-expressing Δ*invG S.* Typhimurium (B, D; red) and incubated with BODIPY-cholesterol (pseudo-colored black) as described in Fig. 2-1. (E) MFI of BODIPY-cholesterol was measured from uninfected (filled) and infected (open) WT (circles) and FAK$^{-/-}$ BMDMs (squares) as previously described. WT Uninf. n=20 cells; WT Infected n=27 cells; FAK$^{-/-}$ Uninf. n=25 cells; FAK$^{-/-}$ Infected n=16 cells. (F) WT and FAK$^{-/-}$ BMDMs were infected (open bars) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3. (G) iBMDMs were either untreated (filled bars) or pretreated with 10 μM T0901317 (open bars) for 24 hours prior to and maintained throughout infection. Cells were then infected at an MOI of 100 for 5 or 24 hours. *Abca1* expression was measured via qPCR as previously described. n=7. (H-K) Control (H-I) or T0901317-pretreated BMDMs (J-K; +T090) were uninfected (H, J) or infected with RFP-expressing Δ*invG* (I, K) and incubated with BODIPY-cholesterol as described above. (L) MFI of BODIPY-cholesterol was measured from uninfected (filled) or infected BMDMs (open) in the presence (+T090; squares) or absence (-Tx; circles) of T0901317 treatment as described above. -Tx Uninf. n=20 cells; -Tx Infected n=30 cells; +T090 Uninf. n=10 cells; +T090 Infected n=10 cells. (M) Control (-Tx) and T0901317-treated (+T090) BMDMs were infected with infected (open bars) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3. **p<0.005, ***p<0.0005, ns=not significant.

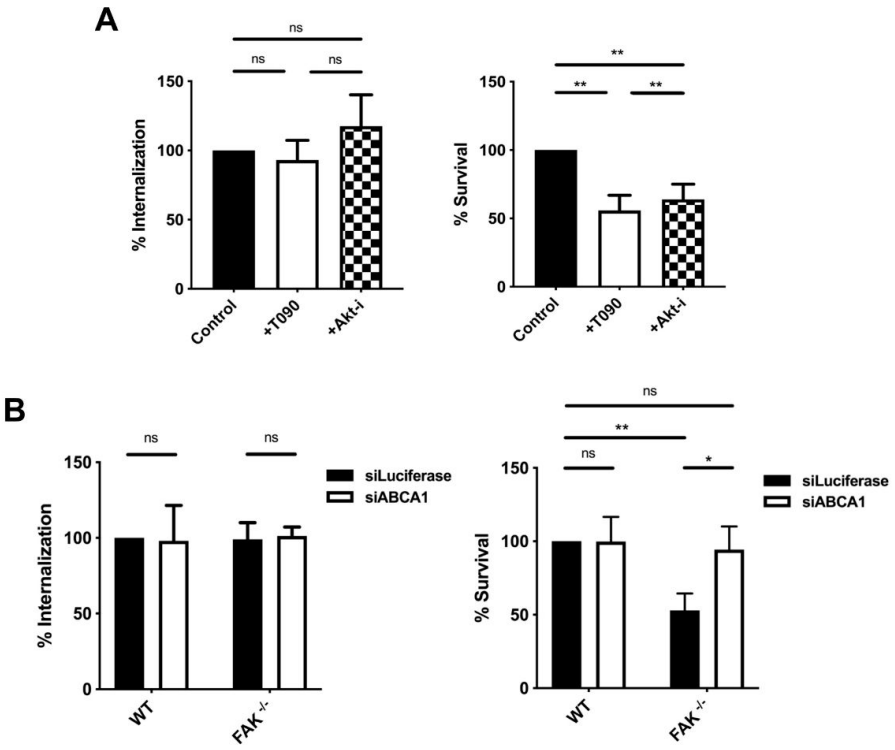

A
ns
ns
ns
150
100
50
0
% Internalization
Control
+T090
+Akt-i
**
**
**
150
100
50
0
% Survival
Control
+T090
+Akt-i
B
ns
ns
150
100
50
0
% Internalization
WT
FAK-/-
siLuciferase
siABCA1
ns
**
ns
*
150
100
50
0
% Survival
WT
FAK-/-
siLuciferase
siABCA1

Figure 2-4 - Suppression of *Abca1* enhances *Salmonella* survival within macrophages. Internalization and survival rates were determined using a standard gentamicin protection assay as described in the materials and methods. For all conditions, cells were infected with Δ*invG S.* Typhimurium at an MOI of 75 for 0.5, 1, or 18 hours prior to lysing cells and plating to enumerate CFUs. (A) iBMDMs were untreated (filled bars) or pretreated with either 10 μM T0901317 for 24 hours (open bars; +T090) or with 10 mM triciribine (checked bars; +Akt-i) for 1 hour prior to and maintained throughout infection. Rates normalized to untreated control infection. Internalization rate = CFU at 1 hour/CFU at 0.5 hour. Survival rate = CFU at 18 hour/CFU at 1 hour. (B) WT or $FAK^{-/-}$ BMDMs were transfected with siLuciferase (filled bars) or siABCA1 (open bars) oligonucleotides prior to infection. Rates normalized to WT BMDM siLuciferase control infection. All values are mean ± SD and n=3. *$p<0.05$, **$p<0.005$, ns=not significant.

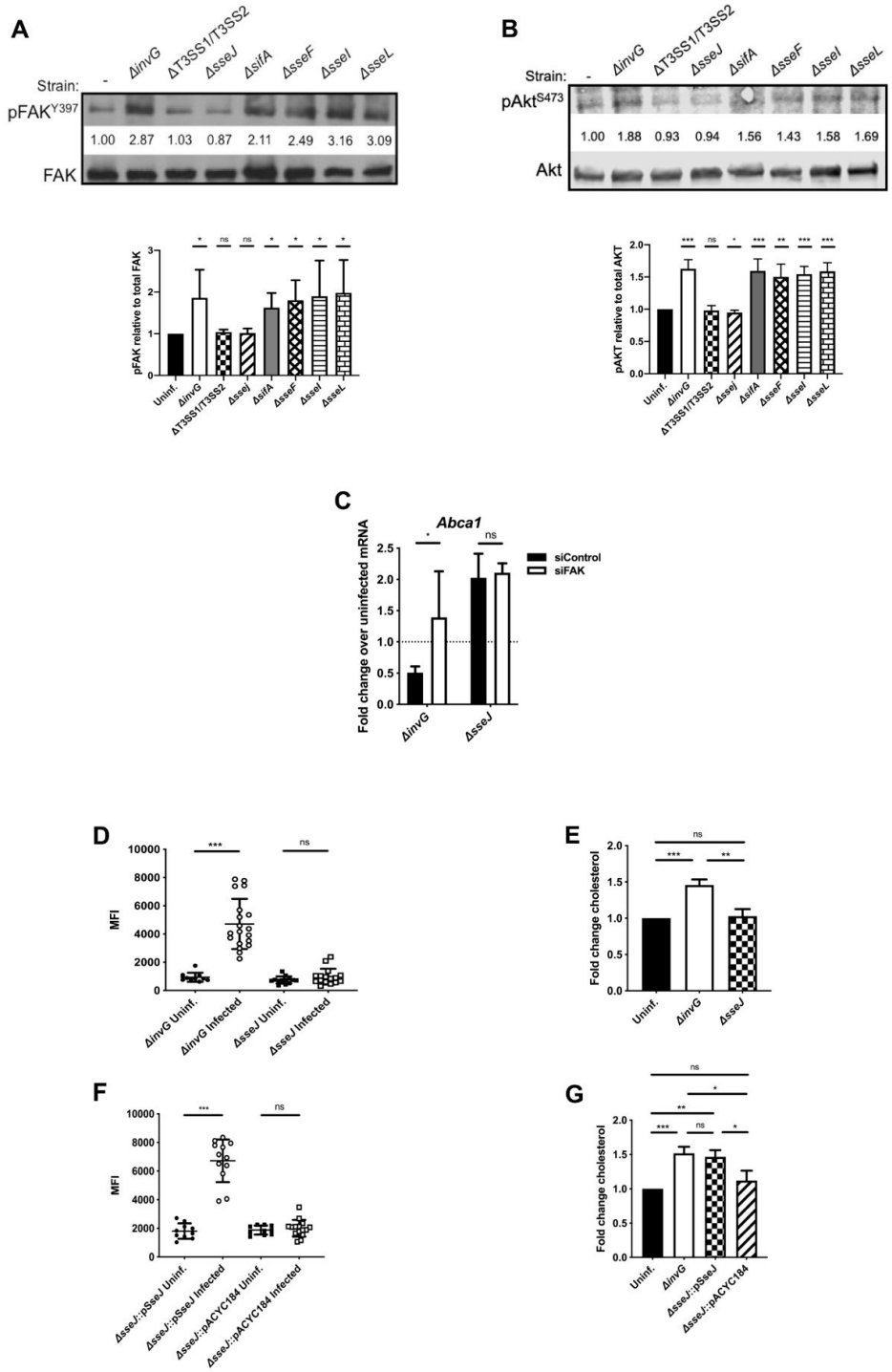

A
Strain: - ΔinvG ΔT3SS1/T3SS2 ΔsseJ ΔsifA ΔsseF ΔsseI ΔsseL
pFAK^Y397
1.00 2.87 1.03 0.87 2.11 2.49 3.16 3.09
FAK
pFAK relative to total FAK
Uninf. ΔinvG ΔT3SS1/T3SS2 ΔsseJ ΔsifA ΔsseF ΔsseI ΔsseL
B
Strain: - ΔinvG ΔT3SS1/T3SS2 ΔsseJ ΔsifA ΔsseF ΔsseI ΔsseL
pAkt^S473
1.00 1.88 0.93 0.94 1.56 1.43 1.58 1.69
Akt
pAKT relative to total AKT
Uninf. ΔinvG ΔT3SS1/T3SS2 ΔsseJ ΔsifA ΔsseF ΔsseI ΔsseL
C
Abca1
siControl
siFAK
Fold change over uninfected mRNA
ΔinvG ΔsseJ
D
MFI
ΔinvG Uninf. ΔinvG Infected ΔsseJ Uninf. ΔsseJ Infected
E
Fold change cholesterol
Uninf. ΔinvG ΔsseJ
F
MFI
ΔsseJ::pSseJ Uninf. ΔsseJ::pSseJ Infected ΔsseJ::pACYC184 Uninf. ΔsseJ::pACYC184 Infected
G
Fold change cholesterol
Uninf. ΔinvG ΔsseJ::pSseJ ΔsseJ::pACYC184

Figure 2-5 - The T3SS-2 effector SseJ is essential for activation of FAK and Akt, downregulation of *Abca1*, and cholesterol accumulation in macrophages.

(A-B) iBMDMs were infected with the indicated *S.* Typhimurium effector mutant strains at an MOI of 100 for 24 hours. Cells were lysed and immunoblotted for (A) total and phospho-FAK^{Y397} or (B) total and phospho-Akt^{S473}. Quantification of pFAK:total FAK (A) and pAkt:total Akt (B) normalized to uninfected control lysates averaged across multiple replicates is shown below representative western blots, mean ± SD. n=4. Values represent the ratio of phospho/total protein for each condition and are normalized to uninfected controls. (C) iBMDMs were either mock-depleted (siControl; filled bars) or FAK-depleted (siFAK; open bars) as described in Fig. 2-2. Cells were then infected with Δ*invG* or Δ*sseJ S.* Typhimurium at an MOI of 100 for 5 hours. *Abca1* expression was measured via qPCR. Values are normalized to uninfected levels (dotted line), mean ± SD. n=4. (D) MFI of BODIPY-cholesterol was measured from uninfected (filled) or infected BMDMs (open) with RFP-expressing Δ*invG* (circles) or RFP-expressing Δ*sseJ* (squares) mutants as previously described. Δ*invG* Uninf. n=10 cells; Δ*invG* Infected n=19 cells; Δ*sseJ* Uninf. n=13 cells; Δ*sseJ* Infected n=16 cells. (E) WT BMDMs were infected with Δ*invG* (empty bar) or Δ*sseJ* (checked bar) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3. (F) MFI of BODIPY-cholesterol was measured from uninfected (filled) or BMDMs infected (open) with RFP-expressing Δ*sseJ*::pSseJ (circles) or RFP-expressing Δ*sseJ*::pACYC184 (squares) as described above. Δ*sseJ*::pSseJ Uninf. n=10 cells; Δ*sseJ*::pSseJ Infected n=12 cells; Δ*sseJ*::pACYC184 Uninf. n=10 cells; Δ*sseJ*::pACYC184 Infected n=15 cells. (G) WT BMDMs were infected with Δ*invG* (empty

bar), Δ*SseJ*::pSseJ (checked bar), or Δ*sseJ*::pACYC184 (slashed bar) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3. (H, I) iBMDMs were uninfected (filled bar) or infected with Δ*invG* (open bar), Δ*sseJ*::pSseJ (checked bar), Δ*sseJ*::pACYC184 (slashed bar), or Δ*sseJ*::H384N (grey bar) at an MOI of 100 for 24 hours. Cells were lysed and immunoblotted for (H) total and phospho-FAK^{Y397} or (I) total and phospho-Akt^{S473} as described above. n=3. (J) Cholesterol was measured from WT BMDMs infected with Δ*invG* (empty bar), Δ*sseJ*::pSseJ (checked bar), Δ*sseJ*::pACYC184 (slashed bar), or Δ*sseJ*::H384N (grey bar) as described above. n=3. *p<0.05, **p<0.005, ***p<0.0005, ns=not significant.

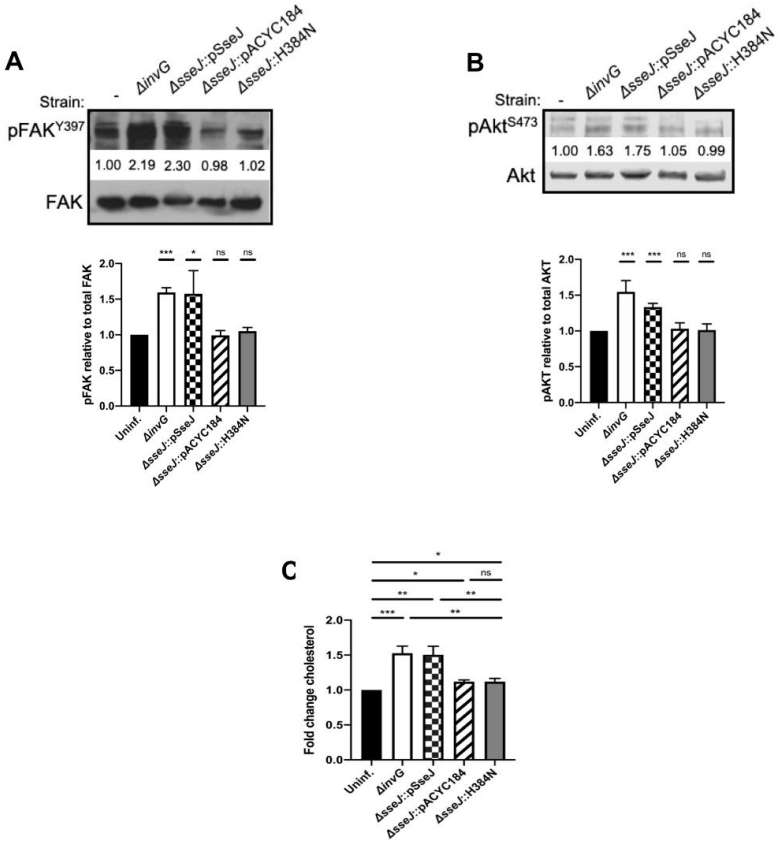

A
Strain:
-
ΔinvG
ΔsseJ::pSseJ
ΔsseJ::pACYC184
ΔsseJ::H384N
pFAK^Y397
1.00 2.19 2.30 0.98 1.02
FAK
pFAK relative to total FAK
***
*
ns
ns
Uninf.
ΔinvG
ΔsseJ::pSseJ
ΔsseJ::pACYC184
ΔsseJ::H384N
B
Strain:
-
ΔinvG
ΔsseJ::pSseJ
ΔsseJ::pACYC184
ΔsseJ::H384N
pAkt^S473
1.00 1.63 1.75 1.05 0.99
Akt
pAKT relative to total AKT
***
***
ns
ns
Uninf.
ΔinvG
ΔsseJ::pSseJ
ΔsseJ::pACYC184
ΔsseJ::H384N
C
Fold change cholesterol
*
*
ns
**
**
***
**
Uninf.
ΔinvG
ΔsseJ::pSseJ
ΔsseJ::pACYC184
ΔsseJ::H384N

Figure 2-6 - The catalytic activity of SseJ is critical for FAK/Akt signaling and cholesterol accumulation.

(A, B) iBMDMs were uninfected (filled bar) or infected with Δ*invG* (open bar), Δ*sseJ*::pSseJ (checked bar), Δ*sseJ*::pACYC184 (slashed bar), or Δ*sseJ*::H384N (grey bar) at an MOI of 100 for 24 hours. Cells were lysed and immunoblotted for (A) total and phospho-FAK^{Y397} or (B) total and phospho-Akt^{S473}. Quantification of pFAK:total FAK (A) and pAkt:total Akt (B) normalized to uninfected control lysates averaged across multiple replicates is shown below representative western blots, mean ± SD. n=3. Values represent the ratio of phospho/total protein for each condition and are normalized to uninfected controls. (C) WT BMDMs were infected with Δ*invG* (empty bar), Δ*sseJ*::pSseJ (checked bar), Δ*sseJ*::pACYC184 (slashed bar), or Δ*sseJ*::H384N (grey bar) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3. *p<0.05, **p<0.005, ***p<0.0005, ns=not significant.

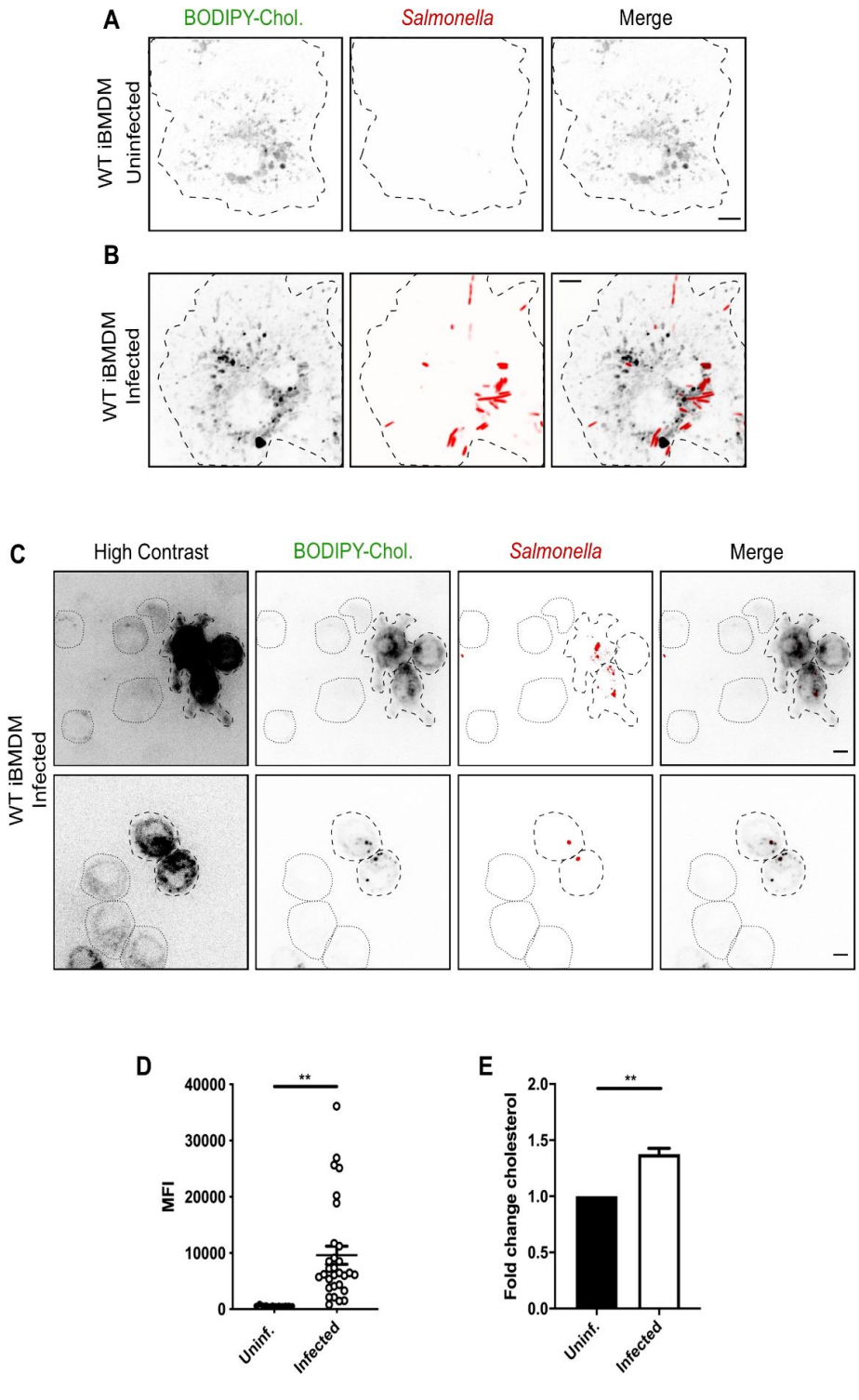

A
BODIPY-Chol.
Salmonella
Merge
WT iBMDM Uninfected
B
WT iBMDM Infected
C
High Contrast
BODIPY-Chol.
Salmonella
Merge
WT iBMDM Infected
D
MFI
40000
30000
20000
10000
0
**
Uninf.
Infected
E
Fold change cholesterol
2.0
1.5
1.0
0.5
0.0
**
Uninf.
Infected

Figure 2-S1 - *Salmonella* Typhimurium induces cholesterol accumulation in immortalized macrophages.

Unless otherwise stated, n refers to the number of individual experimental replicates. (A-B) Immortalized murine macrophages (iBMDMs) were infected with RFP-expressing Δ*invG S.* Typhimurium (red) and incubated with BODIPY-cholesterol (pseudo-colored black) as described in Fig. 2-1. (C) Two representative lower magnification images of a population of uninfected (dotted outlines) and infected (dashed outlines) cells from imaging described above. Image contrast was digitally increased to better show uninfected cells (High Contrast panels). Scale bar represents 5 μm. (D) MFI of BODIPY-cholesterol was measured from uninfected (filled circles) or infected iBMDMs (open circles). Each data point represents a single cell from a representative experimental replicate, mean ± SD. Uninf. n=20 cells; Infected n=35 cells. (E) iBMDMs were infected (open bars) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3. **$p<0.005$.

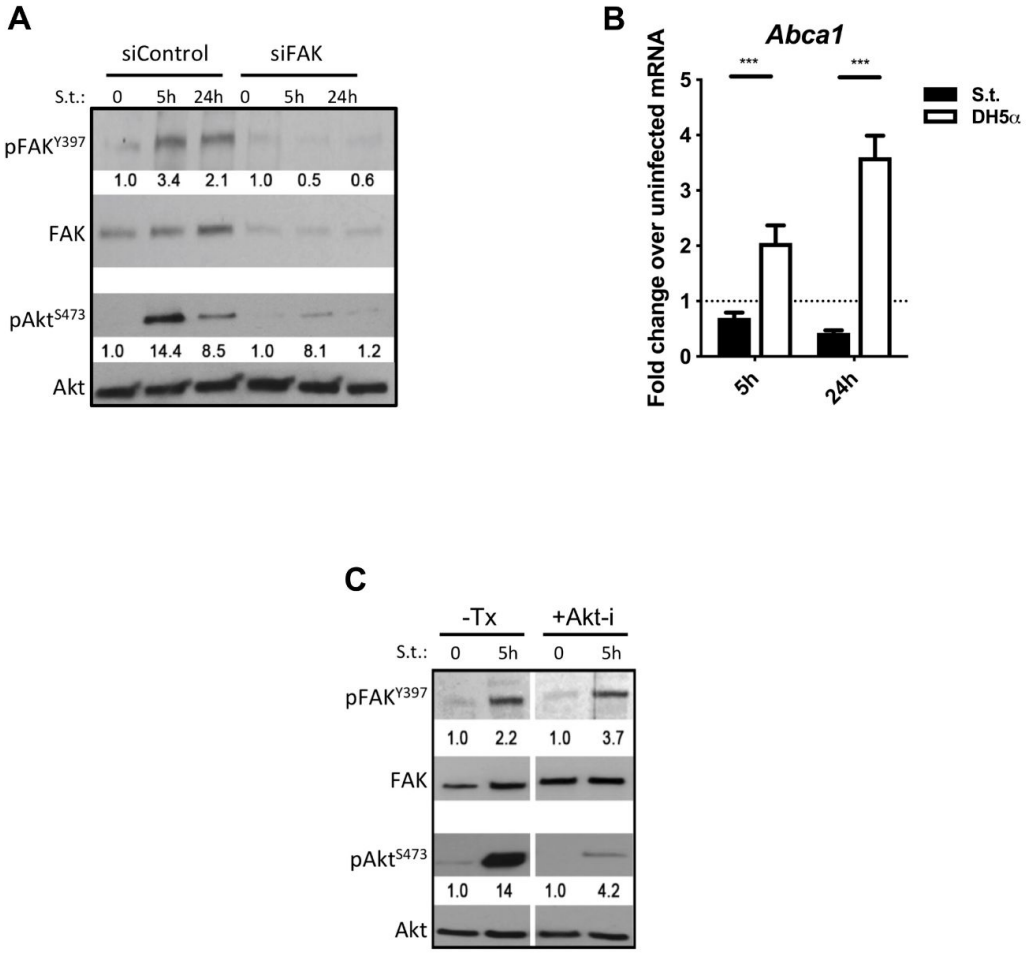

A
siControl
siFAK
S.t.: 0 5h 24h 0 5h 24h
pFAK^Y397
1.0 3.4 2.1 1.0 0.5 0.6
FAK
pAkt^S473
1.0 14.4 8.5 1.0 8.1 1.2
Akt
B
Abca1
***
***
Fold change over uninfected mRNA
5
4
3
2
1
0
5h
24h
S.t.
DH5α
C
-Tx
+Akt-i
S.t.: 0 5h 0 5h
pFAK^Y397
1.0 2.2 1.0 3.7
FAK
pAkt^S473
1.0 14 1.0 4.2
Akt

Figure 2-S2 - *Salmonella* actively suppresses *Abca1* expression in a FAK- and Akt-dependent manner

(A) FAK is essential for *S.* Typhimurium-mediated Akt signaling. iBMDMs were transfected with non-targeting siRNA (siControl) or siFAK oligonucleotides 48 hours prior to infection with Δ*invG S.* Typhimurium (S.t.) at an MOI of 100 for 5 or 24 hours. Representative western blot of cells lysed and immunoblotted to detect phospho-FAK^{Y397}, total FAK, phospho-Akt^{S473}, and total Akt. Values represent the ratio of phospho/total protein for each condition and are normalized to uninfected controls. (B) *Abca1* expression is not downregulated by *E. coli*. iBMDMs were either infected with Δ*invG* (S.t.; filled bars) or DH5α *Escherichia coli* (open bars) at an MOI of 100 for 5 or 24 hours. *Abca1* expression was determined via qPCR and normalized to uninfected levels (dotted line). (C) Pharmacological inhibition of Akt inhibits Akt, but not FAK phosphorylation during infection. iBMDMs were either uninfected (0h) or infected with Δ*invG* for 5h in the presence (+Akt-i) or absence (-Tx) of triciribine (10 μM). Cell lysates were immunoblotted as described above. n=4. ***$p<0.0005$.

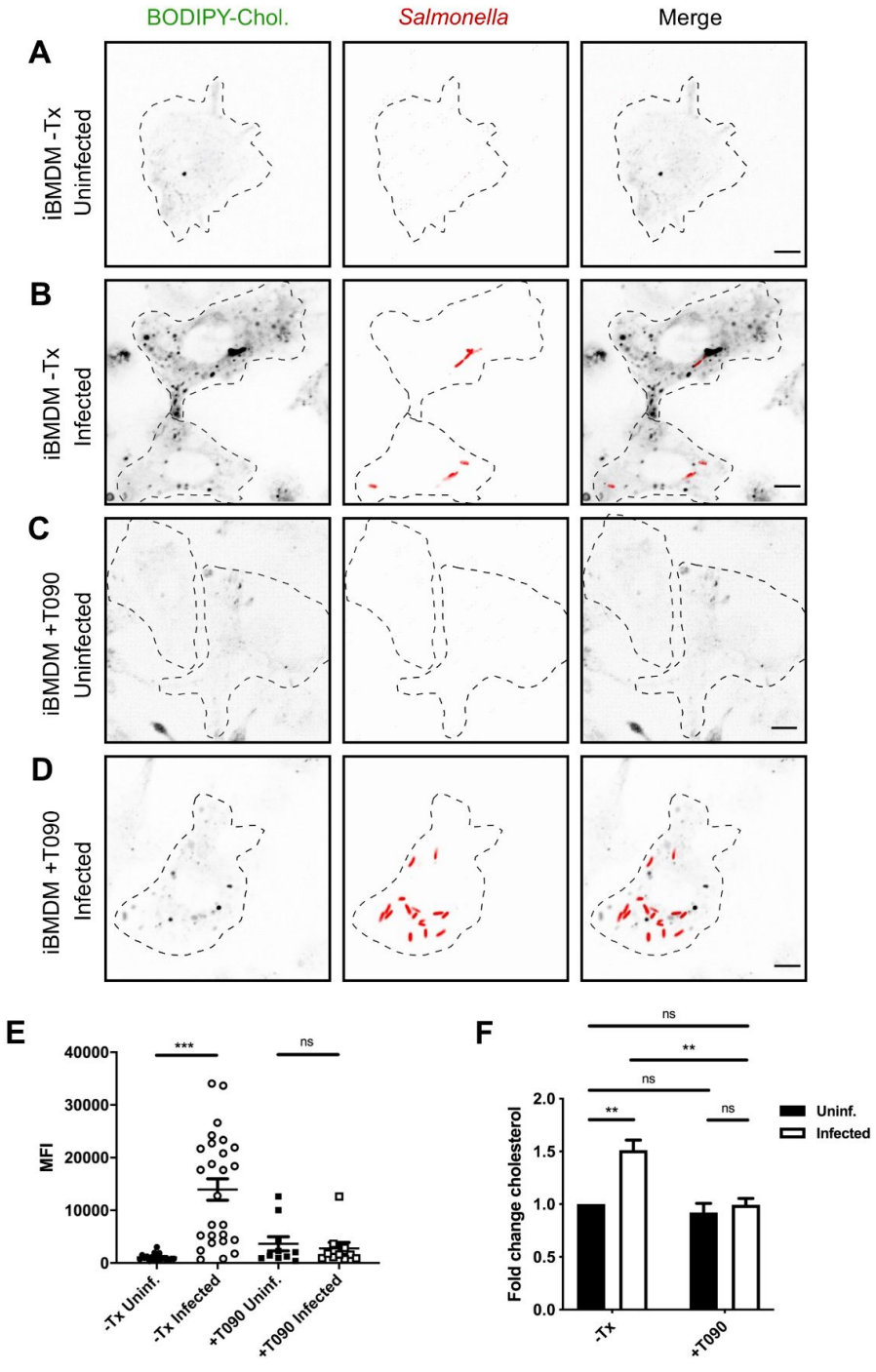

BODIPY-Chol.
Salmonella
Merge
A
iBMDM -Tx Uninfected
B
iBMDM -Tx Infected
C
iBMDM +T090 Uninfected
D
iBMDM +T090 Infected
E
MFI
40000
30000
20000
10000
0
***
ns
-Tx Uninf.
-Tx Infected
+T090 Uninf.
+T090 Infected
F
ns
**
ns
**
ns
Fold change cholesterol
2.0
1.5
1.0
0.5
0.0
Uninf.
Infected
-Tx
+T090

Figure 2-S3 - Failure to downregulate *Abca1* prevents cholesterol accumulation in infected macrophages.

(A-D) Control (A-B) or T0901317-pretreated iBMDMs (C-D; +T090) were uninfected (A, C) or infected with RFP-expressing Δ*invG S.* Typhimurium (red) (B, D) and incubated with BODIPY-cholesterol (pseudo-colored black) as previously described. (E) MFI of BODIPY-cholesterol was measured from uninfected (filled) or infected iBMDMs (open) in the presence (+T090; squares) or absence (-Tx; circles) of T0901317 pretreatment as previously described. -Tx Uninf. n=20 cells; -Tx Infected n=30 cells; +T090 Uninf. n=10 cells; +T090 Infected n=10 cells. (F) Control (-Tx) and T0901317-treated (+T090) iBMDMs were infected with Δ*invG* (open bars) at an MOI of 100 for 18 hours before endogenous cholesterol was extracted and quantified. Values are normalized to uninfected levels (filled bars), mean ± SD. n=3. **p<0.005, ***p<0.0005, ns=not significant.

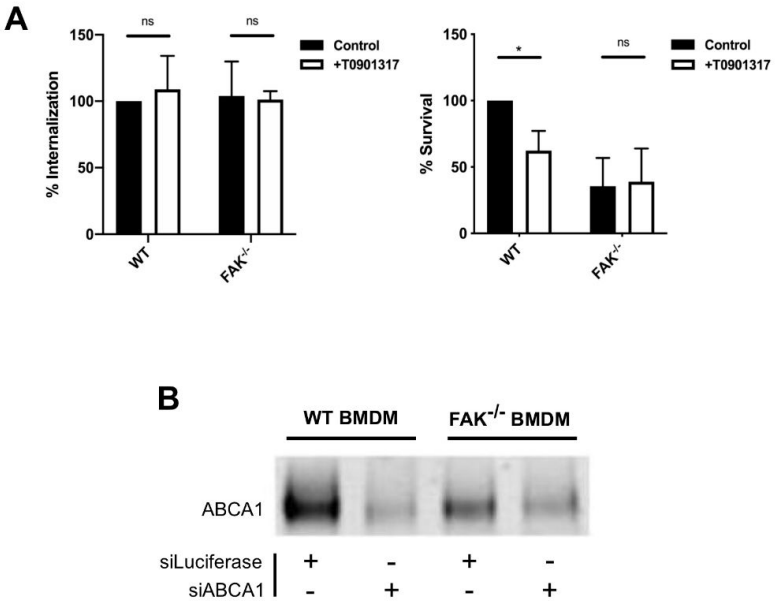

A
ns
ns
Control
+T0901317
% Internalization
150
100
50
0
WT
FAK-/-
*
ns
Control
+T0901317
% Survival
150
100
50
0
WT
FAK-/-
B
WT BMDM
FAK-/- BMDM
ABCA1
siLuciferase + - + -
siABCA1 - + - +

Figure 2-S4 - Pharmacological induction of *Abca1* attenuates *Salmonella* survival within macrophages.

(A) Internalization and survival rates were determined using a standard gentamicin protection assay as previously described. WT or $FAK^{-/-}$ BMDMs were untreated (filled bars) or pretreated with 10 μM T0901317 (open bars) for 24 hours and maintained throughout infection with Δ*invG S.* Typhimurium at an MOI of 75 for 0.5, 1, or 18 hours. Rates are normalized to untreated control infection in WT BMDMs. Internalization rate = CFU at 1 hour/CFU at 0.5 hour. Survival rate = CFU at 18 hour/CFU at 1 hour. All values are mean ± SD and n=3. (B) Confirmation of *Abca1* knockdown by siRNA. Representative western blot of WT or $FAK^{-/-}$ BMDMs transfected with siLuciferase or siABCA1 oligonucleotides. Whole cell lysates containing equivalent amounts of total protein were prepared and were immunoblotted for ABCA1. *$p<0.05$, ns=not significant.

Figure S5

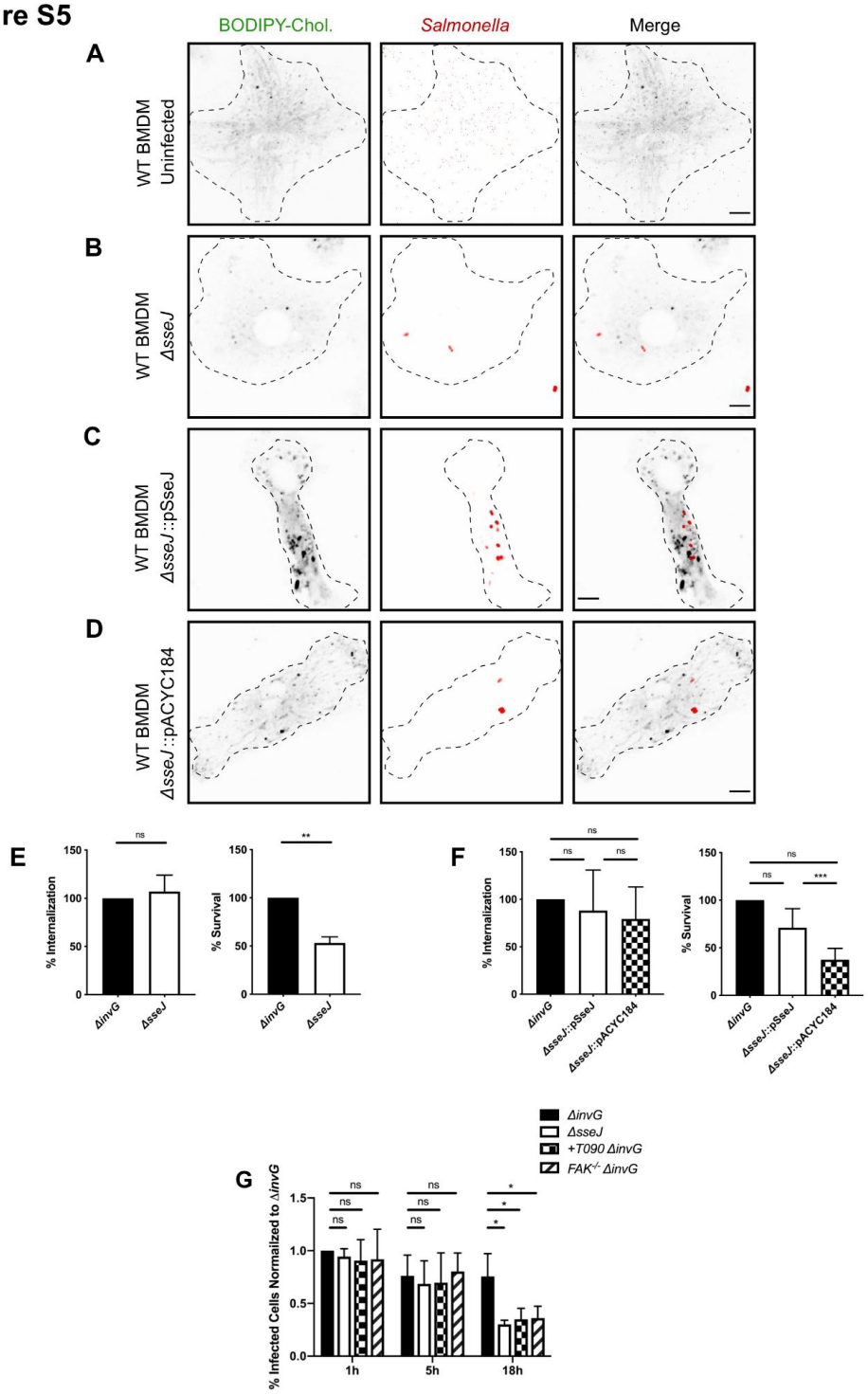

BODIPY-Chol.
Salmonella
Merge
A
WT BMDM Uninfected
B
WT BMDM ΔsseJ
C
WT BMDM ΔsseJ::pSseJ
D
WT BMDM ΔsseJ::pACYC184
E
% Internalization
% Survival
ΔinvG
ΔsseJ
F
ΔsseJ::pSseJ
ΔsseJ::pACYC184
G
ΔinvG
ΔsseJ
+T090 ΔinvG
FAK-/- ΔinvG
% Infected Cells Normalized to ΔinvG
1h
5h
18h

Figure 2-S5 - SseJ enhances *Salmonella* survival by promoting cholesterol accumulation.

(A-D) Images in support of Figs. 2-5, D and 2-5, F. WT BMDMs were infected with (B) RFP-expressing Δ*sseJ*, (C) RFP-expressing Δ*sseJ*::pSseJ, or (D) RFP-expressing Δ*sseJ*::pACYC184, *S.* Typhimurium (red) at an MOI of 50 and incubated with BODIPY-cholesterol (pseudo-colored black) as previously described. Uninfected cells (A) were imaged from the same dish as Δ*sseJ*-infected cells. (E, F) Internalization and survival rates were measured by a standard gentamicin protection assay as previously described. (E) WT BMDMs were infected with Δ*invG* (filled bars) or Δ*sseJ* (open bars). (F) WT BMDMs were infected with Δ*invG* (filled bars), Δ*sseJ*::pSseJ (open bars), or Δ*sseJ*::pACYC184 (checked bars). Mean ± SD. n=3. (G) WT or $FAK^{-/-}$ (slashed bars) BMDMs were infected at an MOI of 75 for 1, 5, or 18 hours with RFP-expressing Δ*invG* (filled bars) or RFP-expressing Δ*sseJ* (open bars) or pretreated with 10 μM T0901317 for 24 hours prior to and throughout infection with RFP-expressing Δ*invG* (checked bars; +T090 Δ*invG*). The percentage of infected cells within a population was measured and normalized to Δ*invG* at 1 hour post-infection. Mean ± SD. Each experimental replicate analyzed a minimum of 400 cells per condition. n=3. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, ns=not significant.

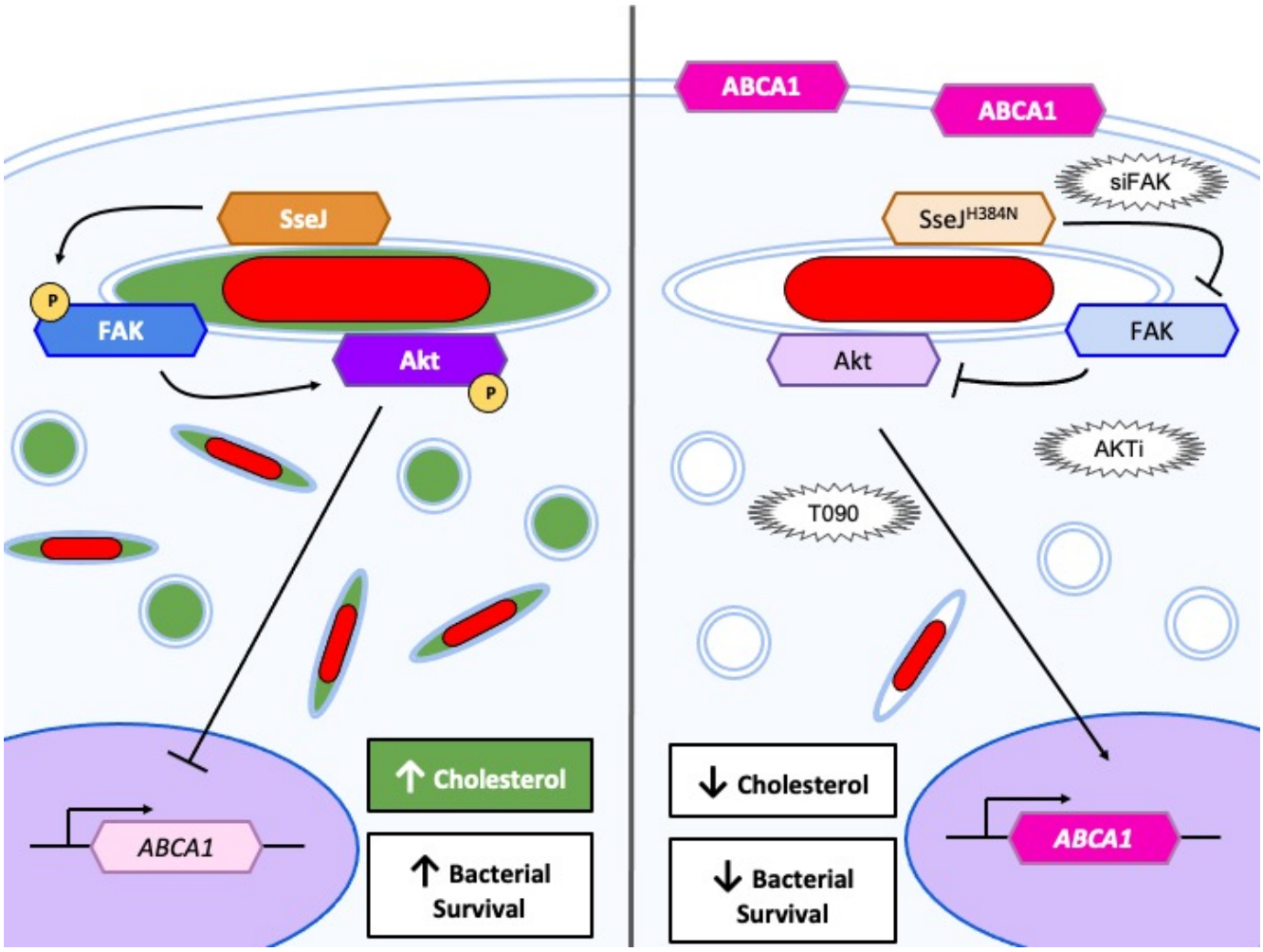

ABCA1
ABCA1
SseJ
P
FAK
Akt
P
siFAK
SseJH384N
FAK
Akt
AKTi
T090
ABCA1
↑ Cholesterol
↑ Bacterial Survival
↓ Cholesterol
↓ Bacterial Survival
ABCA1

Figure 2-0 - Graphical abstract for Greene, *et al. Cell Microbiol* 2021.

Model for the SseJ-mediated activation of FAK and Akt, leading to the suppression of Abca1 and the accumulation of cholesterol.

Materials and Methods

Ethics statement

All experiments in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Virginia (Protocol number 3488). All efforts were made to minimize animal suffering during the course of these studies.

Mice

The generation of myeloid-specific conditional FAK knockout mice and their control littermates have been described previously [336]. Mice were kept in pathogen-free conditions and allowed free access to food and water.

Cell culture

The hind leg bones of mice were isolated, and bone marrow-derived macrophages (BMDMs) were extracted. BMDMs were plated onto untreated petri dishes and cultured in RPMI (Genesee Scientific) media containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin/amphotericin B (PSF), and 10% L929-conditioned media as a source of colony stimulating factor-1 (CSF-1). iBMDM cells were a kind gift from Dr. Jonathan Kagan (Harvard University, Boston, MA). iBMDMs were grown on cell-culture treated petri dishes (Fisher) and cultured in Dulbecco's modified Eagle medium (DMEM; Genesee Scientific) supplemented with 10% FBS and 1% PSF.

Bacterial strains and culture

The *Salmonella* Typhimurium SL1344 strain *ΔinvG* mutant (T3SS1-deficient), and the *ΔorgA*::tet, *ΔspiA*::kan (double T3SS1 and T3SS2 mutant; ΔT3SS-1/ΔT3SS-2) strain have been described previously [286; 337; 338]. The T3SS2 effector mutants (*ΔsifA, ΔsseF, ΔsseI, ΔsseL,* and *ΔsseJ*) were all on a *ΔorgA* (T3SS1-deficient) background and were all a generous gift from Dr. Denise Monack (Stanford University, Stanford, CA). Bacteria were grown under non-invasion-inducing conditions. Briefly, a single colony was inoculated into LB broth with the proper antibiotics and grown statically overnight. Bacteria were harvested the following morning when their OD600 reached 0.6-0.8. Experiments using non-pathogenic Escherichia coli strain DH5α followed the same protocol.

Bacterial infection

30 minutes prior to infection, cells were washed in PBS and cell culture media was replaced with fresh infection media containing 10% heat-inactivated FBS without antibiotics. Bacteria were diluted in this medium and cells were then infected with the indicated bacterial strain and multiplicity of infection (MOI) for 30 minutes. After 30 minutes, cells were washed in PBS and fresh infection medium containing gentamicin (100 μg/ml; Fisher Scientific) was added for 60 minutes to kill extracellular bacteria. After 60 minutes (90 minutes total), the concentration of gentamicin was reduced to 10 μg/ml for the remainder of the assay.

Microscopy

BMDMs were plated on fibronectin-coated (5 μg/ml) glass-bottom 3.5 mm imaging dishes (Mat-Tek Corp.), or fibronectin-coated glass coverslips in a 24-well dish. Cells were infected with the indicated bacterial strain at an MOI of 50. For BODIPY-

cholesterol imaging, at 16h post-infection, cells were rinsed twice with PBS and serum-free DMEM containing 10 µg/mL gentamicin was added to the cells. 30 minutes later, cells were incubated with serum-free DMEM (10 µg/mL gentamicin) containing 0.5 µM BODIPY-cholesterol (TopFluor Cholesterol; Avanti Polar Lipids) for 45 minutes, rinsed twice in PBS, and chased for 45 minutes in phenol red-free imaging medium (140 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES pH 7.4, with 10% heat-inactivated FBS and 0.45% glucose) containing 10 µg/mL gentamicin. Cells were held at 37 °C and imaged at approximately 18h post-infection using a 40x or 60x objective fitted to a Nikon TE 2000 microscope equipped with Yokogawa CSU 10 spinning disc and 512X512 Hamamatsu 9100c-13 EM-BT camera. Green fluorescence was excited with a 488 nm/100 mW diode laser (Coherent) and collected by a BP 527/55 filter. Red fluorescence was excited with a 561 nm/100 mW diode laser (Coherent) and collected by a BP 615/70 filter. Multicolor images were acquired sequentially and compiled using FIJI software [339]. Grayscale image LUTs were inverted to generate a white background with black fluorescence. For RFP-*Salmonella* images, LUT color was edited to generate red fluorescence on a white background while maintaining the same scaling as found in grayscale images. Images were merged using minimum fluorescence intensity to avoid conflicts between the different color schemes and did not notably change image appearance. Exposure time and capture settings were identical between infected and uninfected cells and cells with or without T0901317 treatment. All images (except Fig. S1C) were captured using a 60x objective and unless otherwise stated, no manipulation of brightness or contrast occurred for BODIPY-cholesterol images. RFP-Δ*invG* was digitally adjusted for brightness and contrast to allow for better identification in merged

images with black puncta. For low magnification imaging using a 40x objective, BODIPY-cholesterol image contrast was digitally increased to better show uninfected cells (High Contrast panels).

For percent infection experiments, cells were fixed at the indicated time point in 4% PFA and co-stained with an anti-*Salmonella* (Thermo) and AlexaFluor 647-phalloidin (Invitrogen). Images were captured using a 40x objective on a Nikon C1 Plus Confocal microscope. Mean fluorescence intensity of images was analyzed using NIS-Elements software (Nikon).

Endogenous cholesterol quantification assay

Cholesterol extraction was performed using the Bligh and Dyer method as previously described [315]. In brief, cells were lysed in a chloroform:methanol mixture and lipids were harvested and pelleted from the organic layer. Lipids were resuspended in an isopropanol:NP40 solution and cholesterol was analyzed following a protocol from Robinet, *et a*l., 2010 [316]. Lipid samples plated in a 96-well dish and were treated with bovine catalase (Sigma) before addition of cholesterol oxidase (Sigma), HRP (Sigma), and ADHP (Amplex Red; Invitrogen). Fluorescence was measured using a Cytation 1 plate reader (BioTek).

Drug treatments

Cells were pretreated either overnight with 10 μM T0901317 (Tocris) or for 1h with 10 mM triciribine (Sigma) prior to infection. Treatment was maintained throughout the course of infection.

siRNA and plasmid nucleofection

20 nM siRNA oligonucleotides targeting murine *Abca1* (Dharmacon), FAK (Dharmacon), siLuciferase control (Dharmacon), and non-targeting controls (Invitrogen) were nucleofected into macrophages using program Y-001 on an Amaxa Nucleofector II. Nucleofection was performed 48 hours prior to infection.

Antibodies

Immunoblot analyses were performed using the following antibodies: FAK (Santa Cruz Biotechnology, Inc.), pFAK^{Y397}, Akt, pAkt^{S473}, HRP-linked anti-mouse IgG, HRP-linked anti-rabbit IgG (all Cell Signaling), IRDye® 680RD anti-mouse IgG, IRDye® 800CW anti-mouse IgG, IRDye® 680RD anti-rabbit IgG, and IRDye® anti-rabbit IgG (all LI-COR Biosciences). The mouse Abca1 antibody was a generous gift from Dr. David Castle (University of Virginia, Charlottesville, VA). Immunofluorescence staining was performed using rabbit anti-*Salmonella* (Thermo), donkey anti-rabbit AlexaFluor 488, and AlexaFluor 647 phalloidin (both Invitrogen).

Western blotting

Cells were seeded at 1.5x10^6 cells/well onto 6-well culture dishes 18h prior to infection at an MOI of 100. Cells were then rinsed twice with PBS and lysed in Triton X-100 lysis buffer (50 mM Tris pH 7.4, 1% Triton X-100, 150 mM NaCl, and 2 mM EDTA) supplemented with 1 mM sodium vanadate, 50 mM sodium fluoride, and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride and 1 mg/ml each of pepstatin, leupeptin, and antipain). Samples were loaded onto 4-20% SDS-polyacrylamide gels and probed using the indicated antibodies. Densitometry was performed using Image Studio Lite software (LI-COR Biosciences).

RNA extraction and qPCR

Cells were seeded at $1.5x10^6$ cells/well onto 6-well culture dishes 18h prior to infection at an MOI of 100. Cells were infected at an MOI of 100 for 30 minutes, followed by gentamicin treatment for the indicated time points. RNA was extracted from iBMDMs using a Trizol (Ambion) extraction protocol as per the manufacturer's instructions. cDNA production was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time qPCR analysis was performed using the ABI PRISM SDS7000 sequence detection system (Applied Biosystems) and the following Applied Biosystems TaqMan primer-probe sets: Mm00442646_m1 Abca1, Mm00437390_m1 Abcg1, and Hs99999901_s1 18S ribosomal subunit. PCR was performed using SYBR Green master mix (Applied Biosystems) as recommended by the manufacturer. The ΔΔCT method was used to quantify all relative mRNA levels as described (ABI user guide, 1997), using 18S RNA as the reference and internal standard.

Gentamicin protection assay

The gentamicin resistance assay has been described previously [326]. Briefly, cells were seeded at $1x10^5$ cells/well onto 24-well culture dishes 18h prior to infection at an MOI of 75. Cells were then lysed at 30 minutes, 1h, or 18h post-infection in 0.2% Triton X-100 in PBS and resuspended in additional PBS. CFUs were enumerated by plating 100 µL lysate dilutions onto LB agar.

SseJ complementation vector

Primers (IDT) were designed to specifically amplify SseJ (NCBI gene accession number NP_460590.1) and its putative promoter sequence [245] out of the *Salmonella* Typhimurium SL1344 genome. N-terminus primer: 5′-

CCGCGCGGATCCGTCAGATAATATGTACCAGGC-3′; C-terminus primer: 5′-CGCCTCGACTTCAGTGGAATAATGATGAGC-3′. The SseJ PCR product and pACYC184 prokaryotic expression vector were digested using BamHI and SalI restriction enzymes (New England Biolabs) and ligated together using T4 DNA ligase (New England Biolabs) to generate the pSseJ complementation vector. NEB Q5 Site-Directed mutagenesis (New England Biolabs) was used to generate the H384N catalytically inactive SseJ vector derived from the SseJ complementation vector described above. Primers (IDT) were designed with a single point mutation, converting a His residue to an Asn residue. N-terminus primer: 5'-CGACCTTGTCaatCCAACCCA-3'; C-terminus primer: 5'-TTGAAGACGTATTGCGGAC-3'. The pACYC184 vector was a kind gift from Melissa Kendall (University of Virginia, Charlottesville, VA).

Statistical analysis

Student's t test was used for the comparison of 2 independent groups. Two-way ANOVA with Tukey's multicomponent post-test was used when comparing more than 2 independent groups. All tests were performed with Graphpad Prism8, and p-values of 0.05 were considered statistically significant.

Acknowledgements

The authors thank Denise Monack for the gift of T3SS2 effector mutants, John Kagan for immortalized murine macrophages and Melissa Kendall for the pACYC184 vector. We also thank David Castle for

ABCA1 antibody, help with the fluorometric cholesterol assay, and many discussions about lipid

transport and processing. This work was funded by an NIH grant to JEC, RO1DK58536.

The authors declare no conflicts of interest.

Chapter 3:

Discussion and Future Perspectives

Chapter 3: Discussion and Future Perspectives

Novel Insights into *Salmonella*-Mediated Cholesterol Accumulation

The research detailed in **Chapter 2** investigated the molecular mechanism driving cholesterol accumulation during *Salmonella* Typhimurium infection of host macrophages. Prior to this study, previous work in the Casanova lab demonstrated *S.* Typhimurium induces signaling through the FAK/Akt/mTOR signaling axis to inhibit the autophagic clearance of bacteria [41]. Additionally, *S.* Typhimurium had previously been found to moderately increase cholesterol concentrations during infection of immortalized macrophages [241; 242], however the mechanism(s) driving cholesterol accumulation remained unknown. Here, we confirmed this finding in primary murine macrophages as measured by both endogenous cholesterol, as well as a fluorescently labeled cholesterol analog. Furthermore, we determined that the T3SS-2 effector SseJ induced signaling through FAK and Akt, resulting in the downregulation of *Abca1* and subsequent increase in intracellular cholesterol [**Figure 2-2**]. Pharmacological induction of *Abca1* caused cholesterol concentrations to remain near uninfected levels [**Figure 2-3**]. Importantly, *Abca1* suppression and the subsequent accumulation of cholesterol were important for bacterial survival [**Figure 2-4**], revealing a novel role for SseJ in promoting *S.* Typhimurium survival through the FAK- and Akt-dependent inhibition of ABCA1-mediated cholesterol efflux.

These findings pose three major unanswered questions that will serve as the basis for future investigations by the Casanova lab, as well as several minor questions. First, how does SseJ activity lead to the recruitment and activation of FAK? It is possible that the acyltransferase activity of SseJ alters the SCV membrane composition or produces

deacylated lysophospholipids to promote the recruitment and phosphorylation of FAK at the SCV surface. Additionally, SseJ may bind and promote FAK autophosphorylation directly, or indirectly through another FAK-binding partner, talin-1. Second, how does *Salmonella* induce such a robust accumulation of cholesterol without any deleterious effects to the host cell? Since continued biosynthesis of cholesterol (specifically cholesterol precursors) is important for *Salmonella* survival, *Salmonella* infection appears to prevent the ER from sensing the accumulation of cholesterol by retaining cholesterol in the endocytic network. How this occurs remains unclear, although it may involve the manipulation of multiple host cholesterol transport proteins. Third, how does cholesterol accumulation improve *Salmonella* survival in macrophages? One possibility is that cholesterol accumulation may prevent the fusion of SCVs with host lysosomes and interfere with lysosomal enzyme transport. Alternatively, elevated cholesterol concentrations may interfere with host autophagy and prevent the autophagic killing of *Salmonella* in macrophages. A more thorough discussion of these questions will be presented below.

Discrepancies with Earlier Studies - Lack of Detectable Cholesteryl Ester Production

These results are largely congruent with previous findings; however, our results differed from those of Nawabi, *et al*. in the detection of cholesteryl esters (CEs) during *S.* Typhimurium infection [251]. Our preliminary studies using thin-layer chromatography to investigate levels of total and esterified cholesterol showed that Δ*invG* infection failed to increase cholesteryl ester levels in primary macrophages compared to uninfected controls. Furthermore, preliminary immunofluorescence experiments also failed to observe an increase in lipid droplet formation during Δ*invG* infection [**Figure 3-1**]. In contrast,

Nawabi, *et al*. previously reported an SseJ-dependent increase in CEs during *S.* Typhimurium infection of immortalized macrophages [251]. Several differences in experimental setup may account for this discrepancy. First, our model for macrophage infection utilizes *ΔinvG S.* Typhimurium. While this strain is on a SL1344 background, the deletion of InvG, completely eliminates T3SS-1 function. Conversely, Nawabi, *et al*. used wild-type SL1344 *S.* Typhimurium, suggesting that one or more SPI-1 effector proteins translocated through T3SS-1 may potentiate SseJ-mediated CE production. Secondly, Nawabi, *et al*. utilized immortalized RAW 264.7 macrophages, whereas we used primary murine macrophages for *S.* Typhimurium infection. Although immortalized macrophages provide a common model for studying host-pathogen relationships, immortalized cell lines often contain significant differences in cell signaling and metabolism. Furthermore, *S.* Typhimurium has been shown to induce different proteomic responses between immortalized and primary macrophages [340], suggesting that the production of cholesteryl esters may differ depending on host cell type. Indeed, lipid droplets extracted from *Salmonella*-infected Hep2 epithelial cells do not contain elevated levels of cholesteryl esters [341], further suggesting that *Salmonella*-induced cholesteryl ester formation may be cell type-dependent.

In eukaryotic cells, excess free cholesterol is transported to the ER where it is esterified by ER-resident ACAT and subsequently stored in the form of lipid droplets [**Figure 1-5**]. SseJ serves the same function from the cytosolic surface of SCVs and other endosomal compartments [210; 250]; however, the fate and trafficking of cholesteryl esters produced at endosomal compartments remains unclear. The lack of CE and lipid droplet production in our preliminary experiments suggests that free cholesterol within *ΔinvG*-

infected primary macrophages fails to reach the ER for esterification by host ACAT [**Figure 3-1**]. Furthermore, the lack of detectable SseJ-mediated cholesterol esterification suggests that: 1) cholesterol within the endocytic network fails to reach the exofacial leaflet of the membrane and instead remains inaccessible to SseJ within the endosomal lumen, 2) cholesterol on the cytosolic face of the endosome is bound by various host proteins such as mTOR (see below) and is therefore inaccessible to SseJ, or 3) CEs generated by SseJ are rapidly hydrolyzed back into free cholesterol. Although more work needs to be done to clarify CE production (or lack thereof) in our model, the absence of CE accumulation during infection of primary macrophages implies a significant disruption of host cholesterol trafficking to the ER, as discussed further below.

Potential Mechanisms for SseJ-Mediated FAK Activation

Previous work from our lab revealed that *S.* Typhimurium activates the FAK/Akt signaling axis in macrophages [41]. While we identified SseJ as being necessary for FAK activation during *S.* Typhimurium infection, the mechanism(s) of activation remain unknown [**Figure 2-5**; **Figure 2-6**]. Unstimulated FAK exists in the cytosol in an autoinhibited conformation with the N-terminal FERM domain binding to the catalytic domain [259]. Intriguingly, the phospholipid composition of the plasma membrane has been shown to affect FAK signaling, with FAK activation preferentially occurring in $PI(4,5)P_2$-rich domains [263]. Although SCVs have been shown to contain both PI(3)P and $PI(4,5)P_2$ at early stages of infection [342], the lipid composition of the SCV during later stages of infection remains unclear. However, *Salmonella* induces extensive actin polymerization surrounding the SCV during later stages of infection to maintain bacterial survival [343]. As $PI(4,5)P_2$ is important for actin polymerization [346], this suggests the

presence of $PI(4,5)P_2$ on the SCV membrane. Furthermore, $PI(4,5)P_2$ is converted into PIP_3 by PI3K following the binding of PI3K to phosphorylated FAK [344]. Akt then binds PIP_3-rich membranes where it is phosphorylated by PDK1 [345]. Since Akt is recruited to SCVs following *Salmonella* infection [41], it is highly likely that $PI(4,5)P_2$ is present on the SCV membrane and may facilitate FAK and Akt activation. Notably, both the catalytic and autoinhibitory FERM domains directly bind multiple phosphoinositide species. However, phospholipid binding does not affect activation of isolated catalytic domain, suggesting that phospholipid-rich membranes release FAK autoinhibition, promoting autophosphorylation and subsequent Src-dependent phosphorylation [347; 348]. As SseJ deacylase activity has been demonstrated by the generation of cholesteryl esters in cells and the removal of short chain fatty acid groups from a butyrate ester substrate [250; 251], it is possible that many lipid species contribute as substrates for SseJ activity. Therefore, while it is unlikely that SseJ is directly producing $PI(4,5)P_2$ on the SCV, the esterification of cholesterol and the deacylation of charged phospholipids may alter the lipid packing of the SCV membrane and generate a permissive environment for the recruitment of Src and FAK and the release of FAK autoinhibition. Determining the lipid composition of the SCV will be important for addressing this possibility.

The use of in vivo probes towards various phosphoinositide species [349] will allow for tracking phospholipid dynamics on the SCV in the presence or absence of SseJ. Additionally, complementing our $FAK^{-/-}$ macrophages with mutant FAK constructs designed to disrupt $PI(4,5)P_2$ binding [347] would reveal whether phosphoinositides regulate FAK recruitment and activation during *S.* Typhimurium infection. Alternatively,

since phospholipid binding stabilizes uninhibited FAK [348], FAK signaling induced by other mechanisms may be reinforced by changes in the SCV phospholipid composition.

Catalytic activity of SseJ produces cholesteryl esters and deacylated phospholipids, also referred to as lysophospholipids. Lysophospholipids come in a diverse array of species dependent upon the phospholipid substrate and act as second messengers for the regulation of many cellular pathways [350; 351]. Lysophosphatidic acid (LPA) is one of the better characterized species of lysophospholipids. LPA is generated through the phospholipase- or acyltransferase-dependent hydrolysis or deacylation of phosphatidylcholine. LPA then binds one of six LPA G protein-coupled receptors to stimulate signaling through Gα protein pathways [352]. Signaling through LPA_1 receptor stimulates $G_{12/13}$ to induce RhoA-induced actin cytoskeleton rearrangements, while G_i stimulation promotes PI3K/Akt signaling [353; 354]. This mechanism of FAK activation is unlikely, as LPA receptor-mediated activation of RhoA at the plasma membrane would require the transport of SseJ-generated lysophospholipids from the SCV to the plasma membrane or recruitment of LPA receptors to the SCV. It is possible that LPA and other lysophospholipids are removed from the SCV membrane by fatty acid binding protein (FABP) and packaged within microsomes for intracellular transport and extracellular secretion [355]; however, it is unlikely that autocrine signaling by secreted lysophospholipids regulates cholesterol homeostasis, as uninfected cells neighboring infected cells rarely demonstrate cholesterol accumulation [**Figure 2-1**; **Figure 2-S1**].

Potential mechanisms for lysophospholipid-mediated FAK activation remains unclear, although one promising potential mechanism involves the cytosolic serine/threonine kinase protein kinase C (PKC) as an intermediate. LPA has been

demonstrated to stimulate multiple PKC isoforms, with the magnitude of activation dependent upon the phospholipid composition of host membranes [356; 357]. Multiple PKC isoforms can induce FAK activation through a variety of mechanisms, including direct phosphorylation of FAK/Src, and the indirect activation through the stimulation of cytoskeletal rearrangements [358; 359]. Therefore, lysophospholipid species generated by SseJ acyltransferase activity may play an important functional role in stimulating FAK/Akt signaling. Of the two possibilities presented here, the direct activation of FAK by LPA-induced PKC signaling seems much more likely than the indirect activation of FAK through PKC-dependent cytoskeletal rearrangements. The timing of FAK recruitment to the SCV correlates to the timing of FAK activation during *Salmonella* infection [41], suggesting that FAK activation occurs at the SCV surface instead of peripheral compartments (e.g., focal adhesions). As cytoskeletal rearrangements are conventionally thought to induce FAK activation by providing a mechanical force to separate FAK autoinhibition, it is unlikely that actin polymerization on an unanchored SCV would provide force to relieve FAK autoinhibition.

As the complete characterization of lipid substrates for SseJ remains unclear, the lysophospholipids produced also remain unknown. Ongoing lipidomics studies in our laboratory will seek to identify the lipid species being consumed and produced following the infection of macrophages with wild-type or *ΔsseJ S*. Typhimurium. This will provide valuable insight into the potential role of lysophospholipids in FAK activation and *Salmonella* pathogenesis.

Alternatively, a simple explanation for SseJ-mediated FAK activation may be the direct or indirect binding between these two proteins recruiting FAK to the SCV, causing

a conformational shift in FAK leading to autophosphorylation. Recent proteomics studies have attempted to identify host-pathogen protein-protein interactions during *S.* Typhimurium infection. Although one specific study did not report an interaction between FAK and bacterial effectors, it did identify the binding between SseJ and talin-1 [360]. Talin-1 binds F-actin, as well as the c-terminal FAT domain of FAK [361; 362], suggesting that SseJ may indirectly interact with FAK through the recruitment of talin-1 to the actin-rich SCV. Immunofluorescence microscopy investigating the potential colocalization of FAK and talin-1 on SCVs during infection with *ΔinvG, ΔsseJ,* and catalytically inactive SseJ *S.* Typhimurium strains will determine whether talin-1 is essential for the recruitment of FAK to the SCV. Additionally, immunoprecipitation of talin-1, FAK, and our various SseJ constructs will determine whether FAK and SseJ biochemically interact.

Together, these future studies will provide further insight into the mechanism(s) of SseJ-mediated FAK activation. I anticipate that the catalytic activity of SseJ promotes FAK activation through the modulation of the SCV membrane, causing the enrichment of $PI(4,5)P_2$ on the SCV surface. I anticipate that $PI(4,5)P_2$ on the SCV membrane promotes the recruitment and autophosphorylation of FAK, and also serves as the substrate for PI3K-dependent PIP_3 production, which leads to the subsequent recruitment and phosphorylation of Akt. Therefore, I anticipate that phosphoinositide probes will detect a significant accumulation of $PI(4,5)P_2$ on the SCV surface compared to host endosomes, and that complementation with a $PI(4,5)P_2$ binding-deficient FAK mutant will not be able to restore Akt phosphorylation and *Abca1* suppression during infection of $FAK^{-/-}$ macrophages.

Functional Implications of FAK- and Akt-Dependent Cholesterol Accumulation

As previously mentioned, numerous disease-causing microorganisms target and/or manipulate host cell cholesterol homeostasis for invasion and survival. Certain bacterial species, such as *Anaplasma, Ehrlichia, and Neisseria gonorrhoeae,* have been shown to closely associate with and/or directly incorporate host-derived cholesterol into the bacterial membrane [363; 364]. In contrast, cultured *Salmonella* preparations were almost entirely devoid of cholesterol when grown in a cholesterol-spiked medium [364], suggesting that the accumulating cholesterol seen during *S.* Typhimurium infection is not being directly incorporated into the bacterial membrane, but likely remains within host cell compartments [**Figure 2-1**] where it impacts various host processes to promote bacterial survival.

Salmonella Infection Prevents the ER from Sensing Cholesterol

Proper cholesterol transport between intracellular compartments is critical for maintaining host cholesterol homeostasis. In the ER membrane, cholesterol 25-hydroxylase converts cholesterol into 25-hydroxycholesterol (25HC). As 25HC and other oxysterols are potent activators of LXR signaling and cholesterol efflux [365], the persistent suppression of *Abca1* and sustained cholesterol accumulation within *Salmonella*-infected cells suggests that oxysterols are not being generated due to the lack of cholesterol transport into the ER. The accumulation of cholesterol within the endocytic network we see during infection is highly similar to the distribution in NPC1-deficient cells, where cholesterol is entrapped within the endosomal lumen [185; **Figure 2-1**]. Furthermore, inhibition of cholesterol biosynthesis does not disrupt *Salmonella*-induced cholesterol accumulation in vesicular compartments [241], suggesting that *Salmonella* actively redirects newly acquired extracellular cholesterol, not *de novo* synthesized cholesterol, into

the SCVs and host endosomal network. This phenotype is similar to that seen in cells with impaired endosomal cholesterol efflux, where cholesterol biosynthetic signaling is maintained in cholesterol-rich conditions (see below).

In otherwise healthy cells, excessive accumulation of free cholesterol leads to a robust, cytotoxic ER stress response. The abundance of free cholesterol in the normally cholesterol-poor ER membrane increases the packing of phospholipids, causing membrane stiffening and impaired ER protein function. Alterations in ER membrane fluidity and the dysfunction of integral membrane proteins likely contribute to the free cholesterol-induced unfolded protein response and the upregulation of C/EBP homologous protein (CHOP), a transcription factor which upregulates pro-apoptotic genes and induces inflammatory cell death [366; 367].

Furthermore, the sensing of elevated cholesterol concentrations within the ER inhibits SREBP2 cleavage and downregulates cholesterol biosynthesis [199]. Since *Salmonella* requires precursors of SREBP2-dependent cholesterol biosynthesis, inducing ER cholesterol starvation may be an efficient way for the bacteria to maintain synthesis of these precursors while avoiding cytotoxic stress. Together, the observations by us and others are highly suggestive of a cholesterol trafficking defect preventing cholesterol from reaching the ER. Measuring SREBP2 signaling through *HMGCR* expression and the cleavage of SREBP2 during infection with *ΔinvG* and our SseJ mutant strains will confirm whether *Salmonella* impairs host ER sensing of cholesterol and increases cholesterol biosynthesis. The dispersed distribution of BODIPY-cholesterol seen during *ΔsseJ* infection suggests that cholesterol is not retained in the endocytic network. Therefore, I

anticipate that *ΔsseJ* infection increases the sensing of host cholesterol by the ER and will result in the reduction of SREBP2 cleavage and *HMGCR* expression.

These experiments will provide valuable insight into whether SseJ and/or the enzymatic activity of SseJ is important for preventing cholesterol sensing by the ER. The potential mechanisms for how cholesterol transport may be impaired will be further discussed below.

ER-SCV Membrane Contacts are Likely Sites of Lipid Transport

The ER makes contacts with numerous organelles through the formation of membrane contact sites (MCS). MCS utilize protein complexes between ER and target organelles to tether the compartments in close proximity without membrane fusion and are frequent sites of non-vesicular lipid transport [368; 369]. Previous studies and our unpublished work reveal that SCVs contain the intra-endosomal cholesterol transport protein NPC1 and colocalize with both the ER MCS tether protein VAP-A and the ER-endosomal cholesterol exchanger OSBP1 [258; 360; **Figure 3-2**]. The depletion of VAP-A has been shown to decrease SCV integrity and increase the cytosolic escape of *Salmonella* [258], revealing the importance of ER-SCV contacts in maintaining SCV stability. In contrast, expansion of endosome-ER contacts is sufficient to drive cholesterol efflux out of the endocytic network and to the ER [190]. As VAP-A is critical for the recruitment of lipid transfer proteins at endosome-ER MCS to promote endosomal cholesterol efflux, I hypothesize that *S*. Typhimurium interacts with and recruits multiple host lipid transport proteins to the SCV to facilitate cholesterol removal from the ER. Determining the importance of ER-SCV MCS in cholesterol distribution during infection is complicated by the importance of VAP-A in mediating ER contacts with numerous

organelles to maintain normal host functions. Therefore, investigating the role and regulation of specific lipid transport proteins recruited to the SCV may prove more insightful.

Regulation of Lipid Transport Proteins by Cholesterol

The lack of cholesterol associated with *ΔsseJ* SCVs raises an interesting question: is cholesterol accumulation on a whole-cell level required for the enrichment of cholesterol within the endocytic network or does *Salmonella* actively manipulate intracellular cholesterol transport at baseline cholesterol concentrations? Our findings presented in **Figure 2-3** suggest that cholesterol is not preferentially enriched within endosomal compartments of cholesterol-depleted (i.e., T0901317 treated) cells infected with *ΔinvG Salmonella*. Therefore, while it is likely that SseJ directly impacts endosomal cholesterol trafficking through the recruitment of lipid transport proteins, the enrichment of cholesterol within the endocytic network seems to require the suppression of cholesterol efflux.

Endosomal cholesterol transport is a highly regulated process requiring the coordinated functions of many lipid transport proteins, such as NPC1, OSBP1 and related proteins (ORPs), and GRAMD1 proteins. NPC1 is an integral membrane protein found in late endosomes and lysosomes that tethers the ER on late endosomes by binding to VAP-A on the ER membrane. NPC1 directly binds the cholesterol transport protein GRAMD1b to promote cholesterol efflux out of late endosomes/lysosomes and towards the ER [190]. In the absence of NPC1 (Niemann Pick disease), endosomes become engorged with cholesterol and cholesterol transport to the ER is impaired [370]. This reduction of ER cholesterol results in the sustained signaling through the SREBP2 cholesterol biosynthesis pathway, even in the presence of increased cellular cholesterol levels [185; 371]. Infection

of NPC1$^{-/-}$ HeLa cells with *S.* Typhimurium infection did not further increase cholesterol near the SCVs compared to wild-type cells, supporting my hypothesis that SseJ is establishing an NPC1$^{-/-}$-like phenotype by actively maintaining cholesterol in the endocytic network by interfering with cholesterol transport. In contrast, cholesterol localization towards *ΔsseJ* SCVs (and cholesterol staining in general) in wild-type cells was significantly impaired compared to wild-type SCVs, indicating that SseJ is required for both cholesterol accumulation and the relocalization of intracellular cholesterol to the SCV [**Figure 2-S5**; 360].

Interestingly, SseJ was found to directly interact with both NPC1 and OSBP1 and recruit them to the SCV [258; 360]. OSBP1 is a Golgi-localized cholesterol transport protein which binds VAP-A to facilitate the exchange of ER cholesterol for PI(4)P in the Golgi [192]. In the absence of OSBP1 recruitment, SCVs were destabilized, and *Salmonella* escaped into the host cell cytoplasm [258]. Notably, Kolodziejek, *et al*. did not address whether OSBP1-mediated cholesterol transport was important for SCV integrity; however, I hypothesize that OSPB1-mediated cholesterol transport would direct cholesterol from the ER to the SCV to further enhance endosomal cholesterol accumulation while maintaining ER cholesterol starvation and cholesterol biosynthesis.

The OSBP-like protein ORP1L localizes to late endosomes through the binding of PI(4)P, where it serves as a cholesterol sensor and facilitates cholesterol transport from the exofacial leaflet of endosomal membranes to the ER [372]. Single-cell RNA sequencing has shown that *S.* Typhimurium downregulates ORP1L expression in macrophages [373]. ORP6 has also been implicated in the transport of cholesterol from endosomes to the ER, as the depletion of ORP6 causes endosomal accumulation of cholesterol [374], similar to

what we see during *Salmonella* infection [**Figure 2-1**]. Of note, one proposed mechanism for ORP1L and ORP6 cholesterol transport is the exchange of endosomal cholesterol for ER PI(4)P. However, the ER membrane is largely devoid of PI(4)P due to its hydrolysis by the resident PI(4)P phosphatase, Sac1 [375]. This suggests either the presence of a currently unknown counterlipid, or unidirectional cholesterol transport without the requirement for lipid exchange. Alternatively, endosomal cholesterol transport to the ER has recently been proposed to move through the PM as an intermediate [186], suggesting that ORP1L and ORP6 may facilitate endosome-to-ER cholesterol transport by delivering cholesterol to the PM for subsequent PM-to-ER transport by GRAMD1 proteins [188]. Remarkably, the transcriptional regulation of *ORP6* shares many components with the regulation of *ABCA1* and is upregulated in response to elevated cholesterol [374]. Due to these similarities, I hypothesize that *Salmonella* infection downregulates *ORP6* to suppress cholesterol transport out of the endocytic network and prevent the ER from sensing elevated concentrations of free cholesterol. To date, the role of ORP6 in *Salmonella* infection remains unknown. Future studies will measure ORP6 expression in uninfected and infected macrophages. Additionally, as ORP6 overexpression has been shown to enhance cholesterol transport to the ER and promote cholesterol efflux, I anticipate that the overexpression of ORP6 during *Salmonella* infection would prevent cholesterol accumulation and reduce bacterial survival.

A recent trend of proteomics studies identifying *Salmonella*-host protein-protein interactions, accompanied by future targeted studies using proximity labeling for SseJ and other SCV-associated effectors will likely identify additional lipid and cholesterol transport proteins that localize to the SCV. Follow-up studies determining the functionality of these

transport proteins will need to be performed, as the proteins could be sequestered and inhibited in the SCV membrane, preventing their lipid transport activity. Investigation into the role of MCS and lipid transport proteins during infection will require a combination of cytosolic cholesterol labels (e.g., the D4 domain of perfringolysin O [376]), transport- or cholesterol binding-deficient mutants, knockdowns, *Salmonella* effector mutant strains, and eventually effector-binding mutants. However, careful use of proper controls will be necessary to ensure that any resulting phenotypes are specifically due to the loss of function on the SCV and not the generalized disruption of host lipid transport. I expect that interfering with cholesterol transport out of the ER via OSPB1 will reduce cholesterol labeling on the cytosolic face of the SCV and have only moderately deleterious effects on *Salmonella*-induced cholesterol accumulation, as the only source of ER cholesterol during infection is presumably from biosynthesis. Conversely, I anticipate the ectopic expression of proteins directing cholesterol out of the endosomal network (ORP1L, ORP6) will result in the significant attenuation of *Salmonella* survival and increased cytotoxicity of the host cell due to the loss of cholesterol retention within the endocytic network.

Cholesterol-induced Mistrafficking of Hydrolases and Lysosomal Dysfunction

The classical dogma of SCV maturation proposes the complete isolation of SCVs from host lysosomes, as SCV-lysosome fusion would result in bacterial death. Endosome-lysosome fusion events occur through SNARE proteins, a family of membrane-associated proteins which form membrane complexes to bring target membranes in close proximity in order to facilitate fusion [377]. Elevated cholesterol impairs endosome-lysosome membrane fusion by sequestering SNARE proteins in cholesterol-rich membranes as inactive complexes, impairing their trafficking and function [323]. Thus, the enrichment of

cholesterol within SCV and endosomal compartments may prevent lysosomal fusion events, explaining the dearth of lysosomal cargo found associated with SCVs.

While avoidance of lysosomal fusion was largely inferred from the absence of M6PR-transported lysosomal hydrolases within the SCV [53; 54], it is becoming more widely appreciated that SCVs do, in fact, interact with host lysosomes during the course of infection [62; 63]. Unexpectedly, SCV fusion with host lysosomes does not result in efficient killing of *S.* Typhimurium by antimicrobial lysosomal hydrolases. How *Salmonella* survives fusion with lysosomes remains unclear; however, one possibility is the inhibition of lysosome acidification. Cholesterol feeding of macrophages has previously been shown to inhibit vacuolar H^+-ATPase (v-ATPase) activity, preventing lysosome acidification and cholesterol transport to the ER [328]. As the acidification of lysosomes is necessary for the activation of most lysosomal hydrolases and inhibition of microbial metabolism, *Salmonella*-induced cholesterol accumulation likely inhibits the acidification of phagosomes to prevent bacterial killing following SCV-lysosome contact. This hypothesis can easily be detected by immunofluorescence with pH sensitive dyes (like pHrodo) during infection in the presence or absence of T0901317 treatment.

Alternatively, the depletion of lysosomal hydrolases from the lysosome may allow for *Salmonella* survival following SCV-ER contact. M6PRs facilitate the delivery of lysosomal hydrolases and cathepsins to pre-lysosomal compartments, followed by recycling back to the TGN for reloading with more cargo. Disrupting M6PR recycling prevents the delivery of hydrolases to the lysosome and promotes the mistrafficking and secretion of M6PR cargo out of the cell [378]. Cholesterol accumulation within NPC1-depleted cells reduces intracellular levels of M6PR either through degradation or

downregulation of expression [329]. Furthermore, PI(4)P and cholesterol accumulation in endosomes causes the aberrant trafficking of M6PR away from the endocytic network [379]. These findings demonstrate that under cholesterol-rich conditions, M6PR recycling is impaired. This causes the gradual reduction of M6PR from the Golgi, reducing the amount of M6PR cargo being transported to lysosomes, which subsequently leads to the secretion of excess lysosomal hydrolases and M6PR cargo out of the cell.

While *Salmonella* has previously been shown to inhibit recycling of M6PRs and increase secretion of lysosomal enzymes in a SifA-dependent manner [58], the role of cholesterol in M6PR mistrafficking remains unclear. Therefore, I hypothesize that cholesterol accumulation in *Salmonella*-infected macrophages prevents the recycling of M6PRs and promotes the aberrant secretion of M6PR-dependent lysosomal hydrolases, preventing the degradation of *Salmonella* following lysosomal fusion. As NPC2 is delivered to the endocytic network via M6PR [184], the disruption of M6PR recycling would further enhance endosomal cholesterol accumulation by inhibiting NPC-dependent cholesterol transport.

Measuring intracellular and secreted lysosomal hydrolases or monitoring cathepsin-dependent proteolysis in lysosomes or secreted fractions would allow for us to probe the role of cholesterol accumulation in M6PR mistrafficking. If cholesterol accumulation leads to the secretion of lysosomal hydrolases as hypothesized, the levels of extracellular pro-cathepsin concentrations and possibly proteolytic activity would be elevated in wild-type infection compared to *ΔsseJ* or T0901317-treated cells. Furthermore, I expect that the exogenous addition of cholesterol to host cells would enhance cathepsin secretion. Finally, I anticipate that treating T0901317-treated or *ΔsseJ*-infected cells with

lysosomal hydrolase and cathepsin inhibitors would partially restore bacterial survival. Together, these future experiments will clarify the role of cholesterol in the regulation of M6PR transport.

The Role of Cholesterol Accumulation in Regulating Autophagy

Canonical autophagy is a highly important cellular process for degrading and recycling foreign and endogenous cellular material in response to cellular stress and nutrient starvation. Elevated cholesterol has previously been shown to impair autophagic flux. In NPC1-deficient cells, Rab7a and SNARE proteins are redistributed to cholesterol-rich endosomal membranes, inhibiting the fusion of autophagosomes with lysosomes and preventing degradation of autophagic cargo [324]. Thus, the enrichment of cholesterol within host endosomes may impair membrane fusion and autophagic flux. As a form of selective autophagy, xenophagy induces antimicrobial effects by utilizing conventional autophagic machinery to target and eliminate intracellular pathogens [380]. mTORC1 serves as the master regulator of autophagy, with active mTORC1 inhibiting core autophagy components and suppressing autophagic flux. While our lab had previously established that the FAK- and Akt-mediated suppression of autophagy increases *Salmonella* survival, the role of cholesterol accumulation in regulating autophagy remains unknown.

Although mTORC1 is canonically regulated by nutrient sensing within the cell, cholesterol has also been shown to activate mTORC1 and suppress autophagy. In an elegant series of works by the Roberto Zoncu group, cholesterol accumulation in endosomes was demonstrated to drive active mTORC1 to cholesterol-rich endosomal membranes and prevent lysosomal degradation of autophagic cargo. Lim, *et al*.

demonstrated that OSBP1 localizes to mTORC1^{+} late endosomes/lysosomes where it facilitates cholesterol transfer from the ER to the cytosolic surface of late endosomes/lysosomes. This transfer required the association of OSBP1 with VAP-A and VAP-B and the formation of ER/endosome MCS. Inhibition of OSBP1 or the depletion of cellular cholesterol prevented cholesterol accumulation on the cytosolic surface of endosome/lysosomes, impaired mTORC1 activation on lysosomes, and increased autophagic flux [327]. These findings suggest that the SseJ-mediated recruitment of OSBP to the SCV may enhance cholesterol transport from the ER to the surface of the SCV, allowing for the recruitment and aberrant activation of mTORC1, suppressing autophagy and enhancing bacterial survival.

The lumenal accumulation of cholesterol within NPC1$^{-/-}$ cells has also been shown to enhance mTORC1 activation and reduce autophagic flux [325; 381]. Exactly how lumenal cholesterol stimulates mTORC1 activation remains unclear, but it may require the disruption of the lysosomal glycocalyx. The glycocalyx is composed of numerous membrane glycoproteins which are modified by polysaccharides to increase the thickness of the lysosomal membrane to protect against the degradative enzymes within [382]. Remarkably, modifications of the lysosomal glycocalyx increased cholesterol efflux out of NPC1-deficient endosomes [184], suggesting that *Salmonella*-mediated disruption of the lysosomal glycoprotein barrier may allow for NPC1-independent transport of cholesterol out of the endosomal lumen. However, a much more plausible mechanism for the recruitment of mTORC1 to SCVs in the absence of surface cholesterol enrichment is the direct recruitment of mTORC1 to the SCV surface. In support of this mechanism, co-IP of SCV proteins identified that multiple proteins, including SifA and SseJ, interact with

mTOR [360], suggesting that effector-mediated binding may be the primary source of mTORC1 recruitment.

In this work, we show that ABCA1 depletion in FAK-deficient macrophages restored *Salmonella* survival [**Figure 2-4**]. As it is well established that FAK depletion enhances the autophagic killing of *Salmonella* [41], these findings suggest that *Abca1* suppression and/or cholesterol accumulation enhances *Salmonella* survival independent of autophagy, or that they act downstream of Akt to suppress the autophagic clearance of *S.* Typhimurium. Teasing apart the relationship between cholesterol and autophagy during *Salmonella* infection is one of the main future directions of this project.

Current and previous findings from our lab are largely in support of our hypothesis that the SseJ-induced cholesterol accumulation enhances bacterial survival by impairing autophagic targeting of intracellular *S.* Typhimurium and reducing autophagic flux within host cells. To this end, another member of the Casanova lab, Holly Torsilieri, has continued this line of research and generated intriguing preliminary data suggesting cholesterol accumulation may be the primary driver of mTORC1 activation and inhibition of autophagy. In agreement with previous results, she found that mTOR signaling is enhanced during *S.* Typhimurium infection of macrophages. Furthermore, preliminary immunofluorescence data suggests that mTOR preferentially localizes to SCVs compared to other endolysosomes. In contrast, mTOR signaling and localization to SCVs was reduced in the absence of cholesterol accumulation due to either T0901317 treatment or *ΔsseJ* infection [Torsilieri. Unpublished]. These findings suggest that cholesterol may be important for the passive localization of mTORC1 to all endosomes, while the bacteria

actively recruit mTORC1 to the SCV surface; however, more work will need to be done to confirm the significance of these initial findings.

Future mTOR and LC3 (a marker for autophagosomes) localization and mTOR immunoprecipitation studies using the catalytic SseJ mutants generated in our above research will allow us to determine whether SseJ recruits and activates mTOR and inhibits LC3 localization to SCVs during infection. These same studies with T0901317 treatment or cholesterol feeding will continue to probe the role of cholesterol in mTORC1 recruitment and autophagosome formation. It will be interesting to determine whether mTORC1 is recruited to and whether LC3 is blocked from SCVs during infection of ABCA1-depleted FAK$^{-/-}$ macrophages. As silencing *Abca1* has been shown to rescue *Salmonella* survival in FAK$^{-/-}$ macrophages [**Figure 2-4**], I expect SCVs to be increased in mTOR localization and depleted of LC3 in ABCA1-depleted FAK$^{-/-}$ cells. As LC3 is targeted to SCVs in FAK$^{-/-}$ cells during the early stages of infection [41], is intriguing to speculate that SseJ-dependent signaling through FAK/Akt/mTOR may initially suppress global autophagic responses within target host cells, while the subsequent downregulation of *Abca1* and increase in intracellular cholesterol seen during later stages of infections leads to the sustained targeting of mTORC1 to the SCVs for the specific inhibition of autophagic killing of *S.* Typhimurium.

Further investigation into the role of SseJ and cholesterol in regulating autophagy will utilize our catalytically inactive SseJ mutants and probes for autophagic flux (GFP-LC3-RFP-LC3ΔG) to monitor autophagic activity within untreated and T0901317-treated cells during infection. This will allow us to determine whether SseJ activity and/or cholesterol accumulation is the primary driver of the suppression of autophagy.

Alternatively, loading FAK-depleted and Akt-inhibited cells with cholesterol will allow us to test whether increased cholesterol is sufficient to overcome the loss of FAK/Akt signaling and suppress autophagic flux independent of ABCA1 suppression. Conversely, cholesterol depletion of *ΔinvG*-infected wild-type macrophages is expected to increase autophagic flux. Experiments using a combination of these approaches will provide valuable insight into the role of cholesterol in regulating the autophagic targeting of *S.* Typhimurium.

The Role of Autophagy in Regulating Cholesterol Homeostasis

Just as cholesterol has been demonstrated to play an important role in the regulation of mTORC1 and autophagy, mTOR signaling itself directly impacts lipid metabolism. Inhibition of mTORC1 has been shown to prevent SREBP1-dependent fatty acid synthesis, while mTORC1 activation enhances SREBP2 signaling by inhibiting cholesterol trafficking out of the lysosomes to the ER through an unknown mechanism [383; 384]. In contrast, the induction of autophagy independent of mTOR activity enhanced cholesterol transport to the ER and suppressed SREBP2 signaling [384], suggesting that mTORC1 activity may contribute to the retention of cholesterol in endosomes following *Salmonella* infection.

Furthermore, the combined work from our lab demonstrates that *S.* Typhimurium-induced FAK/Akt/mTOR signaling suppresses the autophagic killing of bacteria and that SseJ-induced FAK/Akt signaling also led to the suppression of cholesterol efflux through the downregulation of *Abca1* [41; **Figure 2-2**]. In unpublished work, Kate Owen additionally found that inhibition of mTOR by rapamycin treatment prevented *S.* Typhimurium suppression of *Abca1* [**Figure 2-0**]. Subsequently, our model may be

expanded such that SseJ stimulates the FAK/Akt/mTOR signaling axis to downregulate *ABCA1* and inhibit the autophagic clearance of *S.* Typhimurium [**Figure 3-4**]. However, given that the mTOR inhibitor rapamycin significantly attenuates bacterial survival [41], future work will have to confirm the role of mTOR in *Abca1* suppression by infecting with a higher MOI to ensure equal numbers of surviving bacteria.

Although we show that SseJ-induced FAK/Akt (and possibly mTOR) signaling is necessary for the downregulation of *Abca1* and the subsequent accumulation of intracellular cholesterol [**Figure 2-0**], the direct mechanism of *Abca1* suppression remains unclear. Frechin, *et al*. propose a mechanism through which Akt stimulates the phosphorylation of the FOXO3 and TAL1 transcription factors, inhibiting their activity and the transcription of *Abca1* [228]. Furthermore, active mTOR and reduced autophagy has been shown to directly inhibit LXR and ABCA1 upregulation [385]. Therefore, it is likely that both mTOR and Akt contribute to the downregulation of *Abca1* during *Salmonella* infection [**Figure 3-3**].

Determining the mechanism behind *Abca1* downregulation and the role of autophagy in *S.* Typhimurium virulence is of high interest to the future direction of this project. I hypothesize that *Salmonella*-induced mTORC1 activity contributes to the sustained inhibition of cholesterol efflux and cholesterol starvation of the ER, allowing for the continued synthesis of cholesterol precursors necessary for survival. While mTORC1 activation may inhibit cholesterol transport out of the endocytic network, I expect this to be secondary to the inhibition of cholesterol transport proteins. Furthermore, I hypothesize that *Salmonella*-induced *Abca1* downregulation occurs through the coordinated efforts of Akt and mTORC1: Akt-dependent FOXO3 and TAL-1 inhibition results in the initial

downregulation of *Abca1*, while increased mTORC1 activation maintains *Abca1* suppression by rendering host cells insensitive to LXR signaling. I expect that *ΔinvG* infection will increase the phosphorylation of FOXO3 and reduce TAL1 protein levels, while infection with SseJ mutant strains will reduce the phosphorylation of FOXO3 and increase TAL1 protein levels in the nucleus.

Outstanding Questions

Although our findings detail mechanisms for the SseJ-dependent cholesterol accumulation during *Salmonella* infection of macrophages, these results raise additional questions which we have not addressed above:

1) *What is the role of biosynthetic cholesterol precursors in Salmonella survival?*

Previous studies have demonstrated that signaling through the cholesterol biosynthetic pathway is important for *Salmonella* survival within macrophages. Cholesterol synthesis is a multi-enzyme process where acetyl-CoA is converted through a series of precursors into cholesterol [**Figure 1-3**]. Interestingly, while statins (an early inhibitor of the cholesterol biosynthetic pathway) inhibit bacterial survival, the inhibition of lanosterol (the precursor immediately prior to cholesterol) had no impact on bacterial survival or the manipulation of intracellular cholesterol levels [241; 242]. These findings are remarkable on two fronts. First, although *Salmonella* infection is accompanied by a large increase in intracellular cholesterol accumulation, cholesterol biosynthetic signaling is maintained. This indicates that the ER remains starved of cholesterol even with the significant increase in intracellular cholesterol, suggesting an inhibition of cholesterol transport to the ER. Second, the terminal production of cholesterol is unnecessary for survival, but the intermediates of cholesterol synthesis are critical. Importantly, many cholesterol precursors

themselves have important biological functions, including the prenylation of proteins. Prenylation is a common post-translational modification which covalently attaches either a geranylgeranyl or a farnesyl group to the prenylation motif of target proteins. This results in the targeting of otherwise cytosolic proteins, including Rab GTPases, to cellular membranes. Geranylgeranylation of Rabs promotes their membrane targeting to properly position Rabs on the appropriate membrane to facilitate their function [386]. Non-geranylgeranylated Rab7 and Rab5 accumulate in the cytoplasm [387], indicating that the synthesis of geranyl pyrophosphate and proper prenylation of Rab proteins are critical for maintaining cellular function. Additionally, Rho GTPases also rely on prenylation for proper functioning in cell proliferation and actin polymerization [388]. Therefore, avoidance of ER cholesterol sensing and maintenance of cholesterol biosynthesis may increase geranyl and farnesyl pyrophosphate to promote the prenylation of Rho and Rab GTPases during infection.

Quantification of prenylation during *ΔinvG* and *ΔsseJ* infection will allow for determining whether SseJ activity alters host protein prenylation [389], while preventing prenylation by treating cells with geranylgeranyltransferase and farnesyltransferase inhibitors will reveal whether sustained production of prenylated Rho and Rab proteins is necessary for *Salmonella* survival. Since *Salmonella* infection has been shown to induce cholesterol biosynthesis, I anticipate that infection also increases the prenylation of host proteins. Furthermore, in the absence of SseJ, I anticipate that host cholesterol biosynthesis and prenylation decreases due to the loss of cholesterol retention in the endocytic network.

2) How does ABCG1-mediated cholesterol efflux impact Salmonella survival?

We found that *Salmonella* infection downregulated the expression of both *Abca1* and *Abcg1* in host macrophages; however, unlike *Abca1*, *Abcg1* downregulation occurred independent of FAK and Akt [**Figure 2-2**]. ABCA1 is thought to account for more than 50% of macrophage cholesterol efflux compared to 20% occurring though ABCG1 [220]. Therefore, the downregulation of *Abcg1* may play a secondary role to *Abca1* downregulation in promoting cholesterol accumulation during *S.* Typhimurium infection. However, since LXR signaling induces both *Abca1* and *Abcg1*, we were unable to separate out the individual contributions of their downregulation in *Salmonella*-induced cholesterol accumulation. Notably, the knockdown of ABCA1 alone was sufficient to restore *Salmonella* survival in FAK-deficient macrophages to levels seen during infection of wild-type cells [**Figure 2-4**], suggesting that ABCG1 expression alone is insufficient to promote cholesterol accumulation and bacterial survival. Future studies will rely on the treatment of *Abca1*- and *Abcg1*-depleted cells with T0901317 to parse out the individual roles of these proteins in facilitating cholesterol efflux during *Salmonella* infection. Given the importance of ABCA1 in cholesterol efflux from macrophages, I anticipate that T0901317 treatment of *Abca1*-depleted cells will only partially reduce cholesterol accumulation during *Salmonella* infection and that *Abcg1* depletion will have limited, if any, impact on *Salmonella* survival.

3) Does cholesterol impact inflammatory signaling in response to infection?

Cholesterol is becoming widely recognized as an important regulator of antimicrobial inflammation through the clustering of TLR4 in cholesterol-rich lipid rafts [390; 391]. LPS pretreatment of macrophages has been shown to increase *ABCA1* expression [392]. This

agrees with our findings that DH5α *E. coli* infection increases *Abca1* expression compared to uninfected controls [**Figure 2-S2**], suggesting that the host innate immune response to Gram-negative bacteria upregulates ABCA1-dependent cholesterol efflux, and that *Salmonella* actively subverts this response to increase cholesterol concentrations. While it is unlikely that the accumulation of cholesterol within the endocytic network impacts plasma membrane TLR4 signaling, TLR4 can also be internalized through endocytosis and induce signaling within endosomes [393]. Endosomal TLR4 signaling through IRF-3 has previously been shown to prevent LXR-induced *ABCA1* expression [394], raising the possibility that the accumulation of cholesterol within the endocytic network may enhance intracellular TLR4 signaling to prevent LXR signaling in cholesterol-engorged cells.

The role of cholesterol accumulation in TLR4 signaling can be assessed by infecting untreated or T0901317-treated cells with wild-type *S.* Typhimurium and quantifying JNK or p38 activation and the expression of IFNβ as a measure of PM and endosomal TLR4 signaling, respectively. If cholesterol potentiates endosomal TLR4 signaling, I anticipate elevated expression of IFNβ in untreated cells compared to T9001317-treated cells following infection. To determine whether endosomal TLR4 signaling assists in the downregulation of *Abca1*, *Abca1* expression can be measured during infection of TRIF- or TRAM-deficient cells, or cells treated with an IRF-3 inhibitor [395]. However, as SseJ downregulates *Abca1* in a FAK-dependent manner [**Figure 2-5**], I do not expect the loss of endosomal TLR4 signaling to dramatically impact *Abca1* expression during *Salmonella* infection.

Fitting the Pieces Together - A Proposed Model for *Salmonella*-Induced Cholesterol Accumulation

While our research reveals the mechanism behind *Salmonella*-induced cholesterol accumulation, the specific function for cholesterol in promoting bacterial survival remains uncertain. While the above discussion attempts to provide context into how SseJ, FAK, Akt, and cholesterol may play a role in a wide array of host processes, here, I propose a model which attempts to explain the functional outcomes of the experimental observations we have made [**Figure 3-4**].

Following infection of macrophages, SseJ is translocated into the host cell cytosol where it localizes to SCVs. Here, SseJ modifies the local lipid environment, generating cholesteryl esters and lysophospholipids from cholesterol and phospholipids. Through changes in membrane lipid composition or direct protein-protein interactions, SseJ recruits and promotes the autophosphorylation of FAK on the SCV membrane. The subsequent stimulation of the FAK/Akt/mTOR signaling axis suppresses cholesterol export by downregulating *Abca1*, driving cholesterol accumulation, and inhibiting autophagy within infected cells. In parallel, SseJ recruits lipid transport proteins, such as OSBP1 and NPC1, to the SCV membrane, while inhibiting the transcription of other transport proteins, such as ORP1 and ORP6. Through the manipulation of host lipid transport proteins, *Salmonella* disrupts endocytic transport of cholesterol, retaining intracellular cholesterol within the endocytic network and maintaining ER cholesterol starvation.

In this proposed model, the SCV replaces the ER as the central hub of regulation of host lipid transport and starves the ER of LDL-derived cholesterol by accumulating cholesterol in endosomal compartments. Retention of cholesterol in the endosomal lumen

would prevent oxysterol production and therefore explain the lack of LXR signaling and *Abca1* upregulation during infection. Furthermore, retaining cholesterol in the endocytic network keeps ER cholesterol concentrations low, preventing cytotoxic stress responses and maintaining SREBP2 signaling to produce the biosynthetic cholesterol precursors necessary for *S.* Typhimurium survival. Finally, through the establishment of VAP-A contact sites, OSBP1 removes cholesterol from the ER and delivers it directly to the cytosolic surface of the SCV. While I anticipate the majority of cholesterol in the ER to be biosynthetic in origin, the recruitment of OSPB1 to the SCV would also allow for the removal of any cholesterol that reaches the ER through low-level transport from the PM or endosomal compartments. Thus, OSBP1-mediated transport ensures sustained ER cholesterol starvation and allows for the recruitment of mTOR to cholesterol-rich SCV surfaces to inhibit autophagy and promote *Salmonella* survival.

Together, this model suggests that *Salmonella* Typhimurium hijacks host macrophage lipid trafficking, redirecting the regulation of cholesterol sensing and transport away from the ER and into the endocytic network, allowing for the substantial increase in intracellular cholesterol concentrations and the avoidance of host stress responses.

Summary

Prior to this study, the mechanism behind cholesterol accumulation during *Salmonella* Typhimurium infection remained unknown. The work presented in this thesis fills this gap in our understanding, detailing the host-pathogen interactions governing the manipulation of cholesterol homeostasis by *S.* Typhimurium. Furthermore, the findings detailed in this work provide valuable insights into the potential roles of cholesterol accumulation in regulating host cholesterol transport, inflammation, and autophagic flux in response to infection. We found that the T3SS-2 effector, SseJ activates the FAK/Akt signaling axis to downregulate expression of the host cholesterol transport protein ABCA1. Importantly, suppression of *Abca1* increases intracellular cholesterol levels which appear to accumulate in the endocytic network and enhances *S.* Typhimurium survival within primary murine macrophages. Importantly, pharmacological induction of *Abca1* during infection significantly attenuated bacterial survival, while *Abca1* depletion in FAK-deficient macrophages restored survival to near wild-type levels. Together, these findings reveal a novel function for SseJ and identifies the host proteins manipulated to promote cholesterol accumulation during *Salmonella* infection.

Our lab has previously shown that *S.* Typhimurium activates FAK and Akt in a T3SS-2-dependent manner; however, the bacterial factor(s) which activated this pathway remained unclear. Here, we show that the catalytic activity of SseJ induces FAK and Akt phosphorylation through a currently unidentified mechanism. SseJ is a deacylase and glycerophospholipid:cholesterol acyltransferase, suggesting that the manipulation of membrane lipid content or the generation of deacylated lysophospholipids may stimulate the activation of FAK. We discovered that *S.* Typhimurium downregulates *Abca1*

expression in an SseJ/FAK/Akt-dependent manner, while also downregulating *Abcg1* independent of FAK and Akt. Although the direct mechanism behind Akt-mediated downregulation of *Abca1* remains unknown, it is thought to involve the Akt-dependent inhibition of FOXO3 and TAL1. Collectively, our findings reveal the importance of ABCA1 suppression and subsequent cholesterol accumulation for *S.* Typhimurium survival in macrophages. By better understanding the molecular mechanisms of how *Salmonella* Typhimurium manipulates host cholesterol homeostasis, we hope to begin laying the groundwork for identifying alternative approaches to treat *Salmonella* infections.

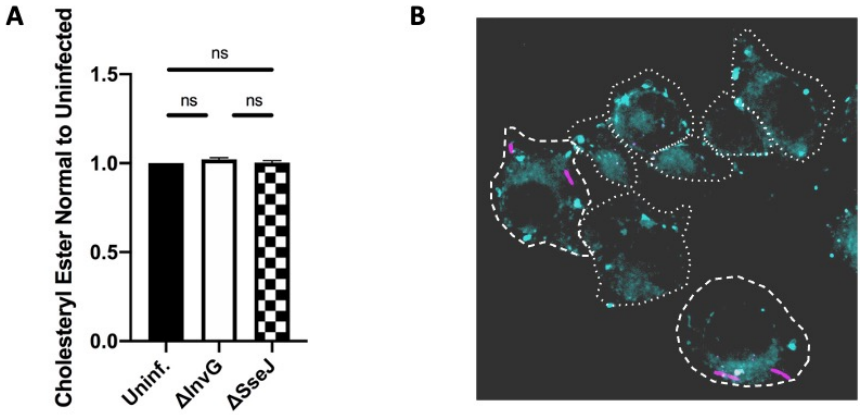

A
B
Cholesteryl Ester Normal to Uninfected
1.5
1.0
0.5
0.0
ns
ns
ns
Uninf.
ΔInvG
ΔSseJ

Figure 3-1. *S.* Typhimurium Fails to Induce Cholesteryl Esters in Primary Macrophages.

A) BMDMs were grown on a 6-well dish and infected with *ΔinvG* or *ΔsseJ S*. Typhimurium for 18 hours at an MOI of 100 or left uninfected as a control. Lipids were isolated and pelleted as described in the materials and methods section of **Chapter 2**. Lipid pellets from all three conditions were resuspended in a 1:1 mixture of chloroform:methanol and spotted on silica-coated glass TLC plates, along with a 1:1 cholesterol:cholesteryl ester standard. Once adsorbed, plates were eluted in a neutral-lipid resolving elution mixture of Hexane: Diethyl Ether: Acetic Acid (75:25:1.5). After drying, plates were developed overnight in an iodine crystal-containing tank. Plates were immediately scanned, and densitometry was performed on the resulting lipid cholesteryl ester spots. Values are normalized to uninfected control cells and averaged across 3 separate experimental replicates, mean ± SD. Statistics were performed as in **Chapter 2**. ns=not significant. B) Infected BMDMs were grown on fibronectin-coated coverslips and infected with RFP-expressing *ΔinvG S*. Typhimurium (magenta) for 18 hours prior to fixation in 4% PFA. Cells were then stained with 5 μg/mL unconjugated BODIPY (cyan) for 15 minutes to label lipid droplets. Images were captured using a 60x objective as described in the materials and methods section of **Chapter 2**. Infected cells (dashed outline) appear highly similar to uninfected cells (dotted outline), suggesting that *ΔinvG* infection of BMDMs does not increase lipid droplet formation.

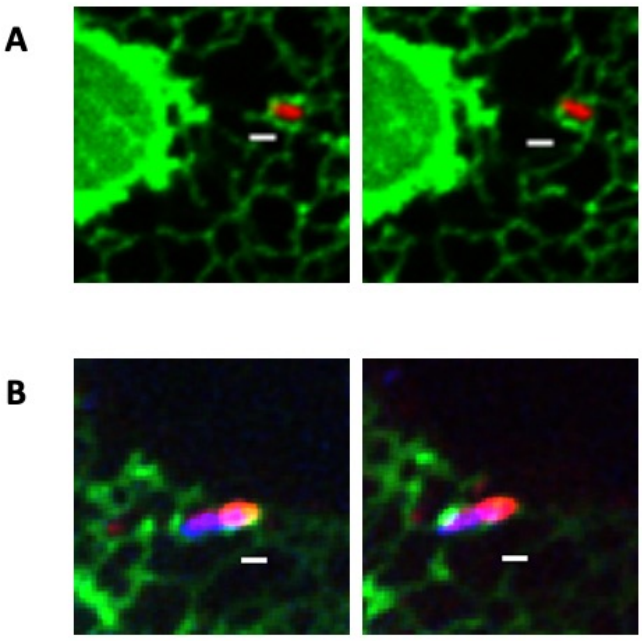

A
B

Figure 3-2. SCVs Colocalize with the ER Integral Membrane Proteins Sec61 and VAP-A.

COS-7 cells were plated on fibronectin-coated glass-bottom imaging dishes and infected with SL1344 *S.* Typhimurium at an MOI of 50 for 4 hours. Live-cell imaging was performed as described in the materials and methods of **Chapter 2**. Images were captured using a 60x objective. Images are representative stills from a 15 minute imaging period tracking a single cell. Scale bars = 1 μm. A) COS-7 cells were transfected with GFP Sec61 as a marker for the ER and infected with RFP SL1344. B) COS-7 cells were transfected with GFP VAP-A and RFP-CD63 as a marker of late endosomes/lysosomes to label the SCV. Cells were incubated with 0.1 μg/mL Hoechst (blue) for 90 minutes prior to imaging to label host nuclei and bacteria. Videos showed extensive ER-SCV contacts that were highly dynamic and were maintained over the course of the 15 minute imaging experiment.

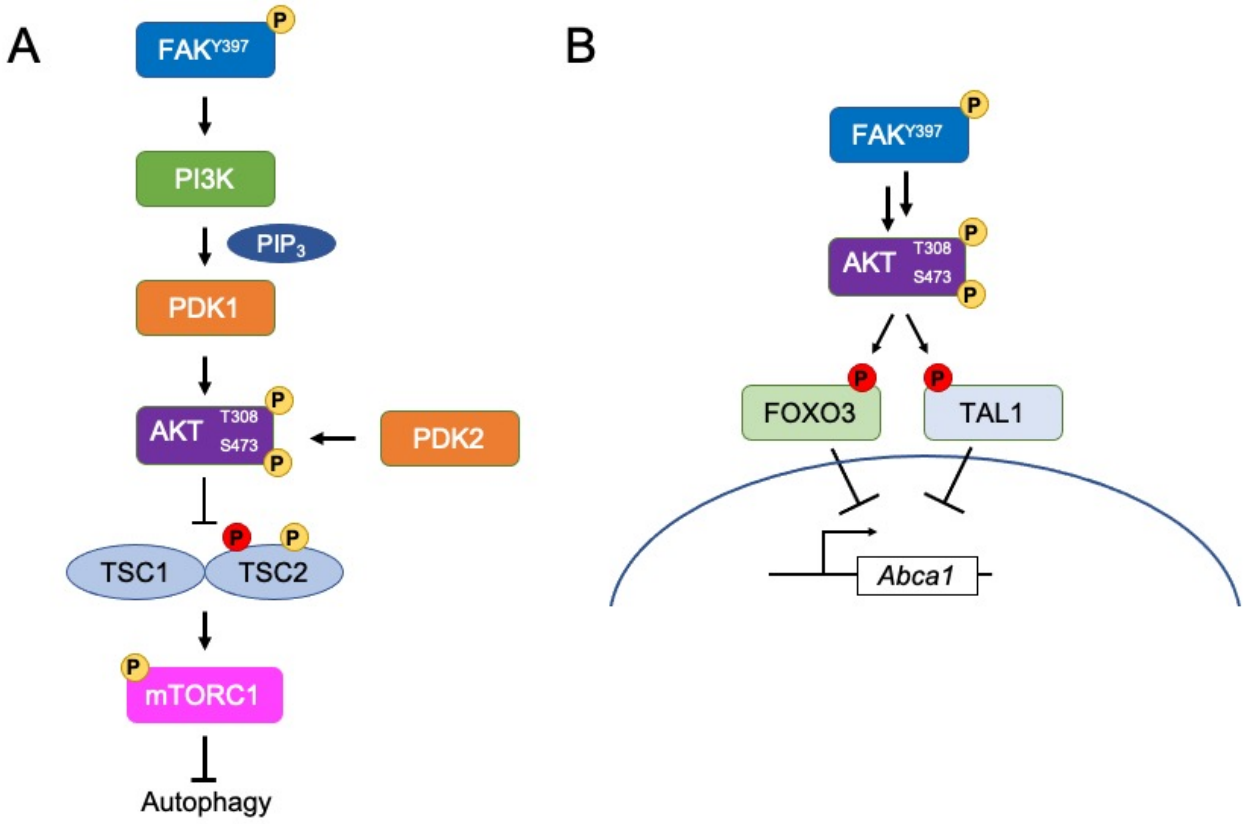

A
FAKY397
P
PI3K
PIP3
PDK1
AKT
T308
S473
P
PDK2
TSC1
TSC2
mTORC1
Autophagy
B
FAKY397
AKT
T308
S473
FOXO3
TAL1
Abca1

Figure 3-3. Proposed Mechanism for Akt-Mediated Downregulation *Abca1* and Suppression of Autophagy.

Inhibitory phosphorylation shown by red P, activating phosphorylation shown by yellow P. A) The mechanism of FAK- and Akt-mediated activation of mTORC1 and suppression of autophagy. FAK phosphorylation of Tyr 397 (which we show occurs due to the catalytic activity of SseJ) leads to recruitment and activation of PI3K through the phosphorylation of the p85 subunit of PI3K. Subsequently, PI3K converts $PI(4,5)P_2$ on the target membrane to PIP_3. Both PDK and Akt bind PIP_3 on the target membrane, allowing for PDK1 to phosphorylate Akt on the Thr308 residue in the Akt "activation loop" for partial activation, while dual phosphorylation of Thr308 and Ser473 (via PDK2, also referred to as TORC2) fully activates Akt [344; 396]. Akt activation subsequently phosphorylates and inactivates TSC2, destabilizing the Rheb GAP TSC1/TSC2 complex. Inactive TSC2 allows for GTP-bound Rheb to activate mTORC1 and promotes mTOR phosphorylation at Ser2481 [397]. Subsequently, mTORC1 activation suppresses autophagy and leads to increased *Salmonella* survival [Model based on 41; 266; 398]. (B) Proposed model for Akt-mediated suppression of *ABCA1* during *Salmonella* infection. Akt is activated in a SseJ- and FAK-dependent manner as detailed in (A). Subsequently, active Akt phosphorylates and inhibits the transcription factors Tal1 and FOXO3. Phosphorylated Tal1 leads to the degradation of the protein, while phosphorylation of FOXO3 prevents its nuclear translocation. As Tal1 and FOXO3 bind the promoter region of *ABCA1*, inhibitory phosphorylation by Akt prevents ABCA1 transcription [Model based on 228].

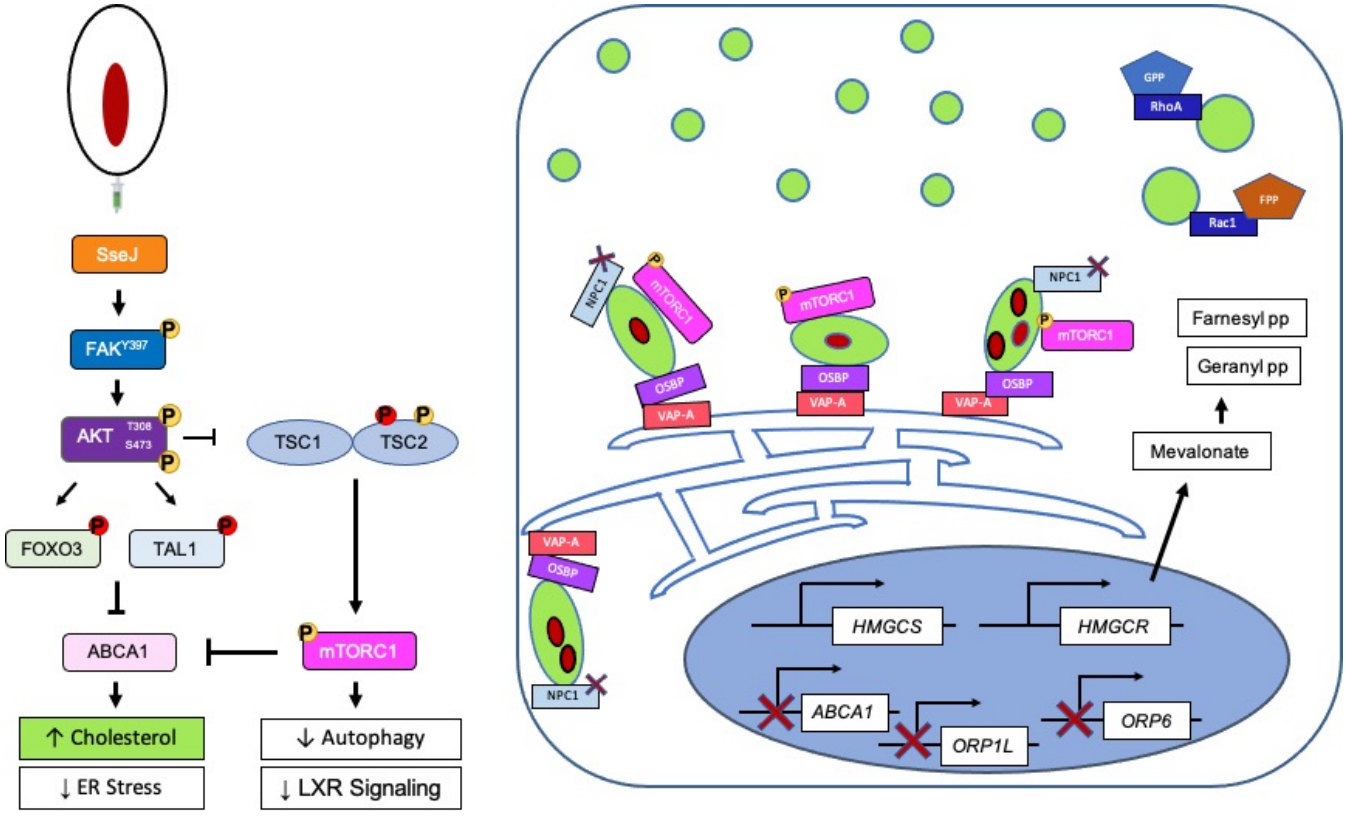

SseJ
FAK Y397
AKT T308 S473
TSC1
TSC2
FOXO3
TAL1
ABCA1
mTORC1
↑ Cholesterol
↓ ER Stress
↓ Autophagy
↓ LXR Signaling
GPP
RhoA
FPP
Rac1
NPC1
mTORC1
OSBP
VAP-A
Farnesyl pp
Geranyl pp
Mevalonate
HMGCS
HMGCR
ABCA1
ORP1L
ORP6

Figure 3-4. Graphical Representation of How Cholesterol Accumulation May Promote *Salmonella* Survival.

Following infection of macrophages, the acyltransferase activity of SseJ modifies the SCV lipid environment, generating cholesteryl esters and lysophospholipids. These changes in membrane lipid composition and/or a direct protein-protein interaction recruits and promotes the autophosphorylation of FAK on the SCV membrane. Stimulation of the FAK/Akt/mTOR signaling axis suppresses cholesterol export by downregulating *Abca1*, driving cholesterol accumulation, and inhibiting autophagy within infected cells. In parallel, SseJ recruits lipid transport proteins, such as OSBP1 and NPC1, to the SCV membrane, while inhibiting the transcription or activity of other transport proteins, such as ORP1L and ORP6. Through this mechanism, *Salmonella* manipulates cellular cholesterol homeostasis by inhibiting cholesterol efflux, disrupting endocytic transport of cholesterol, and maintaining ER cholesterol starvation.

In this proposed model, the SCV becomes the cellular hub of host lipid transport and starves the ER of extracellular-derived cholesterol by preventing cholesterol efflux from the endosomal network. Retention of cholesterol in the endosomal lumen would prevent oxysterol production, inhibiting LXR signaling and *Abca1* upregulation following cholesterol accumulation. Furthermore, retaining cholesterol in the endocytic network prevents cytotoxic stress responses and maintains SREBP2 signaling to produce the biosynthetic cholesterol precursors necessary for *S.* Typhimurium survival. Finally, OSBP1 removes cholesterol from the ER and delivers it directly to the cytosolic surface of the SCV, facilitating the recruitment of active mTORC1 to cholesterol-rich SCV membranes and maintaining ER starvation by removing any cholesterol that reaches the

ER through low-level transport from the PM or endosomal compartments. Through these combined mechanisms, I hypothesize that *Salmonella* Typhimurium hijacks the host intracellular cholesterol transport to accumulate cholesterol in endosomes, suppress autophagy, prevent ER stress responses, and increase bacterial survival in macrophages.

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