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UNIVERSITY OF CALIFORNIA SAN DIEGO

The Interaction of Enteric Bacterial Effectors with the Host Engulfment Pathway Regulates Host Immune Responses

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Mitchel Ngai-Loong Lau

Committee in Charge

Professor Soumita Das, Chair
Professor Christopher Wills, Co-Chair
Professor Alisa Huffaker

2018

The thesis of Mitchel Ngai-Loong Lau is approved, and it is acceptable
In quality and form for publication on microfilm and electronically

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Co-Chair

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Chair

University of California San Diego

2018

Dedication

This Thesis is dedicated to my mom, dad, sister, my friends, and the members of the Das lab for their

patience, support and encouragement

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Acknowledgments

I would first like to thank Professor Alisa Huffaker and Professor Christopher Wills for they accepting me as their Teaching Assistants and taking the time to be part of my Thesis Committee.

Secondly, I would like to thank Soumita Das for overseeing my entire research career at University of California, San Diego. She has been my mentor throughout this entire process, and this thesis would not have been possible without her guidance, support, and patience.

I want to thank Dr. Majid Ghassemian from the Biomolecular & Proteomics Mass Spectrometry Facility, for the Liquid Chromatography-Mass Spectrometry and Dr. Pradipta Ghosh, Department of Medicine for the confocal microscopy of *Salmonella* containing Vacuoles

I also would like to thank past-lab members Courtney Tindle, Rama Pranadinata, and Eileen Lim for laying the groundwork of my thesis today, and assisting me in the early start of my research career. I would also like to thank current Lab Members Researchers Stella-Rita Ibeawuchi, Ibrahim M. Sayed Ibrahim, Julian Tam, and Riane Bandong for aiding in my experiments. Thank you Tiffany Tran for the extensive revisions thesis editing. I also want to thank Amber Ablack from the Crowe Lab for her assistance in and the GI Division for all of the shared equipment and reagents.

Last, but not least, I would like to thank my family: Billie Lau, Jerry Lau, and Amber Lau. Thank you for guiding me throughout my life and helping me reach this pivotal moment in my life. Thank you for all your patience, love, and support. Even when there was long times if non-communication, I am always reminded of your faith in me.

ABSTRACT OF THE THESIS

The Interaction of Enteric Bacterial Effectors with the Host Engulfment Pathway Regulates Host Immune Responses

by

Mitchel Ngai-Loong Lau

Masters of Science in Biology

University of California San Diego 2018

Professor Soumita Das, Chair

Professor Christopher Wills, Co-Chair

The clearance of enteric bacteria by phagocytes is an essential role of host response against infection. Previously, we have reported that BAI1 (Brain Angiogenesis Inhibitor 1) binds bacterial Lipopolysaccharide (LPS) and facilitates the engulfment of

gram-negative bacteria by utilizing ELMO1 (Engulfment and cell motility protein 1). Interestingly, ELMO1 interacts with *Salmonella* effector SifA that controls bacterial survival. Therefore, we hypothesized that the interaction between ELMO1 and SifA affects the pathogenesis of enteric infection, the bacterial clearance, and provides differential immune responses between pathogens and commensals. In silico analysis of bacterial database reveals that *Salmonella* effector SifA interacts with ELMO1 in the WxxxE signature motif which is present in several effectors from other enteric pathogens but is absent in commensals. In addition, SifA is involved in bacterial vacuole integrity. With ELMO1, SifA is involved in the endosomal-lysosomal pathway by interacting with the late endosomal protein Rab9. A pulldown of GST-SifA was performed with murine macrophage cell lysate followed by the identification of host proteins via Liquid-Chromatography Mass Spectroscopy. SifA interacts with Ribophorin 1(RPN1), which is involved in the transport of newly synthesized proteins across the membrane of the rough endoplasmic reticulum, and MOGS, a glycoside hydrolase that functions within the N-glycosylation pathway. Our data showed that ELMO1-depleted cells had significantly delayed bacterial clearance, and ELMO1 is a novel cytosolic sensor that determines bacterial pathogenesis and provides new insight to bacterial clearance via mechanisms of the endosomal-lysosomal system.

I. Introduction

Gastro-enteric infection and Salmonella as a model of enteric bacteria

Foodborne and waterborne infections are a common health problem. In the United States, foodborne and waterborne infections are responsible for a great number of illnesses, deaths and substantial economic loss associated with food recall (MMWR 2013). *Salmonella* is one of the prevalent pathogenic bacteria that infects 1.2 million people a year and causes a majority of gastrointestinal hospitalizations and deaths (Petri et al, 2008). *Salmonella* is a flagellated, gram-negative, facultative intracellular bacterium; it is taken up via contaminated food and can infect a broad range of hosts, animals and humans. Most people can clear *Salmonella* independently, but people with compromised immune systems experience reoccurring instances of *Salmonella* infection (Buchwald and Blaser, 1984; Greig *et al*., 2007).

Salmonella enters epithelial cells by bacteria-mediated invasion pathway using the SPI-1 (*Salmonella* Pathogenicity Island-1) genes (Agbor and McCormick, 2011; Galan and Wolf-Watz, 2006). Upon activation, SPI-1 causes the transcription and production of effectors such as SopB, SopE/E2 that cause the ruffling of the epithelial lining, leading to their intake into intestinal cells (Broz et. Al 2012). Following invasion, *Salmonella* reaches the lamina propria where *Salmonella* encounter phagocytes. The effectors from SPI-2 genes are responsible for survival of bacteria inside macrophages. Once engulfed, *Salmonella* reside in a protected *Salmonella* containing vacuole (SCV) by modulating the pH and preventing the fusion with host cell endosomes and hence of lysosomal enzymes are avoided (Buchmeier and Heffron, 1991; Uchiya et al., 1999).

Pattern recognition receptors and microbial sensing

Within the lamina propria, the area next to the basal surface of the intestines, macrophages and other defense cells immediately recognize pathogens like *Salmonella* via Pattern Recognition Receptors (PRR) (Akira et al, 2004). PRRs recognize certain pathogen-associated molecular pattern molecules (PAMPs) on pathogens that cause their engulfment and activate immune responses (Figure 1). Recognition of PAMPs are critical to the fate of host responses, as their main function is to survey and degrade bacteria from the area (Kagan et al 2012). For example, the PRR, Toll-Like Receptor 4 (TLR4) binds to LPS of bacteria to recruit Myeloid differentiation primary response 88 (MyD88), initiating NF-κB-mediated pro-inflammatory cytokines to initiate innate immune responses (Arancibia et al, 2007). Cytosolic PRR Nod1 and Nod2 bind bacterial peptidoglycan (Girardin et Al, 2001). Recently it has been shown that the activation of small Rho GTPases Rac1 and Cdc42 by bacterial effector SopE triggers Nod1 signaling with consequent RIPK2-mediated induction of NF-κB-dependent inflammatory responses (Keestra et al, 2013). Although most of these PRRs and their signaling has been reported in epithelial cells, but there are limited studies available in the role of phagocytes following *Salmonella* internalization.

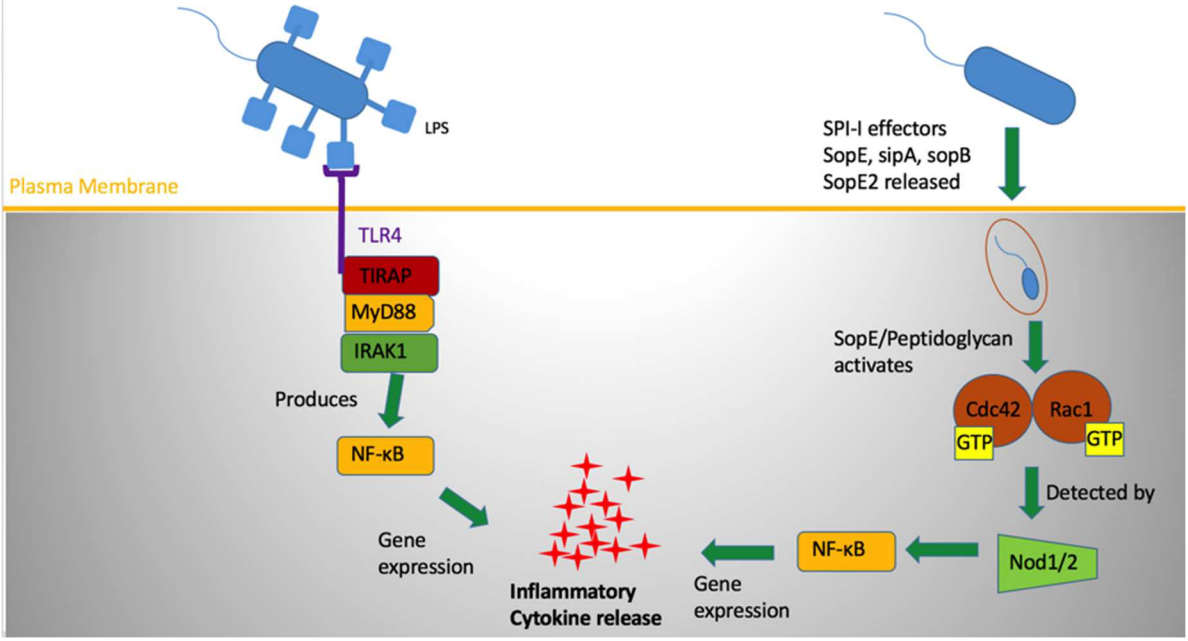

Figure 1. The role of PRRs in *Salmonella* pathogenesis: TLR4 binds to LPS of *Salmonella* to recruit TIRAP, MyD88, and IRAK1. Nod1 and Nod2, cytosolic PRRs, are able to detect *Salmonella* infection by detecting the activation of Rac1 and Cdc42 from bacterial effectors SopE and peptidoglycan. When activated, MyD88,Nod1, and Nod2 initiate a signaling cascade that leads to NF-κB gene expression, releasing proinflammatory cytokines to promote immune responses.

BAI1/ELMO1 as a new pattern recognition receptor

Previous data from our lab identified BAI1 (Brain Angiogenesis Inhibitor 1) as a new pattern recognition receptor that preferentially binds gram-negative bacterial Lipopolysaccharide (LPS) (Das et al., 2011; Das et al., 2014). Unlike TLR4 (Toll like receptor 4) which binds the lipid A region of LPS, BAI1 recognizes the core oligosaccharide. The binding of gram-negative bacteria on the BAI1 receptor recruits the cytosolic protein ELMO1 (engulfment and cell motility protein 1)(Das et al., 2011; Das et al., 2014). ELMO1 interacts with Dock180 and together they act as a guanine exchange factor for Rac1 (shown in Fig. 2). Once Rac1 is activated, it rearranges the actin

cytoskeleton of the macrophage to allow engulfment of Gram-negative bacteria such as *Salmonella* (Figure 2)(Das et al., 2011; Das et al., 2014). In addition, ELMO1 has been shown in the engulfment and clearance of apoptotic cells and controlling immune responses(Gumienny et al., 2001,Das et al., 2015).

The role of ELMO1 in inflammatory diseases

Genetic studies showed the association of ELMO1 with inflammatory bowel disease (IBD), Rheumatoid arthritis (RA), kidney disease, and diabetic nephropathy (Parmar et al., 2012; Pezzolesi et al., 2009; Whitaker et al., 2015). Work from our collaborator showed that BAI1-mediated ELMO1 regulates ABCA1 expression that linked to Cardiovascular disease (CVD)(Fond et al., 2015). BAI1-deficient mice has pronounced colitis and lower survival (Lee et al., 2016). Interestingly, ELMO1 regulates pro-inflammatory cytokines (MCP-1, IL1-β, TNF-α), the important cytokines involved in several inflammatory diseases including IBD, CVD and RA. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine, which plays a significant role in the recruitment of mononuclear cells to the site of inflammation, and it is one of the major cytokine involved in inflammatory diseases including IBD (Singh et al., 2016). Our lab recently found that the expression of ELMO1 was elevated in the gut epithelium in IBD, and high ELMO1 expression correlate the elevated expression of pro-inflammatory cytokines such as

MCP-1, which in turn recruits monocytes and generates TNF-α; a major cytokine involved in IBD (Suarez et al 2018).

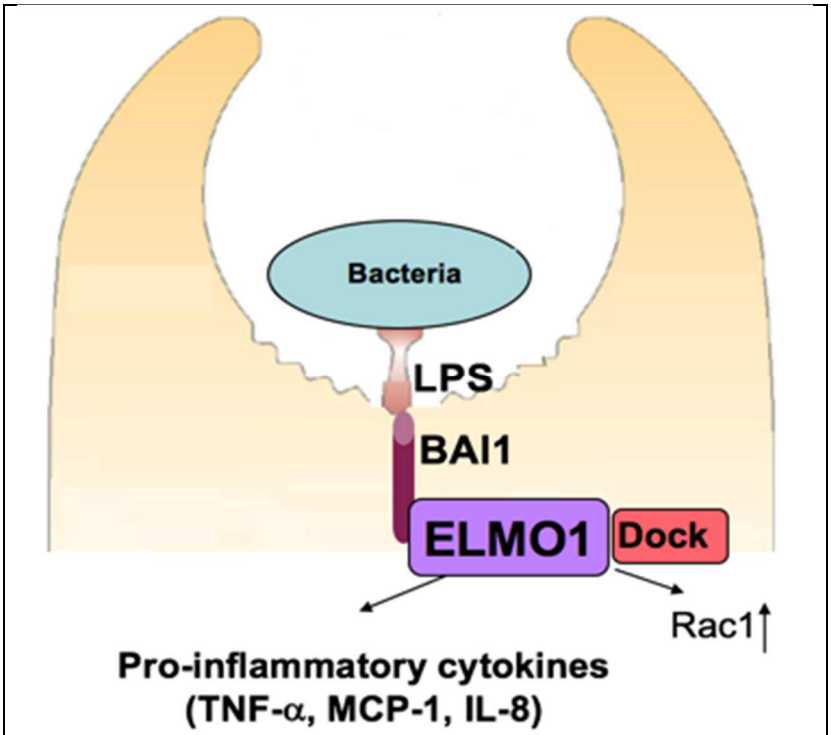

Figure 2. BAI1 and ELMO1-mediated host engulfment pathway:BAI1 detects LPS of Gram-negative bacteria. Binding triggers the recruitment of ELMO1 and Dock180 to BAI1 to act as a bipartite guanine nucleotide exchange factor for the small Rho GTPase Ras-related C3 botulinum toxin substrate 1 (Rac). This causes bacterial internalization with several species of gram-negative bacteria. ELMO1 regulates pro-inflammatory cytokines such as TNF-α during activation. *Salmonella* (SL), upon internalization, releases bacterial effectors via a Type III Secretion System (T3SS) that affect this process.

The interaction of ELMO1 with bacterial effectors

We recently found that ELMO1 generates differential immune responses after encountering pathogen vs a non-pathogen. TNF-α response released against pathogenic *Salmonella* is ELMO1-dependent and is higher in magnitude compared to non-pathogenic *E. coli* infection, which is ELMO1-independent. Our results showed that Salmonella Pathogenicity Island 2 (SPI-2) effector SifA (*Salmonella*-induced filaments A) binds ELMO1 and requires its WxxxE (Trp-xxx-Glu) motif; a signature motif present only in a

family of bacterial effectors produced by several enteric pathogens. Therefore, the major goal of this proposal will be to understand the interaction of ELMO1 with bacterial effector molecules, its impact in regulating host inflammatory responses following engulfment and the possible targeted therapy in inflammatory diseases.

Salmonella's* SifA *maintains SCV formation and abrogates host processes

To prevent the degradation of *Salmonella* within the *SCV*, other SPI-2 effectors are activated during the development of the endosome via acidification (Rappl et. Al, 2003). One of the many effectors that are released is SifA. Upon release of SifA, the SCV develops microtubular structures in a filamentous shape (sifs) and thereby attenuating endosomal function within the SCV (Jackson et al, 2008).

After entering epithelial cells, *Salmonella* resides inside the SCVs, which is formed by recruiting proteins from the endosomal system and preventing the clearance by lysosomal system (LaRock et. Al 2015). The targets are processed by early-late endosome when Rabs, a family of GTPases, are recruited sequentially. For example, early endosomal marker Rab5 is replaced by Rab7 and then replaced by the late endosomal marker Rab9 which signals the endosome for lysosomal degradation (Soldati et Al 1995). Loss of Rab function would lead to the interruption of the endosomal development system; therefore, by interrupting function with these Rabs, *Salmonella* is able to stay inside the SCV and proliferate in the cell. For example, Rab7 binds to the *Salmonella* induced filaments (sifs) during *Salmonella* infection, indicating their role in the

alteration in the Microtubule Organizing Center (MTOC) (McEwan Et. Al 2015). Additionally, Rab7 was shown to be coupled with SifA via Pleckstrin homology domain-containing protein family member 1 (PLEKHM1). PLEKHM1's original role has been

known to interact with Rab7 in the autophagy pathway, but has now been discovered to be used by SifA to not only maintain the SCV, but also interfere with Rab7's function (McEwan Et. Al 2015). In addition, SifA inhibits Rab9 function and the endosomal-lysosomal system. Rab9 helps with the retrograde trafficking of Mannose-6-Phosphate receptor (M6PR) back to the trans-Golgi apparatus and plays a role near the late-stage endosomal development process (McGourty et Al 2012). SifA binds to SKIP and Rab9 to create a complex that sequesters and inhibits Rab9 and interrupts the macrophages' development by interfering with the late checkpoints of endosomal development (Dumont et Al 2010, McGourty et Al 2012). Therefore, SifA also prevents the retro-grade trafficking of M6P receptors, displaying multiple processes that *Salmonella* abrogate to maintain survival in the SCV. A visual representation of the abrogation of Rabs during *Salmonella* infection is displayed in Figure 3.Moreover, with the use of *Salmonella* SifA mutants, the SCV integrity is not able to be maintained, leaving *Salmonella* in the cytosol of the cell to be exposed to other host defenses (Beuzon et al, 2000).

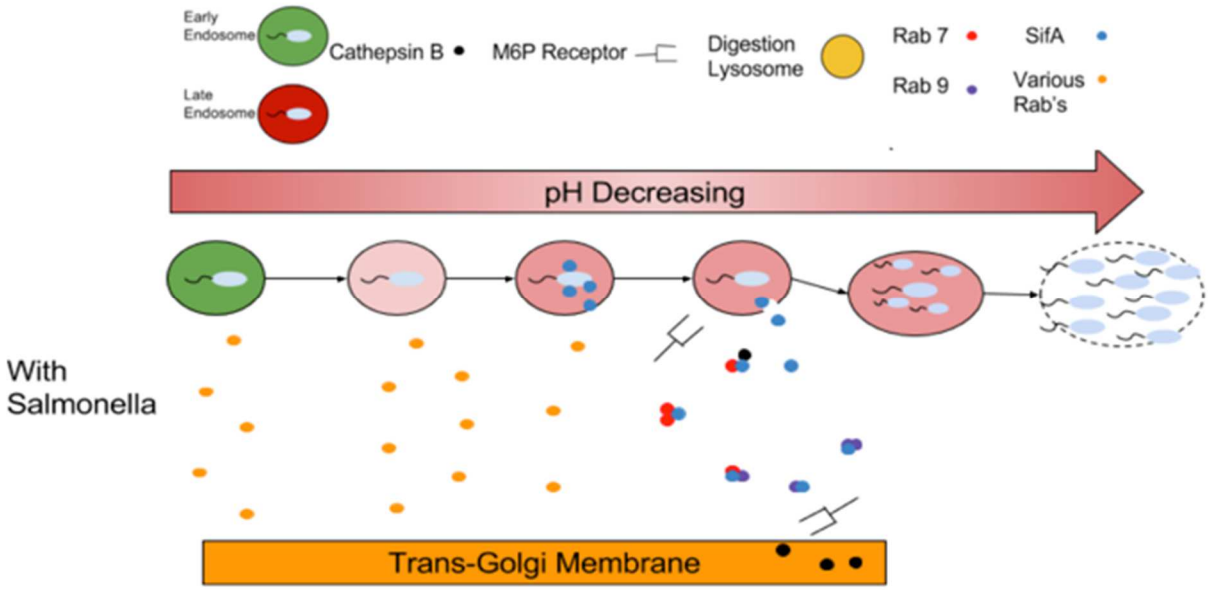

Figure 3. *Salmonella* SifA prevents endosomal development and allows of bacterial survival. During the acidification of the endosome, *Salmonella* releases SifA, which interacts and inhibits the functions of Rab7 and Rab9 GTPases. This interaction prevents the fusion of the lysosome to the Late Endosome, which allows the *Salmonella* to proliferate.

Current Studies and Aims

Despite extensive research on *Salmonella* pathogenesis, bacterial effectors, and the host defenses, there is a lack of knowledge regarding the interplay between host-bacterial interfaces and the hijacking of the host clearance machinery by *Salmonella*. Most of the published literatures rely on the interaction of *Salmonella* with epithelial cells, while data on *Salmonella* with phagocytes is lacking. Our previous studies showed that ELMO1 knockout cells have lower internalization of bacteria inside intestinal phagocytes. Interestingly, bacterial clearance was delayed in ELMO1-depleted macrophages. To understand how the engulfment pathway can discriminate between pathogens and commensals, we infected Control and ELMO1-depleted macrophages with pathogenic *Salmonella* and non-pathogenic *E.coli*. Interestingly, the internalization of pathogenic and

non-pathogenic bacteria is ELMO1-dependent; also, the TNF-α response is low in non-pathogenic bacteria and it is ELMO1-independent, confirming the contribution of pathogenic factors in the regulation of ELMO1. To determine the *Salmonella*-effectors that can regulate ELMO1, an in-silico analysis was performed. It was further verified with the GST pull-down assay that showed that ELMO1 interacts with Salmonella effector SifA. Previously, it has been shown that SifA can inhibit the functions of late endosomal GTPases Rab7 and Rab9 and that it interacts with Rab9 (McEwan et. Al, 2015, Jackson et al, 2008). Previously, we found that ELMO1 interacts with Rab9.

***Hypothesis*:** ELMO1 interacts with *Salmonella* effector SifA, which is involved in bacterial survival, and Rab9 that controls the fusion of endosome with a lysosome. Therefore, we hypothesized that ELMO1 is a cytosolic sensor that interacts with bacterial effectors involved in the pathogenesis of enteric infection, controls bacterial clearance and provides differential immune responses between pathogens and commensals.

II. RESULTS

The *Salmonella* effector SifA contains the unique signature motif WxxxE

Our in silico analysis showed that SifA contains a signature sequence that is unique to pathogenic bacteria but is absent in the commensals. Previously, SifA shared the signature WxxxE motif found in a family of effectors from enteric pathogens (Alto et al., 2006), including IpgB1 of *Shigella* that interacts with ELMO1(Handa et al., 2007). Interestingly, the interaction of ELMO1 with SifA was abolished when the WxxxE motif was mutated, confirming our hypothesis that the WxxxE motif is important for interaction. Further analysis and a BLAST search showed that the effectors from *Yersinia*, *E. coli* and *Citrobacter* also share this motif (Shown in the Table 1 below).

Table 1. The effectors from enteric bacteria contain the conserved WxxxE Motif. The BLAST search with the amino acid sequences showed homologues within enteric pathogens that contain the WxxxE or "Trp-x-x-x-Glu" amino acid sequence.

SifA	ST,SE	TELRKGHLDG**WKAQE**KATYLAAKIQ
	SE'	SEWRKGNLDE**WKTQE**KATYLAAKIQ
	YF	NINQGDKFDM**WKKEE**RTTYLSAVIN
SifB	ST,SE,SE',SE'',SN	AMAEKGNLCD**WKEQE**RKAAISSRIN
IpgB1	SF,E,EC	DSNSGNQLFC**WMSQE**RTSYVSSMIN
IpgB2	SF,EC	EQI-GENITD**WKNDE**KKVYVSRVVN
Map	EP,EC	KQTGSSDTQQ**WFKQE**QITFLSRAVN
	CR	KQTGNGDTQQ**WFRQE**QITFISKTVN
	EH	KQTGSSDTQQ**WFKQE**QITFLSRTVN
EspT	EC	KQTRSGDTQQ**WFQQE**QTTYISRTVN
	CR	LKN-EGKMNE**WMREE**CICFVSRDVN
EspM	EC,CR	RQS-TKDIDE**WIKDE**RIVYPVRVIN
TrcA	EP	RQN-TKDING**WIKDE**RIVYPSRVIN

E. coli (EC,EH,EP), *Citrobacter rodentium* (CR),*Shigella flexneri* (SF), *Salmonella* (ST,SE,SE',SE'',SN) and *Yersinia frederiksenii* (YF).

The interaction of *Salmonella* SifA with ELMO1 and Rab9

Salmonella SifA has been shown to interact with late endosomal GTPase Rab9 to abrogate endosomal maturation (McGourty et. Al, 2012). Our data showed that overexpressed FLAG-tagged ELMO1and EGFP-tagged SifA and found that the interaction between SifA and ELMO1 was dependent on the WxxxE signature motif of SifA. Next, we investigated whether the SifA-ELMO1-Rab9 was present in the same complex using the GST pulldown assay. GST and GST-SifA were overexpressed and bound to Glutathione sepharose beads as shown in Figure 4. Mouse macrophage lysate was added to our beads, incubated, washed, then followed by an immunoblot with ELMO1 and Rab9 as shown in Figure 5. The immunoblot displayed a significant amount of ELMO1 and Rab9 bound to the GST-SifA beads, indicating the presence of the ELMO1, Rab9, and SifA in a complex.

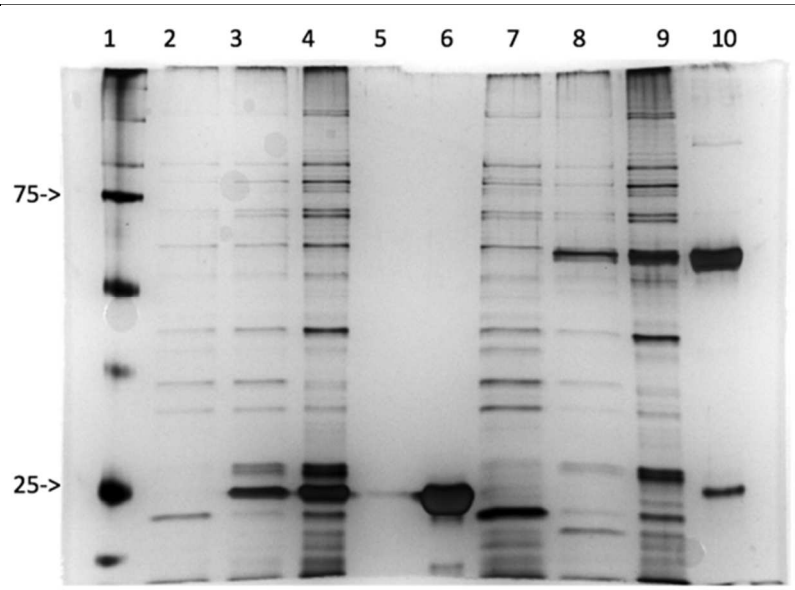

Figure 4. Successful binding of GST and GST-SifA to glutathione beads.

1. Ladder
2.GST uninduced
3. GST induced
4. GST sonicated lysate
5. Gap
6. GST Beads after binding
7.GST-SifA uninduced
8. GST-SifA induced
9. GST-SifA sonicated lysate
10. GST-SifA Beads after binding

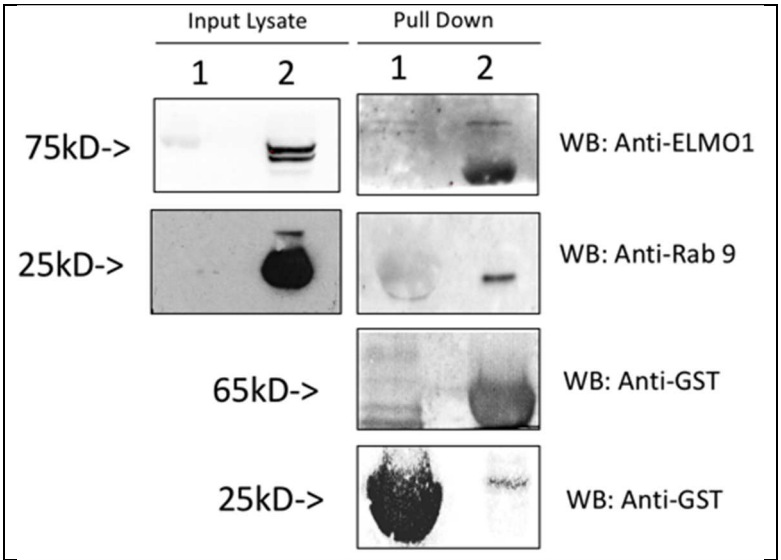

Figure 5. *Salmonella* effector SifA interacts with engulfment protein ELMO1 and Endosomal Protein Rab9.
Input lysate
1. Ladder
2. J774 Cell lysate

Pull Down
I. GST tagged beads with J774 Cell Lysate
II. GST-SifA tagged beads with J774 Cell lysate

ELMO1-SifA interaction is important for the SCV maintenance and bacterial clearance

Previously we had shown that ELMO1 facilitated the engulfment of bacteria, induced pro-inflammatory cytokines and regulated bacterial clearance (Das S, CMGH; Sarkar A JID). *Salmonella* SifA is required for the stability of the SCV (Beuzon et al. 2000), but the relevance ELMO1 in SCV formation is unknown. To investigate the possible relevance of ELMO1 in SCV formation, Control or ELMO1 shRNA cells (J774) were infected with wild-type(WT) or SifA mutant *Salmonella*. After 12 hours, cells were fixed, permeabilized, stained and confocal images were captured to monitor the SCVs. The SCVs were categorized based on the Lysosomal Associated Membrane Protein-1

(LAMP1) surrounding the *Salmonella*. They were evaluated as either complete (80-100% coverage), partial (25-80% coverage), or exposed (0-25% coverage) SCVs. Partial and exposed SCVs signifies abrogated SCV formation. The quality of SCVs were counted and plotted based on Control or ELMO1 shRNA cells and whether they were infected with WT or SifA mutant *Salmonella* as shown on Figure 6. Control shRNA cells infected with WT *Salmonella* contain an average of 10% Exposed, 30% partial and 60% complete SCVs whereas ELMO1 shRNA cells have 45% exposed, 15% partial and 40% complete SCVs. In case of infection with SifA mutant, control shRNA cells contain 45% exposed, 30% partial and 25% complete SCVs whereas ELMO1 shRNA cells have 60% exposed, 15% partial and 35% complete SCVs. Our results showed that either ELMO1 or SifA is involved in the SCV formation as the percentage of exposed SCVs increases and depletion of both ELMO1 and SifA increases the amount of exposed SCVs.

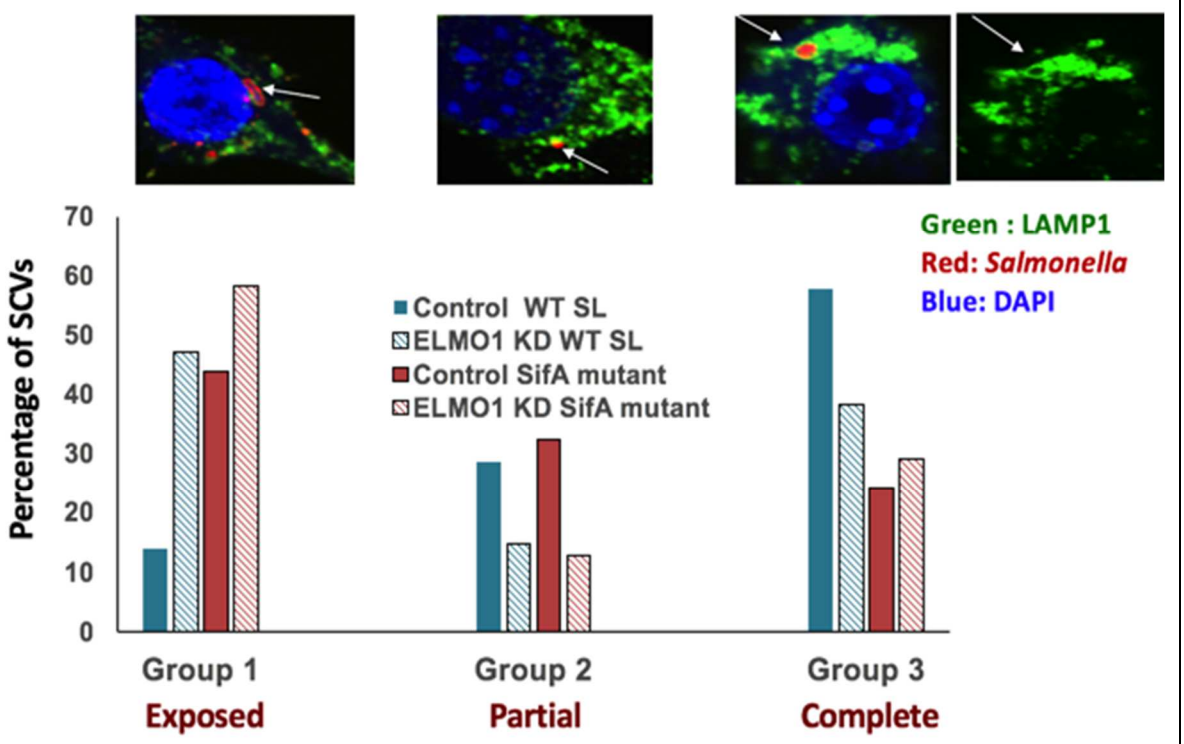

Figure 6. ELMO1 plays a significant role in the maintenance of SCVs. Control and ELMO1 shRNA cells were infected with WT and SifA mutant *Salmonella* for 1 h and treated with gentamicin to eliminate extracellular bacteria. After 12 hours, samples were fixed with formaldehyde, stained for LPS (Red), DAPI (blue), and Lysosome Associated Membrane Protein 1(LAMP1, Green), and visualized by a confocal microscopy. SCVs were evaluated based on the coverage of LAMP1 structures around *Salmonella* units, thereby determining "Exposed", "Partial", and "Complete" SCV formation. The examples of each group was shown on the top.

The interaction of other host proteins with SifA

SifA regulates bacterial survival inside macrophages by controlling the SCV formation. Previous reports showed that the host protein PLEKHM1 localizes with LAMP1 to distinct regions of the SCV where it interacts with SifA (McEwan et al., 2015). SifA also binds SKIP, another host cellular protein, required for SCV tabulation (Leone and Meresse, 2011; Ohlson et al., 2008). To further investigate, the host proteins that can bind SifA, GST-SifA and GST-tagged glutathione sepharose beads were incubated with mouse macrophage cell lysates and visualized by Silver staining (Figure 7). This was

subsequently repeated to identify interacting host proteins of SifA by Liquid-Chromatography Mass Spectrometry (LC-MS). The results were analyzed and sorted on the basis of matching peptide sequences (# of peptides at least two or more) in the database and the significance value (shown in Table 2). The proteins were categorized on the basis of their function. Two proteins from the group of glycosylation/protein modification found higher score for number of peptides: RPN1 (Ribophorin 1) with 18 polypeptides and MOGS (Mannosyl-Oligosaccharide Glucosidase) with 12 polypeptides. These two proteins were further verified by GST pulldown assay (Figure 8A). We also tested whether ELMO1 can interact with these two proteins. We found the interaction of ELMO1 with MOGS when GST-ELMO1 was incubated with macrophage cell lysate but not with the RPN1 (Figure 8B).

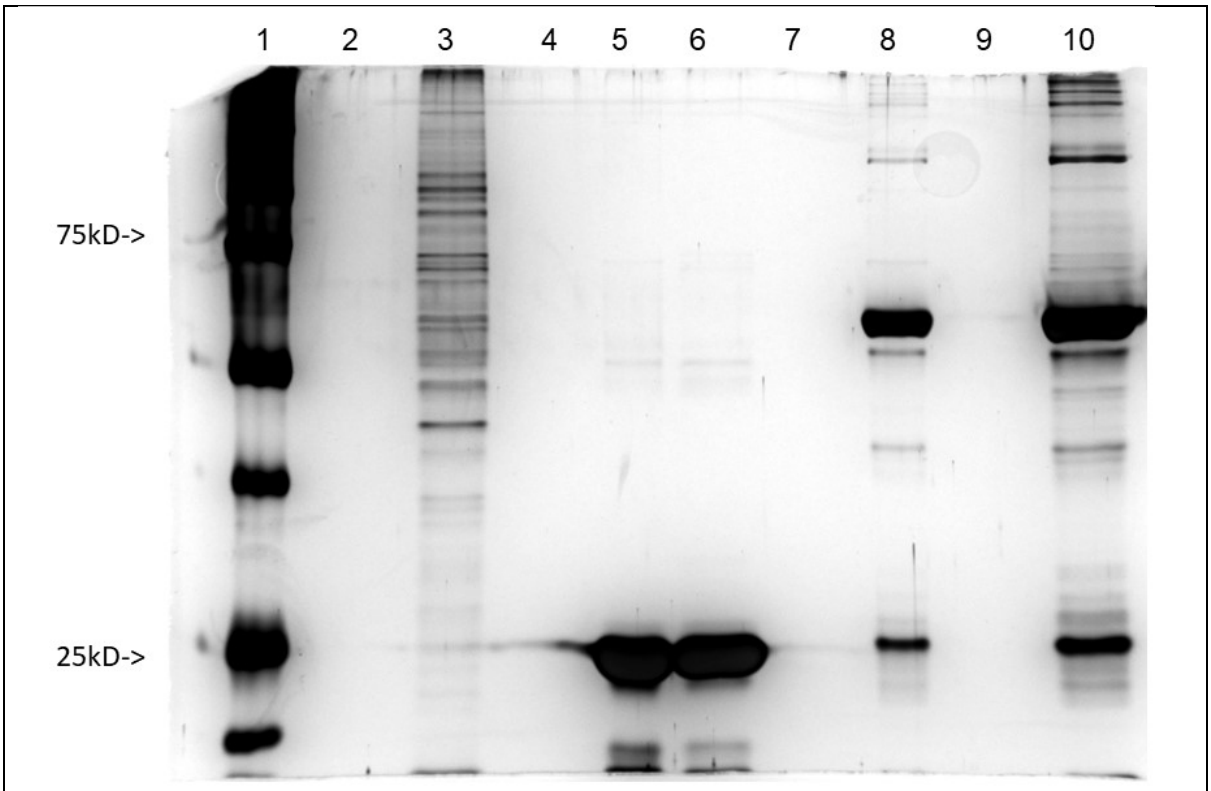

Figure 7. The expression of GST and GST-SifA and their binding with the macrophage cell lysates.

The binding of J774 cell lysate with GST or GST-SifA bead was tested where after binding the bead was boiled, loaded on 10% SDS-PAGE and stained with silver stain.

1. Ladder
2. Gap
3. 600ng J774 lysate
4. Gap
5. GST Beads (no protein)
6. GST Beads (2mg of protein)
7. Gap
8. GST SifA Beads (no protein)
9. Gap
10. GST-SifA (2mg of protein)

Table 2. Categorized and Labeled Protein interactions uniquely found in GST-SifA beads analyzed via Liquid-Chromatography Mass Spectrometry.

Protein Group	Protein ID	Accession	#Peptides	#Unique	#Spec Sample 2	PTM	Avg. Mass	Description
MITOCHONDRIAL PROTEINS								
74	88	Q9R112|SQRD_MOUSE	5	5	10	Carbamidomethylation	50282	Sulfide:quinone oxidoreductase mitochondrial OS=Mus musculus GN=Sqrdl PE=1 SV=3
107	138	Q62425|NDUA4_MOUSE	4	4	14		9327	Cytochrome c oxidase subunit NDUFA4 OS=Mus musculus GN=Ndufa4 PE=1 SV=2
182	139	Q9DCJ5|NDUA8_MOUSE	3	3	6		19992	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8 OS=Mus musculus GN=Ndufa8 PE=1 SV=3
221	173	Q9DC70|NDUS7_MOUSE	3	3	3		24683	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7 mitochondrial OS=Mus musculus GN=Ndufs7 PE=1 SV=1
91	106	Q8K354|CBR3_MOUSE	2	0	20		30953	Carbonyl reductase [NADPH] 3 OS=Mus musculus GN=Cbr3 PE=1 SV=1
58	52	Q922J9|FACR1_MOUSE	8	8	18		59435	Fatty acyl-CoA reductase 1 OS=Mus musculus GN=Far1 PE=1 SV=1
79	41	Q62186|SSRD_MOUSE	4	4	18		18937	Translocon-associated protein subunit delta OS=Mus musculus GN=Ssr4 PE=1 SV=1
133	177	Q8BH59|CMC1_MOUSE	3	3	4	Carbamidomethylation	74570	Calcium-binding mitochondrial carrier protein Aralar1 OS=Mus musculus GN=Slc25a12 PE=1 SV=1
128	141	P16858|G3P_MOUSE	2	2	4	Carbamidomethylation	35810	Glyceraldehyde-3-phosphate dehydrogenase OS=Mus musculus GN=Gapdh PE=1 SV=2
188	237	P47857|PFKAM_MOUSE	2	0	5		85269	ATP-dependent 6-phosphofructokinase muscle type OS=Mus musculus GN=Pfkm PE=1 SV=3
5	18	P51881|ADT2_MOUSE	13	10	92	Carbamidomethylation	32931	ADP/ATP translocase 2 OS=Mus musculus GN=Slc25a5 PE=1 SV=3
51	30	Q9JIY5|HTRA2_MOUSE	11	11	30	Deamidation (NQ)	49348	Serine protease HTRA2 mitochondrial OS=Mus musculus GN=Htra2 PE=1 SV=2
35	27	Q8VEM8|MPCP_MOUSE	10	9	29	Carbamidomethylation	39632	Phosphate carrier protein mitochondrial OS=Mus musculus GN=Slc25a3 PE=1 SV=1
48	47	Q9WUA3|PFKAP_MOUSE	10	7	17	Carbamidomethylation	85455	ATP-dependent 6-phosphofructokinase platelet type OS=Mus musculus GN=Pfkp PE=1 SV=1
39	102	P48962|ADT1_MOUSE	6	3	28	Carbamidomethylation	32904	ADP/ATP translocase 1 OS=Mus musculus GN=Slc25a4 PE=1 SV=4
64	62	Q8BMS1|ECHA_MOUSE	5	5	7	Deamidation (NQ)	82670	Trifunctional enzyme subunit alpha mitochondrial OS=Mus musculus GN=Hadha PE=1 SV=1
G Proteins								
72	43	P62880|GBB2_MOUSE	7	4	15	Carbamidomethylation	37331	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 OS=Mus musculus GN=Gnb2 PE=1 SV=3
63	38	P08752|GNAI2_MOUSE	6	3	22	Carbamidomethylation	40489	Guanine nucleotide-binding protein G(i) subunit alpha-2 OS=Mus musculus GN=Gnai2 PE=1 SV=5
90	54	P62874|GBB1_MOUSE	5	3	11	Carbamidomethylation	37377	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Mus musculus GN=Gnb1 PE=1 SV=3
86	60	Q9DC51|GNAI3_MOUSE	4	1	15		40538	Guanine nucleotide-binding protein G(k) subunit alpha OS=Mus musculus GN=Gnai3 PE=1 SV=3
110	163	Q6R0H7|GNAS1_MOUSE	2	0	11		121505	Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas OS=Mus musculus GN=Gnas PE=1 SV=1
120	159	Q3V3I2|GNAT3_MOUSE	2	0	11		40316	Guanine nucleotide-binding protein G(t) subunit alpha-3 OS=Mus musculus GN=Gnat3 PE=1 SV=2
212	232	P29387|GBB4_MOUSE	2	0	4	Carbamidomethylation	37379	Guanine nucleotide-binding protein subunit beta-4 OS=Mus musculus GN=Gnb4 PE=1 SV=4
641	222	Q8CIM5|GPR84_MOUSE	2	2	2		43717	G-protein coupled receptor 84 OS=Mus musculus GN=Gpr84 PE=1 SV=1
354	153	Q91V41|RAB14_MOUSE	2	1	2	Carbamidomethylation	23897	Ras-related protein Rab-14 OS=Mus musculus GN=Rab14 PE=1 SV=3
N GLYCOSYLATION AND PROTEIN MODIFICATION								
11	3	Q91YQ5|RPN1_MOUSE	18	18	69	Carbamidomethylation	68528	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Mus musculus GN=Rpn1 PE=1 SV=1
30	26	Q80UM7|MOGS_MOUSE	12	12	31		91831	Mannosyl-oligosaccharide glucosidase OS=Mus musculus GN=Mogs PE=1 SV=1
71	39	O70152|DPM1_MOUSE	7	7	17	Pyro-glu from Q	29175	Dolichol-phosphate mannosyltransferase subunit 1 OS=Mus musculus GN=Dpm1 PE=1 SV=1
44	55	Q09200|B4GN1_MOUSE	7	7	16		59212	Beta-1 4 N-acetylgalactosaminyltransferase 1 OS=Mus musculus GN=B4galnt1 PE=1 SV=1
73	86	P11499|HS90B_MOUSE	7	7	12		83281	Heat shock protein HSP 90-beta OS=Mus musculus GN=Hsp90ab1 PE=1 SV=3
55	73	P17879|HS71B_MOUSE	4	0	21	Deamidation (NQ); Deh	70176	Heat shock 70 kDa protein 1B OS=Mus musculus GN=Hspa1b PE=1 SV=3
170	137	Q64518|AT2A3_MOUSE	2	0	2	Carbamidomethylation	113638	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3 OS=Mus musculus GN=Atp2a3 PE=1 SV=3

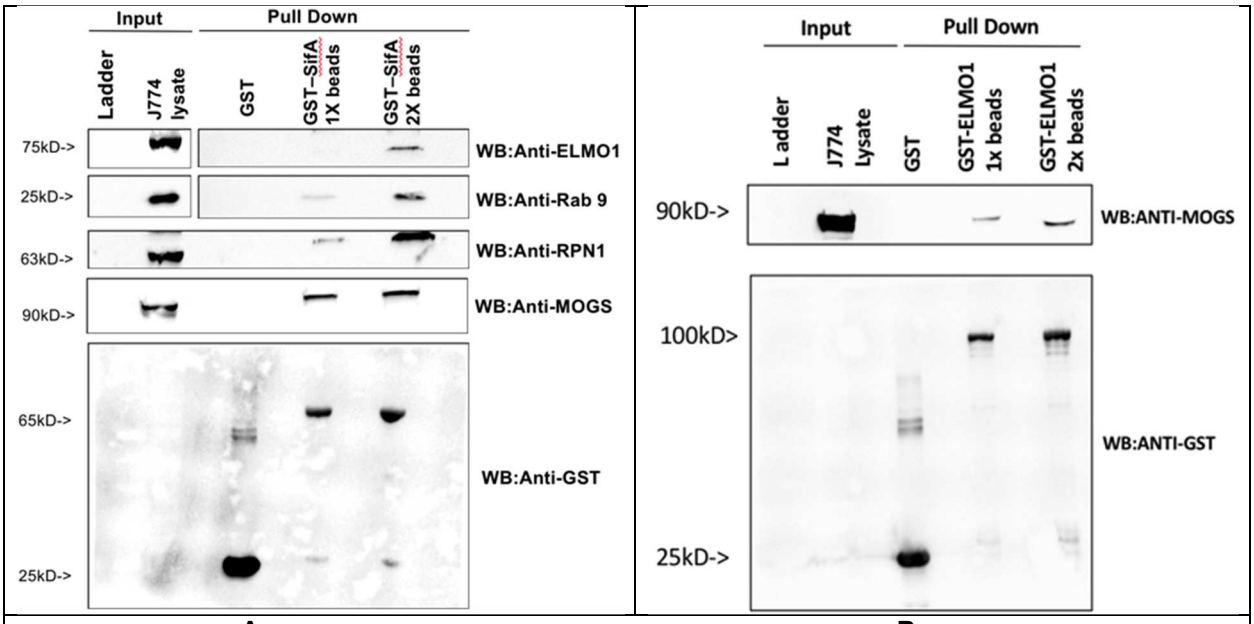

Figure 8. (A) The validation of LC-MS data, binding of SifA with MOGS and RPN1. J774 cell lysate was incubated with GST or GST-SifA tagged Sepharose beads in two different concentrations, loaded on SDS-PAGE and immunoblotted with the antibodies for MOGS, EPN1, ELMO1 and Rab9. The presence of ELMO1 and Rab9 show successful binding of previously known proteins, while the interactions of RPN1 and MOGS show new interactions of *Salmonella* SifA interacting with novel host proteins. **(B) ELMO1 interacts with N-Glycosylation Enzyme MOGS.** J774 cell lysate was incubated with GST or GST-ELMO1 tagged Sepharose beads with two concentrations were loaded on SDS-PAGE and immunoblotted with the antibody for MOGS.

III. DISCUSSION

The BLAST search (Table 1) showed that the SifA, SifB of *Salmonella*; IpgB1, IpgB2 of *Shigella*; Map, EspT and EspM of *E. coli* share the same motif. These effectors regulate cytoskeletal rearrangements of host processes to facilitate invasion of bacteria. Further studies on ELMO1 KO mice using SifA mutant will help us to understand the role of ELMO1 and SifA in *Salmonella* pathogenesis and host immune responses.

Previous literature has concluded that proper SCV development is abrogated with the absence of *Salmonella* SifA, but the relationship between ELMO1 of phagocytes to SCV maintenance has not been investigated (Beuzon et al, 2000). Our data suggested the relevance of ELMO1 in proper SCV formation, since SCV formation was adversely affected in the absence of ELMO1 in macrophages. Since proper SCV formation is required for bacterial survival, the interaction of ELMO1 and SifA is important for SCV formation and bacterial clearance.

Previous reports had shown the relevance of ELMO1 in bacterial clearance. Despite lowered total intake of bacteria into phagocytes, ELMO1 shRNA phagocytes had significantly attenuated bacterial clearance and was also correlated to lowered activity of lysosomal enzymes compared to control phagocytes (Sarkar et. Al, 2017). From our previous understanding of *Salmonella* SifA in bacterial survival, we hypothesized that *SifA*-mutant *Salmonella*'s survival will be less inside of phagocytes due to the loss of its main bacterial effector. However, clearance assays done using SifA-mutant bacteria showed significantly higher survival rates compared to Wild-Type *Salmonella*. It is possible that the interaction between *Salmonella* SifA and macrophages' host proteins are essential to initiate immune responses. In a previous experiment, the production of

pro-inflammatory cytokines were lowered in ELMO1 shRNA cells and further in ELMO1 shRNA cells infected with SifA mutant. Our data suggested that SCV formation is abrogated during SifA mutant *Salmonella* infection, thus agreeing with results reported by Beuzon et al. Without the formation of SCVs, bacteria are free in the cytosol, and can be multiplied further as reflected in our plating assay with higher bacterial count in SifA mutant compared to WT *Salmonella*. Our findings of the interaction of SifA with ELMO1 and Rab9 will further help us to know the involvement of bacterial effector in hijacking the endo-lysosomal machineries.

Our data showed that the interactions between *Salmonella* SifA ELMO1 and late endosomal GTPase Rab9 suggests a novel role of ELMO1 in endosomal development and bacterial clearance. We used an over-expressed system to confirm the interaction of Rab9 with ELMO1, and we were also able to confirm the interaction of endogenous Rab9 and ELMO1 with SifA. This data solidifies the role of ELMO1 in bacterial clearance and endosomal development.

The analysis of LC-MS results showed the interaction of SifA with several host proteins: for example, G Proteins, and N-Glycosylation Proteins. In our study, we focused on the relevance of N-Glycosylation proteins and its role in *Salmonella* infection. N-Glycosylation is a form of protein modification that requires the use of sugars to bind onto amide groups found on certain N-linked amino acids (Breitling et. Al, 2013). Immune cell receptors require the use of N-Glycosylation and other protein modifications to maintain their specific antigen specific binding sites (Monticelli et al, 2016). By interfering or abrogating these post-translational modifications of immune cell receptors, the immune function and cellular responses will be consequently reduced.

The correlation between bacterial infections and N Glycosylation is unknown. Recent reports done on human patients with congenital diseases had reported that N Glycosylation mutations have lowered immunoglobulin levels (in various types of IgGs) that were needed for proper immune responses and function (Monticelli et al, 2016). MOGS is an important enzyme in the production of immune receptors due to its function in removing the distal α1,2-linked glucose from the Glc3Man9GlcNAc2 glycan after its transfer to the nascent polypeptide at the rough endoplasmic reticulum (Breitling et al, 2013). For Congenital Disorders of Glycosylation with (CDG-MOGS), test subjects had undetectably low amounts of IgA and hypogammaglobulinemia, an immune disorder with a reduction in gamma globulins that help fight infection (Monticelli et al, 2016). Due to the effect of mutated MOGS leading to lowered immune function, this indicates the possibility that SifA interacts with MOGS with the goal to lower immune receptor production.

Another N Glycosylation protein that was found in LC-MS data was Ribophorin1(RPN1) which is a protein that regulates substrate delivery to the oligosaccharyltransferase (OST), an enzyme that helps the binding of sugars to the amino acid chain (Wilson et al, 2008). Simple receptors require the presence of RPN1 to have effective protein modification, but organisms with a deleted RPN1 exhibit a fatal phenotype. As MOGS is in close proximity in relation to the OST, this may indicate a possible link between *Salmonella* infection and host N-Glycosylation processes.

Due to the relationship of *Salmonella* SifA, ELMO1, and N-Glycosylation proteins, a novel observation that *Salmonella* interacts with N-Glycosylation during infection, but in what capacity is unknown. Functional assays using saccharide-binding lectin

immunosorbent assays are currently under development to determine the levels of N-Glycosylation during *Salmonella* infection.

Salmonella SifA and SifB, *Shigella* Ipgb1 and Ipgb2, and *E. Coli* and *Citrobacter Rodentium* Map, EspT and EspM, and TrcA bacterial effector proteins were found to have the similar WxxxE amino acid motif (Table 2). These effectors found in enteric pathogens but absent in commensals. The effectors cause cytoskeletal rearrangements of host processes to facilitate invasion of host cells or abrogate host processes. This not only implies that the WxxxE motif is a conserved motif in pathogenic bacterial effectors, but indicates a sequence that immune cells can utilize to discriminate between pathogenic and commensal bacteria, thereby modulating immune responses to provide the appropriate reaction. From unpublished data, it was indicated that *Salmonella* SifA not only requires WxxxE motif to bind to ELMO1, but ELMO1 is only able to produce pro-inflammatory cytokines with the WxxxE sequence present in *Salmonella* SifA.

Further understanding of the role of ELMO1 and SifA in *Salmonella* infection and immune function can allow discovery of therapeutic targets for bacterial infection. This is important because individuals with underdeveloped or compromised immune systems are not always able to clear *Salmonella* infections on their own.

IV. MATERIALS & METHODS

Cell lines and bacterial culture:

Murine macrophage cell line J774 (obtained from American Type Culture Collection, Manassas, VA, USA) and ELMO1 depleted J774 cells were used as phagocytes. ELMO1 depleted shRNA cells were generated as described previously (Das et al. 2015). Cells were maintained in high-glucose-containing DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life-Technologies), 2 mM penicillin-streptomycin (Sigma) and 0.5µg/ml puromycin (Sigma). A wild strain of *Salmonella enterica* serovar Typhimurium (SL1344) and SifA-mutant were used for infections. For this, a single colony was picked up from LB agar plates, inoculated in LB broth and cultured shortly for 6-8 hours on a shaking platform to allow aeration (150rpm) at 37ºC. Finally, the culture was allowed to grow in a static oxygen limiting condition overnight. The multiplicity of infection (MOI) between bacteria and phagocyte were maintained 10:1 or 2.5:1 for all experiments.

Cell lysis and Western Blotting:

J774 Murine Macrophages were lysed in cold lysis buffer (RIPA-Z): 50 mM Tris-HCl, pH 7.4, 0.25% sodium deoxycholate, 100 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1% Triton X with protease inhibitor cocktail and phosphatase inhibitor with (Sigma). Equal amount of proteins were loaded on the SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-rad). Membranes were blocked using 5 % non-fat dry milk (LabScientific Inc.) in Tris Buffer Saline containing 0.05 % Tween -20 (TBST) for 1h at room temperature. Membranes were then incubated with respective primary antibody (ratio mentioned above) in 5 % BSA containing TBST over night at 4ºC. Membranes were

then washed and incubated with appropriate secondary antibody (1:2000, Cell Signaling Technologies) for 1h at room temperature in blocking buffer (5 % non-fat dry milk in TBS containing 0.05 % Tween-20).

Immunofluorescence and confocal staining for SCV formation:

J774 cells were infected with *Salmonella* (SL1344) or the SifA mutant, fixed with 2% formaldehyde, permeabilized with 0.1% saponin and blocked with 3% BSA. LAMP1 (green) was stained with Alexa Fluor 488 anti-mouse LAMP-1 antibody (Biolegend) antibody for overnight at 4C. *Salmonella* (red) was stained with anti-*Salmonella* Antigen (Pierce). Nucleus was stained with Hoecsht (blue). Confocal images were captured using the 100x objective.

Antibodies and other reagents:

Mouse monoclonal antibody (mAb) to ELMO1 (sc-271519), MOGS (sc-374006), and Ribophorin 1 (sc-48367) (1:1000, Santa Cruz Biotechnology) was used for detecting the protein level by Western blotting. Rab9 was purchased from Cell Signaling Technologies (Rabbit mAb 5118S) and used with a dilution of 1:1000; Anti-GST HRP Conjugate (1:2000, Sigma-Aldrich RPN1236V). Alexa Fluor® 488 anti-mouse CD107a (LAMP-1 Antibody in Rat Biolegend #121607) in a dilution of 1:800 and BacTrace® Anti-*Salmonella* CSA-1 Antibody (sera care # 5310-0322, 01-91-99) in a dilution of 1:1000 were used for immunofluorescence.

Transformation and expression of GST, GST-ELMO1 and GST SifA, binding with Glutathione sepharose beads

20 µl of BL21(DE3) Competent *E. Coli* cells were mixed with 50 ng of Plasmid DNA (GST, GST-SifA, and GST-ELMO1), heat shocked, and incubated into SOC media. Selection plates (Kanamycin or Ampicillin) were used to grow colonies that effectively transformed the plasmid and were incubated overnight. Colonies were then selected were allowed to grow into overnight in Luria Broth (LB) cultures with antibiotic to grow bacteria with the plasmid. Growing into a large induction flask, the overnight culture was then grown and expression of protein was induced with IPTG (Sigma) at a concentration of 0.5 mM. After 4 h of induction, bacterial pellet was collected by centrifugation.

To bind the proteins with GST to Glutathione Sepharose beads, the bacterial pellet was resuspended in lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton-X and protease inhibitor cocktail. The bacterial pellet was then sonicated and centrifuged to collect purified protein. After collection of purified protein, each 40µl of rehydrated Glutathione Beads (Sigma Aldrich) were incubated with 500µl of purified GST proteins for three hours. Fused beads were then washed with 50 mM Tris pH 7.6, 150 mM NaCl, 1mM EDTA, 0.5% Triton X and stored in -20C in 50% glycerol and PBS solution. To validate the successful binding of the GST proteins to the beads, the bead complexes were ran through an SDS PAGE and stained using the Silver Stain kit (Thermo Scientific #24612)

Immunopulldown Assay

J774 Murine Macrophages were lysed in RIPA-Z buffer: 50 mM Tris, pH 7.4, 0.25% sodium deoxycholate, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1% Triton X with protease inhibitor cocktail and phosphatase inhibitor. Cells were then centrifuged to obtain purified protein lysate, and was incubated with prepared GST

beads as described above. 4.5 mg of protein was used per 75µl of GST-SifA and GST-ELMO1 beads, and 2 mg of protein was used per 25µl of beads. After incubation, the bead complex was washed with Wash buffer: 50 mM Tris, pH 7.4, 150 mM NaCl, 1mM EDTA, 0.25% Triton-X and were ran through an SDS-PAGE. 50 µg of protein was used as a loading control for protein expression, and bait protein control was tested either Commassie Stain (BioRad) or by Western Blot.

Sample preparation for LC-MS

Protein samples were diluted in TNE (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA) buffer. RapiGest SF reagent (Waters Corp.) was added to the mix to a final concentration of 0.1% and samples were boiled for 5 min. TCEP (Tris (2-carboxyethyl) phosphine) was added to 1 mM (final concentration) and the samples were incubated at 37°C for 30 min. Subsequently, the samples were carboxymethylated with 0.5 mg/ml of iodoacetamide for 30 min at 37°C followed by neutralization with 2 mM TCEP (final concentration). Proteins samples prepared as above were digested with trypsin (trypsin:protein ratio - 1:50) overnight at 37°C. RapiGest was degraded and removed by treating the samples with 250 mM HCl at 37°C for 1 h followed by centrifugation at 14000 rpm for 30 min at 4°C. The soluble fraction was then added to a new tube and the peptides were extracted and desalted using C18 desalting columns (Thermo Scientific, PI-87782). Peptides were quantified using BCA assay and a total of 1 ug of peptides were injected for LC-MS analysis.

LC-MS

Trypsin-digested peptides were analyzed by ultra high pressure liquid chromatography (UPLC) coupled with tandem mass spectroscopy (LC-MS/MS) using

nano-spray ionization. The nanospray ionization experiments were performed using a Orbitrap fusion Lumos hybrid mass spectrometer (Thermo) interfaced with nano-scale reversed-phase UPLC (Thermo Dionex UltiMate™ 3000 RSLC nano System) using a 25 cm, 75-micron ID glass capillary packed with 1.7-µm C18 (130) BEH^{TM} beads (Waters corporation). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of ACN (Acetonitrile) at a flow rate of 375 µl/min for 1h. The buffers used to create the ACN gradient were: Buffer A (98% H_2O, 2% ACN, 0.1% formic acid) and Buffer B (100% ACN, 0.1% formic acid). Mass spectrometer parameters are as follows; an MS1 survey scan using the orbitrap detector (mass range (m/z): 400-1500 (using quadrupole isolation), 120000 resolution setting, spray voltage of 2200 V, Ion transfer tube temperature of 275 C, AGC target of 400000, and maximum injection time of 50 ms) was followed by data dependent scans (top speed for most intense ions, with charge state set to only include +2-5 ions, and 5 second exclusion time, while selecting ions with minimal intensities of 50000 at in which the collision event was carried out in the high energy collision cell (HCD Collision Energy of 30%), and the fragment masses where analyzed in the ion trap mass analyzer (With ion trap scan rate of turbo, first mass m/z was 100, AGC Target 5000 and maximum injection time of 35ms). Protein identification and label free quantification was carried out using Peaks Studio 8.0 (Bioinformatics solutions Inc.)

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