The ELMO1, a Microbial Sensor Regulates Bacterial Clearance and Endo-lysosomal Signaling

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The ELMO1, a Microbial Sensor Regulates Bacterial Clearance and Endo-lysosomal Signaling

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

in

Biology

by

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2019
The thesis of Julian Tam is approved, and it is acceptable
In quality and form for publication on microfilm and electronically

Co-Chair

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University of California San Diego

2019
Dedication

This Thesis is dedicated to my family, my friends, and the fellow members of the Das lab for their patience, support and encouragement.
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ABSTRACT OF THE THESIS

The ELMO1, a Microbial Sensor Regulates Bacterial Clearance and Endo-lysosomal Signaling

by

Julian Tam

Masters of Science in Biology

University of California San Diego 2019

Professor Soumita Das, Chair
Professor Amy Kiger, Co-Chair

The clearance of enteric bacteria by phagocytes is an essential role of the host response against infection. Previously, we have reported that BAI1 (Brain Angiogenesis Inhibitor 1) binds bacterial lipopolysaccharide (LPS) of gram-negative bacteria and mediates its engulfment by activating ELMO1 (Engulfment and cell motility protein 1).
While both pathogenic and commensal Gram-negative bacteria express lipopolysaccharide (LPS), intestinal phagocytes are able to discriminate commensals from enteric pathogens. Using *Salmonella* as a model organism, we showed that ELMO1 interacts with *Salmonella* effector protein called SifA (*Salmonella* induced filaments A) which involves in the survival of bacteria inside macrophages. SifA shares the WxxxE signature motif that present only in enteric pathogens and absent in commensals. The signature motif is important for interaction with ELMO1. Therefore, we hypothesized that the ELMO1-SifA interaction could play a role in the bacterial survival by interfering with host cellular pathways. Pulldown assays with GST-SifA on murine macrophage lysate revealed that SifA interacts with late endosomal protein Rab9. Rab 9 is a host GTPase involved in the retrograde trafficking of CI-MPRs (Cation-independent mannose 6 phosphate receptors) from the late endosome to the trans-Golgi network. Further analysis using GST-Rab9 pulldowns with direct recombinant proteins in solution revealed that increasing concentrations of SifA displaces the strong ELMO1 interaction with Rab9. To elucidate the functional relevance of ELMO1-SifA interaction, immunofluorescence microscopy was performed to monitor the integrity of Salmonella-containing vacuole and the localization of CI-MPR. Our data shows an accumulation of CI-MPR at the surface plasma membrane in the presence of SifA. Bacterial clearance assays using mutant strains of *Salmonella* showed that clearance was delayed in ELMO1-depleted murine macrophages when compared to control macrophages. Interestingly, macrophages infected with *Salmonella* mutant strains lacking SifA had significantly higher intracellular bacteria. Our findings provide new insight on how *Salmonella* modulates host pathways
through its interaction with ELMO1 to create its own replicative niche in the form of *Salmonella* containing vacuoles within host cells.
I. INTRODUCTION

Enteric infections and Salmonella as a model bacteria

Foodborne and waterborne infections are a persistent health issue around the world. In the United States, these infections are responsible for a significant amount of hospitalizations, deaths, and economical cost associated with food recalls (MMWR, 2013). The pathogenic bacteria *Salmonella* is the second leading cause of foodborne and waterborne infections, being accountable for over 1 million cases each year, behind the norovirus (Petri et al., 2008; Scallan et al., 2011). *Salmonella* infects a broad range of hosts after uptake through contaminated food and water. Most people can eventually clear out the infection, but those with weakened or compromised immune systems such as young children and elderly people encounter recurrent infections (Buchwald and Blaser, 1984; Greig et al. 2007). Therefore, it is important to understand the interaction between *Salmonella* and the host response during infection. A model illustrating *Salmonella* pathogenesis is shown in Figure 1.

Upon ingestion, *Salmonella* encounters the epithelial lining, where *Salmonella* mediates its invasion into epithelial cells by expressing genes within SPI-1 (*Salmonella* pathogenicity island 1) (Agbor and McCormick, 2011; Galan and Wolf-Watz, 2006). The production of bacterial effectors such as SopB, SopE, and SopE2 allows *Salmonella* to cross the epithelial lining and invade the cells (Broz et al., 2012). After that, *Salmonella* is engulfed by phagocytes in the lamina propria where another set of genes within SPI-2 (*Salmonella* pathogenicity island 2) are expressed, which help the bacterial survival within host macrophages. *Salmonella* is protected inside the macrophage by forming a phagosomal-like compartment called the SCV (*Salmonella* containing vacuole) through
maintaining an acidic pH, preventing the fusion with the lysosome and reducing the activity of lysosomal enzymes (Buchmeier and Heffron, 1991; Uchiya et al., 1999).

**The host engulfment pathway**

Macrophages sense the presence of pathogens such as *Salmonella* through their pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) (Akira et al., 2004). This interaction between PRRs and PAMPs is important for generating the appropriate response during infection. Previous findings from our lab found that BAI1 (Brain Angiogenesis Inhibitor 1) acts as a PRR to bind the Lipopolysaccharide (LPS) of Gram-negative bacteria (Das et al., 2011; Das et al., 2014). Unlike another PRR called TLR4 (Toll-Like Receptor 4) which recognizes the lipid A region of LPS, BAI1 binds the core oligosaccharide.

Once BAI1 binds to the LPS of Gram-negative bacteria, the receptor recruits the cytosolic protein ELMO1 (Das et al., 2011; Das et al., 2014). ELMO1 then interacts with another cytosolic protein called Dock180 which acts as a guanine nucleotide exchange factor for Rac1 (Figure 2). The activation of Rac1 leads to actin cytoskeleton reorganization, allowing macrophages to engulf Gram-negative bacteria such as *Salmonella*. In addition, ELMO1 has previously been shown to be involved in the phagocytosis of apoptotic cells and the generation of inflammatory responses (Gumienny et al., 2001; Das et al., 2015).

**Role of ELMO1 in inflammatory disease**

Studies have shown that ELMO1 is implicated in different inflammatory diseases such as IBD (inflammatory bowel disease), RA (Rheumatoid arthritis), kidney disease, and diabetic nephropathy (Parmar et al., 2012; Arandjelovic et al., 2019; Pezzolesi et al.,
ELMO1 also regulates the expression of pro-inflammatory cytokines associated with these diseases such as MCP-1, IL1-β, and TNF-α. Recent findings from our lab found that ELMO1 expression was elevated in the gut epithelium of IBD patients compared to healthy subjects. In addition, a positive correlation between ELMO1 and MCP-1 was detected, which leads to the recruitment of monocytes to the site of inflammation. These recruited monocytes then produce more cytokines such as TNF-α, resulting in chronic inflammation of the gut as seen in IBD (Sayed et al., 2018).

**Interaction of ELMO1 with bacterial effectors**

ELMO1 has been reported to interact with *Shigella* effector IpgB1, facilitating its invasion into host cells through membrane ruffling (Handa et al., 2007). A literature search showed that a conserved signature WxxxE (Tryptophan-xxx-Glutamate) motif was found in a family of effectors from enteric pathogens, including SifA from *Salmonella* (Alto et al., 2006). Following this finding, our lab identified more effectors through a BLAST database search that shared this motif. Interestingly, we found that these effectors are present in pathogenic bacteria and not in commensal ones (Table 1). Additionally, mutation of the WxxxE motif in *Salmonella* SPI-2 effector SifA abolished its interaction with ELMO1 (Figure 3).

**Modulation of SCV interaction with host proteins by Salmonella effector SifA**

For *Salmonella* to avoid degradation and replicate within the SCV, the bacteria releases SPI-2 effectors during the maturation process of the endosome. Of the SPI-2 effectors, SifA is essential for the integrity of the SCV, acquisition of LAMP1 (lysosome associated membrane protein 1), and the formation of Sifs (*Salmonella* induced
filaments), a network consisting of tubular structures extending from the SCV (Jackson et al., 2008).

During the initial timeframe of infection, the SCV acquires early endosome markers such as EEA1 (early endosome antigen 1) (Steele-Mortimer et al., 1999). The SCV then loses these markers and acquires late endosomal markers including the lysosomal glycoprotein LAMP1, at this stage the bacteria are able to proliferate within the SCV. In the absence of SifA, the integrity of the SCV is lost and Salmonella is exposed to other host defenses present in the cytosol (Beuzon et al., 2000). The normal maturation process of endosomes during phagocytosis is regulated by a family of small GTPases known as Rabs, which are important for the trafficking of membrane proteins. Therefore, these Rab GTPases are the prime targets for Salmonella to modulate and inhibit their function for the survival of the bacteria (Smith et al., 2007). For example, SifA has been shown to target Rab7 through PLEKHM1 (Pleckstrin homology domain-containing protein family member 1) to recruit membranes along Sifs for proper SCV formation (McEwan et al., 2015). In addition, SifA has been shown to interact with host protein SKIP to sequester Rab9. Since Rab9 is involved with the retrograde trafficking of MPRs (mannose 6 phosphate receptors) to the TGN (trans-Golgi network), lysosomal activity is therefore attenuated as a result (McGourty et al., 2012). A figure demonstrating the modulation of endosomal signaling and disruption of MPR trafficking is shown in Figure 4. SifA also interacts with the multisubunit tethering factor HOPS (homotypic fusing and protein sorting) complex for Salmonella to obtain a constant supply of nutrients and membranes to maintain the integrity of the SCV (Sindhwani et al., 2017). These findings show that
Salmonella is able to exploit host machinery to abrogate various host processes including the endo-lysosomal system.

**Mannose-6-phosphate receptors are involved in trafficking of lysosomal enzymes**

During phagocytosis, lysosomal enzymes such as hydrolases are synthesized and transported to the lysosome, leading to degradation of the internalized component. These enzymes are transported from the trans-Golgi network to the early endosome by MPRs, which recognize and bind mannose 6 phosphate residues present on the enzymes. Upon acidification of the endosome, the ligand dissociates, and the receptors are recycled back to the trans-Golgi network (Dahm et al., 1989). The two types of MPRs are the cation-dependent MPRs (CD-MPRs) and cation-independent MPRs (CI-MPRs). The two types of receptors are functionally similar. Although both receptors are present on the cell surface, only the CI-MPR can bind and internalize extracellular lysosomal enzymes. CI-MPRs are found on the plasma membrane, where they bind any lysosomal enzymes that may have been secreted and are internalized rapidly back to the endosome via endocytosis (Lin et al., 2004). Salmonella has been reported to inhibit retrograde trafficking of MPRs by sequestering Rab9 (McGourty et al., 2012), but the role of the bacteria in endocytosis of CI-MPRs from the plasma membrane has yet to be studied.

Despite many studies done on the pathogenesis of Salmonella, little is known about how the bacteria exploit different host cellular pathways to evade the host immune response. Currently, findings from our lab indicated that ELMO1 helps macrophages to uptake the bacteria and then the bacteria is degraded in an ELMO1—dependent clearance pathway. Since the effector SifA is important in maintaining SCV integrity by interacting with various Rabs throughout the endosomal maturation process, we wanted
to determine whether ELMO1 associates with Rabs. An immunopulldown assay demonstrated that ELMO1 interacts with late endosomal marker Rab9. Due to the interactions between ELMO1, SifA, and Rab9 with each other, we hypothesize that the interaction of ELMO1-SifA is important for the innate immune responses and in regulating endo-lysosomal signaling that controls bacterial clearance. We used biochemical approaches to determine the interaction between ELMO1-SifA and Rab9. Functional approaches with confocal microscopy and bacterial plating was used to know the effect of ELMO1-SifA interaction on bacterial clearance, as discussed in the result and discussions.

Figure 1. The pathogenesis in Salmonella. When Salmonella reaches the epithelial lining of the small intestines, it employs a Type III secretion system (TTSS) to inject SPI-1 (Salmonella pathogenicity island-1) effectors, which promote its invasion into the host cell. Upon internalization, SPI-2 (Salmonella pathogenicity island-2) effectors are expressed which facilitate bacterial survival and the formation of the SCV (Salmonella containing vacuole). When Salmonella is able to across into the lamina propria, it encounters macrophages, which engulf the bacteria and secrete inflammatory cytokines, which increase inflammation and recruitment of macrophages. Once engulfed, Salmonella again express SPI-2 effectors (such as SifA and SifB) to form and reside within the SCV of host macrophages.
Figure 2. BAI1/ELMO1 mediated engulfment in phagocytes. Binding of LPS from Gram-negative bacteria to the receptor BAI1 recruits ELMO1 and Dock180 to the engulfment site. ELMO1 stabilizes Dock180 and together they act as a bipartite guanine nucleotide exchange factor for the small Rho GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1), leading to engulfment of the bacteria. ELMO1 also regulates the expression of pro-inflammatory cytokines such as TNF-α, MCP-1, and IL-8 during activation. Salmonella (SL), upon internalization, releases bacterial effectors via a Type III Secretion System (T3SS) that affect this process. (Das et al., PNAS 2011, Das et al., CMGH 2015, Das et al., Faseb J 2014).
Table 1. WxxxE signature motif is conserved between several bacterial effectors including SifA of Salmonella in enteric pathogens. A BLAST search between amino acid sequences showed homologues within enteric pathogens that contain the WxxxE or “Trp-x-x-x-Glu” amino acid sequence.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Conserved Forms</th>
<th>Signature Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>SifA</td>
<td>ST, SE</td>
<td>TELRKGLLDGW AQEKATYLAQKIQ</td>
</tr>
<tr>
<td></td>
<td>SE′</td>
<td>SEWRKGNLDEWF TQEKATYLAQKIQ</td>
</tr>
<tr>
<td></td>
<td>YF</td>
<td>NINQGDFDMDWEETTYLSAVIN</td>
</tr>
<tr>
<td>SifB</td>
<td>ST, SE, SE′, SE′′, SN</td>
<td>AMAEKGNLCDW EQERKAAISSRN</td>
</tr>
<tr>
<td>IpgB1</td>
<td>SF, E, EC</td>
<td>DSNSGQNQFLCWWMSQERTSYVSSMIN</td>
</tr>
<tr>
<td>IpgB2</td>
<td>SF, EC</td>
<td>EQ1-GENITDWNDEKKVYVSRVNN</td>
</tr>
<tr>
<td>Map</td>
<td>EP, EC</td>
<td>KQTGSDTQWFEQITFLSRAVN</td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>KQTGNDTQWFEQITIFISKTVN</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>KQTGSDTQWFEQITIFLSRTVN</td>
</tr>
<tr>
<td>EspT</td>
<td>EC</td>
<td>KQTTRSGDTQWFOQETTTYISRTVN</td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>IKN-EGKHMNEWMEECICFVSRDVN</td>
</tr>
<tr>
<td>EspM</td>
<td>EC, CR</td>
<td>RQS-TKIDEWIDERIVYPVRVIN</td>
</tr>
<tr>
<td>TrcA</td>
<td>EP</td>
<td>RQN-TKIDENVIDERIVYPVRVIN</td>
</tr>
</tbody>
</table>

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

Figure 3. The WxxxE signature motif is important for ELMO1-SifA interaction. EGFP-SifA, EGFP-SifA_{E201A}, and FLAG-ELMO1 plasmids were transfected into HEK 293 cells. Coimmunoprecipitation was performed with EGFP beads on protein lysate. The samples were run through SDS-PAGE and immunoblotted for FLAG and EGFP. The blot on the left is the input lysate, which was immunoblotted for FLAG and EGFP, showing successful transfection of the plasmid constructs. The right blot is the indicates pulldown with EGFP beads, which was then probed for FLAG to test for ELMO1.
Figure 4. *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.
II. MATERIALS AND METHODS

Cell lines and bacteria culture

Murine macrophage cell line J774 (ATCC) and ELMO1 depleted J774 cells were used as phagocytes. ELMO1 depletion was achieved through shRNA by electroporation as noted previously (Das et al. 2015). Cells were maintained in DMEM with high glucose (Gibco) containing 10% fetal bovine serum (Sigma), 2mM penicillin-streptomycin (Gibco), and 0.5ug/ml puromycin (Gibco). Wild-type Salmonella enterica serovar Typhimurium (SL1344) and ΔSifA mutant strains were used for infections. The bacterial strains were streaked onto LB agar plates with the appropriate antibiotics and a single colony was selected for inoculation in LB broth. The inoculation process consisted of 6-8 hours shaking at 150RPM at 37°C to allow for aeration then transferred to a static condition for overnight incubation. The multiplicity of infection (MOI) between the bacteria and phagocytes was 10:1 or 30:1 for all experiments.

Bacterial clearance assay

Control and ELMO1 depleted J774 macrophages were infected with the Salmonella strains SL1344 and ΔSifA mutant for 30 minutes. After infection, cells were treated with DMEM containing 0.5mg/ml gentamicin for 90 minutes to kill extracellular bacteria. Media was then changed to media containing 0.05mg/ml gentamicin for the later 12h and 24h timepoints. At each timepoint, cells were washed with PBS then lysed with 1% Triton-X 100 in PBS. The lysate was collected, serially diluted in PBS, and plated onto LB agar plates to count for colony forming units (CFU) the next day.
Western blot

J774 cells were lysed with ice cold lysis buffer: 1% NP-40, 0.5% deoxycholate, 1% SDS in 1X PBS with protease and phosphatase inhibitors. The amount of the protein in the lysate was quantified with the Lowry assay (Bio-rad), equal amounts of protein were loaded on SDS-PAGE and transferred onto a nitrocellulose or PVDF membrane (Bio-rad). Membranes were then blocked by 5% non-fat dry milk in 1X Tris buffered saline (TBS) for 1 hour at room temperature with gentle shaking. Membranes were then incubated with the appropriate primary antibody in 5% non-fat dry milk or bovine serum albumin in 1X TBS with 0.05% Tween-20 (TBST) overnight at 4°C. Membranes were then washed the next day and incubated with the secondary antibody in blocking buffer for 1 hour at room temperature with gentle shaking.

Antibodies used for Western blotting and immunofluorescence microscopy

Mouse monoclonal antibody (mAb) to ELMO1 (sc-271519) was used with a dilution of 1:1000 for Western blotting. Rabbit monoclonal antibody to Rab9 (5118S) and CI-MPR (D3V8C) (Cell Signaling Technologies) was used with a dilution of 1:500 for Western blotting. Alexa Fluor 488 anti-mouse LAMP1 antibody was used with a dilution of 1:500 for immunofluorescence microscopy. Rabbit monoclonal antibody to CI-MPR supplied from Dr. Stuart Kornfeld from Washington University School of Medicine in St. Louis was used with a dilution of 1:200 for immunofluorescence microscopy. Alexa Fluor 594 goat anti-rabbit antibody was used with a dilution of 1:500 as a secondary for immunofluorescence microscopy.
Transformation and expression of plasmids and sequential binding onto Glutathione-sepharose beads

50-100 ng of plasmid DNA containing the desired bait protein was added to 50ul BL21 competent *E. coli* cells, which were then heat shocked and incubated in S.O.C. media. Cultures were then grown overnight on LB agar plates containing ampicillin or kanamycin for selection. Transformed colonies were selected and grown in LB broth with antibiotics overnight. Next day, bacteria cultures were grown into a large induction flask. IPTG was added to a final concentration of 0.5mM to induce the expression of protein for 4 hours at room temperature. After induction, bacteria cell pellet was collected through centrifugation.

Bacteria cell pellet was resuspended with lysis buffer containing 50mM Tris/HCl, 150mM NaCl, 10% glycerol, 5mM MgCl₂, 1mM EDTA, and protease inhibitor. The resuspension was then sonicated and Triton-X 100 was added afterwards to a final concentration of 0.5%. Lysate was kept on a rotator for 30 minutes at 4°C and centrifuged to obtain the purified protein in the supernatant. The supernatant was then incubated with the Glutathione sepharose beads (Sigma) for three hours at 4°C. The protein-bead complex was then washed with lysis buffer and stored at -20°C in 50% glycerol in PBS solution. Validity of the beads were tested with SDS-PAGE and stained with Coomassie Blue (Bio-rad).

**GST immunopulldown assay**

Control and ELMO1 depleted J774 murine macrophage were lysed as described and incubated with the GST bead complexes overnight at 4°C on a rotator. A concentration of 1mg protein was used per 50ul of beads for each incubation. After
overnight incubation, the beads were washed with lysis buffer and ran through SDS-PAGE. Target protein expression was then detected by western blot.

**Immunofluorescence microscopy for CI-MPR localization**

Control and ELMO1 depleted J774 murine macrophages were infected with the *Salmonella* strains SL1344 and ΔSifA, fixed with 4% paraformaldehyde in PBS for 10 minutes, and blocked with 0.1% saponin in PBS with 2% BSA. LAMP1 (green) was stained with Alexa Fluor 488 anti-mouse LAMP1 antibody and CI-MPR (red) was stained with Alexa Fluor 594 goat anti-rabbit antibody.

**GST-Rab9 competition assay**

GST-Rab9WT and GST-Rab9CA proteins were purified and bound Glutathione-sepharose beads overnight at 4°C. For the competition assay, equal amounts of GST-Rab9WT and GST-Rab9CA beads were independently incubated with 40µg of His-ELMO1 and increasing proportionate concentrations of His-SifA (1:1-1:3). GST beads alone served as negative control. Nucleotide mix (50µM GMP-PNP, 1mM DTT, Na₃VO₄) was added to all samples and GST binding buffer was added to total volume of 350µL. Samples were incubated for 4 hours at 4°C then centrifuged at 17,200 x g for 10 seconds. Supernatant was saved and the beads were washed four times with GST wash buffer. 5X reducing sample buffer was then added and the beads were boiled for 10 minutes at 100°C and centrifuged. The supernatant was collected, and the previous step was repeated. The supernatant was then collected and combined with the supernatant of the previous step. The samples were then run on 15% SDS-PAGE and target proteins were detected by Western blot.
III. RESULTS

The interaction between ELMO1, SifA, and Rab9

Previous reports have shown that *Salmonella* effector SifA inhibits the function of the late endosomal protein Rab9, negatively affecting the endosomal maturation process (McGourty et al. 2012). As ELMO1 interacts with SifA and helps in the bacterial clearance, here we tested whether ELMO1 can interact with Rab9 or not. In the GST-pulldown assay, we have found that *Salmonella* effector SifA interacts with the late endosomal protein Rab9 and ELMO1 endogenously from the J774 macrophage lysate (Figure 5). In addition, ELMO1 interacts with Rab9 endogenously.

We then wanted to determine whether the interactions between ELMO1 and SifA were direct, so we incubated GST and GST-SifA proteins bound to glutathione sepharose beads with His-ELMO1 in solution and found that ELMO1 interacted directly with SifA (Figure 6).

Next, we wanted to investigate the relationship and interplay between the proteins in the ELMO1-SifA-Rab9 complex. To assess this point, a direct competitive assay approach was used in which purified GST-tagged wild-type Rab9 and GST-tagged constitutively active Rab9 mutant were bound to glutathione sepharose beads and incubated with His-ELMO1 and a non-hydrolyzable form of GTP. Interestingly, ELMO1, but not SifA, bound to Rab9-GTP in isolation. As increasing concentrations of His-SifA were introduced into the ELMO1-Rab9 complex, the binding between ELMO1 and Rab9-GTP decreased (Figure 7). Thank you to Mahitha Shree from Dr. Pradipta Ghosh’s lab for providing the data for the competition assay experiment.
**ELMO1-SifA interaction is important for the SCV maintenance and bacterial clearance**

In collaboration with Mitchel Lau, a member from our lab, control and ELMO1 depleted J774 macrophages were infected with wild-type and ΔSifA mutant *Salmonella* strains. After 12 hours, the infected cells were fixed, permeabilized, and stained. Immunofluorescence microscopy images were captured to monitor the *Salmonella* containment within the SCVs. The SCVs were categorized based on LAMP1 surrounding the *Salmonella* (Figure 8). 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 and ELMO1-depleted macrophages and whether they were infected with wild-type or ΔSifA *Salmonella*. Control macrophages infected with wild-type *Salmonella* contained an average of 10% exposed, 30% partial and 60% complete SCVs, whereas ELMO1 depleted macrophages contained 45% exposed, 15% partial and 40% complete SCVs. In case of infection with ΔSifA mutant, control macrophages contain 45% exposed, 30% partial and 25% complete SCVs whereas ELMO1 depleted macrophages had 60% exposed, 15% partial and 35% complete SCVs. Our results showed that the percentage of exposed SCVs increases in the absence of ELMO1 and SifA, suggesting their importance in the formation and maintenance of the SCV.

**Salmonella clearance is delayed in ELMO1 depleted macrophages in vitro**

Previously, our lab has found that the clearance of *Salmonella* was significantly delayed after infection on ELMO1 depleted J774 cells when compared to control J774 cells (Sarkar et al. 2017). Here we show that the bacteria count for *Salmonella* mutant
strain ΔSifA is significantly higher in ELMO1 depleted J774 macrophages when compared to control J774 macrophages at the 12 and 24 hour timepoints, indicating a delayed clearance in ELMO1 depleted macrophages. In addition, the number of intracellular bacteria after normalization to entry in macrophages infected with *Salmonella* mutant strain ΔSifA is significantly higher when compared to wild-type *Salmonella* (Figure 9).

**Retrograde trafficking of CI-MPRs is abrogated in the absence of ELMO1 or presence of SifA**

In order to analyze the downstream effect of the interaction between Rab9, ELMO1 and SifA on CI-MPR trafficking, control and ELMO1 depleted macrophages were infected with wild-type and ΔSifA *Salmonella*. After infection, cell lysates were collected and detected for CI-MPR by western blot. The amount of proteins was normalized to tubulin. Our preliminary data suggests that at both 45 minute and 10-hour timepoints, ELMO1 depleted macrophages had less CI-MPR present compared to control macrophages. In addition, for all samples infected with wild-type *Salmonella*, there was also a decreasing trend in CI-MPR levels. Interestingly, CI-MPR levels was higher in ΔSifA mutant *Salmonella* infected cells than wild-type *Salmonella* infected cells (Figure 10).

Next, we stained infected cells to visualize CI-MPR localization throughout the cell. In ELMO1 depleted macrophages, there was a decrease in CI-MPR fluorescence compared to control macrophages after infection with both wild-type and ΔSifA *Salmonella*. In addition, there a decrease in LAMP1 staining in ELMO1 depleted macrophages infected with ΔSifA, suggesting their importance in SCV formation (Figure 11). We then wanted to measure the localization of CI-MPRs at the plasma membrane after infection. In control and ELMO1 depleted macrophages infected with wild-type
Salmonella, there was an increase in CI-MPR levels at the surface compared to ΔSifA mutant after infection for 45 minutes. (Figure 12). These experiments were done in collaboration with Dr. Gajanan Katkar from Dr. Pradipta Ghosh’s lab.

Figure 5. SifA interacts with endogenous ELMO1 and Rab9. GST and GST-SifA bound to glutathione-sepharose beads were incubated with J774 murine macrophage lysate. The samples were loaded onto SDS-PAGE gel and immunoblotted for ELMO1, Rab9, and GST. From left to right, the lanes shown are ladder, protein lysate, GST pulldown, and GST-SifA pulldown.

Figure 6. SifA interacts with directly with ELMO1. GST and GST-SifA bound to glutathione-sepharose beads were incubated with purified recombinant protein His-ELMO1. The samples were loaded onto SDS-PAGE gel and immunoblotted for His. On the top panels, the left lane is GST pulldown and the right lane is the GST-SifA pulldown. The amount of GST beads loaded was visualized through Ponceau stain. The input bands indicate the percentage of the concentration loaded onto the pulldown assays.
Figure 7. Increasing concentrations of SifA disrupts ELMO1 interaction with Rab9-GTP. Wild-type GST-Rab9 (WT) and constitutively active GST-Rab9 (QL) beads were incubated with purified recombinant protein His-ELMO1, His-SifA, and GMP-PNP, a non-hydrolyzable form of GTP. The samples were then immunoblotted after SDS-PAGE for His. ELMO1 initially binds to Rab9-GTP with strong affinity. As increasing amounts of SifA are added to complex, ELMO1 band intensity decreases, suggesting that SifA is displacing ELMO1 and competing for interaction with Rab9-GTP. The amount of GST and GST-Rab9 beads loaded was measured with Ponceau staining.
Figure 8. ELMO1 and SifA have significant roles in the maintenance of SCVs. Control and ELMO1 shRNA macrophages were infected with wild-type and ΔSifA mutant *Salmonella* for 1 hour and treated with gentamicin to eliminate extracellular bacteria. After 12 hours, samples were fixed, permeabilized, and stained for LPS (red), DAPI (blue), and LAMP1 (green). The samples were then visualized through 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 are shown on the top.
Figure 9. The number of intracellular bacteria was higher in macrophages infected with ΔSifA Salmonella strain. Control and ELMO1 depleted macrophages were infected with wild-type and ΔSifA Salmonella strains for 30 minutes, treated with gentamicin, then measured for intracellular bacteria as indicated by the clearance assay in the Methods section. The counts were normalized to entry at 30 minutes after 12 and 24 hours of infection and plotted as the fold change to control shRNA macrophage infected with wild-type Salmonella.

Figure 10. CI-MPR is degraded in the absence of ELMO1 or the presence of SifA. Control and ELMO1 depleted macrophages were infected with WT (SL) and ΔSifA (SifA) Salmonella strains for 45 minutes or 10 hours. Infected cells were lysed and the samples were loaded on SDS-PAGE and immunoblotted for CI-MPR and tubulin as the loading control. The intensity of the bands was quantified and adjusted for amount of loading control protein tubulin, as shown by the bar graphs on the right.
Figure 11. ELMO1 plays an important role in trafficking of CI-MPRs. Control and ELMO1-depleted macrophages were infected with WT Salmonella and ΔSifA (SifA) Salmonella strains for 10 hours. The samples were then fixed with 2% paraformaldehyde in PBS, permeabilized, and stained for CI-MPR (red), LAMP1 (green), and nuclei via DAPI (blue).
Figure 12. *Salmonella* effector SifA leads to accumulation of CI-MPR at the surface. Control and ELMO1-depleted macrophages were infected for 45 minutes with WT (SL) and ΔSifA (SifA) *Salmonella* strains. The samples were fixed and stained for surface levels of CI-MPR (red).
IV. DISCUSSION

Previous reports have shown that Salmonella effector SifA sequesters Rab9 through host protein SKIP via its Pleckstrin homology (PH) domain (McGourty et al. 2012). Data from our lab have revealed that Salmonella effector SifA interacts with Rab9 and ELMO1. We then hypothesized that these three proteins may form a complex and play a role in formation of the SCV. A direct interaction in biochemical assay showed that SifA competes for binding to Rab9 with ELMO1 and that ELMO1 binds strongly to Rab9-GTP. We then visualized the downstream effects of the ELMO1-SifA-Rab9 complex through immunofluorescence microscopy. Studies have reported that Rab9 in its GTP bound state functions in recycling CI-MPRs from the late endosome back to the trans-Golgi network, so as to prevent the receptors from reaching the lysosome and being degraded. In addition, CI-MPRs are found at the plasma membrane, where they can retrieve any lysosomal enzymes that may be accidentally secreted and are internalized rapidly (Ghosh et al., 2003). Together, these findings suggest that ELMO1 and SifA have opposing effects on the retrograde trafficking of CI-MPRs from the late endosome. By interfering the interaction of ELMO1 with Rab9-GTP and keeping Rab9 inactive, SifA can prevent the recycling of CI-MPRs from the late endosome, therefore attenuating lysosomal enzyme activity at the SCV and allowing for bacterial survival. The mechanism by which SifA prevents the retrieval of CI-MPRs from the plasma membrane remains unknown and further studies will be needed to explore this pathway. A figure summarizing Salmonella effector SifA’s role in the host engulfment pathway is shown in Figure 13.

Previous data has shown that ELMO1 is involved in bacterial clearance. Internalization of bacteria and subsequent bacterial clearance are significantly attenuated
in the absence of ELMO1. From our understanding of SifA’s role in maintaining the integrity of the SCV, we expected the number of intracellular bacteria to be lower in infections with the ΔSifA Salmonella mutant strain. Interestingly, our bacterial clearance data shows that the number of intracellular bacteria was higher in infections in these strains when compared to wild-type Salmonella in our J774 murine macrophage model. When the SCV integrity is compromised, it is possible that free Salmonella are able to hyper-replicate in the cytosol. A study done by Beuzon et al. has shown that the replication of Salmonella (SL12023) increased in the cytosol of the epithelial HeLa cells but decreases in that of RAW murine macrophages. The difference in findings could be due the usage of different Salmonella strains and cell lines and shows the difference in host cellular responses to Salmonella infection. It is possible that initiation of immune responses is dependent on the interaction between host proteins and virulence factors such as SifA.

Our unpublished data has shown that ELMO1 interacts with the cytosolic host protein NOD2. Like BAI1, NOD2 is a PRR that recognizes muramyl dipeptide (MDP), a component of the bacterial peptidoglycan cell wall (Kawai and Akira, 2009). NOD2 mutations have also been reported to be implicated in Chron’s disease (Ogura et al., 2001). In addition, recent studies have shown that NOD2 promotes MCP-1 signaling to recruit monocytes in response to bacterial infection (Kim et al., 2012). Further experiments identifying the regions in which ELMO1 and NOD2 interact and testing for downstream effects on cytokine signaling can help us understand their roles in the innate immune response and inflammation.
In order to modulate host responses to ensure the survival of *Salmonella* within the SCV, SifA interacts with various host proteins. Unpublished data using a mass spectrometry approach after pulldown assays with GST-SifA identified host proteins from J774 macrophage lysate which were then sorted according to their functions. We found proteins from the N-glycosylation/trafficking and G-PCRs. N-glycosylation is the process in which an oligosaccharide is added to Asparagine residues of newly synthesized proteins in the endoplasmic reticulum. The addition of glycans to nascent polypeptides is essential for proper function, folding, and trafficking (Breitling et al., 2013). It is possible that *Salmonella* would interact with proteins involved in N-glycosylation to disrupt development of proteins that may affect its survival such as lysosomal enzymes. The capacity of the relationship between these novel interactions remain unknown and should be explored in future experiments.

Immunocompromised individuals such as children and older people are most at risk of recurrent *Salmonella* infections as they cannot effectively clear out bacteria. Our results suggest that ELMO1 is a novel cytosolic sensor that determines bacterial pathogenesis by interacting with bacterial effectors and provides new insight to the degradation of bacteria via mechanisms of the endosomal-lysosomal system.

This thesis, in part is currently being prepared for submission for publication of the material. Tam, Julian; Lau, Mitchel; Katkar, Gajanan; Ibrahim, Ibrahim; Pranadinata, Rama; Shree, Mathitha; Ghosh, Pradipta; Das, Soumita. “The ELMO1, a Microbial Sensor Regulates Bacterial Clearance and Endo-lysosomal Signaling”. The thesis author was the primary investigator and author of this material.
Figure 13. *Salmonella* effector SifA inhibits CI-MPR trafficking. *Salmonella* is engulfed by macrophages through a BAI-ELMO1 mediated process into the early endosome. The early endosome undergoes a maturation process into the late endosome. During this process, CI-MPRs are trafficked from the *trans*-Golgi network to the early endosome and release attached lysosomal enzymes at the late endosome. The CI-MPRs are then recycled from the late endosome back to the *trans*-Golgi network. CI-MPRs are also found at the surface, where they are endocytosed into the early endosome. *Salmonella* effector SifA inhibits the retrograde trafficking and the endocytosis of CI-MPRs, leading to CI-MPR degradation at the lysosome, resulting in decreased lysosomal activity and increased bacterial survival.
V. REFERENCES


