Microbial Sensing by ELM01 Mediates Intestinal Inflammation

Author
Vega, Kevin

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Microbial Sensing by ELMO1 Mediates Intestinal Inflammation

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

in

Biology

by

Kevin Vega

Committee in charge:

Professor Peter Ernst, Chair
Professor Emily Troemel, Co-Chair
Professor Soumita Das
Professor Alisa Huffaker

2017
The thesis of Kevin Vega is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

__________________________________________

Chair

University of California, San Diego

2017
DEDICATION

I dedicate this work to my parents, sisters, and grandparents for the sacrifices they made so that I can follow my dreams.
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ABSTRACT OF THE THESIS

Microbial Sensing by ELMO1 Mediates Intestinal Inflammation

by

Kevin Vega

Master of Science in Biology
University of California, San Diego, 2017
Professor Peter Ernst, Chair
Professor Emily Troemel, Co-Chair

 Millions of people worldwide suffer from food borne illness caused by the Salmonella bacterial species. Disease caused by Salmonella infection is more devastating and likely fatal in immunocompromised individuals or those in economically disadvantaged conditions. These infections start at the gastrointestinal epithelium and translocate subsequently into the lamina propria, where they encounter immune and inflammatory cells such as macrophages.
Following infection, macrophages release pro-inflammatory cytokines, such as IL-1β, to coordinate inflammatory responses that help clear the infection. We previously demonstrated that engulfment and cell motility protein 1 (ELMO1) expressed by macrophages is essential for the induction of many pro-inflammatory cytokine responses. Here, the role of ELMO1 in the regulation of IL-1β was investigated following *Salmonella* infection. The murine macrophage cell line J774 was stably transfected with control or ELMO1 shRNA, resulting in a 90% decrease in ELMO1 expression. In turn, cells were infected with *Salmonella enterica* Serovar typhimurium. Mature IL-1β levels, detected by western blot and ELISA, decreased significantly after *Salmonella* challenge in ELMO1-inhibited cells. Furthermore, luciferase assays showed a 2-fold increase in NFκB activation, a transcription factor regulating many pro-inflammatory cytokines including IL-1β, in ELMO1 overexpressed cells. Additionally, co-transfection of ELMO1 and NOD2, a cytosolic bacteria-sensing protein, revealed an increase in NFκB activation. In contrast, ELMO1 did not reproducibly regulate the post-translational activation of IL-1β by caspase 1. These results suggest ELMO1 regulates the NFκB pathway and the subsequent expression of pro-inflammatory cytokines after bacterial infection. Understanding the mechanisms by which ELMO1 regulates inflammation can reveal new therapeutic targets for people suffering from chronic inflammation and related immune-mediated disorders.
I.

Introduction
Gastrointestinal systems maintain homeostatic and dynamic interfaces between commensal microbes, pathogenic organisms, and host immune responses. Specifically, the intestinal immune system maintains optimum levels of antimicrobial activity without resulting in a constant state of inflammation (1). In healthy individuals, intestinal inflammation is induced in part by local macrophages in response to host-pathogen interactions. In contrast, clearance of apoptotic cells by these same macrophages has been shown to have no pro-inflammatory consequences, leading us to believe pathogens have unique characteristics that macrophages recognize to initiate the appropriate immune response (2).

Pathogens are recognized by macrophages via Pattern Recognition Receptors (PRRs) expressed on the surface of immune cells (3-5). PRRs recognize and bind specific Pathogen-Associated Molecular Patterns (PAMPs) exhibited by bacteria (3-5). Binding of a PAMP to a PRR signals the macrophage to engulf the bound ligand, forming an intracellular phagosome and subsequently activating downstream signals that result in the release of inflammatory cytokines (6). Previous studies investigating the role of PRRs have been performed in vitro using endotoxin levels representative of millions of bacterial encounters per host cell. In reality, much fewer encounters are necessary to induce disease (6). Our lab previously identified a PRR called Brain Angiogenesis Inhibitor 1 (BAI1) which recognizes the phosphatidylserine of apoptotic cells, as well as the core oligosaccharide of the LPS coat surrounding gram-negative bacteria, such as that of Salmonella (7).
Upon ligand binding, the cytosolic domain of BAI1 recruits engulfment and cell motility protein 1 (ELMO1) and dedicator of cytokinesis 180 (Dock180) (8). Together, this complex acts as a bipartite guanine nucleotide exchange factor (GEF) for the GTPase Rac1, which triggers cytoskeleton actin to collapse and rebuild in manner to allow engulfment (9). Previous findings revealed ELMO1 is not only critical for maximal bacterial engulfment by macrophages, but its inhibition results in a significant decrease in the levels of multiple pro-inflammatory cytokines (6). However, the mechanisms by which ELMO1 mediates pro-inflammatory cytokine response after pathogenic encounter remains obscure.

Interestingly, Das et al observed a decrease in IL-1RA, an inhibitor that diminishes sensitivity towards IL-1β, in ELMO1-shRNA J774 macrophages (6). IL-1β is the circulating form of IL-1, a pro-inflammatory cytokine and potent inducer of the body’s immune response. Mutations in the IL-1β gene that result in overproduction of the cytokine or downregulation of its antagonist have been shown to lead to, or worsen, inflammatory diseases ranging from rheumatoid arthritis to inflammatory bowel disease (IBD) (10). Thus, IL-1β is tightly regulated in healthy individuals, and is normally produced by infected and damaged cells (10). IL-1β expression is mediated by the NFκB pathway, as its promoter region contains several κB transcription factor binding sites upstream of the gene (11). IL-1β is synthesized as an inactive pro form, which is cleaved by activated caspase 1 in conjunction with the inflammasome NLRP3 (10).
ELMO1 is not known to directly activate the NFκB pathway. Therefore, we initially hypothesized ELMO1 can crosstalk with macrophage cytosolic sensor proteins to initiate the appropriate immune response after bacterial engulfment. We identified two protein targets for the investigation of ELMO1-mediated crosstalk: NOD1 and NOD2. NOD1 is capable of sensing the activation state of Rac1, a target of bacterial effector proteins, to initiate an immune response (12). Additionally, NOD1 and NOD2 have LRR domains that recognize the bacterial wall component diaminopimelic acid (DAP) and muramylidipeptide (MDP), respectively (13, 14). Upon activation, NOD1/2 activate the NFκB pathway via RIP2 phosphorylation, as well as upregulate caspase 1 auto-activation (15), to initiate a host immune response (16). Thus, if ELMO1 is regulating IL-1β, it may be delivering the bacterial cargo close to these NOD molecules. Therefore, I focused on NOD1/2 as candidates for ELMO1 crosstalk, as well as probing for a synergistic effect on IL-1β response.

In this study, we found that ELMO1 is critical for maximal IL-1β production, where its silencing led to a 50% decrease of active IL-1β. Our preliminary data from luciferase assays of ELMO1 and NOD2 co-transfected HEK cells shows an upregulation of NFκB activity after Salmonella infection. Interestingly, an ELMO1-NOD1 additive effect was not detected. Furthermore, caspase 1 levels were not changed significantly between control and ELMO1-deficient cells, suggesting caspase 1 expression is independent of ELMO1. These studies suggest ELMO1 has a critical role in immune signaling after pathogen encounter.
Figure 1: BAI1 Recognizes Phosphatidylserine of Apoptotic Epithelial cells and Lipopolysaccharide of Gram-Negative Bacteria. Ligand binding triggers the recruitment of ELMO1 and Dock180 to the cytosolic portion of BAI1, which act as a bipartite guanine-nucleotide exchange factor (GEF) and activate Rac1. N terminus of BAI1 binds the LPS core oligosaccharide which is distinct from the TLR4 as the TLR4/MD2/CD14 complex binds Lipid A. After binding BAI1, the bacteria are internalized with the help of the BAI1 interacting partner ELMO1. Here, we will elucidate the role of ELMO1 in host inflammatory regulation, specifically by investigating cross-talk with the bacterial sensors NOD1 and NOD2, in order to elucidate downstream transcription and activation of pro-inflammatory cytokines.
II.

Materials and Methods
Intestinal Macrophage Isolation

Intestinal macrophages were isolated as done previously (6, 7). Briefly, Control and ELMO1-deficient (myeloid-specific) mice were rendered unconscious in a CO\textsubscript{2} chamber and euthanized via cervical dislocation. Small intestines were immediately harvested and kept in Hank’s Buffered Salt Solution (HBSS) with 5% fetal bovine serum (FBS). Fat and Peyer’s patches were removed from the intestinal walls, followed by opening of the intestine. Feces and mucus were washed from the intestinal lumen using Dulbecco’s Phosphate Buffered Saline (PBS). Tissues were dissociated using gentleMACS Dissociator (MACS Miltenyi), and non-viable cells were removed using the Dead Cell Removal Kit (MACS Miltenyi). Samples were then incubated with magnetically-labeled CD-11b microbeads (MACS Miltenyi), and positive cells were eluted into a separate collection tube for further experimentation.

Cell Lines and Bacterial Culture:

The murine macrophage cell line J774 (American Type Culture Collection (ATCC), Manassas, VA, USA), control shRNA, and ELMO1-depleted shRNA J774 cells (a kind gift from Dr. Ravichandran, University of Virginia) were used as phagocytes. ELMO1-depleted shRNA cells were produced as mentioned previously (6, 7). Cells were maintained in high-glucose-containing DMEM (Life Technologies, Carlsbad) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM penicillin-streptomycin (Sigma) and 0.5μg/ml puromycin (Sigma). HEK-293 (ATCC) cells were also maintained in high-glucose containing DMEM (Life Technologies) supplemented with 10% fetal bovine serum
(Life Technologies) and 2 mM penicillin-streptomycin (Sigma). Wild type *Salmonella enterica* serovar Typhimurium (SL1344) was used for infection. To perform this, an isolated colony was picked from LB agar plates, inoculated in LB broth, and cultured for 6 hours on a shaking platform to allow for aeration, as done previously. An aliquot was then transferred to fresh LB broth, and was grown in static, oxygen-limited conditions overnight. The ratio between bacteria and phagocytes was maintained at 10:1 for all experiments.

**RT-PCR**

RNA was isolated from control and ELMO1 shRNA cells following infection with *Salmonella* for the respective time periods using the Total RNA Kit (Omega bio-tek). RNA was reverse transcribed with the RT-PCR Kit from Quanta Biosciences. Quantitative RT-PCR was performed using buffers containing SYBR green (Biotool), and the forward and reverse primers of ELMO1, TLR4, BAI1, MCP1, TNFα, and GAPDH were as follows:
<table>
<thead>
<tr>
<th>Primers</th>
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<th>Reverse</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>GAPDH (mouse)</td>
<td>gctttgcttataatagggactgc</td>
<td>ccatatgtctacgggacga</td>
</tr>
</tbody>
</table>

**ELISA**

Control and ELMO1 shRNA J774 macrophages were infected with *Salmonella* 1344. Supernatants were collected and assayed for IL-1β by ELISA (BD Biosystems). Gentamycin Protection Assay was performed to assess intracellular infection, in which cells were treated with a high concentration of gentamycin (10µM) for 90 min after 1 hour of infection. Media was then changed to a low gentamycin concentration (1µM) for the remainder of the experiment.

**Immunopulldown:**

Human Embryonic Kidney (HEK) 293 cells were transfected with control vector, HA-tagged NOD1, HA-tagged NOD2, and FLAG-tagged ELMO1. 3µg of total DNA was used for transfection of each condition. Cells were uninfected or infected with *Salmonella* 1344. Cells from each treatment condition were lysed using 50mM Tris, pH 7.4, .05% sodium deoxycholate, 100mM NaCl, 10mM MgCl₂, 10% glycerol, 1 mM DTT, 1% NP-40, 1% Triton-X, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktail.
Samples were incubated with anti-FLAG agarose beads (Sigma) and separated by SDS-PAGE.

**Western Blotting:**

Control and ELMO1 shRNA cells were lysed in cold RIPA buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1mM EDTA, 0.25% sodium-deoxycholate, 1% NP40) containing fresh protease inhibitor cocktail (Sigma). Equal protein amounts were loaded into each lane of an SDS-PAGE, separated, and then transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked using 5% non-fat dry milk (Lab Scientific Inc.) dissolved in Tris-Buffered Saline containing 0.05% Tween-20 (TBST) for 1 hour at room temperature. Membranes were then incubated with the appropriate primary antibody in 5% milk or BSA dissolved in TBST overnight at 4°C. 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 TBST).

**Luciferase Assay**

HEK293 cells were transfected with pcDNA control vector, ELMO1, NOD1, NOD2, and pNifty luciferase plasmid. Total transfected DNA was 300ng for each condition. Lipofectamine 2000 (Thermo Fisher) transfection reagent to total DNA was used at a 3:1 ratio. Cells were transfected in OptiMEM (Thermo Fisher) reduced serum media for 6 hours, followed by addition of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS). Media was changed
to DMEM with 10% FBS 18 hours after transfection. Cells were infected using *Salmonella* 1344 for 5 hours, after which the cells were lysed with 1x passive lysis buffer (PLB) (Promega). Protein concentrations were measured using a photometer, and Luciferase Assay Reagent 2 (LARII) (Promega) was added. Luminescence was recorded using a spectrophotometer.

**Antibodies and other reagents:**

Cell lysates were probed using rabbit polyclonal antibody to caspase 1 (1:500, Santa Cruz Biotechnology), rabbit anti-HA antibody (Cell Signaling, 1:1000), mouse anti-rabbit secondary (1:2000, Cell Signaling), horse anti-mouse secondary (1:2000, Cell Signaling), rabbit anti-β-Actin antibody (Cell Signaling, 1:1000), and monoclonal mouse anti-FLAG (1:1000, Sigma).
III.

Results
Intestinal Macrophages Express a significant amount of ELMO1 and BAI1

A previous report from our lab demonstrated ELMO1 significantly increases bacterial engulfment in intestinal macrophages (6) (Figure 1). To elucidate the involvement of ELMO1 in bacterial sensing, we first isolated intestinal macrophages from mice. Flow cytometry analysis of F4/80 expression by our cell sample confirmed the purity of intestinal macrophages to be greater than 84% (Figure 2A). To phenotype the intestinal macrophages, isolated intestinal RNA and performed RT-PCR. Apart from BAI1, toll-like receptor 4 (TLR4) is the only other known macrophage receptor that recognizes the LPS coat of gram-negative bacteria (21). Therefore, we examined the expression levels of ELMO1, TLR4, and BAI1 in intestinal macrophages. Analysis revealed undetectable levels of TLR4 from our CD11b+ macrophages, yet levels were readily detected from our J774 macrophage positive control (Figure 2B).

ELMO1 Silencing Decreases IL-1β Production after Salmonella Infection

To determine the effect of ELMO1 inhibition on engulfment, Das, et al reported the decrease of pro-inflammatory cytokines TNFα and MCP1 (6). Interestingly, their proteome profiler array utilizing the supernatants of Salmonella infected ELMO1-shRNA macrophages also revealed a decrease of IL-1RA, an inhibitor known to diminish the effect of the pro-inflammatory cytokine IL-1β (17). Therefore, we sought to determine the role of ELMO1 in IL-1β regulation. We infected control shRNA and ELMO1-shRNA J774 macrophages with Salmonella for various lengths of time (Figure 3). Cell lysates were harvested and run on SDS-PAGE and immunoblotted using an IL-1β antibody. Densitometric analysis
revealed a significant decrease of active IL-1β production ELMO1-deficient macrophages (Figure 3A). Additionally, we collected the supernatants from the previous samples for an IL-1β ELISA (Figure 3B). The ELISA data also showed a significant decrease of IL-1β from the supernatant of ELMO1-deficient macrophages.

**ELMO1 Interacts with NOD1/2**

ELMO1 is not known to directly induce the transcription of cytokine genes, so I postulated that its role in engulfment might juxtapose the microbial cargo adjacent to a PRR (18). Therefore, we investigated the possibility of ELMO1 interacting with macrophage cytosolic sensor proteins. NOD1 and NOD2 were ideal candidates for investigation due to their known capability for sensing over-activated Rac1 (which is activated by ELMO1) as well as their recognition of the bacterial cell wall components DAP and MDP (12, 16). To investigate a possible ELMO1-NOD1/2 interaction, we first transfected HEK293 cells with plasmids encoding HA-NOD1, HA-NOD2, and a vector control plasmid (Figure 4). Western blot analysis of the cell lysate using an HA antibody revealed HEK293 cells successfully expressed HA-NOD1 and HA-NOD2 (Figure 4A). Next, cells were transfected with FLAG-ELMO1 and HA-NOD1, FLAG-ELMO1 and HA-NOD2, or vector control and were either uninfected or infected with *Salmonella*. Cell lysates were harvested and incubated with anti-FLAG beads, after which proteins were separated by SDS-PAGE and immunoblotted using an antibody recognizing HA (Figure 4B-C). Western blot analysis revealed an ELMO1-NOD1/2 interaction,
which magnified after bacterial challenge, implying a possible cooperative role for the complex.

*The ELMO1-NOD2 Interaction Increases NFκB Activity*

Similar to its cooperative role for engulfment, we hypothesized ELMO1 interacts with NOD1/2 to initiate an immune response. IL-1β is regulated by the NFκB inflammatory pathway, containing several regulatory kB binding sites upstream of the gene (12). To test for a cooperative effect involving ELMO1, we transfected HEK293 cells with plasmids encoding FLAG-ELMO1, HA-NOD1/2, and the κB induced reporter plasmid firefly luciferase. Cells were then left uninfected or infected with *Salmonella*. The luciferase assay revealed cells transfected to express ELMO1 alone had a 2-fold increase of NFκB activity compared to infected control (Figure 5). A significantly increased NFκB response was detected in transfected cells expressing NOD1 or NOD2, which increased further in response to infection (Figure 5). The NFκB activity levels of the ELMO1-NOD1 co-transfected cells were comparable to that of the NOD1 transfected cells, revealing no significant additive effects. In contrast, the ELMO1-NOD2 co-transfected cells revealed a 40% increase of NFκB activity compared to cells transfected with NOD2 alone, suggesting an additive effect (Figure 5).

*ELMO1 Does Not Mediate IL-1β via Caspase 1.*

The processing of IL-1β to its mature state can be catalyzed by caspase 1 (10). To investigate whether ELMO1 changes IL-1β expression by post-translation modification via caspase 1, we infected control and ELMO1-shRNA J774
macrophages with *Salmonella* for various time points. Cell lysates were separated via SDS-PAGE and immunoblotted using a caspase 1 antibody. The results revealed no major change in the conversion of pro-caspase 1 to active caspase 1 between our ELMO1 silenced cells and control, suggesting the effects of ELMO1 on IL-1β were independent of caspase 1.

*ELMO1 and IL-1β are Upregulated in Active Intestinal Inflammation in Humans*

Our data thus far revealed an upregulation of host defense genes after infection. In order to determine if there was any relevance to humans, we assayed for the RNA encoding ELMO1-related inflammatory molecules in human intestinal tissues. Since *Salmonella*-infected human tissues are not readily available, we compared gene expression in colonic tissue from subjects with or without chronic inflammation (colitis associated with Crohn’s disease). RT-PCR analysis showed the levels of TNFα, MCP1, ELMO1, and IL-1β all increased in patients with active disease and thus, mimicking the *in vitro* observations.
Figure 2: Intestinal Macrophages exhibit ELMO1 and BAI1, but no detectable TLR4. Intestinal macrophages from wild type mice were enriched with CD11b magnetic beads and isolated via magnetic column separation. J774 macrophage cell line was used as a positive control. A) FACS using F4/80 staining was used to determine the macrophage percentage of our isolated cell sample (left) as compared to our J774 cell line control (right). B) Gene expression levels from positive control cell line J774 (left) were compared to those of isolated CD11b-positive intestinal macrophages (right). Expression was determined using Real Time RT-PCR. Data is representative of at least 3 independent experiments.
Figure 3: ELMO1 Silencing Decreases IL-1β Activation After Infection. Non-specific shRNA-treated J774 macrophages (control) and ELMO1-shRNA treated J774 macrophages (ELMO1) were infected with Salmonella enterica Serovar typhimurium at a MOI of 10 for the indicated time points. A) Equal amounts of lysate (50 µg) were loaded onto SDS-PAGE and immunoblotted using an IL-1β antibody. The same blot was stripped and blotted with a β-Actin antibody. The blots were used for the densitometric analysis and the ratio of active to pro form of IL-1β was detected and mentioned. The blot shown is the representative of 3 individual experiments. B) Supernatants collected from the previous experiments were used to quantify secreted IL-1β by ELISA (R&D). Data is representative of 3 independent experiments and * indicates p values below 0.05.
Figure 4: FLAG-ELMO1 Interacts with HA-NOD1/2 Expressed in HEK293 cells. HEK293 cells transfected with FLAG-ELMO1 and HA-NOD1 or FLAG-ELMO1 and HA-NOD2. Portions of collected cell lysate were incubated with anti-FLAG beads for immunopulldown. Samples were run on SDS-PAGE and probed using the indicated antibody.
Figure 5: ELMO1 and NOD2 Synergize to Upregulates NFκB Activation after Infection. HEK 293 cells were transfected with vector control, ELMO1, NOD1, and NOD2 along with pNifty luciferase reporter plasmid containing an NFκB inducible promoter. Following transfection, HEK 293 cells were either uninfected (Un) or infected with *Salmonella* (SL) for 5 hours. Cell lysate was prepared using passive lysis buffer (Promega) and firefly luciferase was measured by luminescence. Measurements were normalized using equal protein concentrations. Data shown is representative of 3 individual experiments.
Figure 6: ELMO1 Silencing has no Major Effect on Caspase 1 Expression. Control and ELMO1-shRNA J774 macrophages were cultured and infected with Salmonella for the noted time points. Cell lysates were collected and equal protein amounts were run on SDS-PAGE. Immunoblotting was performed using a caspase 1 antibody (Santa Cruz), and the same blot was stripped and re-blotted with a β-Actin antibody (Cell Signaling). Densitometry comparing active caspase 1 to β-Actin is shown and representative of 3 independent experiments.
Figure 7: Pro-Inflammatory Gene Levels are Increased in Patients with Active Intestinal Inflammation. In collaboration with UCSD-IBD Center, RNA was isolated from the intestinal biopsy specimens (4-6 per group) from the healthy and Crohn’s Disease patients. Real Time RT PCR was done with the specific genes and 18S rRNA was used as an internal control. Values represent mean ± SEM and are significant with a p value of <0.05.
IV.

Discussion
According to the CDC, millions of people worldwide suffer from some form of chronic inflammation. Inflammatory diseases of the intestine caused by pathogenic bacteria significantly reduce the quality of life of an individual, and can be deadly for those living in economically disadvantaged communities where healthcare may not be readily available. With the increased emergence of antibiotic-resistant bacteria, the need to identify a broad inflammatory regulator that can be targeted for novel therapeutic treatments is critical.

In this thesis, I investigated the role of ELMO1, a cytosolic protein, on mediating pro-inflammatory responses after pathogen engulfment. To do this, we measured the levels of IL-1β in the cytosol and supernatant of J774 macrophages expressing control or ELMO1 shRNA. The key finding of my investigation was requirement for ELMO1 in the production of active IL-1β. This conclusion is based on the observation that diminishing ELMO1 expression using shRNA significantly reduced the production of IL-1β as detected by western blot analysis and ELISA. In a previous study, Das et al demonstrated a decrease of pro-inflammatory cytokines, namely MCP1 and TNFα, secreted from ELMO1-deficient macrophages (6). This report is consistent with our findings, in that downregulation of ELMO1 leads to a decrease of pro-inflammatory cytokines, in our case IL-1β.

MCP1, TNFα, and IL-1β all stimulate unique aspects of inflammation (19, 20). Additionally, the ligands, proteins, and pathways leading to the production and
secretion of each of these cytokines are unique (19, 20). To gain further evidence in support of our BAI1-ELMO1-mediated inflammatory response pathway, we quantified the levels of TLR4, BAI1, and ELMO1 mRNA from our control J774 cell line and isolated intestinal macrophages via quantitative RT-PCR. Interestingly, the levels of TLR4, the only other known macrophage receptor capable of recognizing the LPS coat of gram-negative bacteria, were undetectable in our intestinal macrophage samples. A search of published literature revealed similar findings, although the reasoning for this has yet to be elucidated (21, 22). One possible explanation is that the absence of TLR4 would limit the possibility of excessive inflammation stimulated by the vast amount of LPS produced by the adjacent gram-negative microbes. In contrast, BAI1 and ELMO1 were readily detected in our intestinal macrophage sample, suggestive of a TLR4 independent, BAI1-ELMO1 dependent inflammatory response.

Our observations, along with those collected previously, suggest that ELMO1 mediates the production of various pro-inflammatory cytokines. However, no investigation has revealed ELMO1 as a direct activator of immune pathways. Therefore, we hypothesized ELMO1 may interact with cytosolic sensor proteins that initiate immune cascades, possibly by recruiting them to the site of engulfment in order to increase detection efficiency. We initially focused our search for ELMO1 interacting proteins by searching the literature for sensors known to interact with the ELMO1-mediated engulfment machinery. NOD1 and NOD2, cytosolic proteins known to sense both bacterial peptidoglycan components and the activation state of Rac1, emerged as potential targets for investigation. Our immunopulldown
revealed an ELMO1-NOD1/2 interaction in uninfected cells, shifting our focus to the downstream effects of this interaction.

Once activated, NOD1/2 initiate the NFκB pathway, resulting in the production and secretion of κB transcription factor dependent cytokines, which include IL-1β (23). To investigate the direct effect of the ELMO1-NOD1/2 finding on NFκB activity, and indirectly on IL-1β, we transfected a luciferase reporter plasmid containing a κB inducible promoter, along with ELMO1 and NOD1/2, into HEK cells. Our luciferase assay showed an increased additive effect in ELMO1-NOD2 transfected cells, with the highest NFκB activity detected in Salmonella infected conditions. This finding inversely correlates with our observed decrease of active IL-1β in ELMO1-deficient cells, suggesting ELMO1 increases NOD2-mediated activation of the NFκB pathway. Of note, we did not measure an NFκB activity increase of the same magnitude in the ELMO1-NOD1 condition as we did in the ELMO1-NOD2 treatment. A major distinction between NOD1 and NOD2 is the extra caspase activation and recruitment domain (CARD) expressed by NOD2, and thus might be the cause for an increased response (16).

Caspase 1 is a known post-translational modifier of IL-1β, cleaving the inactive pro form into the active form (10). Activation of caspase 1 has been shown to be upregulated after NOD1 stimulation (15). With the observation of decreased active IL-1β in ELMO1-deficient macrophages, our inability to reproduce a western blot in which caspase 1 levels also decreased was initially surprising. Instead, the levels of active caspase 1 in Salmonella infected, ELMO1-deficient macrophages,
did not substantially deviate from that of our control shRNA cells. Once more, a possible explanation to this effect would be ELMO1-deficient macrophages have impaired sensing of engulfed bacteria, leading to a smaller synthesis of IL-1β, in turn suggesting ELMO1 has its greatest influence on the events leading up to transcription of pro-inflammatory genes.

To gain insight into the in vivo involvement of ELMO1 in a human intestinal inflammation model, we obtained mucosal biopsy specimens from the colon of subjects with active Crohn’s disease and healthy controls (subjects in remission). Crohn’s disease is believed to be a result of excessive inflammatory responses to luminal microbes in genetically susceptible individuals. Since we are interested in elucidating the involvement of ELMO1 in immune signaling after bacterial engulfment in the intestine, we thought it appropriate to extend our investigation to this disease. Quantitative RT-PCR analysis of the active Crohn’s disease patients showed a substantial increase of all the previously in vitro tested proteins when compared to patients in remission. Although this disease is different than the changes induced by Salmonella, these results suggest that the molecules are present in both types of conditions, and thus, may reflect a common role in regulating host responses to microbes.

For future experiments, utilizing an IL-1β promoter in a similar luciferase assay would yield a more definitive result as to the effect of ELMO1 and NOD1/2 on IL-1β production. Work by other groups has implied that proteins such as cathepsin B and caspase 8 may influence IL-1β activity levels (24). Therefore,
investigation of these proteins and how they interact with ELMO1 may influence IL-1β levels.

Taken together, our results indicate a role for ELMO1 in the sensing of bacteria and the subsequent initiation of the immune response, possibly through physical interaction or crosstalk with the cytosolic sensor proteins NOD1/2. The interaction of ELMO1 with these proteins may be a target for the control of chronic inflammation and disease arising from this response. An increased understanding of the ELMO1-mediated immune response is critical for the identification of new therapeutic targets in patients suffering from inflammatory disorders, particularly those in which microbes may initiate or perpetuate disease.
V.
References


