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ELMO1 Regulates Autophagy Induction and Bacterial Clearance During Enteric Infection

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Macrophages are specialized phagocytic cells involved in clearing invading pathogens. Previously we reported that engulfment and cell motility protein 1 (ELMO1) in macrophages mediates bacterial internalization and intestinal inflammation. Here we studied the role of ELMO1 in the fate of internalized targets. ELMO1 is present in the intracellular vesicles and enhances accumulation of the protein LC3B following engulfment of *Salmonella* or treatment with autophagy-inducing rapamycin. The protein ATG5 and the kinase ULK1 are involved in classical autophagy, while LC3-associated phagocytosis is ULK1 independent. ATG5 but not ULK1 cooperated with ELMO1 in LC3 accumulation after infection, suggesting the ELMO1 preferentially regulated LC3-associated phagocytosis. Because LC3-associated phagocytosis delivers cargo for degradation, the contribution of ELMO1 to the lysosome degradation pathways was evaluated by studying pH and cathepsin B activity. ELMO1-depleted macrophages showed a time-dependent increase in pH and a decrease in cathepsin B activity associated with bacterial survival. Together, ELMO1 regulates LC3B accumulation and antimicrobial responses involved in the clearance of enteric pathogens. This paper investigated how innate immune pathways involving ELMO1 work in a coordinated fashion to eliminate bacterial threats. ELMO1 is present in the phagosome and enhances bacterial clearance by differential regulation of lysosomal acidification and enzymatic activity.

Keywords. Engulfment; autophagy; *Salmonella*; cathepsin; bacterial clearance.

Food-borne infections are a common health problem globally where enteric bacteria *Salmonella* play an important role. Most people clear *Salmonella*, but those with compromised immunity can experience reoccurring bouts of infection and become chronic carriers [1, 2]. *Salmonella* enter epithelial cells by bacteria-mediated invasion pathway by using the genes encoding *Salmonella* pathogenicity island 1 (SPI-1) and subsequently reach the lamina propria, where they encounter phagocytes [3, 4]. The effectors from SPI-2 genes are responsible for survival of bacteria inside macrophages. Once engulfed, *Salmonella* reside in a protected *Salmonella*-containing vacuole by modulating the pH and preventing fusion with host cell endosomes and activity of lysosomes, to avoid clearance [5, 6].

Pattern-recognition receptors are the initial line of defense against *Salmonella*, binding microbial products or pathogen-associated molecular patterns and activating both innate and adaptive immune responses. Pattern-recognition receptors bind microbial ligands irrespective of their pathogenicity; for example, Toll-like receptor 4 (TLR4) binds lipopolysaccharide from pathogens or commensals. Previously, we reported that the inhibitor BAI1 binds lipopolysaccharide and that it is unique from TLR4 because BAI1 binds core oligosaccharide of lipopolysaccharide directly [7]. The cytosolic region of BAI1 interacts with ELMO1, which, in turn, activates Dock180, and together they act as a guanine nucleotide exchange factor for Rac1 [7, 8]. Recently, we showed that ELMO1 is particularly important in the intestine, where it plays an important role in regulating intestinal inflammation [9, 10]. While phagocytes control the outcome of bacterial infection, it is not entirely clear how they regulate bacterial clearance following their engulfment.

Autophagy is one of the mechanisms whereby host cells degrade invading bacteria [11]. Interestingly, there is evidence that the autophagy pathway has evolved the ability to degrade invading pathogens that have escaped into the cytosol of a cell [12]. The protein LC3 is a surrogate marker for autophagy, and the active, lower-molecular-weight form of LC3, LC3B-II, is a marker of autophagy. Conventional autophagy is characterized by the formation of a double-membrane vacuole, the autophagosome, which is embedded with LC3B-II [13]. In contrast, LC3-associated phagocytosis is a noncanonical form of autophagy in which pathogens are engulfed into single-membrane vacuoles that become embedded with LC3B-II and degrade bacteria at a faster rate than conventional autophagy [13]. LC3-associated phagocytosis shares proteins associated with conventional autophagy, such as the protein ATG5, to recruit LC3B-II to the membrane; however, LC3-associated phagocytosis is independent of the kinase ULK1 [14]. Pattern-recognition receptors can activate

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either LC3-associated phagocytosis or autophagy pathways [14–16], so we checked the effect of ELMO1 in both of the pathways.

Using *Salmonella* as a model, we studied the role of ELMO1 in bacterial clearance and found impairment in the absence of ELMO1. Moreover, ELMO1 is present in isolated phagosomes after engulfment and regulates LC3B accumulation in response to infection or rapamycin. ATG5 but not ULK1 enhances this response, thereby implicating ELMO1 in the control of LC3-associated phagocytosis. Following *Salmonella* infection, ELMO1 differentially regulates the acidification of phagolysosomes and the hydrolytic enzymatic activity of lysosomal enzymes in a time-dependent manner. The reduced cathepsin B activity correlates with the delayed clearance of *Salmonella* in ELMO1-depleted macrophages. Taken together, our results support the notion that, subsequent to engulfment, ELMO1 regulates lysosomal signaling that enhances bacterial clearance.

METHODS

Descriptions of methods not specified below are available in the Supplementary Materials.

Cell Lines and Bacterial Culture

The murine macrophage cell line J774 (American Type Culture Collection, Manassas, VA) and J774 cells with stable suppression of ELMO1 were used as phagocytes [9]. Cells were maintained in high-glucose-containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum [9]. *Salmonella enterica* serovar Typhimurium (SL1344) was used for infections. *Salmonella* from the LB agar plate was inoculated into LB broth and cultured for 6–8 hours as described previously [7, 9, 17].

Maintenance of Mice and Animal Infection

ELMO1-knockout mice were generated [9, 18] and bred at the University of California–San Diego by mating heterozygotic breeders to yield offspring with shared exposure to the environmental microbiota. The Institutional Animal Care and Use Committee at the University of California–San Diego approved all animal experiments with wild-type and ELMO1-knockout mice.

Antibodies

Rabbit LC3B monoclonal antibody (1:1000; Cell Signaling Technologies) and rabbit LC3B polyclonal antibody (1:200; Novus Biologicals) were used to detect LC3B either by Western blotting and immunofluorescence, respectively. Cathepsin B (1:1000; Cell Signaling Technologies) was used for Western blotting.

Western Blotting

Both cells and phagosomes were lysed in ice-cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails (Sigma). Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked using 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1 hour at room temperature. Membranes were then incubated with respective primary antibody in 5% bovine serum albumin containing TBST overnight at 4ºC.

Preparation of Phagosomal Fractions

Phagosomal fractions were prepared from adherent macrophages in accordance with a previously published protocol for magnetic separation [19], with minor modifications as mentioned in details in the Supplementary Materials.

Downregulation of ATG5 and ULK1

ATG5 and ULK1 expression was suppressed using mouse ATG5 and ULK1 small-interfering RNA (siRNA; On-Target Plus siRNA), respectively, and as a control siRNA we used On-Target Plus Non-Targeting Pool (Dharmacon, GE Life Sciences). Control and ELMO1 small-hairpin RNA (shRNA) J774 cells were plated on 6-well plates and transfected with 80 nM siRNA (control siRNA, ATG5 siRNA, or ULK1 siRNA) in 2 mL of OptiMEM medium, using lipofectamine RNAiMAX transfection reagent (Thermo-Fisher Scientific) according to the supplier's protocol.

Bacterial Clearance by Plating Assay

A total of 5×10^5 cells were seeded with control and ELMO1 shRNA cells in 24-well plates. Cells were infected with *Salmonella* at a multiplicity of infection of 10 for 30 minutes. Extracellular bacteria were killed with gentamicin (500 µg/mL) for 90 minutes at 37°C, followed by low-dose gentamicin (50 µg/mL) for the rest of the experiment. At indicated times, cells in each well were washed with phosphate-buffered saline (PBS) and lysed in 1% Triton X-100 in PBS for 15 minutes at 37°C, followed by serial dilution and plating onto LB agar plates. The number of colony-forming units (a measure of bacterial clearance and survival) was normalized at each time point to the number of colony-forming units at bacterial entry at 30 minutes and was used to plot the graph for bacterial clearance.

Measurement of Cathepsin B Activity

J774 macrophages (either control or ELMO1-depleted) were plated on the 96-well plate with a cell density of 10^5 cells/well. After infection, cells were washed with PBS. Lysates were tested for enzymatic activity, using the cathepsin B–specific (Bachem) fluorogenic substrate Z-Arg-Arg-AMC (which was dissolved in dimethyl sulfoxide to generate a 40-mM stock). Cells were lysed with lysis buffer (100 mM sodium acetate [pH 5], 1 mM ethylenediaminetetraacetic acid [EDTA], 4 mM DTT, and 0.5% Triton X), and equal volumes of lysates were mixed with reaction buffer (100 mM sodium acetate [pH 5], 1 mM EDTA, 4 mM DTT, and 100 µM Z-Arg-Arg-AMC) in a white 96-well flat-bottomed reaction plate. The fluorescence intensity was measured in a plate reader, with an excitation of 320, an emission of 460, and a cutoff of 420.

Statistical Analysis

Results are expressed as mean values ± standard deviations. Results were compared using a 2-tailed Student *t* test and were considered significant if *P* values were <.05. The statistical analysis was done using Excel or GraphPad biostatistics packages.

RESULTS

ELMO1 Is Involved in Bacterial Clearance and Is Present in the Phagosome

Previously, we have shown that ELMO1 increases bacterial internalization following infection in ELMO1-depleted macrophages [9]. Here we assayed bacterial survival, starting 3 hours after infection, in control and ELMO1-depleted shRNA cells (J774) (Figure 1A and Supplementary Figure 1*A*). Despite the attenuated internalization of *Salmonella* in ELMO1-depleted cells at 3 hours, the bacterial count was higher in ELMO1 shRNA cells, starting at 6 hours (Supplementary Figure 1*A*). At 12 hours, a significantly higher number of bacteria was present in ELMO1-depleted cells, which similar findings at 24 hours, indicating delayed clearance of bacteria by ELMO1-depleted macrophages (Figure 1A).

After engulfment of a target, the phagosomes mature from early to late endosomes. Before performing functional assays, the presence of ELMO1 in phagosomes was tested. The phagosomes were isolated from control and ELMO1 shRNA cells. ELMO1 was detected at 75 kD in cytosolic and phagosomal fractions by Western blot (Figure 1B). ELMO1-depleted shRNA cells were used as a negative control.

ELMO1 Induces Autophagy by Accumulating LC3B

After engulfing the target, phagocytes induce autophagy to clear the target/pathogen [14, 20]. Phagocytosis/endocytosis and autophagy are closely related events and share a number of common interacting protein partners [21, 22]. To understand the role of ELMO1-mediated engulfment in autophagy induction, control and ELMO1 shRNA cells were infected with *Salmonella* for 1 hour and 3 hours (Figure 2A). The level of LC3B-II in ELMO1-depleted cells was less than that in control cells (Figure 2A). The densitometric analysis from 3 independent experiments confirmed the significant 2-fold reduction of the LC3B-II level in ELMO1 shRNA cells (Figure 2B). Control and ELMO1 shRNA cells were treated with various concentrations of rapamycin, an mTOR inhibitor and autophagy inducer, for 2 hours (Figure 2C). Densitometric analysis revealed a significant, 3-fold reduction in the LC3B-II level at a 200-nM rapamycin concentration (Figure 2D). This reduction started at 2 hours and was maintained up to 20 hours, indicating that ELMO1-mediated autophagy induction by rapamycin is dose and time dependent (Supplementary Figure 1*B*). The presence of LC3B-mediated puncta was detected by confocal microscopy (Figure 2E), and the percentage of ELMO1 shRNA cells with puncta was decreased after rapamycin treatment (Figure 2F). The nonphagocytic cells, HEK-293 cells, were transfected with either full-length BAI1 or ELMO1 and treated with rapamycin to assess LC3B accumulation. Enhanced accumulation of LC3B was observed after ELMO1 overexpression, as well as in BAI1-overexpressing cells (Supplementary Figure 1*C*).

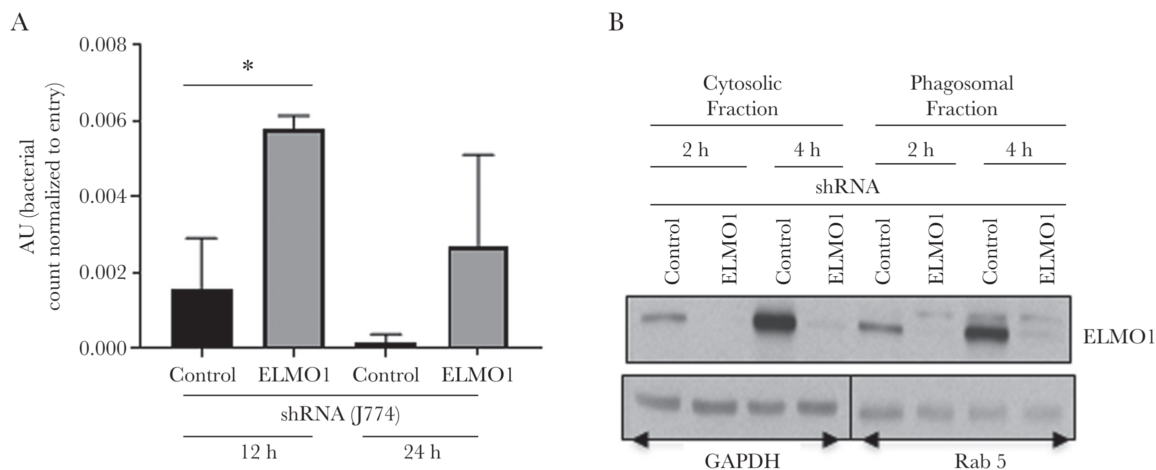

Figure 1. ELMO1 regulates bacterial clearance and is present in the phagosomal fraction. *A*, The delayed clearance of *Salmonella* in ELMO1-depleted macrophages was evaluated after infection of control and ELMO1 small-hairpin RNA (shRNA) J774 cells with *Salmonella* enterica serovar Typhimurium (SL1344) at a multiplicity of infection of 10 for 30 minutes. Extracellular bacteria were killed by treatment with gentamicin. At 12 and 24 hours, cells were lysed using 1% Triton X-100 in phosphate-buffered saline, serially diluted, and plated for counting of colony-forming units. The graph was plotted for bacterial clearance and survival at the indicated times. Data at each time point were normalized to the number of colony-forming units at bacterial entry. Data are from 3 independent experiments. **P* ≤ .05. AU, arbitrary units. *B*, Control and ELMO1 shRNA cells were incubated with polystyrene magnetic beads for 2 and 4 hours. Phagosomal extracts were prepared using a magnetic separator and were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was performed for ELMO1 for both cytosolic and phagosomal fractions. For equal loading, GAPDH (cytosolic fraction) and Rab 5 (phagosomal fraction) were used. The blot is representative of 3 independent experiments.

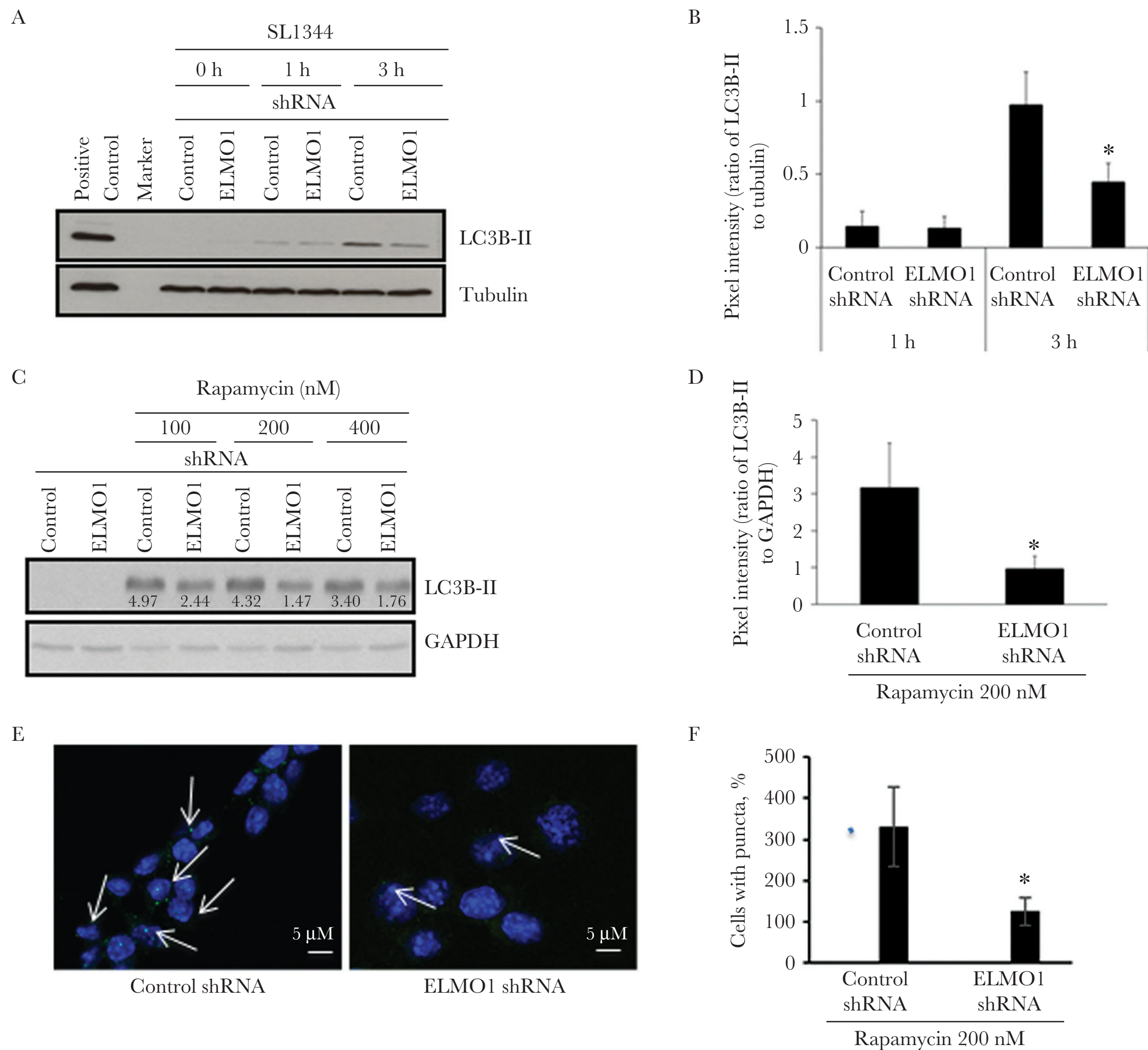

Figure 2. ELMO1 regulates autophagy induction by LC3. *A*, Control and ELMO1 small-hairpin RNA (shRNA) J774 cells were infected with *Salmonella* for the indicated time and immunoblotted with LC3B antibody. HeLa cells treated with 50 µM chloroquine was used as a positive control. The same blot was stripped and reprobed with α-tubulin antibody to confirm equal loading. The ratio of LC3B-II to α-tubulin was determined to measure the amount of LC3B-II accumulation. *B*, Representative blots after densitometric analysis from 3 independent experiments are shown. $^*P \leq .05$. *C*, Control and ELMO1 shRNA (J774) cells were treated with different concentrations (100, 200, and 400 nM) of rapamycin for 2 hours. Whole-cell lysates were prepared and immunoblotted with LC3B antibody. Equal loading was confirmed by reprobing the blot with GAPDH antibody. *D*, Densitometric analysis from 3 independent experiments, showing significant differences between control and ELMO1 shRNA groups. $^*P \leq .05$. *E*, Control and ELMO1 shRNA cells were treated with rapamycin (200 nM) for 2 hours, fixed, and stained with anti-LC3 antibody and then with Alexa Fluor 488 secondary antibody. Cells were counterstained with Hoechst (blue), and fluorescence intensity was measured by confocal microscopy. The LC3B puncta was marked with white arrows. *F*, The number of puncta and the number of cells were counted in 5–7 different fields, and the percentage of cells with puncta formation was plotted from 3 independent experiments. $^*P \leq .05$.

To assess autophagy induction via the accumulation of autophagic vacuoles, both control and ELMO1 shRNA cells were incubated with monodansylcadaverine, a specific autophagolysosomal marker that fluoresces following interactions with membrane lipids [23, 24]. The lowered fluorescence in ELMO1 shRNA cells suggested ELMO1-dependent autophagy induction and autophagosome formation (Supplementary Figure 1*D*).

ELMO1 Is Involved in LC3-Associated Phagocytosis

After engulfment of a pathogen, phagocytes clear the target by either conventional autophagy or LC3-associated phagocytosis. Because ULK1 and ATG5 are required for LC3B-II accumulation in autophagy [14, 21, 25], we checked the involvement of ELMO1 in these pathways, using control and ELMO1 shRNA cells infected with *Salmonella*, after downregulation of the autophagy proteins ATG5 and ULK1. To identify which pathway ELMO1 might be involved in, macrophages were infected with *Salmonella*, following ATG5 or ULK1 knockdown. As expected, the accumulation of LC3B-II was lowered in ELMO1-depleted macrophages but not in the control macrophages when transfected with control siRNA (Figure 3A). The densitometric analysis showed that, in the

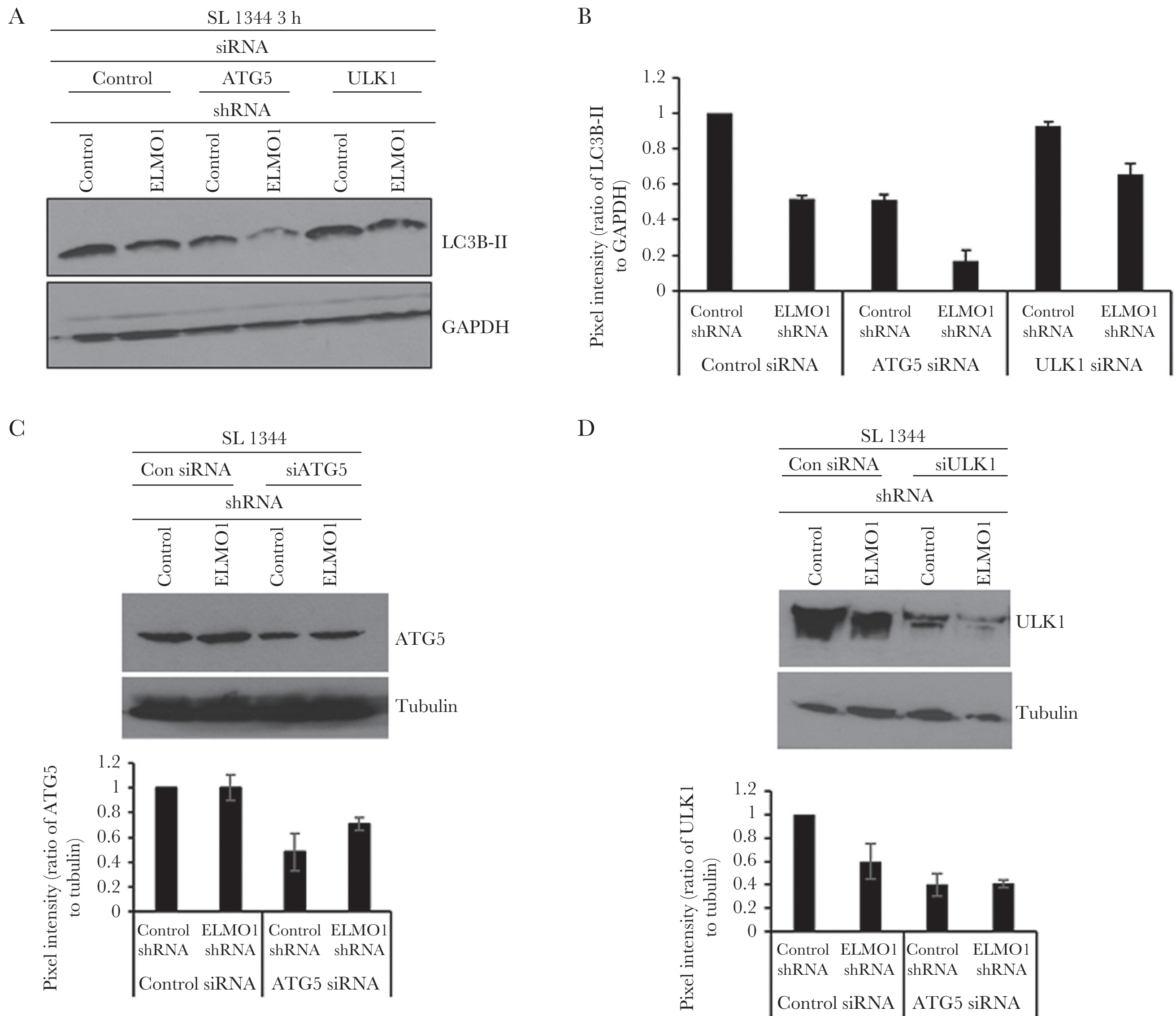

Figure 3. ATG5-dependent and ULK1-independent accumulation of LC3B. *A*, Control and ELMO1 small-hairpin RNA (shRNA) J774 cells were transfected with control, ATG5, or ULK1 small-interfering RNA (siRNA) and infected with *Salmonella*. The top panel shows probing for LC3B-II, and the bottom panel shows GAPDH, used as the loading control. *B*, Densitometric analysis of LC3B-II from 3 independent experiments. *C* and *D*, Downregulation of ATG5 and ULK1 was demonstrated by Western blot.

ATG5-knockdown macrophages, there was downregulation of LC3B-II in both control and ELMO1-depleted macrophages, but the downregulation was compounded in ELMO1-depleted macrophages (Figure 3B). The ULK1-knockdown macrophages had LC3B-II levels comparable to those in the control macrophages (Figure 3A and 3B), indicating that early LC3 accumulation triggered by *Salmonella* infection is independent of ULK1. The level of downregulation of ATG5 and ULK1 was confirmed by Western blot (Figure 3C and 3D). Interestingly, LC3-associated phagocytosis recruits LC3B-II to the phagosome independent of ULK1 [14]. The ULK1-independent LC3B-II generation following ELMO1-mediated *Salmonella* engulfment indicates the involvement of LC3-associated phagocytosis.

ELMO1 Regulates Proteolysis in Phagocytes

After target engulfment, phagosomes undergo sequential fusion with early and late endosomes [26, 27]. Phagosomal acidification is important for the activity of many lysosomal hydrolytic enzymes. ELMO1-regulated proteolytic activity was tested in control and ELMO1 shRNA cells following infection with *Salmonella* or nonfluorescence magnetic beads preloaded with DQ-BSA, a green boron-dipyrromethene dye (BODIPY) conjugated to bovine serum albumin. DQ-BSA is normally nonfluorescent, and upon proteolysis and subsequent dequenching it starts generating bright green fluorescence that can be assessed by confocal microscopy (Figure 4A), as well as by flow cytometry (Figure 4B). An increase in green fluorescence in ELMO1 shRNA cells as compared to

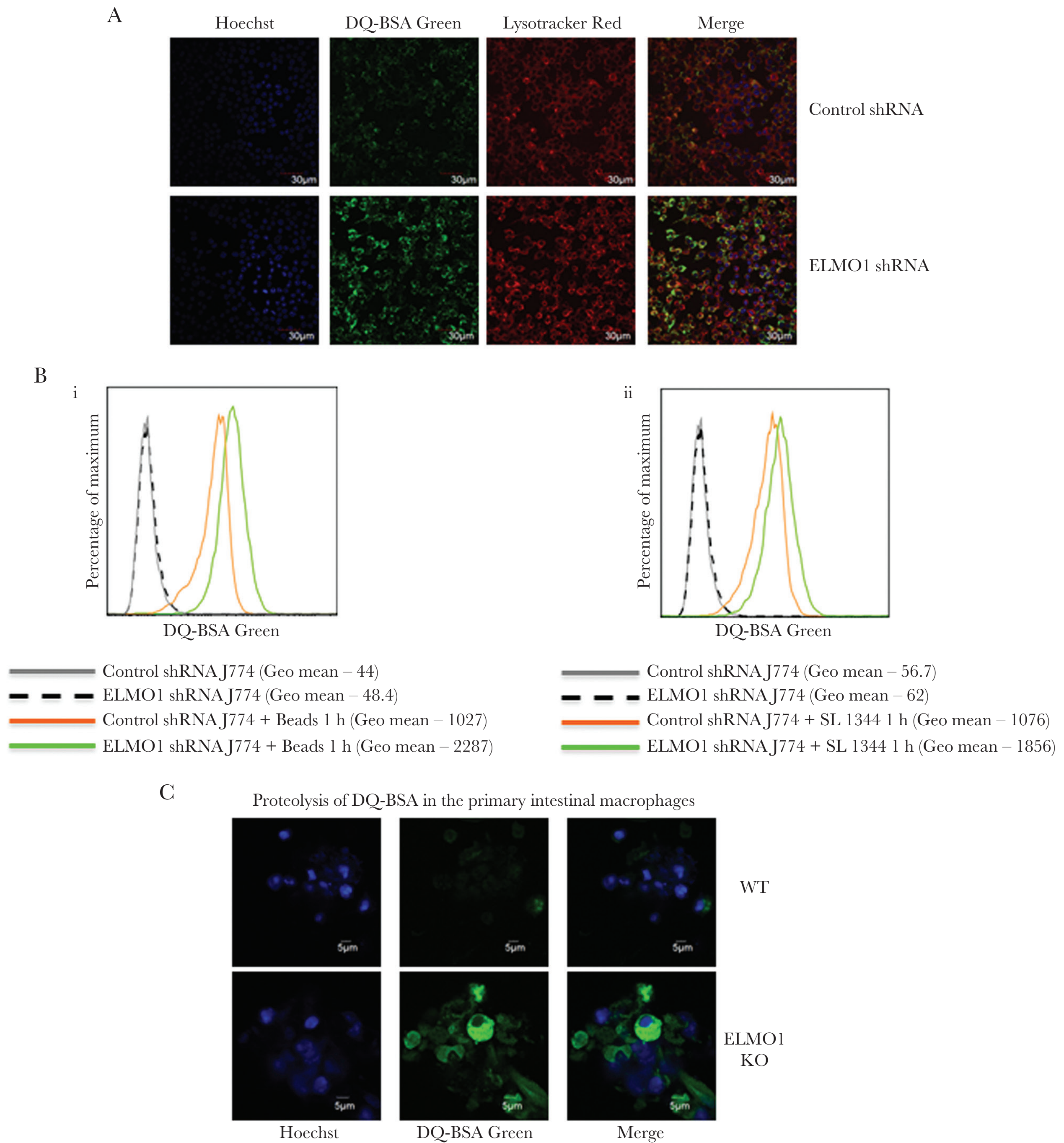

Figure 4. Inhibition of endosomal proteolysis by ELMO1 in murine macrophage cell line and in primary intestinal macrophages. *A*, Control and ELMO1 small-hairpin RNA (shRNA) cells were first preloaded with Lysotracker Red (1:1000) for 2 hours in complete medium, washed, and incubated with magnetic beads (in a 1:10 ratio) in the presence of DQ-BSA green (10 µg/mL) for 1 hour. Cells were washed, mounted without fixing, and analyzed by confocal microscopy. Nuclei were counterstained with Hoechst, shown in blue. *B*, Control and ELMO1 shRNA cells were treated either with polystyrene magnetic beads (1:10) or with *Salmonella* (SL1344) coincubated with DQ-BSA green (10 µg/mL) for 1 hour. Cells were then washed and immediately analyzed by flow cytometer without fixing. A representative histogram shows the intensities of DQ-BSA green and geometric means for each sample. *C*, Intestinal macrophages isolated from control and ELMO1-knockout mice were incubated with magnetic beads (in a 1:10 ratio) in the presence of DQ-BSA green (10 µg/mL) for 1 hour. Cells were washed, mounted without fixing, and analyzed by confocal microscopy. Nuclei were counterstained with Hoechst, shown in blue.

control cells were observed with either beads (Figure 4A and 4B) or *Salmonella* (Figure 4B), suggesting a slightly increased proteolytic capacity/environment due to impaired expression of ELMO1.

ELMO1-Mediated Proteolysis in Primary Intestinal Macrophages Isolated From the Murine Intestine

To confirm our in vitro findings, we extended our study in the in vivo ELMO1 knockout mouse model. We isolated intestinal

macrophages that mimic physiologically intestinal antigen-presenting cells and checked their purity by staining with the murine macrophage marker F4/80 (Supplementary Figure 1*E*). The murine macrophage cell line J774 was used as a positive control. These intestinal macrophages from control and ELMO1-knockout mice were incubated for 1 hour with polystyrene beads preloaded with DQ-BSA, and the fluorescence was checked by microscopy. An increase in green fluorescence in ELMO1-knockout mice was observed (Figure 4C), correlating with our in vivo findings.

Effect of Acidification on *Salmonella* Gene Expression

The acidic pH inside the phagocytes can induce expression of the gene encoding *Salmonella* SPI-2 [28–30]. The expression of several SPI-2 effectors was checked, and interestingly the expression of *sseA* and *sscB*, responsible for *Salmonella*-induced filament production [31], had changed in a time-dependent manner (Supplementary Figure 2). Six hours after *Salmonella* infection, both genes were significantly upregulated in control macrophages but not in ELMO1-depleted macrophages (Supplementary Figure 2).

Differential Regulation of Lysosomal Acidification and Cathepsin Activity by ELMO1 in Phagocytes

The nascent phagosome undergoes maturation by fusion and fission events with endosomes and lysosomes to generate an acidic, highly hydrolytic mature phagolysosome. We used LysoTracker, an acidophilic lysosomotropic dye that shows color only when the pH is acidic. We also measured the activity of cathepsin B, a lysosomal cysteine protease active at low pH [32]. LysoTracker Green–preloaded control and ELMO1 shRNA J774 cells were incubated with Magic Red cathepsin B after incubation with either polystyrene magnetic beads or *Salmonella* and examined by confocal microscopy. We found an increase in red fluorescence intensity for cathepsin B after 1 hour of target engulfment, as well as colocalization (yellow suggests colocalization of active cathepsin B with Lysotracker Green), in ELMO1 shRNA cells as compared to control cells, suggesting an increase in cathepsin B activity (Figure 5A and 5B).

Control and ELMO1 shRNA cells were tested for the endogenous level of cathepsin B (Figure 5C), with ELMO1-depleted cells having greater levels of cathepsin B at 1 and 3 hours after infection. At 6 hours, levels of cathepsin B were comparable in control and ELMO1 shRNA cells. Twelve hours after infection, the cathepsin B level was higher in control cells.

Because cathepsin B plays a significant role in bacterial clearance [32, 33], we tested the activity of cathepsin B (Figure 5D). *Salmonella* infection is unique in downregulating lysosomal degradation. We also found that, following infection, cathepsin B activity was decreased. Interestingly, ELMO1 shRNA cells showed higher cathepsin B activity, which was maintained up to 1 hour after infection. Three and 6 hours after infection, the activity of cathepsin B was comparable; after 12 hours, the activity was lower in ELMO1 shRNA cells, with significantly lower activity after 24 hours. The specificity of the assay was indicated with cathepsin B inhibitor (Figure 5D). Less accumulation of cathepsin B and the associated activity in ELMO1 shRNA cells later times after infection correlate with the bacterial clearance data in Figure 1A.

Intracellular *Salmonella* resides inside a modified phagosome, the *Salmonella*-containing vacuole [34]. These vacuoles share an endosomal marker whereby *Salmonella* regulates the degradation pathway to stay inside the niche. We tested the late endosomal marker, LAMP1, which is recruited to the vacuole to degrade bacteria, in control and ELMO1 shRNA cells (Figure 5E). The RGB analysis showed a dense distribution of LAMP1 surrounding the red fluorescent bacteria in control shRNA cells, compared with ELMO1 shRNA cells. A similar finding was was indicated by Lysotracker staining at 24 hours (Supplementary Figure 3), when ELMO1 shRNA cells had decreased acidity.

DISCUSSION

This study showed that the host-signaling molecule ELMO1 is involved in crosstalk with the bacterial degradation pathway and regulates endolysosomal signaling involved in the clearance pathway. We are the first to show that ELMO1 is present in the phagosomal fraction. Our observations (Figure 6) include several interesting features: (1) ELMO1 induces LC3B accumulation following infection; (2) ELMO1-depleted cells had higher acidification and proteolysis, followed by higher cathepsin B activity during early infection, possibly occurring via a compensatory pathway in phagocytes; and (3) during the late infection, ELMO1-depleted cells showed decreased cathepsin B activity and lower lysosomal acidification, correlating with reduced bacterial clearance.

ELMO1-dependent accumulation of LC3B was observed following *Salmonella* infection or treatment with rapamycin, suggesting that ELMO1-mediated autophagy induction is not only dependent on target engulfment. ELMO1 activates Rac GTPases, which is important for actin-mediated engulfment, migration, and other cellular processes [35–37]. It could be an important factor in autophagosome formation, because the actin cytoskeleton participates in the formation of autophagosomes during starvation-induced autophagy [38]. Moreover, ELMO1 is involved in LC3-associated phagocytosis, but it is possible that, upon engulfment, cargo could also be carried in vesicles, such as phagosomes and autophagosomes, or undergo LC3-associated phagocytosis, because all of these processes occur simultaneously during the clearance of pathogens.

It is unclear whether the induction of autophagy favors hosts or pathogens [20, 39]. Following uptake, internalized bacteria move from phagophores into the autophagosomes before undergoing lysosomal degradation. Autophagy can be an important

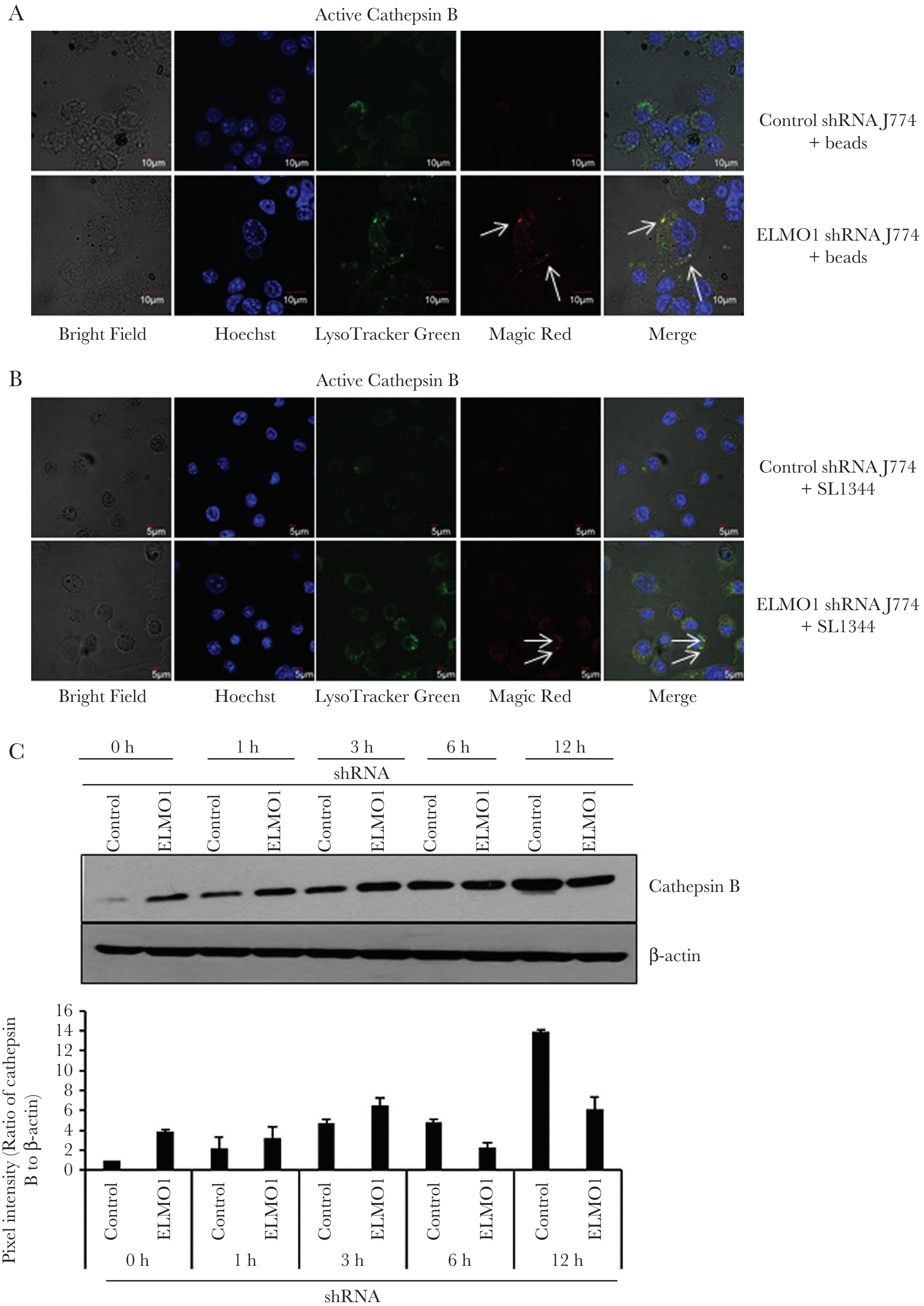

Figure 5. ELMO1 regulates cathepsin activity and phagosomal acidification. *A* and *B*, Control and ELMO1 small-hairpin RNA (shRNA) cells were first preloaded with Lysotracker Green (1:1000) for 2 hours. Cells were then washed and incubated with either beads (*A*) or *Salmonella* (*B*) for 1 hour, washed, and incubated with Magic red cathepsin B for 30 minutes (white arrows show sites with cathepsin B activity). Nuclei were counterstained with Hoechst, shown in blue. *C*, Control and ELMO1 shRNA cells were infected with *Salmonella* for the indicated times, were lysed, and underwent Western blotting for detection of endogenous cathepsin B. A representative figure from 3 independent experiments is shown, and findings of the densitometric analysis are plotted below the blot. *D*, Some of the control or ELMO1 shRNA cells used in panel *C* were plated in 96-well plates, infected, and lysed at the indicated times. The generation of free AMC from cleavage of the cathepsin B substrate Z-Arg-Arg-AMC was detected by fluorescence. The value from the fluorescence plate reader for each condition was normalized to the uninfected control shRNA value and plotted as arbitrary units (AU). Data are from 3 independent experiments. $^{*}P \leq .05$. Con, control. *E*, The distribution of the late lysosomal marker LAMP1 (green) was detected in control and ELMO1 shRNA cells after infection with *Salmonella* and labeling with *Salmonella* lipopolysaccharide (red). The line tracing the fluorescence images has been selected.

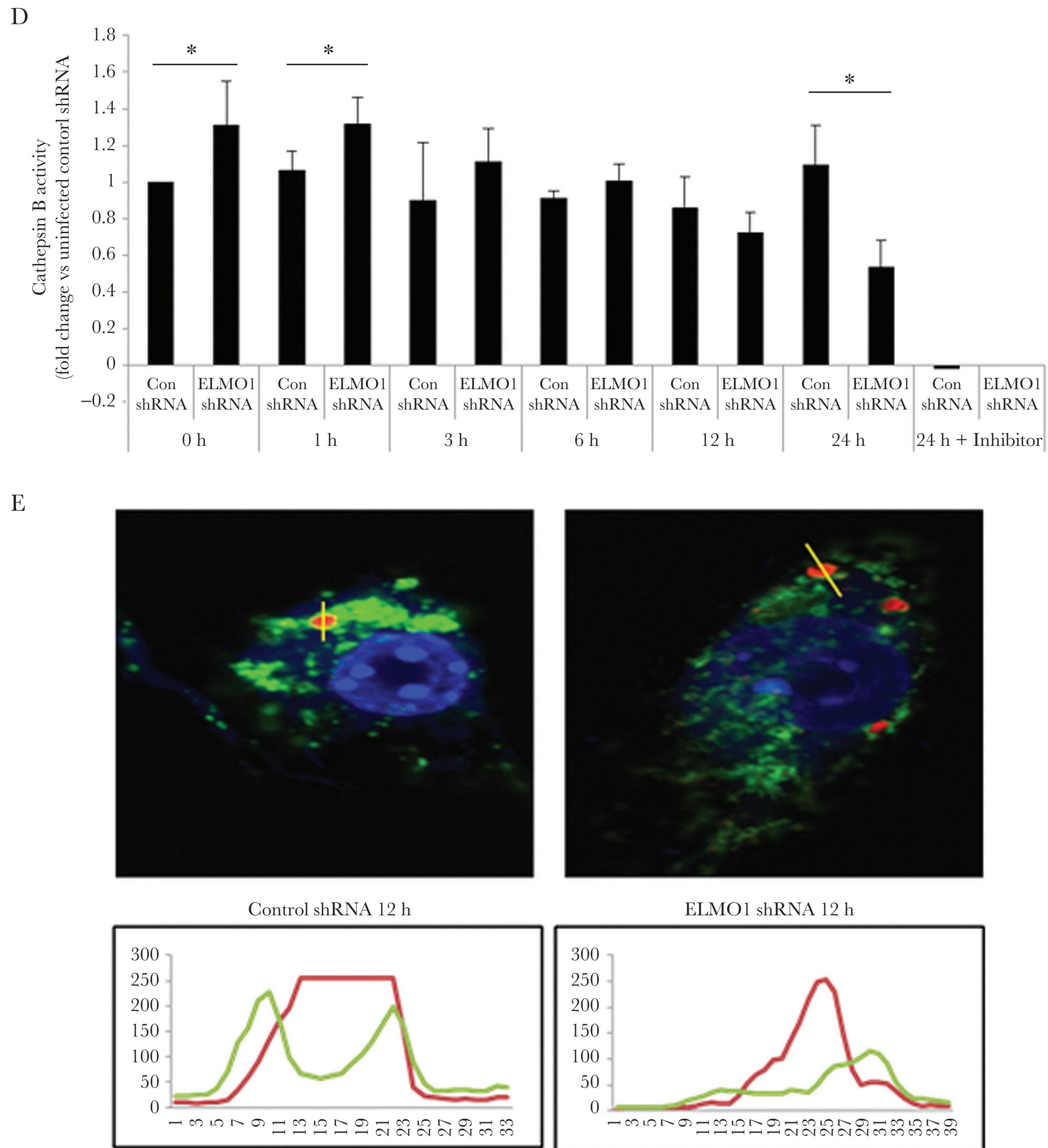

Figure 5. Continued

marker of infection and stimulate the innate immune response by targeting intracellular bacteria to the cytosol and controlling their growth when they reside in the phagosome [40]. *Salmonella* resides inside *Salmonella*-containing vesicles [34], which are unique in nature and modify the host response pathway to prevent acidification of and fusion with lysosomes and subsequent degradation. The downstream signaling in host-microbe interaction depends on the balance between host immune systems and microbes. Therefore, it is necessary to understand how enteric pathogens are cleared by phagocytes. This study delineates a new function of the cytosolic sensor ELMO1 after engulfment of enteric pathogens.

Autophagy is involved in both innate and adaptive immunity because it controls major histocompatibility complex class II antigen presentation. Bacterial sensors such as TLRs are well studied for their roles in recognizing microbes, autophagy induction, and initiating immune responses [41, 42]. However, very little is known about the mechanism by which they influence the phagosomal maturation/bacterial clearance. TLR4 plays an important role in bacterial pathogenesis, but there is controversy regarding the contribution of TLR4 to phagosomal maturation [43, 44]. It is possible that the BAI1-ELMO1 pathway functions as a standby option to TLR activities because TLR activities are compromised while maintaining gut homeostasis. The accumulation of LC3B

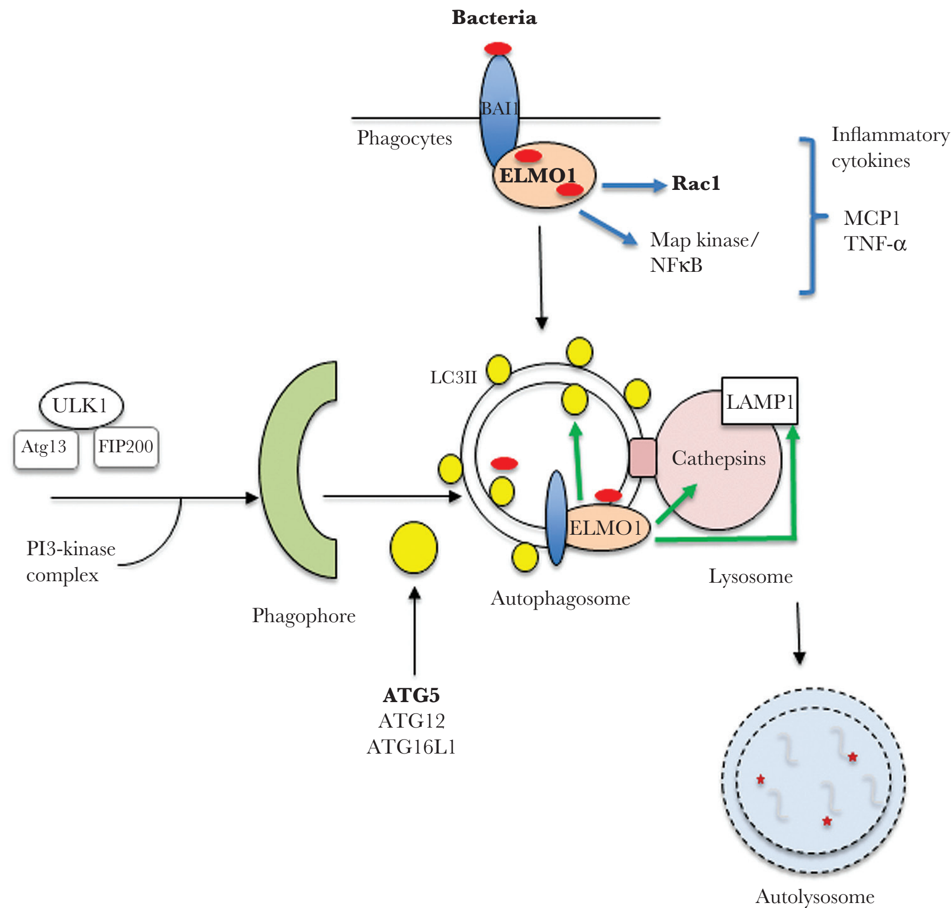

Figure 6. A simplified model showing the role of ELMO1 in the autophagy induction and endosomal pathway to degrade targets following engulfment. ELMO1 is a cytosolic protein that binds BAI1, the pattern-recognition receptor on bacterial lipopolysaccharide. Previous work (blue arrows) showed that ELMO1 internalizes bacteria, activates Rac1, and generates proinflammatory cytokines, using Map kinases and the nuclear factor κB (NF-κB) pathway [9]. Here, the role of ELMO1 in bacterial clearance and autophagy induction is established. Autophagy starts in the cytoplasm by formation of a double-membrane structure, the phagophore, where the ULK1 complex (ULK1/2, Atg13, and FIP200) initiates the induction of autophagy. The elongation and expansion steps in autophagosome formation involve several proteins, including Atg5. BAI1/ELMO1 is present in the phagosome following engulfment of the particle. The autophagosome sequesters LC3 and fuses with lysosomes to form an autolysosome containing lysosomal hydrolases (cathepsins) and LAMP1. The engulfed materials are degraded in the autolysosomes by the lysosomal enzymes. The effect of ELMO1 on autophagy induction, LAMP1 sequestration, and cathepsin B are shown by green arrows.

is partially regulated by BAI1 (Supplementary Figure 1*B*), and, of greater interest, ELMO1-mediated autophagy induction was markedly absent when BAI1 was mutated in a region where it interacts with ELMO1 (data not shown).

The higher acidification of ELMO1-depleted cells basally and during early infection possibly occurs through the compensatory effect of ELMO1 downregulation. We have previously observed that the absence of one phagocytic signaling molecule can be taken care of by another signaling partner. The outcome also depends on the balance between the host signaling partner and the target molecule [9]. Interestingly, following entry of the target, the signaling axis moved, and ELMO1-depleted cells had lower lysosomal activity, to protect the host, and exhibited delay in the clearance of engulfed bacteria. A recent report showed that the inhibition of cathepsin B decreased *Chlamydia muridarum* clearance in RAW macrophages [45]. This finding concurs with our data that there was less accumulation of cathepsin B and delayed *Salmonella* clearance following infection. It is also possible that bacterial effectors interact with ELMO1 and modulate the signaling, which needs further investigation. We also found that the endogenous cathepsin B level started to decrease in ELMO1 shRNA cells 6 hours after infection and was significantly lower at 12 hours, whereas cathepsin B enzymatic activity decreased in ELMO1 shRNA cells beginning at 12 hours and was more prominent at 24 hours. This is possible because the endogenous cathepsin B must undergo enzymatic modification to become activated [46].

The lysosomal degradation is extremely essential for pathogen elimination. However, in the course of coevolution, intracellular pathogens have evolved multiple mechanisms to overcome this lysosomal activity. The regulation of acidification, proteolysis, and enzymatic activity by ELMO1 is important to the function of this cytosolic sensor in host defense. Moreover, using an inhibitor of cathepsins, we noticed an increase in the number bacteria that survived (data not shown), indicating the

contribution of lysosomal enzymes to bacterial clearance. The mechanistic regulation of the lysosomal enzymes by ELMO1 needs detailed investigation in the future. Here we used murine macrophages, but future work involving human phagocytes is important to understand the mechanism that may be linked to inflammatory diseases. The regulation of lysosomal enzymes, followed by bacterial clearance by ELMO1, indicates a possible new aspect of host innate signaling that can be targeted in infections associated with antimicrobial resistance and inflammatory diseases.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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A. S., C. T., R. P., S. D., and P. B. E. read the literature and designed the experiments. A. S., C. T., R. P., and S. D. performed the experiments. A. S., C. T., R. P., S. D., and P. B. E. analyzed and interpreted the data. S. R., L. E., and T. S. contributed to reagents/materials/analysis tools and techniques. L. E., T. S., and P. B. E. provided suggestions to S. D. about the work and manuscript writing. S. D. wrote the manuscript and organized the project, with assistance from A. S. and P. B. E., and received helpful comments and edit from all authors. All authors had access to the study data and approved the final manuscript.

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