Autophagy Controls Salmonella Infection in Response to Damage to the Salmonella-containing Vacuole

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Salmonella enterica serovar Typhimurium (S. Typhimurium) is a facultative intracellular pathogen that causes disease in a variety of hosts. S. Typhimurium actively invade host cells and typically reside within a membrane-bound compartment called the Salmonella-containing vacuole (SCV). The bacteria modify the fate of the SCV using two independent type III secretion systems (TTSS). TTSS are known to damage eukaryotic cell membranes and S. Typhimurium has been suggested to damage the SCV using its Salmonella pathogenicity island (SPI)-1 encoded TTSS. Here we show that this damage gives rise to an intracellular bacterial population targeted by the autophagy system during in vitro infection. Approximately 20% of intracellular S. Typhimurium colocalized with the autophagy marker GFP-LC3 at 1 h postinfection. Autophagy of S. Typhimurium was dependent upon the SPI-1 TTSS and bacterial protein synthesis. Bacteria targeted by the autophagy system were often associated with ubiquitinated proteins, indicating their exposure to the cytosol. Surprisingly, these bacteria also colocalized with SCV markers. Autophagy-deficient (atg5−/−) cells were more permissive for intracellular growth by S. Typhimurium than normal cells, allowing increased bacterial growth in the cytosol. We propose a model in which the host autophagy system targets bacteria in SCVs damaged by the SPI-1 TTSS. This serves to retain intracellular S. Typhimurium within vacuoles early after infection to protect the cytosol from bacterial colonization. Our findings support a role for autophagy in innate immunity and demonstrate that Salmonella infection is a powerful model to study the autophagy process.

Salmonella enterica serovar Typhimurium (S. Typhimurium)§ is a Gram-negative bacterial pathogen that invades and multiplies within the cells of its host. It is responsible for a variety of host-specific diseases, including gastroenteritis in humans and a systemic disease resembling typhoid fever in permissive mouse models (1, 2). Inside host cells, S. Typhimurium replicates within a vacuolar compartment termed the Salmonella-containing vacuole (SCV). Maturation of this phagosome-like compartment is altered by the bacteria to create a niche favorable for replication (3–5). For example, delivery of NADPH oxidase and inducible nitric-oxide synthase to the SCV is blocked, along with fusion of the SCV with lysosomes (2, 6–10). Late stages of infection in vitro are characterized by the formation of tubular membranous extensions emanating from the SCV called Salmonella-induced filaments (Sifs) (11–13).

To establish an intracellular niche, S. Typhimurium delivers bacterial effector proteins into the host cell via needle-like appendages on the bacterial surface termed type III secretion systems (TTSS) (5, 14, 15). The TTSS encoded in Salmonella pathogenicity island (SPI)-1 is necessary for the invasion of non-phagocytic cells and the establishment of gastroenteritis in animal models (1, 14, 16). After the early stages of infection, the SPI-1 system is down-regulated (17, 18), and expression of the SPI-2 TTSS is induced to continue modification of the SCV and intracellular growth (17, 19–22). SifA is an effector protein of the SPI-2 TTSS and is necessary for both maintenance of the SCV and for Sif formation. ΔsifA S. Typhimurium lose the SCV at late times postinfection (6–8 h) and enter the cytosol (11, 23–27).

TTSSs are used by many Gram-negative pathogens to deliver bacterial effector proteins into host cells (14), and these needle-like structures have been shown to cause damage to host cell membranes. For example, TTSSs form pores in membranes to allow protein delivery (28) and can cause contact-dependent lysis of sheep red blood cells (28, 29). Roy et al. (30) have suggested that the S. Typhimurium SPI-1 TTSS can damage the SCV early after invasion, and have proposed a vacuole repair mechanism in which lysosomes are recruited to the damaged vacuoles in a calcium-dependent manner.

Recently, we and others (23, 24, 31) have observed that a small but significant proportion of wild-type S. Typhimurium escape from the SCV early after invasion in vitro, though the mechanism(s) of this escape are currently unclear. The existence of cytosolic S. Typhimurium suggests that the mechanism proposed by Roy et al. (30) is not sufficient to retain the entire bacterial population within vacuoles. We have previously shown that bacteria that enter the cytosol in such a manner are targeted by the ubiquitin system (31). The consequences of this are still unknown, though it may play a role in MHC class I presentation in macrophages.

Macroautophagy (hereafter referred to as autophagy) is a cellular process that mediates the degradation of long-lived proteins and unwanted organelles in the cytosol by delivering them to the lysosome (32–34). Autophagy is regulated by a multitude of factors, including nutritional status, hormones and intracellular signaling pathways (34–36). Recent evidence has suggested that the autophagy pathway inter-
acts with intracellular bacteria in a variety of ways (33, 37). Some bacterial species subvert autophagy and reside within autophagosome-like vacuoles, including Brucella abortus (38, 39), Porphyromonas gingivalis (40), Coxiella burnetti (41), and Legionella pneumophila (42, 43). It has been suggested that these bacteria block fusion of their autophagosomes with lysosomes, thereby providing a niche permissive for growth (33).

Recent studies also suggest a role for autophagy in host cell defense. Group A Streptococcus pyogenes is able to invade cells and lyse the phagosome using Streptolysin O. Once within the cytosol, autophagy targets this pathogen and effectively restricts its growth (44). Rickettsiae spp. have been observed in double-membrane autophagosome-like structures, correlating with bacterial destruction and decreased replication (45, 46). Additionally, autophagy targets Mycobacterium tuberculosis-containing phagosomes in interferon-γ-activated macrophages to overcome the block in phagosomal maturation imposed by this pathogen (47). The above studies suggest that autophagy is able to target bacteria in the cytosol and within vacuoles. However, the mechanisms by which autophagy targets intracellular bacteria are still unclear.

To date, a possible interaction of S. Typhimurium with the autophagy system has not been tested. Here we demonstrate a role for autophagy in controlling infection by this pathogen. Our results suggest that autophagy targets S. Typhimurium in SCVs damaged by the SPI-1 TTSS. We propose that the host cell uses autophagy to restrict the growth of S. Typhimurium in the cytosol during infection.

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Cell Culture**—Wild-type S. Typhimurium SL1344 (48) and the isogenic mutants Δsfa (27) and Δsra (11) were used for these studies. Wild-type-expressing GFP was previously described (19). ΔinvA and ΔlnvA expressing the Yersinia Inv protein (ΔinvA/pinv) in the 14028S background (49, 50) were also used. HeLa human epithelial cells and mouse embryonic fibroblasts (MEF) were maintained in Dulbecco’s modified Eagle’s growth medium (HyClone) supplemented with 10% fetal bovine serum (Wisent) at 37 °C in 5% CO₂ without antibiotics. Cells were seeded in 24-well tissue culture plates at 5.0 × 10⁴ cells/well 16–24 h before use.

**Plasmids and Transfection**—GFP-LC3 was generated as previously described (51). Genelucie (Oncogene Research Products, San Diego, CA) and FuGene 6 (Roche Applied Science) transfection reagents were used according to the manufacturers’ instructions.

For visualization of wild-type bacteria during co-infection studies, monomeric red fluorescent protein (mRFP) was expressed in S. Typhimurium. Bacteria were transformed with the plasmid pBR-RFP (52). The region upstream from rpsM in S. Typhimurium was PCR-amplified using the following primers: rpsMf (5'-ATG GAA TTC TTT TTT AAG AAG GAG ATA TAC ATA TGG CTT CCT CCG AGG AGC TTG GC-3') and rpsMr (5'-ATG AAG CTT TTA GGC GCC GGT GGA GTG CCG-3'). The PCR product was digested with EcoRI and HindIII (sites underlined in RpsMf and RpsMr) and cloned into pBR322 to construct pBR-RFP. The region upstream from rpsM in S. enterica was PCR-amplified using the following primers: prpsMf (5'-ATG CAA TTG ATA CTA TGA AGC AAG CCG-3') and prpsMr (5'-ATG CAA TGG CTA TTT AAT ATG TAC GTA CTA T-3'). The PCR product was then digested with MfeI (sites underlined in prpsMf and prpsMr) and cloned into the EcoRI site lying upstream of mrfp in pBR-RFP. A clone where the rpsM promoter orientation was driving expression of mrfp was selected, confirmed by nucleotide sequencing and designated pBR-RFP.

**Bacterial Infections**—Late-log S. Typhimurium cultures were used for infecting cells and prepared using a method optimized for bacterial invasion (53). Briefly, wild-type and mutant bacteria were grown for ~16 h at 37 °C with shaking and then subcultured (1:33) in LB without antibiotics for 3 h. Bacterial inocula were prepared by pelleting at 10,000 × g for 2 min, diluted 1:100 in PBS, pH 7.2, and added to cells for 10 min at 37 °C. After infection, extracellular bacteria were removed by extensive washing with PBS and addition of 100 μg/ml gentamicin to the medium. At 2 h postinfection, the gentamicin concentration was decreased to 10 μg/ml. ΔinvA/pinv bacteria were grown to late-log, harvested, and adjusted to an A₆₀₀ of 1. The bacteria were then diluted 1:40 in PBS pH 7.2 and centrifuged onto cells for 1 min at 1000 rpm. The rest of the infection was performed as with wild-type S. Typhimurium.

For ΔinvA co-infection experiments, wild-type S. Typhimurium expressing mRFP (52) and ΔinvA were added to the cells at the same time in a 1:5 ratio as previously described (50). The rest of the infection was performed as with wild-type S. Typhimurium. To inhibit bacterial protein synthesis, 200 μg/ml chloramphenicol was added directly to the growth medium and kept on throughout the rest of the infection. The phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin was similarly added to a final concentration of 100 nM. The gentamicin-protectection replication assay was performed as previously described (11).

**Immunofluorescence and Confocal Microscopy**—Cells were fixed with 2.5% paraformaldehyde in PBS, pH 7.2, for 10 min at 37 °C. Fixed cells were permeabilized and blocked in 0.2% saponin (Calbiochem, San Diego, CA) and 10% normal goat serum for 12–24 h, and stained as previously described (11). Samples were mounted on slides and analyzed using a Zeiss Axiosvert confocal microscope and LSM 510 software. Confocal images were imported into Adobe Photoshop and assembled in Adobe Illustrator. For three-dimensional representations, confocal Z-stacks were deconvolved and movies assembled with Volocity software (Improvision). Colocalization and enumeration studies were performed on a Leica DMI2RE fluorescence microscope by direct visualization. At least 100 bacteria or cells were counted per condition in each experiment, and at least three independent experiments were performed. The mean ± S.D. are shown in the figures.

**Antibodies**—Rabbit polyclonal antibodies to S. Typhimurium O antigen serum Group B were from Difco Laboratories (Kansas City, MO). Mouse anti-human LAMP-1 antibody (clone H4A43) was developed by J. Thomas August and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD, National Institutes of Health and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Mouse anti-human MHC-1 antibody was from Serotec (Raleigh, NC) and mouse anti-human CD44 from Sigma. Mouse anti-ubiquitinated proteins (clone FK2) was from Affiniti Research Products Ltd. (Plymouth Meeting, PA). All secondary antibodies used were AlexaFluor conjugates from Molecular Probes (Burlington, Ontario). DAPI (Molecular Probes) was used according to the manufacturer’s instructions.

**RESULTS**

The Autophagy System Targets a Population of S. Typhimurium following Invasion—S. Typhimurium invades non-phagocytic cells and modifies the SCV to create a specialized vacuolar niche permissive for intracellular growth (3–5). As previously noted, not all intracellular S. Typhimurium remain within SCVs, but rather a significant proportion escape into the cytosol early after invasion (23, 24). These cytosolic bacteria are targeted by the ubiquitin system and become associated with ubiquitinated proteins (31). Therefore, we used immunostaining
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FIGURE 1. The autophagy system targets a population of S. Typhimurium following invasion. A, HeLa cells were transfected with GFP-LC3 (green in Merge), infected with wild-type S. Typhimurium and fixed at 1 h postinfection. Cells were then costained with a polyclonal antibody to S. Typhimurium (blue in Merge) and a monoclonal antibody to ubiquitinated proteins (red in Merge) as indicated. Representative confocal Z-slices are shown. The lower panels show higher magnification of the boxed areas in the upper panels. Size bar indicates 5 μm. For three-dimensional representation, see supplementary Movie S1. B, HeLa cells were transfected with GFP-LC3 and infected as in A. Cells were fixed at 1 h postinfection and costained with a polyclonal antibody to S. Typhimurium and a monoclonal antibody to ubiquitinated proteins (Ub’d Protein). The percentage of GFP-LC3- or GFP-LC3+ bacteria colocalizing with ubiquitinated proteins was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each condition. The average ± S.D. is shown for three independent experiments. Asterisk indicates a significant difference (p < 0.05) as determined by the two-tailed Student’s t test. C, HeLa cells were transfected with GFP-LC3 and infected as in A. Cells were fixed at the indicated time points and costained with DAPI (to label DNA) and a monoclonal antibody to ubiquitinated proteins, or with a polyclonal antibody to S. Typhimurium and a monoclonal antibody to LAMP-1. The percentage of ubiquitinated protein+ (UBP+) LAMP-1+ (LAMP-1+UBP+) or GFP-LC3+ bacteria was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each time point. The average ± S.D. is shown for three independent experiments.

for ubiquitinated proteins as a marker for bacteria exposed to the cytosol (Fig. 1A).

To examine autophagy during S. Typhimurium infection, HeLa cells were transfected with a GFP fusion to the N terminus of microtubule-associated light chain-3 (LC3) (51). LC3 is conjugated onto the membrane of autophagosomes and is a well-characterized marker of these structures (33, 51). Cells were infected with wild-type S. Typhimurium under conditions optimized for SPI-1 TTSS expression and bacterial invasion (53). As shown in Fig. 1A, a population of intracellular S. Typhimurium colocalized with GFP-LC3 (see also supplementary Movie S1). Treatment of cells with the PI3K inhibitor wortmannin, also a well-known inhibitor of autophagy (33), blocked GFP-LC3 colocalization with intracellular S. Typhimurium (data not shown). Approximately 50% of GFP-LC3+ bacteria colocalized with ubiquitinated proteins, compared with only ~5% for GFP-LC3− bacteria (Fig. 1B). This suggests that S. Typhimurium autophagy is occurring in the cytosol, or a compartment that has access to the ubiquitin-conjugating enzymes present in the cytosol.

Next, we examined the kinetics of S. Typhimurium autophagy (Fig. 1C). Colocalization of S. Typhimurium with GFP-LC3 peaked at 1 h postinfection, with up to ~20% of total intracellular bacteria associated with the autophagy marker. Autophagy of S. Typhimurium was transient, as GFP-LC3 colocalization was not observed above background levels (3–5% of total bacteria) after 2 h postinfection. However, bacterial colocalization with ubiquitinated proteins peaked at 4 h postinfection (~24% of the total bacterial population). Thus, autophagy of S. Typhimurium is an early event, and occurs prior to maximal escape by the bacteria into the cytosol, possibly when damage to the SCV is first initiated.

Lysosomal-associated membrane protein-1 (LAMP-1) was rapidly acquired by the SCV during the first hour postinfection (Fig. 1C), as previously described (23–25). Only 75–80% of wild-type S. Typhimurium colocalized with LAMP-1 between 2 and 12 h postinfection. However, S. Typhimurium did not colocalize with GFP-LC3 during this period despite the fact that up to 20% of intracellular bacteria were LAMP-1− and apparently present in the cytosol. These findings demonstrate that the autophagy response to S. Typhimurium is limited, because it does not target cytosolic bacteria after 1 h postinfection.

Autophagy Restricts the Intracellular Growth of S. Typhimurium—Autophagy of S. Typhimurium would be expected to have two important consequences. First, autophagosomes would be expected to deliver these bacteria to lysosomes for degradation. Because of technical limitations, we were unable to directly demonstrate that S. Typhimurium subject to autophagy are killed in lysosomes. However, a second, and perhaps more important consequence of S. Typhimurium autophagy, is that it would remove these bacteria from the cytosol. In this scenario, autophagy would protect the host cell cytosol, which has been shown to be permissive for S. Typhimurium replication in some cell types, including HeLa cells (23, 25). In support of this notion, wortmannin (which inhibits autophagy) promotes the release of S. Typhimurium from vacuoles, and allows them to grow rapidly in the cytosol of HeLa cells, (23, 50).

To directly test the role of autophagy in restricting intracellular growth by S. Typhimurium, we utilized a mouse embryonic fibroblast (MEF) cell line harboring a knock-out of the atg5 locus (atg5−/−). Atg5 is essential for the early steps in autophagosome formation, and cells lacking this protein are completely deficient in macroautophagy (54). Wild-type and atg5−/− MEFs were transfected with GFP-LC3 and infected with S. Typhimurium. As in HeLa cells, ~20% of intracellular bacteria colocalized with GFP-LC3 by 1 h postinfection in wild-type MEFs (Fig. 2, A and B). However, S. Typhimurium colocalization with GFP-LC3 was sustained in wild-type MEFs compared with HeLa cells, and did not start declining until after 4 h postinfection. This suggests that autophagosome maturation occurs slowly in wild-type MEFs. Indeed, autophagosomes containing the Gram-negative bacterial pathogen S. pyogenes were found to mature with a similar time course in this cell type (44). As expected, autophagy of S. Typhimurium was not observed in atg5−/− MEFs during the 12-h infection period (Fig. 2, A and B).

atg5−/− MEFs exhibited an increase in bacterial association with ubiquitinated proteins compared with wild-type cells at 8 h postinfection, although the difference was not statistically significant (Fig. 2C). However, the increase in the number of bacteria associated with ubiquitinated proteins in the autophagyl deficient cells indicated an increase in the number exposed to the cytosol. In agreement with this, atg5−/− MEFs also showed a significant decrease in bacteria within LAMP-1+ compartments compared with wild-type cells (Fig. 2D).

To assess bacterial replication, we counted the number of intracellular S. Typhimurium over an 8-h infection period using immunofluorescence. The results are expressed in Fig. 2E as the percentage of infected cells containing a certain number of intracellular bacteria (see legend). Intracellular growth during the 8-h course of infection was indicated by...
Autophagy restricts the intracellular growth of S. Typhimurium. A wild-type (WT MEF) and autophagy-deficient (atg5−/− MEF) mouse embryonic fibroblasts were transiently transfected with GFP-LC3 (green in Merge) and infected with S. Typhimurium. Cells were fixed at 1 h postinfection and costained with a monoclonal antibody to S. Typhimurium (blue in Merge) and a monoclonal antibody to LAMP-1 (red in Merge) as indicated. Representative confocal Z-slices are shown. Arrows indicate intracellular S. Typhimurium colocalizing with GFP-LC3 in wild-type MEFs. Arrowheads indicate S. Typhimurium not colocalizing with GFP-LC3 in autophagy-deficient MEFs. Size bars indicate 5 μm. B, wild type (●) and atg5−/− (□) MEFs were transfected with GFP-LC3 and infected as in A. Cells were fixed at the indicated time points and stained for S. Typhimurium with DAPI. The percentage of GFP-LC3+ bacteria was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each time point. The average ± S.D. is shown for three independent experiments. Asterisks indicate a significant difference from wild-type MEF numbers (p < 0.05) as determined by the two-tailed Student’s t test. C, wild type (●) and atg5−/− (□) MEFs were transfected with GFP-LC3 and infected as in B. Cells were fixed at the indicated time points and costained for S. Typhimurium with DAPI and a monoclonal antibody to ubiquitinated proteins (Ub’d protein). The percentage of Ub’d protein+ bacteria in each cell type was enumerated as in B, D, and E. MEFs were transfected with GFP-LC3, infected, and fixed as in B. D, MEFs were costained with a polyclonal antibody to LAMP-1 (red) and a monoclonal antibody to ubiquitinated proteins (Ub’d protein). The percentage of LAMP-1+ bacteria in each cell type was enumerated. E, wild type (●) and atg5−/− (□) MEFs were transfected with GFP-LC3 and infected as in B. Cells were fixed at the indicated time points, and stained with a polyclonal antibody to S. Typhimurium. The number of intracellular bacteria was enumerated as in at least 100 infected cells. These numbers were grouped according to the legend, and the amount expressed as a percentage of the total infected cell population. The average ± S.D. is shown for three independent experiments. Asterisks indicate a significant difference from wild-type MEF numbers (p < 0.05) as determined by the two-tailed Student’s t test.

A decrease in the percentage of cells with low numbers of intracellular bacteria concomitant with an increase in the percentage with high numbers of bacteria. At early times (1–4 h postinfection) the number of intracellular bacteria/cell was similar in wild-type and atg5−/− MEFs. However, at 8 h postinfection, autophagy-deficient MEFs showed a greater percentage of cells with high numbers of intracellular S. Typhimurium compared with wild-type MEFs (~62% versus ~35%, respectively, for >20 bacteria/cell). Similar results were obtained by scoring bacterial colony forming units from infected cell lysates in a gentamicin-protection assay (data not shown). Because increased bacterial replication in atg5−/− MEFs was observed in conjunction with an increase in the number of cytotoxic bacteria in autophagy-deficient cells (Fig. 2, C and D), we propose a role for autophagy in restricting the cytosolic replication of S. Typhimurium by preventing bacteria from entering this compartment.

S. Typhimurium Autophagy Requires the SPI-1 TTSS and Bacterial Protein Synthesis—Bacterial TTSSs have been shown to form pores within cellular membranes (28, 29), and the SPI-1 TTSS has been proposed to damage the SCV (30). It is possible that autophagy targets SCVs damaged by the SPI-1 TTSS after invasion. To test this, we infected GFP-LC3 transfected HeLa cells with a S. Typhimurium strain harboring a deletion in the invA gene. invA encodes an essential component of the SPI-1 TTSS, and thus mutants are completely deficient in delivery of SPI-1 effectors and in formation of the translocation needle (55–57). To complement invasion of host cells, the invA mutant was transformed with a plasmid expressing the Yersinia Invasin (Inv) protein, previously shown to be sufficient for bacterial uptake into phagosomes and subsequent delivery to lysosomes (49, 50). SPI-1 TTSS-deficient bacteria expressing Yersinia Inv (∆invA/pinv) did not efficiently enter the cytosol after invasion, as indicated by a minimal association of ubiquitinated proteins with the bacterial surface (Fig. 3A). Similarly, these bacteria were not targeted by the host autophagy system (Fig. 3, B and C). As expected, the ∆invA/pinv

FIGURE 2. Autophagy restricts the intracellular growth of S. Typhimurium. A, wild-type (WT MEF) and autophagy-deficient (atg5−/− MEF) mouse embryonic fibroblasts were transiently transfected with GFP-LC3 (green in Merge) and infected with S. Typhimurium. Cells were fixed at 1 h postinfection and costained with a polyclonal antibody to S. Typhimurium (blue in Merge) and a monoclonal antibody to LAMP-1 (red in Merge) as indicated. Representative confocal Z-slices are shown. Arrows indicate intracellular S. Typhimurium colocalizing with GFP-LC3 in wild-type MEFs. Arrowheads indicate S. Typhimurium not colocalizing with GFP-LC3 in autophagy-deficient MEFs. Size bars indicate 5 μm. B, wild type (●) and atg5−/− (□) MEFs were transfected with GFP-LC3 and infected as in A. Cells were fixed at the indicated time points and stained for S. Typhimurium with DAPI. The percentage of GFP-LC3+ bacteria was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each time point. The average ± S.D. is shown for three independent experiments. Asterisks indicate a significant difference from wild-type MEF numbers (p < 0.05) as determined by the two-tailed Student’s t test. C, wild type (●) and atg5−/− (□) MEFs were transfected with GFP-LC3 and infected as in B. Cells were fixed at the indicated time points and costained for S. Typhimurium with DAPI and a monoclonal antibody to ubiquitinated proteins (Ub’d protein). The percentage of Ub’d protein+ bacteria in each cell type was enumerated as in B. D, and E. MEFs were transfected with GFP-LC3, infected, and fixed as in B. D, MEFs were costained with a polyclonal antibody to LAMP-1 (red) and a monoclonal antibody to ubiquitinated proteins (Ub’d protein). The percentage of LAMP-1+ bacteria in each cell type was enumerated. E, wild type (●) and atg5−/− (□) MEFs were transfected with GFP-LC3 and infected as in B. Cells were fixed at the indicated time points, and stained with a polyclonal antibody to S. Typhimurium. The number of intracellular bacteria was enumerated as in at least 100 infected cells. These numbers were grouped according to the legend, and the amount expressed as a percentage of the total infected cell population. The average ± S.D. is shown for three independent experiments. Asterisks indicate a significant difference from wild-type MEF numbers (p < 0.05) as determined by the two-tailed Student’s t test.

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mutant trafficked to LAMP-1+ compartments to a greater extent than wild-type S. Typhimurium (Fig. 3D).

To further test the involvement of the SPI-1 TTSS in autophagy of S. Typhimurium, GFP-LC3 transfected HeLa cells were co-infected with ΔinvA bacteria and wild-type S. Typhimurium expressing mRFP. This method has previously been shown to allow efficient uptake of non-invasive ΔinvA bacteria by the dynamic ruffling of the plasma membrane caused by wild-type invasion (49, 50). After uptake, the wild-type and ΔinvA bacteria enter distinct vacuoles, and presumably undergo different intracellular trafficking routes as ΔinvA bacteria do not replicate after co-infection (50). Therefore, the only difference between the two bacterial populations early after invasion is lack of the SPI-1 TTSS secretion apparatus by the ΔinvA S. Typhimurium. Wild-type S. Typhimurium were visualized by RFP fluorescence, and total bacteria were stained with an anti-S. Typhimurium antibody. In this way, the ΔinvA and wild-type populations could be differentiated by immunofluorescence.

Following co-infection, the ΔinvA S. Typhimurium mutant did not associate with ubiquitinated proteins (Fig. 4A). In contrast, ~17% of the wild-type S. Typhimurium expressing mRFP colocalized with ubiquitinated proteins at 2 h postinfection (Fig. 4A). In this co-infection model, ~27% of wild-type S. Typhimurium colocalized with the autophagy marker GFP-LC3 (Fig. 4, B and C). However, co-infected ΔinvA bacteria did not associate with GFP-LC3, indicating they were not targeted by the autophagy system (Fig. 4, B and C). These results are consistent with a direct role for the SPI-1 TTSS apparatus in causing damage to the SCV and the subsequent recognition of S. Typhimurium in the cytosol by the ubiquitin and autophagy systems.

Next, we investigated whether SCV damage by intracellular S. Typhimurium is an active process. For this, we infected cells for 10 min with wild-type S. Typhimurium and then washed extracellular bacteria from the cultures. Infected cells were then treated with the cell-permeant antibiotic chloramphenicol to block bacterial protein synthesis. As shown in Fig. 5A, chloramphenicol treatment effectively blocked the association of ubiquitinated proteins with intracellular bacteria. Autophagy of S. Typhimurium was also dependent upon bacterial protein synthesis (Fig. 5B). Consistent with these results, the percentage of LAMP-1+ bacteria increased significantly with chloramphenicol treatment (Fig. 5C). Thus, escape of S. Typhimurium from SCVs and subsequent recognition by the ubiquitin and autophagy systems is an active process that requires bacterial protein synthesis.

The Autophagy System Targets S. Typhimurium within Damaged Vacuoles—The rapid kinetics of S. Typhimurium autophagy suggested that this process may be occurring prior to completion of bacterial escape into the cytosol. To test whether autophagy targets SCVs damaged by the SPI-1 TTSS, we measured colocalization of GFP-LC3+ bacteria with known SCV markers. Major histocompatibility complex-1 (MHC-1) is present on the SCV early after invasion and is recycled from this compartment within 3 h (58, 59). CD44 follows a similar route and is present on the SCV during the first hour of infection (58, 59). As shown in Fig. 6A, significantly less GFP-LC3+ bacteria colocalized with both of these early SCV markers than GFP-LC3+ bacteria. However, a large fraction of GFP-LC3+ S. Typhimurium (~90% of the levels for GFP-LC3+ bacteria) did colocalize with these SCV markers. Confocal microscopy revealed that staining for these SCV markers was often not continuous around GFP-LC3+ bacteria (Fig. 6B and supplementary Movie S2). In fact, these early SCV markers usually exhibited patchy staining, and regions around bacteria lacking SCV marker staining were often found to contain GFP-LC3.

Colocalization studies with the SCV marker LAMP-1 revealed no significant difference between the GFP-LC3+ and GFP-LC3+ bacterial populations (Fig. 6A). Confocal microscopy of this staining revealed that, similar to early SCV markers, LAMP-1 often showed discontinuous staining in conjunction with GFP-LC3 fluorescence (Fig. 6C and supplementary Movie S3). However, there were also cases of continuous weak LAMP-1 staining in areas of high GFP-LC3 fluorescence. LAMP-1 staining could represent the normal SCV maturation process, or maturation of autophagosomes containing S. Typhimurium into autolysosomes (33, 60). LAMP-1+ vesicles were often observed in the vicinity of GFP-LC3+ bacteria and other small GFP-LC3+ vesicles (Fig. 6C, small arrows), suggesting interaction of bacteria-containing autophagosomes with the endocytic pathway, as in other models of autophagy (33, 61, 62). Together, our findings suggest the autophagy system targets an intracellular bacterial population present within damaged
SCVs, and thus exposed to the cytosol through gaps in the SCV membrane.

**Autophagy Does Not Target Cytosolic ΔsifA Bacteria at Late Times Postinfection**—We have shown that autophagy of intracellular S. Typhimurium occurs within the first hour of infection and is dependent upon the SPI-1 TTSS. At late times postinfection, SCV maturation and maintenance is dependent on the SPI-2 TTSS (17, 19–22). Maturation of the SCV includes the formation of long, membranous structures called Sifs, a process initiated 4–5 h postinfection (12, 13, 63) (Fig. 7A). The function of Sifs is still unclear, but their formation correlates with rapid bacterial growth in host cells and is regulated by many SPI-2 TTSS effector proteins (12).

To test a role for the autophagy system in Sif formation, MEFs were infected with wild-type S. Typhimurium for 8 h, then fixed and stained for LAMP-1 to visualize Sifs. In atg5−/− MEFs, Sif formation was dra-

![FIGURE 5. S. Typhimurium autophagy requires bacterial protein synthesis.](image)

A. HeLa cells were transiently transfected with GFP-LC3, infected with wild-type S. Typhimurium and fixed at the indicated time points. Chloramphenicol was either omitted (●) or added directly to the medium at 10 min postinfection (□). Cells were costained for LAMP-1 and the percentage of LAMP-1 staining, and the large arrowhead indicates a GFP-LC3 bacterium without discontinuous LAMP-1 staining. Small arrows indicate LAMP-1− vacuoles in close approximation to GFP-LC3− bacteria and vesicles. Size bar indicates 5 μm. For three-dimensional representation, see supplementary Movie S3.

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![FIGURE 6. The autophagy system targets S. Typhimurium within damaged vacuoles.](image)

A. HeLa cells were transiently transfected with GFP-LC3, infected with wild-type S. Typhimurium and fixed at 1 h postinfection. Cells were costained with a polyclonal antibody to S. Typhimurium and monoclonal antibodies to CD44, MHC-1, or LAMP-1 as indicated. The percentage of GFP-LC3+ (green in Merge) and infected as in A. Cells were fixed at 1 h postinfection, and stained with a polyclonal antibody to S. Typhimurium (blue in Merge) and a monoclonal antibody to MHC-1 (red in Merge) as indicated. Representative confocal Z-slices are shown. The lower panels show higher magnification of the boxed areas in the upper panels. Notice the patchiness of MHC-1 staining in areas of high GFP-LC3 fluorescence. Size bar indicates 5 μm. For three-dimensional representation, see supplementary Movie S2.

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B. HeLa cells were transfected with GFP-LC3, infected with wild-type S. Typhimurium (green in Merge) and fixed at 1 h postinfection. Cells were costained with a polyclonal antibody to S. Typhimurium (blue in Merge) and a monoclonal antibody to LAMP-1 (red in Merge) as indicated. Representative confocal Z-slices are shown. The lower panels show higher magnification of the boxed areas in the upper panels. The large arrowhead indicates a GFP-LC3+ bacterium without LAMP-1 staining, and the large arrow indicates a GFP-LC3+ bacterium with discontinuous LAMP-1 staining. Small arrows indicate LAMP-1− vacuoles in close approximation to GFP-LC3− bacteria and vesicles. Size bar indicates 5 μm. For three-dimensional representation, see supplementary Movie S3.
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FIGURE 7. Autophagy does not target cytotoxic ΔsifA S. Typhimurium at late times postinfection. A, HeLa cells were infected with wild-type S. Typhimurium and fixed at 10 h postinfection. Cells were costained with a monoclonal antibody to S. Typhimurium (green in Merge) and a monoclonal antibody to LAMP-1 to visualize Sifs (red in Merge) as indicated. Representative confocal Z-slices are shown. Arrow indicates a Sif. Size bar indicates 5 μm. B, wild-type and autophagy-deficient (atsg5−/−) MEFs were infected with S. Typhimurium and fixed at 8 h postinfection. In parallel experiments, autophagy-deficient cells were transfected with a plasmid expressing Atg5 (Δatg5) prior to infection. Cells were costained with a monoclonal antibody to S. Typhimurium and a monoclonal antibody to LAMP-1 to visualize Sifs. The percentage of infected cells exhibiting the Sif phenotype was enumerated. At least 100 cells were counted for each condition. The average ± S.D. is shown for three independent experiments. Asterisk indicates a significant difference (p < 0.05) as determined by the two-tailed Student’s t test. C, HeLa cells were transiently transfected with GFP-LC3, infected with wild-type (○), ΔssaR (□), or ΔsifA (●) S. Typhimurium, and fixed at the indicated time points. In parallel experiments, chloramphenicol was added to ΔsifA-infected cells at 8 h postinfection (shaded ○). Cells were costained for S. Typhimurium with DAPI and a monoclonal antibody to ubiquitinated proteins (Ub’d protein), and the percentage of Ub’d protein− S. Typhimurium was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each time point. The average ± S.D. is shown for three independent experiments. D, HeLa cells were transiently transfected with GFP-LC3, infected with wild-type (○), ΔssAR (□), or ΔsifA (●) S. Typhimurium, and fixed at the indicated time points. The average ± S.D. is shown for three independent experiments. E, HeLa cells were transfected, infected, and fixed as in C. Cells were costained for S. Typhimurium with DAPI, and the percentage of GFP-LC3− S. Typhimurium was enumerated as in C. Asterisks indicate a significant difference from wild-type numbers (p < 0.05) as determined by the two-tailed Student’s t test.

FIGURE 8. Cells infected with S. Typhimurium can mount an autophagic response at late times postinfection. A, HeLa cells were transiently transfected with GFP-LC3, infected with wild type, ΔsifA, or ΔsifA S. Typhimurium for 8 h. The cells were then re-infected with wild-type bacteria expressing mRFP (WT-RFP) and fixed after another 1 h of infection. Total bacteria were stained with a polyclonal antibody to S. Typhimurium, and the percentage of wild-type bacteria expressing mRFP associated with GFP-LC3 enumerated by fluorescence microscopy only in preinfected cells. At least 100 bacteria were counted for each time point. The average ± S.D. is shown for three independent experiments. B, HeLa cells were transfected with GFP-LC3 (green in Merge) and infected with wild type or ΔsifA bacteria for 8 h as indicated. The cells were then re-infected with wild-type bacteria expressing mRFP (WT-RFP; red in Merge) and fixed after another 1 h of infection. Total bacteria were stained with a polyclonal antibody to S. Typhimurium (blue in Merge). Representative confocal Z-slices are shown. An arrow indicates wild-type bacteria expressing mRFP colocalizing with GFP-LC3 (yellow in Merge), and arrowheads indicate preinfection bacteria that do not colocalize with GFP-LC3. Size bars indicate 5 μm.
S. Typhimurium for 8 h. The cells were then reinfectected with wild-type bacteria expressing mRFP, and autophagy of these latter bacteria was assessed after 1 h of infection. Control experiments demonstrated that the mRFP-expressing strain, as well as a strain expressing GFP, colocalized with GFP-LC3 to a greater extent than non-expressing bacteria (~34 and ~33% versus ~23%, respectively, at 1 h; data not shown). Only cells containing both bacteria expressing mRFP and the preinfected strain were counted. An 8-h preinfection with wild-type ΔssaR or ΔsifA bacteria did not prevent autophagy of wild-type bacteria expressing mRFP, although GFP-LC3 colocalization was slightly lower than that observed without preinfection (Fig. 8, A and B). This suggests that host cells are still capable of mounting an autophagy response even at late times postinfection. Therefore, the lack of autophagy of ΔsifA cytosolic bacteria may be caused by another reason, such as the down-regulation of a target molecule or the difference in how the loss of the SCV is achieved. 

DISCUSSION

This study is the first examination of the role of autophagy in S. Typhimurium pathogenesis. We found that a subset (~20%) of intracellular S. Typhimurium were targeted by the host autophagy system in HeLa cells by 1 h postinfection. However, the majority of S. Typhimurium were not targeted. Although some bacteria, like L. pneumophila and B. abortus, preferentially inhabit modified autophagosomes (33, 37–41, 43), there is no evidence that S. Typhimurium utilizes a similar mechanism.

In Fig. 9 we present a model depicting how in vitro infection might lead to four different populations of intracellular S. Typhimurium. The majority of bacteria reside within LAMP-1+ SCVs after invasion and alter SCV maturation to allow intracellular growth and Sif formation (population 1). However, our data presented here and that of Roy et al. (30) suggest that the SCV can be damaged by the SPI-1 TTSS following invasion. Roy et al. (30) have proposed a mechanism that repairs damaged SCVs through calcium release and recruitment of lysosomes to the damaged SCV. Their model predicts a population of intracellular bacteria present within vacuoles and killed by lyosomal proteases (population 2). Here we show that intracellular S. Typhimurium can be targeted by the autophagy system (population 3). Several lines of evidence suggest that the autophagy system targets S. Typhimurium in damaged SCVs: (i) bacterial autophagy was dependent on the SPI-1 TTSS; (ii) bacteria targeted by the autophagy system colocalized with ubiquitinated proteins, indicating their exposure to the cytosol; (iii) bacteria in autophagosomes colocalized with SCV markers; and (iv) bacteria were not subject to autophagy at late times postinfection, or when delivered to the cytosol via mutation of the sifA gene. As the autophagosome marker LC3 is present, this population of intracellular bacteria is likely distinct from the one recruiting mature lysosomes for phagosomal repair. However, it is possible that both autophagy and the lysosomal repair pathway may act on the same damaged SCV at the same time.

The fate of S. Typhimurium targeted by the autophagy system is still unclear. However, our studies of autophagy-deficient MEFs demonstrated that autophagy restricts the intracellular growth of S. Typhimurium in the cytosol and therefore represents a host defense mechanism. These findings are consistent with previous observations that the PI3K/autophagy inhibitor wortmannin enhances the intracellular growth of S. Typhimurium (23). S. Typhimurium has adapted to growth within a vacuolar compartment in host cells. Thus, it is perhaps not too surprising that these bacteria are targeted by host cell systems that...
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restrict bacterial growth in the cytosol. This agrees with Nakagawa et al. (44) who demonstrated that S. pyogenes, another pathogen not adapted to life within the host cytosol, is targeted by the autophagy system in host cells. On the other hand, Shigella flexneri is a facultative intracellular bacterium adapted to life in the cytosol of host cells (65). This pathogen has evolved a specific mechanism to avoid autophagy in this compartment: the TTSS effector IcsB blocks interaction of the bacterial protein IcsA with host Atg5. In accordance with this model, the intracellular growth of mutants lacking icsB is restricted by the autophagy system (66).

Autophagy of intracellular S. Typhimurium is rapid, transient, and maximal at 1 h postinfection. However, previous studies have shown that a population of S. Typhimurium exists in the cytosol without a limiting membrane at later times postinfection (population 4) (23, 24, 31). Bacteria that escape autophagy and the lysosome repair pathway (30) are nonetheless subject to recognition by the ubiquitin system, possibly promoting bacterial antigen presentation on MHC class I molecules (31). As a proportion of intracellular S. Typhimurium associate with both ubiquitinated proteins and the autophagosome marker LC3 (Fig. 1B), it is possible that ubiquitination is involved in autophagy recognition of these bacteria. It has previously been shown that a temperature-labile mutant form of the mammalian E1 ubiquitin-activating enzyme halts long-lived protein degradation induced by starvation (67). As well, Komatsu et al. (68) have suggested that the ubiquitination of protein aggregates may serve as a regulatory signal for autophagy. However, we have not observed any indication that this may be the case for autophagy recognition of S. Typhimurium. The kinetics of autophagy and ubiquitin association with intracellular bacteria suggests separate pathways with maximal recognition levels at 1 and 4 h postinfection, respectively (this work and Ref. 31). Further work needs to be done to clarify the role of ubiquitination in autophagy recognition of S. Typhimurium.

In this study, we were surprised to find that ΔsifA S. Typhimurium were not targeted by autophagy after loss of the SCV. There are several possible explanations for this observation. First, ΔsifA bacteria may actively avoid recognition in the cytosol. However, ΔsifA S. Typhimurium treated with chloramphenicol were not targeted by autophagy (Fig. 7D), suggesting this is not the case. Second, the autophagy system may be inactive when ΔsifA bacteria enter the cytosol. Contrary to this theory, we showed that autophagy is able to target mRFP-expressing bacteria after an 8-h preinfection with another strain (Fig. 8, A and B). Third, it is possible that ΔsifA S. Typhimurium are not targeted by autophagy because the bacterial molecule(s) necessary for recognition is/are down-regulated at the time ΔsifA bacteria enter the cytosol. The S. Typhimurium SPI-1 translocase SipB has been implicated in driving autophagy when overexpressed, and in inducing autophagy-mediated cell death in infected macrophages (69). However, SipB has not been demonstrated to be an actual autophagy target. Pull-down experiments in S. Typhimurium-infected HeLa cells did not show any direct interaction between a GST-tagged SipB construct and either LC3 or Atg5.5

A fourth possibility is that the damaged SCV itself is the target for autophagy recognition. Autophagy recognition of damaged phagosomes could represent a general innate immune mechanism for protecting the cytosol from pathogen colonization. This would negate the need for a specific bacterial target for the autophagy machinery. Indeed, Marten et al. (70) have shown that infection of activated mouse macrophages by the intracellular parasite Toxoplasma gondii results in the recruitment of p47 GTPases to the parasitophorous vacuole (PV). These are host molecules involved in the resistance of interferon-activated mouse macrophages to intracellular pathogens. Relocalization of p47 GTPases to the PV during T. gondii infection results in vesiculation and disruption of the PV membrane. LC3-labeled vesicles accumulate near disrupted PVs, suggesting the induction of autophagy in response to vacuole damage (70). As well, p47 GTPases are recruited to phagosomes during Mycobacterium tuberculosis infection of mouse macrophages (47). This recruitment induces autophagy as an innate immune mechanism to overcome the block in phagosomal maturation imposed by the pathogen (47). Together with our results, these observations suggest that phagosome damage, induced by the pathogen or the host, may provide a signal for autophagy recognition. Indeed, damaged organelles have long been known to be targeted for degradation by autophagy (71, 72). While the mechanisms that govern autophagy of damaged organelles remain unknown, it is noteworthy that this occurs in all eukaryotic cells, from yeast to man. We propose that mammalian cells utilize the conserved ability of autophagy to recognize damaged organelles to target microbial invaders, including S. Typhimurium.

Our study demonstrates that autophagy impacts on the intracellular growth of S. Typhimurium in epithelial cells and fibroblasts in vitro. Importantly, these cell types are encountered by S. Typhimurium during SPI-1 mediated invasion of the host intestinal surface in vivo. Autophagy is rapidly emerging as an important component of the innate immune response in eukaryotic cells (73). Despite being originally identified as a bulk degradative process, the specificity of autophagy is becoming more apparent. Our study demonstrates that S. Typhimurium autophagy is a dynamic, rapid and localized response. Therefore, S. Typhimurium invasion will be a useful model system to study the mechanisms and consequences of autophagy in future experiments.

Acknowledgments—We thank Dr. Noboru Mizushima for providing atg5−/− MEFs. We thank Michael Woodside for assistance with confocal microscopy, Dr. Nat Brown for providing the pBR-RFP 1 vector, and Drs. Nicola Jones, Mauricio Terebiznik, and Michele Swanson, as well as members of the Brumell laboratory, for helpful discussions and critical reading of the manuscript. Infrastructure for the Brumell laboratory was provided by a New Opportunities Fund from the Canadian Foundation for Innovation and the Ontario Innovation Trust.

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