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β1 Integrin-Dependent Engulfment of Yersinia enterocolitica by Macrophages Is Coupled to the Activation of Autophagy and Suppressed by Type III Protein Secretion

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Autophagy is a central lysosomal degradation process that is essential for the maintenance of cellular homeostasis. Autophagy has furthermore emerged as integral part of the host immune response. Autophagic processes promote the separation and degradation of intracellular microorganisms which contributes to the development of innate and adaptive immunity. Some pathogenic microbes have therefore evolved mechanisms to evade or impede autophagy. We analyzed the effects of the enteropathogenic bacterium Yersinia enterocolitica on autophagy in macrophages. Yersinia use a number of defined adhesins and secreted proteins to manipulate host immune responses. Our results showed that Y. enterocolitica defective in type III protein secretion efficiently activated autophagy in macrophages. Autophagy was mediated by the Yersinia adhesins invasin and YadA and particularly depended on the engagement of β1 integrin receptors. Several autophagy-related events followed β1 integrin-mediated engulfment of the bacteria including the formation of autophagosomes, processing of the marker protein LC3, redistribution of GFP-LC3 to bacteria-containing vacuoles, and the segregation of intracellular bacteria by autophagosomal compartments. These results provide direct evidence for the linkage of β1 integrin-mediated phagocytosis and autophagy induction. Multiple microbes signal through integrin receptors, and our results suggest a general principle by which the sensing of an extracellular microbe triggers autophagy. Owing to the importance of autophagy as host defense response, wild-type Y. enterocolitica suppressed autophagy by mobilizing type III protein secretion. The subversion of autophagy may be part of the Y. enterocolitica virulence strategy that supports bacterial survival when β1 integrin-dependent internalization and autophagy activation by macrophages are deleterious for the pathogen. The Journal of Immunology, 2009, 183: 5847–5860.
Here, we investigated the influence of the Gram-negative bacterium *Yersinia enterocolitica* on autophagy in infected host cells. *Yersinia* is a prototypical bacterial pathogen that establishes disease through the modification of host immune responses. There are three human pathogenic *Yersinia* spp. *Yersinia pestis* is the causative agent of bubonic plague, whereas *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are enteropathogenic bacteria eliciting enteritis, ileitis and mesenteric lymphadenitis (24). The *Yersinia* outer surface protein invasin is central for the pathogenicity of enteropathogenic *Yersinia*. Invasin clusters and activates eukaryotic β1 integrin receptors which promotes uptake of the bacteria into the host cell (25–27). The invasin-mediated internalization process is important for the initial phase of infection, enabling enteropathogenic *Yersinia* to penetrate the host mucosa and traverse to the underlying lymphoid tissue of the Peyers’ patches. Once having overcome the epithelial barrier, *Yersinia* spp. pursue a virulence principle that allows their extracellular survival and multiplication in the host lymphoid tissue. A plasmid-encoded type III protein secretion system is activated to mediate the delivery of bacterial virulence factors inside host cells (28–31). These proteins, termed *Yersinia* outer proteins (Yop), suppress or modify cellular signal transduction pathways that are normally aimed to activate protective immunity. Several Yops interfere with signaling processes that control the dynamics of the actin cytoskeleton and thereby promote phagocytosis resistance (28–31). This is achieved by the modulation of the functions of Rho-GTPase members (YopE, YopT, and YopO/YpkA) and by the dephosphorylation of focal adhesion complex proteins (YopH). YopE, YopT, YopO/YpkA, and YopH act in concert to prevent the ingestion of *yersinia* by phagocytic cells (28–31). Moreover, invasins suppress the production of proinflammatory cytokines and trigger apoptosis in macrophages. Both effects are brought about by YopP/YopJ which inhibits proinflammatory and antiapoptotic NF-κB and MAPK signaling (28–31). *Yersinia*-induced apoptosis further involves the activation of TLR-dependent proapoptotic signals (32).

We wondered whether the immunomodulatory activities of *Yersinia* may also influence autophagy. We focused in our study on macrophages which are a major target cell of *Yersinia* virulence (33). It is demonstrated that yersiniae are capable of signaling autophagy in macrophages. The activation of autophagy was conferred by the *Yersinia* adhesin invasin and *Yersinia* adhesin A (YadA) and specifically involved β1 integrin receptors. This is a first hint that the engagement of integrin receptors by a bacterial microbe is linked to the activation of autophagy. Integrin-mediated phagocytosis may reflect a general pathway of autophagy activation by extracellular microorganisms. The execution of autophagy in response to *Yersinia* infection was implicated in host defense networks that assisted pathogen elimination. It was therefore consequent that wild-type *Yersinia* counteracted autophagy by the activation of type III protein secretion. We suggest that the subversion of autophagy is critical for *Yersinia* virulence in interaction with host immune cells.

**Materials and Methods**

*Yersinia* and *Escherichia coli* strains

The *Y. enterocolitica* strains used in this study were the serogroup O8 wild-type strain WA (34), mutants of WA deficient for single (WA-ΔyopP, WA-ΔyopE) and multiple (WA-ΔyopP/ΔyopE, WA-ΔyopP/ΔyopO) effector Yops (35–37), and a virulence plasmid-cured derivative of WA termed WA-C (34). Strain WA-C-Δinv additionally lacks the *Yersinia* adhesion invasin (38). Strain WA-ΔlcrD harbors the *Yersinia* virulence plasmid, but is defective in the secretion of any Yop because of insertional inactivation of the gene for LcrD which is an essential element of the *Yersinia* type III secretion system (38, 39). WA-ΔlcrD expresses both invasin and the plasmid-encoded adhesin YadA. Furthermore, we utilized *Yersinia* strains that overproduce either YopE or YopP as sole effector Yop. These strains harbor one plasmid encoding the Yop secretion and translocation machinery (pTTSS, in which TTSS is type III protein secretion system) and a second plasmid encoding the YopE or YopP module (35, 40). The resulting strains WA-ATSS-YopE and WA-ATSS-YopP produce YopE or YopP, respectively, but no other effector Yops. To localize yersiniae in cells expressing GFP-LC3 the *Yersinia* strains WA, WA-C, WA-C-Δinv, and WA-ΔlcrD were transfected with plasmid pLAC-RFP which constitutively labels the bacteria with RFP (41). Additionally, we investigated cellular effects induced by the *E. coli* strain HB101-pINV1914 expressing the *Y. enterocolitica* inv gene (*E. coli* inv+*) and by its invasin-negative parent *E. coli* laboratory strain HB101 (42).

**Cells, stimulation, and infection conditions**

Murine J774A.1 macrophages were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS and 5 mM l-glutamine (43). The human embryonic kidney (HEK) 293 cell line and embryonic fibroblast-like G2D5 cells and their derivatives, G2D5B1A cells stably transfected with cDNA expressing the β1 integrin receptor, were cultured in DMEM cell growth medium containing 10% heat-inactivated FCS (44). G2D5 and G2D5B1A cells were kindly provided by Dr. R. Fässler and Dr. T. Sakai (Max-Planck-Institute of Biochemistry, Martinsried, Germany; Ref. 44). Elicited peritoneal macrophages were obtained from female mice 3 days after i. p. inoculation of 10% proteose peptone broth as described (45). Bone marrow cells were prepared from femurs and tibiae of female 6- to 8-wk-old mice and grown in DMEM supplemented with heat-inactivated 10% FCS, 5% horse serum, 2 mM-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 10 ng/ml M-CSF (PeproTech). Medium was changed every 2 days, and bone marrow-derived adherent macrophages were used for infection after 7 days of culture. TLR2-deficient mice provided by Tularik (46) were backcrossed with TLR4-defective C57Bl/6j mice (Charles River Laboratories) to obtain TLR2- and TLR4-double-defective TLR2−/−TLR4−/− mice (45) for macrophage preparation. Human peripheral blood monocytes were isolated and differentiated into macrophages by treatment with GM-CSF (PeproTech) as described previously (43).

For infection, overnight bacterial cultures grown at 27°C were diluted 1/20 in fresh Luria-Bertani broth and grown for another 2 h at 37°C. Shift of the growth temperature to 37°C initializes activation of the *Yersinia* type III secretion machinery for efficient translocation of Yops into the host cell upon cellular contact (38). To equalize and synchronize infection, bacteria were normally seeded on the cells by centrifugation at 400 × g for 5 min with a quantity of 20 bacteria per cell, unless stated otherwise. For incubation times longer than 90 min, bacteria were routinely killed by addition of gentamicin (100 μg/ml) after 90 min of infection to prevent bacterial overgrowth. Where indicated, the cells were treated with one of the following compounds 30 min before infection, unless stated otherwise: 50 nM bafilomycin A1 (Tocris Bioscience), 75 μM chloroquine (Sigma-Aldrich), 20 μM cytochalasin D (Sigma-Aldrich), 10 μg/ml E64d (Sigma-Aldrich), 1 μg/ml LPS from *E. coli* O55:B5 (Sigma-Aldrich), 10 mM 3-methyladenine (3-MA; Sigma-Aldrich), 10 μg/ml pepstatin A (Sigma-Aldrich), 50 μM PPI (Tocris Bioscience), 50 μg/ml rapamycin (Calbiochem, EMD), 50 μM trypstatin AG 1478 (Sigma-Aldrich). Anti-β1 integrin/CD29-blocking (clone Ha2/5) and isotype control Abs (BD Biosciences), or the Rhodamine-phosphate-inactivating *Clostridium difficile* toxins B and B-F (47, 48) were administered to J774A.1 macrophages 1 or 3 h before infection, respectively. The purified clostridial toxins were provided by Dr. H. Genth (Institute for Toxicology, Hannover Medical School, Hannover, Germany). Cells were always maintained at 37°C in a humidified atmosphere (5% CO2).

**Western immunoblotting**

For assessment of the cellular LC3 levels and the conversion of naive LC3-I to processed and lipidated LC3-II, cells were solubilized with radioimmunoprecipitation buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), and phosphatase and protease inhibitors (Roche). The lysates were cleared by centrifugation, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with a rabbit polyclonal Ab directed against LC3B (Cell Signaling Technology). LC3-I is less sensitive to detection by this Ab than is lipidated LC3-II. This is the case for many anti-LC3 Abs (7). Use of the anti-LC3 Ab therefore facilitates the detection of LC3-II in Western blots. Immunofluorescence experiments indicative of autophagic processes. Immunoreactive bands were visualized using an appropriate secondary Ab and ECL detection reagents (Amersham Pharmacia Biotech). Equal protein loading of the gels was controlled by detecting actin with...
mouse mAb (Millipore) in the cellular lysates. Data are from one experiment representative of at least three performed. Phospho-specific anti-p38 immunoblotting was performed as described above using mAb directed against phosphorylated Thr<sup>180</sup> and Tyr<sup>182</sup> of p38 (Cell Signaling Technology). The total cellular pool of p38 was labeled by stripping the membrane and successive immunoblotting with global anti-p38 Ab (Cell Signaling Technology).

**Fluorescence microscopy of LC3**

To assess intracellular GFP-LC3 localization, J774A.1 cells were transiently transfected with a pEGFP-LC3 construct kindly provided by Drs. J. C. Howard and S. Martens (Institute for Genetics, University of Cologne, Cologne, Germany; Ref. 49). J774A.1 cells were grown on glass coverslips and transfected with ExGene 500 (Fermentas) following the manufacturer’s instructions (35). Twenty hours posttransfection, cells were infected with *Yersinia* strains. Bacteria were killed by addition of gentamicin 90 min after onset of infection. Following additional incubation periods, cells were washed with PBS to remove nonadherent and nonphagocytosed bacteria, fixed with 4% paraformaldehyde, mounted in Mowiol containing 0.18% p-phenylenediamine (Sigma-Aldrich) as anti-fading reagent, sealed with nail polish, and visualized by confocal microscopy (Zeiss Axiovert 200M) using Volocity 4.3 software (Improvision). The mean numbers of cells displaying at least five autophagic aggregates or vacuoles upon *Yersinia* infection vs total numbers of cells were microscopically quantified from three experiments.

For immunofluorescence localization of endogenous LC3, cells were seeded on glass coverslips in 24-well plates 1 day before infection. After 3–4 h of infection, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with ice-cold acetone for 5 min, blocked with 2% normal goat serum and 4% BSA in PBS, and incubated with anti-LC3B (Cell Signaling Technology) followed by Alexa 488-labeled anti-GFP IgG (Molecular Probes). The anti-LC3B Ab preferentially labels autophagosome-associated LC3-II (7). Coverslips were mounted in Mowiol and processed for confocal microscopy as indicated above.

**TEM**

Cell suspensions were transferred into cellulose capillary tubes (50), fixed with 2.5% glutaraldehyde in PBS, washed, and postfixed for 30 min with 1% OsO<sub>4</sub> in PBS. For ultrathin sectioning, the samples were gradually dehydrated with ethanol and embedded in Epon resin. Ultrathin sections were cut with a LKB Ultratome III and poststained with 2% uranyl acetate and lead citrate. The sections were examined with a Philips CM 120 transmission electron microscope at 80 kV, and a MultiScan 794 camera (Gatan) was used for acquisition of electron micrographs.

**Quantification of bacteria-cell interaction, bacterial uptake, and killing**

To discriminate between intra- and extracellularly located bacteria, *yersiniae* were stained usually 1 h after onset of infection by applying a double-immunofluorescence technique as described (38, 51). Briefly, extracellularly located bacteria were marked first with anti- *Y. enterocolitica* O8 rabbit serum (SIFIN) and visualized with Alexa 568-labeled anti-rabbit IgG (Molecular Probes). This technique allows determination of the numbers of both cell-associated (red and green fluorescence) and phagocytosed (exclusively green fluorescence) bacteria. For every strain investigated, three separate experiments were performed, and 100 cells from each experiment were analyzed under a fluorescence microscope. Mean percentages of phagocytosed vs total numbers of bacteria per cell were determined.

For evaluation of the bacterial killing rates, J774A.1 macrophages were infected as indicated. Multiplication of the bacteria was stopped after 90 min of infection by the addition of the bacteriostatic antibiotic tetracycline (2 μg/ml). Following a total incubation time of 3.5 h, the entire samples containing both macrophages and bacteria were treated on ice with Triton X for 30 min. Bacteria were counted after 36 h of incubation at 26°C. Control samples, fed with the same input of bacteria but containing no macrophages, were treated in parallel under the same conditions. The killing rates were quantified as the mean percentages of killed bacteria with respect to the total number of bacteria ± SD from four independent experiments.

**Results**

**Avirulent plasmid-cured *Y. enterocolitica* induces autophagy in macrophages**

To assess whether *Yersinia* could influence autophagy, we analyzed the processing and accumulation of LC3-II in *Y. enterocolitica*-infected J774A.1 macrophages. The conversion of LC3-I to lipidated LC3-II is a hallmark of autophagy and indicates autophagosome formation (7, 8, 10, 52). Lipidated LC3-II can be distinguished from the nonlipidated LC3-I form by higher electrophoretic mobility. It was revealed that the avirulent, plasmid-cured *Yersinia* strain WA-C triggered the generation of LC3-II in a time-dependent manner starting 2 h after onset of infection (Fig. 1). Treatment with chloroquine served as positive control mediating LC3-II accumulation through the blockade of lysosomal degradation (Fig. 1, right). The conversion of LC3-I to LC3-II was not observed in either uninfected cells or in cells infected with the wild-type strain WA which harbors the virulence plasmid-encoded *Yersinia* type III protein secretion system. This indicates that type III protein secretion may prevent the induction of autophagy otherwise conferred by avirulent, plasmid-cured *yersiniae*.

An alternative method to monitor induction of the autophagic pathway is to study the localization of LC3 in stimulated cells (7, 8, 10, 52). The conversion of LC3-I to LC3-II correlates with the recruitment of LC3 to the autophagosomal membrane. GFP-fused LC3 is therefore widely used as a tool to label and detect autophagic organelles (7, 8, 10, 52). J774A.1 macrophages were transfected with a plasmid encoding enhanced GFP-tagged LC3 and localization of GFP-LC3 upon *Yersinia* infection was analyzed. Fig. 2A shows that GFP-LC3 accumulated in punctate dots in the cytoplasm of macrophages infected with the avirulent strain WA-C which is typical for autophagosome formation. Efficient accumulation of GFP-LC3 in aggregates was marginally detected in uninfected and infected cells and during infection with the wild-type strain WA (Fig. 2A). After 3 h of infection with WA-C, GFP-LC3 additionally localized in circular structures (Fig. 2A). This suggests that GFP-LC3 redistributes to intracellular vacuoles containing ingested bacteria. We labeled bacteria with RFP and found that GFP-LC3 actually accumulated around WA-C in the infected cells, starting ~2 h after onset of infection (Fig. 2, B and C). This indicates that GFP-LC3 is directed to WA-C-containing phagosomes after autophagy induction. The decoration of the phagosomes membrane with GFP-LC3 may result from fusion events between LC3-positive punctate aggregates and bacteria-comprising membranous compartments. No comparable manifest redirection of cytoplasmic fluorescent GFP-LC3 occurred upon infection with WA (Fig. 2, A

**FIGURE 1.** Virulence plasmid-cured WA-C triggers LC3-II conversion in J774A.1 macrophages. J774A.1 macrophages were left untreated (ø), or infected with the virulence plasmid-cured *Y. enterocolitica* strain WA-C or the wild-type strain WA. At the denoted times after onset of infection, cellular lysates were prepared and subjected to immunoblotting using anti-LC3 Ab. Application of chloroquine (75 μM) served as a positive control, triggering LC3-II accumulation within 3.5 h. Equal loading of the gel with cellular lysates was controlled by successive immunoblotting against actin.
and B). The impaired reallocation of GFP-LC3 correlated with less efficient uptake of WA compared with WA-C (Figs. 2D and 3A). Engulfment of the bacteria by J774.A1 cells was determined by using a double-immunofluorescence method that allowed the discrimination of intra- and extracellular bacteria (38, 51). After 1 h of infection, 82 ± 9% bacteria of WA-C were internalized in contrast to 2.9 ± 1% bacteria of WA (Fig. 3A). Uptake of the bacteria was followed by a remarkable quantitative increase in the appearance of GFP-LC3-positive puncta or vesicles (Fig. 3B). Accordingly, 89.2 ± 5% of WA-C-infected cells showed such autophagy-related structures after 3.5 h (p < 0.0001 in relation to noninfected cells), whereas only a minor increase was recorded for WA (36.3 ± 7%; p < 0.05). Thus, the uptake of WA-C precedes autophagosome formation and recruitment of LC3 to bacteria-containing phagosomes which is paralleled by the transition of LC3-I to LC3-II (Fig. 1).

We additionally analyzed the behavior of endogenous LC3 in Yersinia-infected macrophages by immunofluorescence microscopy (Fig. 4A). The Ab used shows stronger reactivity with lipidated LC3-II, compared with LC3-I and therefore preferentially labels autophagosome-associated LC3-II over cytoplasmic LC3-I (7). Some aggregates of endogenous LC3 typical for autophagosomes were detected in uninfected cells. WA-C infection substantially increased the formation of autophagosomes, whereas less autophagosomal structures were seen after infection with WA (Fig. 4A). Comparable results were obtained on primary human monocyte-derived macrophages (data not shown). The staining of endogenous LC3 consequently confirms the generation of autophagosome-like puncta observed after GFP-LC3 overexpression. The LC3-positive aggregates accumulated adjacent to WA-C, but they did not uniformly enclose the bacteria-bearing vesicles as did GFP-LC3 in the later stages of infection (Fig. 4A vs Fig. 2, B and C). Normally, intravesicular LC3 is rapidly degraded in the autolysosomes after autophagosome-lysosome fusion and cytosoically orientated LC3 is removed from the outer autophagosomal membrane during maturation (8, 52, 53). The rapid turnover of endogenous LC3 could prevent sustained LC3 integration into the autophagosomal compartments. Overexpressed GFP-LC3 could be more stable (9) allowing manifest visualization of LC3 redistribution. Together, these data demonstrate that virulence plasmid-cured yersiniae trigger activation of the autophagic pathway in macrophages which is suppressed by the wild-type strain.

Yersinia invasin essentially contributes to the induction of autophagy

We next attempted to define the bacterial components involved in the induction of autophagy by virulence plasmid-cured yersiniae. Enteropathogenic yersiniae harbor invasin as a prominent outer membrane protein. Invasin mediates adherence to eukaryotic cells by binding to β1 integrins and thereby promotes bacterial uptake into host cells which is accompanied by a proinflammatory reaction (25–27, 54). We compared the autophagic responses induced by WA-C and the invasin-negative mutant WA-C-Δinv. WA-C-Δinv was unable to trigger LC3-II accumulation (Fig. 4B); concomitantly, LC3 did not efficiently aggregate to autophagosomal punctate structures or vacuoles in WA-C-Δinv-infected cells (Figs. 2B and 3B). The impaired autophagic response correlated with less efficient uptake of WA-C-Δinv compared with WA-C. After 1 h of infection, 2.5 ± 5% of bacteria of WA-C-Δinv were internalized, in contrast to 82 ± 9% of WA-C (Fig. 3A). These results identify the invasin protein as important component of Yersinia to trigger autophagy in infected macrophages. The autophagic events followed invasin-mediated cell invasion. We included the compound 3-MA in the Western blot experiments (Fig. 4B). 3-MA acts as
Invasin-dependent phagocytosis precedes increased formation of autophagy-related structures. A, Quantification of bacterial uptake. J774A.1 macrophages were infected with the different \textit{Yersinia} strains at a ratio of 7.5 bacteria per cell for 60 min. The infected cells were then processed by double-immunofluorescence staining to distinguish intra- from extracellular yersiniae. Mean percentages of ingested vs total cell-associated bacteria were determined by analyzing 100 cells from each experiment by fluorescent microscopy. Results are expressed as means from three experiments ± SD. B, Quantification of GFP-LC3-positive, autophagy-related cellular structures. J774A.1 macrophages were transfected with the GFP-LC3 expression plasmid and infected 20 h later with red-fluorescent protein-expressing WA-C, wild-type WA, invasin-negative WA-C-\textit{inv}, or the LcrD mutant deficient in the secretion of all effector Yops (WA-\textit{ΔlcrD}). The number of infected cells exhibiting GFP-LC3-positive aggregates or vesicles was quantified microscopically after 1.5 and 3.5 h of infection. Only cells displaying at least five GFP-LC3-labeled autophagic organelles were included. Means and SDs were calculated from three independent experiments.

Several publications describe the induction of autophagy by TLR receptors (16, 21–23). We therefore asked whether TLR signaling could also be critical for invasin-dependent autophagy. The invasin-negative mutant WA-C-\textit{Δinv} has been demonstrated to act as effective activator of TLR2 and TLR4 (45). However, this strain was in our experiments unable to trigger autophagy in J774A.1 macrophages (Figs. 2B, 3B, and 4). In the same manner, the \textit{E. coli} laboratory strain HB101 did not confer LC3-II accumulation (Fig. 4B, right), unless it was genetically engineered to produce \textit{Y. enterocolitica} invasin (\textit{E. coli}-inv\textsuperscript{+}). These results suggest a major role of invasin in \textit{Yersinia}-induced autophagy that must be differentiated from the autophagic events reported for TLR stimulation. In fact, the treatment of J774A.1 macrophages with LPS did not provoke comparable LC3-II accumulation (Fig. 5A) or GFP-LC3-positive autophagosome formation (data not shown) within the investigated time frame of 3.5 h. When J774A.1 macrophages were exposed to LPS for 16 h, a mild but detectable accumulation of LC3-II became apparent (Fig. 5B). Thus, LPS seems to induce a delayed and reduced autophagic response compared with invasin-bearing bacteria. We furthermore assessed whether the long-term exposure of J774A.1 macrophages to LPS could influence \textit{Yersinia}-dependent autophagy. Prolonged LPS treatment desensitizes macrophages and renders the cells tolerant to subsequent TLR activation and signaling. Accordingly, J774A.1 cells treated with LPS for 16 h no longer responded with p38 phosphorylation (Fig. 5B, bottom) or NF-\kappaB activation (57) to LPS stimulation or \textit{Yersinia} infection. However, despite the blockade of TLR signaling, WA-C significantly induced the accumulation of LC3-II in LPS-tolerized cells (Fig. 5B). Moreover, WA-C triggered the conversion of LC3-I to LC3-II in peritoneal and bone marrow-derived macrophages defective for TLR2 and TLR4 (Fig. 5C). Together, these results indicate that the induction of autophagy by \textit{Yersinia} largely occurs independently from TLR signaling. Rather, \textit{Yersinia} invasin appears to play a prominent role in autophagy activation. Invasin thereby needs to be bacterial surface-exposed because recombinant invasin applied as GST fusion protein to J774A.1 macrophages was not able to trigger a comparable autophagic response (data not shown). This feature is related to the known proinflammatory activity of invasin that depends on the clustering of host cell receptor by corpuscular invasin, but is not mediated by soluble invasin protein (42).

\textit{Yersinia}-induced autophagy involves engagement of \(\beta_1\) integrin receptors

\textit{Y. enterocolitica} invasin binds and activates eukaryotic cells specifically via \(\beta_1\) integrin receptors (26, 27, 54). We attempted to characterize the induction of autophagy by \(\beta_1\) integrins in response to \textit{Yersinia} infection in more detail. For this purpose we investigated a second \textit{Yersinia} strain that expresses the virulence plasmid-encoded YadA additionally to invasin. YadA activates cellular \(\beta_1\) integrin receptors and promotes bacterial uptake into host cells akin to invasin, but receptor interaction is indirect in this case and occurs via linkage with extracellular matrix components, such as collagen or laminin (58–61). WA-\textit{ΔlcrD} bears the virulence plasmid but is impaired in Yop secretion because of deficiency in LcrD, an essential component of the \textit{Yersinia} type III protein secretion system (39). WA-\textit{ΔlcrD} induced GFP-LC3 recruitment similar to WA-C (Fig. 3B). The conversion of LC3-II was even more pronounced after WA-\textit{ΔlcrD} infection (Fig. 6A). WA-C and WA-\textit{ΔlcrD} triggered the transition of LC3-I to LC3-II also in HEK293 cells (Fig. 6A, right). Wild-type WA inhibited LC3-II accumulation in HEK293 cells similar as in macrophages (Fig. 6A, right). This indicates that the mechanisms of autophagy induction and inhibition by \textit{Yersinia} are operative and overlapping in several cell types. However, we focused in our study on interaction of \textit{Yersinia} with macrophages which are, compared with epithelial cells, the primary target cell type of \textit{Yersinia} virulence (33).

We characterized several pharmacological inhibitors of \(\beta_1\) integrin signaling events on their influences on autophagy induced by WA-C and WA-\textit{ΔlcrD}. \(\beta_1\) integrins are linked to intracellular signaling by the activation of Src family tyrosine kinase as early invasin- and YadA-mediated signaling event (58, 59, 61–64).
Invasin-bearing bacteria induce accumulation of endogenous LC3 and conversion of LC3-II in macrophages. A. Aggregation of endogenous LC3 in autophagy-related puncta following WA-C infection. J774A.1 macrophages were infected with avirulent WA-C, wild-type WA, or the invasin-deficient WA-C mutant WA-C-Δinv. The strains harbored a plasmid allowing constitutive red-fluorescent protein expression. The cells were processed for immunofluorescence staining of endogenous LC3 using polyclonal Ab that preferentially labels autophagosome-associated LC3-II. To denote bacteria-containing vacuoles that are surrounded by autophagosomal LC3-positive punctate dots. Light microscopic images superimposed in the background support the demarcation of cell boundaries and adjacent cells. Images in A have identical magnifications. B. LC3-II accumulation following infection with invasin-bearing bacteria. J774A.1 macrophages were left untreated (ø) or infected with virulence plasmid-cured WA-C or the invasin-deficient WA-C mutant WA-C-Δinv. The autophagy inhibitor 3-MA (10 mM) was coadministered during WA-C infection where indicated. Right. Infections were performed with control E. coli HB101 (E. coli), or its derivative producing Y. enterocolitica invasin (E. coli-inv”). Cell lysates were prepared 3.5 h later and subjected to immunoblotting using anti-LC3 Ab. Equal loading of the gel with cell lysates was controlled by successive immunoblotting against actin.

Treatment of J774A.1 macrophages with the general protein tyrosine kinase inhibitor tyrphostin or the selective Src family kinase inhibitor PP2 reduced WA-C- and WA-ΔctrD-dependent LC3-II accumulation without decreasing bacterial adherence (Fig. 6B; Refs. 63 and 64, and data not shown). This suggests that the activity of Src is important for the induction of autophagy after β1 integrin ligation by Yersinia. Because Src also controls cell invasion by yersiniae, we used cytochalasin D to assess the role of bacterial internalization on autophagy induction. Cytochalasin D depolymerizes F-actin and thereby prevents bacterial cell invasion (58, 59, 61). This drug similarly inhibited the conversion of LC3-II triggered by WA-C and WA-ΔctrD (Fig. 6B). Together, these results suggest that the autophagic responses to Yersinia depend on β1 integrin-mediated bacterial uptake. A critical role of β1 integrins in controlling invasin-dependent phagocytosis and autophagy was confirmed by applying a β1 integrin-blocking mAb to Yersinia-infected J774A.1 macrophages. Preincubation of the cells with the anti-β1 integrin/CD29-blocking Ab substantially prevented the accumulation of LC3-II following WA-C infection (Fig. 6C). Isotype control Abs did not display an inhibitory effect. Neither Ab interfered with chloroquine-dependent LC3-II accumulation (data not shown). The uptake of WA-C (81.4 ± 7% phagocytosis) was also reduced by the anti-β1/CD29-integrin Ab (15.5 ± 4% phagocytosis) but not the isotype control (79.8 ± 7% phagocytosis). Thus, β1 integrins control phagocytosis and autophagy in macrophages infected with invasin-bearing yersiniae.
Tophagic responses result from enforced autophagy induction or Yersinia-induced autophagy. A with anti-integrin/CD29-blocking or isotype control Ab (100 μg/ml) in FCS-free medium for 1 h. Thereafter, the medium was replaced by FCS-containing medium, and cells were infected with WA-C as indicated. Cells were left untreated (ø) or were infected with WA-C or WA-ΔlcRD or stimulated with LPS, and processed for LC3 immunoblotting using anti-LC3 Ab. Where indicated, the Src kinase inhibitor PP2, tryphostin, and cytochalasin D, J774A.1 macrophages were subjected to immunoblotting using anti-LC3 Ab. To analyze the invasin and YadA-expressing yersiniae in J774A.1 macrophages and HEK293 cells. Cells were left untreated (ø) or were infected with WA-C or WA-ΔlcRD producing both invasin and YadA but impaired in type III protein secretion as indicated. Cell lysates were prepared 3.5 h after onset of infection and subjected to immunoblotting using anti-LC3 Ab.

**Figure 6.** Interference with β₁ integrin-related activities counteracts Yersinia-induced autophagy. A, LC3-II accumulation triggered by invasin- and YadA-expressing yersiniae in J774A.1 macrophages and HEK293 cells. Cells were left untreated (ø) or were infected with invasin-expressing WA-C, invasin-deficient WA-C Δinv, wild-type WA, or WA-ΔlcRD producing both invasin and YadA but impaired in type III protein secretion as indicated. Cell lysates were prepared 3.5 h after onset of infection and subjected to immunoblotting using anti-LC3 Ab. B. Autophagy inhibition by PP2, tryphostin, and cytochalasin D. J774A.1 macrophages were infected with WA-C or WA-ΔlcRD or stimulated with LPS, and processed for LC3 immunoblotting as in A. Where indicated, the Src kinase inhibitor PP2 (50 μM), the general tyrosine kinase inhibitor tryphostin (try.; 50 μM), or the actin filament-depolymerizing agent cytochalasin D (cyt-D; 20 μM) were coadministrated during infection. Chloroquine (75 μM) served as positive control. C. Impairment of LC3-II accumulation by anti-β₁ integrin-blocking Abs. J774A.1 macrophages were left untreated (ø), or incubated with anti-β₁ integrin/CD29-blocking or isotype control Ab (100 μg/ml) in FCS-free medium for 1 h. Thereafter, the medium was replaced by FCS-containing medium, and cells were infected with WA-C as indicated. Cells were processed for LC3 immunoblotting as in A, D. LC3-II accumulation without disruption of the autophagic flux. J774A.1 macrophages were left untreated (ø), or infected with WA-C or WA-ΔlcRD. Where indicated, the lysosomal protease inhibitors E64d and pepstatin A ( pep. A; both 10 μg/ml) were coadministrated. Cells were then processed for LC3 immunoblotting as in A. The levels of LC3-II were increased in inhibitor-treated cells, suggesting that the dynamic turnover of LC3-II was not affected by infection with WA-C or WA-ΔlcRD. Equal loading of the gels with lysates was controlled by actin immunoblotting.

We furthermore checked whether the β₁ integrin-dependent autophagic responses result from enforced autophagy induction or from the blockade of final autophagic steps that mediate the degradation of LC3-II in the autolysosome along with the autophagosomal cargo. Both principles can be responsible for the intensification of autophagy (7, 53, 65). We treated J774A.1 cells with E64d and pepstatin A, two inhibitors of lysosomal proteases (7, 53, 65). Application of these compounds increased the LC3-II levels in WA-C- and WA-ΔlcRD-infected cells (Fig. 6D). This suggests that Yersinia-induced LC3-II is physiologically destabilized by autolysosomal formation. Thus, the accumulation of LC3-II following Yersinia infection does not result from interruption of the autophagic flux, but originates from the enforced activation of autophagy after β₁ integrin induction.

To additionally corroborate a role of β₁ integrins in Yersinia-related autophagy, we took advantage of the fibroblast-like cell line GD25, which is derived from β₁ integrin-deficient embryonic stem cells and impaired in β₁ integrin expression (44). The GD25 cells were analyzed for LC3-II accumulation in response to infection with avirulent yersiniae along with GD25B1A cells stably transfected with a wild-type β₁A integrin splice variant. In these experiments, the LC3-II levels increased in GD25B1A cells following infection with WA-C and were pronounced after infection with WA-ΔlcRD, compared with noninfected cells (Fig. 7A). The LC3-II levels did not change upon Yersinia infection in the parent GD25 cell line (Fig. 7A). Chloroquine was used as positive control, triggering LC3-II conversion in both cell lines. In the same manner, the invasin-bearing E. coli strain elicited accumulation of LC3-II in GD25B1A cells, but not in GD25 cells (Fig. 7A). The autophagic responses induced by the different strains in GD25B1A cells again correlated with increased bacterial internalization (93.6 ± 2% uptake of WA-C after 3 h, 96.2 ± 2% WA-ΔlcRD, 92.6 ± 2% E. coli-inv+, vs 5.1 ± 3% WA-C-Δinv and 6.8 ± 3% E. coli). These results demonstrate that the activation of autophagy by Yersinia specifically involves the engagement of β₁ integrin receptors and β₁ integrin-mediated bacterial uptake. The GD25 cell line lacking β₁ integrins displayed some basal LC3-II accumulation under nonstimulatory conditions which was even more obvious after 36 h and not apparent for β₁ integrin-expressing GD25B1 cells (Fig. 7B). This result may fit to the previously described observation that lack of integrin engagement and reduced attachment to the extracellular matrix confer autophagy activation in epithelial cells (66). This suggests that β₁ integrins could possibly serve opposed autophagy-related functions depending on the cellular context. They may counteract autophagic processes when providing attachment to extracellular matrix components (66) but promote autophagy during phagocytosis.

Yersinia-mediated, β₁ integrin-linked autophagy involves autophagosome formation and xenophagy

To analyze the invasin and β₁ integrin-dependent autophagic processes at the ultrastructural level, J774A.1 macrophages were infected with plasmid-cured WA-C and characterized by TEM. WA-C was found to be internalized by J774A.1 cells. Most of the bacteria resided intracellularly 3 h postinfection. Furthermore, multiple double-membrane-bound vacuoles were observed in the surroundings of WA-C-containing phagosomes (Fig. 8, A–C). Some of the vesicles were characterized by an electron density equivalent to that of the cytoplasm; others showed increased electron densities indicative of catabolic processes (Fig. 8, A–C). These organelles were reminiscent of macroautophagic vacuoles in various stages of maturation (7, 10). WA-C infection triggered the formation of such autophagosomal vesicles which correspond to the LC3-positive punctate structures detected by fluorescence microscopy. In addition, fusion events between the originated autophagosomes and neighboring phagosomes were conceivable (Fig. 8A, enlarged inset). The formation of such vacuoles was not
obvious in macrophages infected with the wild-type strain WA (Fig. 8D). WA largely resisted phagocytosis and remained predominantly extracellular. If single WA bacteria were internalized no autophagic structures or membranes were in evidence close to the ingested bacteria (data not shown). These data indicate that the ingestion of WA-C largely resisted phagocytosis and remained pre-obvious in macrophages infected with the wild-type strain WA (Fig. 8D).

TEM furthermore revealed that 15–20% of the ingested WA-C bacteria were segregated from the cell cytoplasm by characteristic multilamellar structures (Fig. 8C). The appearance of such multimembrane-bound vesicles is compatible with the enclosure of single, WA-C-encompassing phagosomes by autophagic sequestration membranes in terms of xenophagy (7, 10). In fact, these bacteria-containing vesicles comprised several membrane layers including the phagosomal membrane and the inner and outer membranes of a potential autophagic vacuole (Fig. 8C, enlarged inset). Moreover, several flat and double-membrane-bound structures were detected adjacent to WA-C (Fig. 8B, enlarged inset, arrows). These structures could relate to nucleation organelles that generate phagophore and autophagosomal sequestration membranes to enclose the WA-C-harboring vacuole. These TEM results suggest that a significant portion of WA-C-containing phagosomes is subjected to xenophagy. The xenophagic processes appear to occur additionally to the formation of classical, autophagosomal vacuoles close to ingested WA-C.

**FIGURE 7.** Induction of autophagy by *Yersinia* involves engagement of *β*1 integrins. A, *β*1 integrin-dependent LC3-II accumulation mediated by invasin- and YadA-expressing bacteria. Fibroblast-like, *β*1 integrin-deficient GD25 and derivative GD25β1A cells stably transfected with *β*1A integrin receptor were left untreated (ø), or infected with WA-C, invasin-deficient WA-C-Δinv, or WA-ΔinvD producing invasin and YadA but impaired in type III protein secretion. Alternatively, infections were performed with *E. coli* HB101 (*E. coli*), or the invasin-producing *E. coli* derivative (*E. coli-inv*+). Chloroquine (75 μM) was used as positive control. Cell lysates were prepared 8 h after onset of infection and subjected to immunoblotting using anti-LC3 Ab. B, Increased LC3-II accumulation in *β*1 integrin-deficient GD25 cells. GD25 cells and GD25β1A cells expressing *β*1A integrin receptor were seeded in cell culture wells and left untreated (ø) for 36 h. Then, cell lysates were prepared and processed for immunoblotting as in A. Equal loading of the gels with lysates was controlled by actin immunoblotting.

**FIGURE 8.** *β*1 integrin-dependent engulfment of WA-C triggers the formation of autophagic vacuoles. J774A.1 macrophages were infected with virulence plasmid-cured WA-C (A–C) or the wild-type strain WA (D). Cells were fixed and processed for TEM 3 h after onset of infection. Autophagy-related characteristics were visualized in WA-C-infected cells (A–C), but remained inconspicuous after WA infection (D). The arrays in B denote potential autophagosomal membrane assembly sites. Arrays in C indicate phagosomes that are enclosed by double-membranous autophagic vacuoles; outer and inner autophagosomal and phagosomal membranes are highlighted (C, inset). y, *Yersinia*; av, autophagic vacuole; n, nucleus; m, mitochondria.

*β*1 integrin-dependent autophagy contributes to bacterial elimination

Our data show that yersiniae impaired in type III protein secretion trigger several autophagy-related events by the engagement of *β*1 integrin receptors which are suppressed by wild-type Yersinia. It is well established that autophagy assists host immunity by supporting the elimination of intracellular microbes (3, 6, 13, 15, 16, 21, 23, 67–69). To learn more about the functions and consequences of invasin and *β*1 integrin-dependent autophagy, we determined the killing rates of WA-C and the invasin-negative mutant WA-C-Δinv in J774A.1 macrophages treated with 3-MA or bafilomycin A. 3-MA acts as inhibitor of autophagy at the applied concentration (1, 8, 55) that impairs LC3-II accumulation in WA-C-infected macrophages (Fig. 4B). Bafilomycin A inhibits the vacuolar ATPase to prevent lysosomal acidification and fusion of autophagosomes with lysosomes (4, 70). Both drugs only slightly inhibited invasin-dependent uptake of WA-C into macrophages (Fig. 9A). They did not affect cellular viability in the investigated time frames compared with untreated cells (5–10% dead cells). The bacteriostatic antibiotic tetracycline was added to the killing assays 90 min after onset of infection to suppress continual bacterial replication. Cells were then lysed after a total incubation time of 3.5 h, and the numbers of surviving bacteria were estimated by the determination
of colony forming units. Fig. 9B shows that WA-C was efficiently eliminated by J774A.1 macrophages in these experiments, in contrast to WA-C-Δinv. Invasin-dependent invasion of macrophages therefore appears to promote bacterial killing. The application of 3-MA or bafilomycin A significantly attenuated the elimination of WA-C by J774A.1 macrophages (p = 0.0003 and p = 0.017, respectively). Thus, the induction of autophagy and autolysosome maturation apparently restricts the viability of invasin-bearing, ingested bacteria. This suggests that the activation of autophagy following β₁ integrin-dependent engulfment of the bacteria is a host immune reaction that supports bacterial killing in macrophages. This conclusion fits into the concept that autophagy-related processes are central components of antimicrobial host defense reactions (13, 15, 16, 21, 23, 67, 69). Fig. 9B furthermore demonstrates minor, but significant reduction WA-C-Δinv by J774A.1 cells in a 3-MA-sensitive manner (p = 0.023). The elimination of WA-C-Δinv in this case correlated with a slight increase in bacterial internalization within 3 h of infection (13.1 ± 7% phagocytosis compared with 2.5 ± 5% after 1 h; Fig. 3A). Thus, an invasin-independent pathway may mediate limited uptake of WA-C-Δinv and autophagy with delayed and attenuated kinetics.

β₁ integrin-dependent, invasin-related killing of yersiniae by macrophages could be relevant in the initial phases of *Yersinia* infection. The first pool of yersinia invading the host after oral inoculation has been shown to be rapidly eliminated and killed by phagocytes (71). It is considered that clearance of the bacteria under these conditions results from a delay in type III protein secretion (71, 72). The *Yersinia* type III secretion system is not readily active after mucosal invasion, but requires the sensing of a temperature shift from ambient to body temperature to become activated after orogastric infection. Our data show that wild-type WA yersiniae grown at 27°C are less efficient in the suppression of autophagy (Fig. 9C) and phagocytosis (67.4 ± 3% uptake of WA) compared with WA bacteria that were additionally preactivated for 2 h at 37°C (2.9 ± 1% bacterial uptake; Figs. 3A and 9C). The preactivation step at 37°C was routinely done in all other experiments of this study to initialize activation of the *Yersinia* type III secretion machinery (38). Delayed type III secretion and concomitant enhanced sensitivity to phagocytosis and autophagy could critically contribute to the elimination of freshly ingested yersiniae that have not yet fully adapted their virulence arsenal to the modified environmental conditions in the early invasive stages of *Yersinia* infection.

Wild-type *Yersinia* globally suppresses autophagy induction in macrophages by type III protein secretion

In view of the role of autophagy as host defense mechanism, it was consequent that wild-type *Yersinia* WA suppressed autophagy, whereas WA-C and WA-ΔlcrD generated autophagic responses (Fig. 3B). This underscores the importance of Yop secretion and translocation in the repression of autophagy. The prevention of autophagy by WA parallel blockade of bacterial uptake (Fig. 3A), which suggests that autophagy inhibition by wild-type *Yersinia* and phagocytosis resistance are interconnected. The suppression of phagocytosis by *Yersinia* is brought about by the concerted activities of YopE, YopH, YopT, and YopO, and loss of a single Yop can be compensated by others without substantial reduction of phagocytosis resistance (28–31, 38, 73, 74). *Yersinia* mutants deficient for single effector Yops were in the same manner effective in the suppression of autophagy and phagocytosis (data not shown). This implies that the inhibition of autophagy follows blockade of phagocytosis and similarly depends on several Yop

![FIGURE 9](http://www.jimmunol.org/Downloadedfrom/28-Oct-2017)
activities. Although significant restrictions in autophagy inhibition were not detected for single Yop effector mutants, a candidate that regulates macrophage autophagy in response to Yersinia infection.

FIGURE 10. Autophagy inhibition by *Yersinia* involves YopE and the targeting of Rho-GTPases. A, Inhibition of LC3-II accumulation by Yop mutants and Yop-overproducing yersiniae. J774A.1 macrophages were left untreated (ø) or were infected with plasmid-cured WA-C, wild-type WA, WA mutants defective for single (WA-ΔyopP, WA-ΔyopE) or multiple effector Yops (WA-ΔyopP/ΔyopE, WA-ΔyopP/ΔyopO), strain WA-ΔlcrD impaired in any Yop secretion, or *Yersinia* strains overproducing YopE (WA-TTSS-YopE) or YopP (WA-TTSS-YopP) as sole effector Yops. Cell lysates were prepared 3 h after onset of infection and subjected to immunoblotting using anti-LC3 Ab. Equal loading with cell lysates was controlled by actin immunoblotting. B, Impairment of LC3-II conversion by Rho-GTPase-inactivating toxins from *Clostridium difficile*. J774A.1 macrophages were left untreated (ø), or incubated with the Rho-GTPase-inactivating toxins B (1 ng/ml) or B-F (50 ng/ml) from *C. difficile* (C. diff.) for 3 h. Thereafter, the cells were infected with WA-C or WA-ΔlcrD as indicated. Cells were processed for LC3 immunoblotting as in A.

FIGURE 11. Wild-type *Yersinia* globally affects the autophagic response. J774A.1 macrophages were left untreated (ø) or were infected with wild-type WA, plasmid-cured WA-C, or invasin-deficient WA-C-Δinv. Where indicated, chloroquine (75 μM) was administered to the cells together with WA (50%), or 30 (30%) or 60 (60%) min following WA infection. Rapamycin (50 μg/ml) was applied to the cells simultaneously with WA or WA-C-Δinv. Cell lysates were prepared and subjected to immunoblotting 3.5 h after onset of infection, using anti-LC3 Ab. Equal gel loading with lysates was controlled by actin immunoblotting.

The targeting of Rho-GTPases. A, Inhibition of LC3-II accumulation by Yop mutants and Yop-overproducing yersiniae. J774A.1 macrophages were left untreated (ø) or were infected with plasmid-cured WA-C, wild-type WA, WA mutants defective for single (WA-ΔyopP, WA-ΔyopE) or multiple effector Yops (WA-ΔyopP/ΔyopE, WA-ΔyopP/ΔyopO), strain WA-ΔlcrD impaired in any Yop secretion, or *Yersinia* strains overproducing YopE (WA-TTSS-YopE) or YopP (WA-TTSS-YopP) as sole effector Yops. Cell lysates were prepared 3 h after onset of infection and subjected to immunoblotting using anti-LC3 Ab. Equal loading with cell lysates was controlled by actin immunoblotting. B, Impairment of LC3-II conversion by Rho-GTPase-inactivating toxins from *Clostridium difficile*. J774A.1 macrophages were left untreated (ø), or incubated with the Rho-GTPase-inactivating toxins B (1 ng/ml) or B-F (50 ng/ml) from *C. difficile* (C. diff.) for 3 h. Thereafter, the cells were infected with WA-C or WA-ΔlcrD as indicated. Cells were processed for LC3 immunoblotting as in A.

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apoptosis conferred by YopP within 3–5 h of infection (35). The deficiency of YopP alone did not abrogate autophagy inhibition (WA-ΔyopP; Fig. 10A) although preventing apoptosis (Ref. 35 and data not shown). On the contrary, strain WA-TTSS-YopP triggered apoptosis (35) but was unable to suppress autophagy (Fig. 10A). This excludes that *Yersinia* counteracts autophagy only to favor apoptosis induction. Together, these data suggest the involvement of YopE in the suppression of *Yersinia*-triggered autophagy. YopE is a GTPase-activating protein that inactivates Rho-GTPase family members (28, 31). A potential role of Rho-GTPases in the execution of *Yersinia* and β1 integrin-related autophagy was corroborated by applying the *Clostridium difficile* toxins B and B-F on *Yersinia*-infected cells. Clostridial toxins are accepted tools in cell biology to specifically inactivate Rho-GTPases (48). Toxin B glucosylates and inactivates Rho, Rac, and Cdc42, whereas toxin B-F blocks Rac and R-Ras (47, 48). Pretreatment of J774A.1 macrophages with the toxins prevented the accumulation of LC3-II in response to infection with avirulent WA-C and WA-ΔlcrD (Fig. 10B). The viability of the cells was not affected (data not shown). This suggests that YopE and the inactivation of Rho-GTPases could be involved in the blockade of autophagy during *Yersinia* infection. Rac, which is targeted by both clostridial toxins (47, 48) and by YopE (28, 31), is a likely candidate that regulates macrophage autophagy in response to *Yersinia* infection.

However, *Yersinia*-conf erred prevention of autophagy did not seem to be simply consequence of impaired phagocytosis but rather appeared to involve specific targeting of the autophagy pathway. We assessed the impact of *Yersinia* on autophagy elicited by rapamycin and chloroquine, two pharmacological autophagy enhancers (7, 10). J774A.1 macrophages were treated with either drug following infection with the wild-type strain. Rapamycin is a specific inhibitor of mammalian target of rapamycin, a kinase that negatively regulates autophagy. Mammalian target of rapamycin inhibition by rapamycin consequently increases the autophagic flux. Chloroquine inhibits the lysosomal degradation of autophagosomes and promotes autophagosome accumulation. Both autophagy inducers were substantially attenuated in their capacities to trigger LC3-II conversion in WA-infected cells (Fig. 11). Fig. 11 furthermore shows that infection with WA-C-Δinv did not affect rapamycin-dependent autophagy. This indicates that wild-type
Yersinia actively interferes with the induction of autophagy through type III secretion. Global targeting of the cellular autophagic response may aid Yersinia to evade autophagy-related macrophage defense reactions.

Discussion

Autophagy is emerging as an important host defense mechanism against invading microbes. Bacteria, viruses, and parasites can activate autophagy. However, the cellular pathways involved are often not defined (1, 3, 5, 12, 20). In this study we identify a hitherto unknown principle of autophagy induction.

The enteropathogenic bacterium Y. enterocolitica modifies host immune responses by an array of strategies. Yersinia-mediated immunomodulation also targets autophagy in macrophages. Yersinia-deficient in the secretion of Yops efficiently activated autophagy through the engagement of \(\beta_1\) integrin receptors and cell invasion. The induction of autophagy involved the Yersinia adhesins invasin and YadA which cluster \(\beta_1\) integrins to gain access into eukaryotic cells (26, 27, 58). Many bacteria and viruses stimulate \(\beta_1\) integrins (75–77), and this is a first study demonstrating the induction of autophagy as a result of microbial \(\beta_1\) integrin activation. The activation of autophagy could be a general feature of \(\beta_1\) integrin-dependent phagocytosis. It differs from the silencing of autophagy conferred by the binding of integrins to the extracellular matrix which was previously reported (66).

The autophagic response following \(\beta_1\) integrin-induction and uptake of Yersinia was multifaceted. A significant amount of ingested yersiniae was captured in typical, double-membrane-bound autophagic vacuoles. This indicates the execution of xenophagy, a process by which a bacteria-containing phagosome becomes enclosed in an autophagic compartment (7, 11). Furthermore, multiple smaller autophagosomal vesicles were found in close proximity to the bacteria-bearing phagosomes. These vacuoles developed distinctly from xenophagy and seemed to be able to fuse with adjacent phagosomes. A hint for such fusion events was given by the autophagosome marker GFP-LC3 that first localized to autophagic vesicles and then redistributed to WA-C-containing phagosomes. GFP-LC3 efficiently decorated the phagosomal membranes in the later stages of infection even though most of the bacteria were not enclosed by typical autophagic multimembrane layers. A similar phenomenon has been recently reported in the context of TLR signaling. It was shown that GFP-LC3 is recruited to phagosomes that had engulfed latex beads associated with various TLR ligands, but no typical double-membranous structures were observed (16). The underlying mechanisms remained elusive. Fusion events between autophagosomes and phagosomes could explain the integration of GFP-LC3 into phagosomal membranes (3, 5, 6, 16, 17). Autophagosomes can fuse with endosomes which results in the formation of amphisomes that possess single membrane but stain positive for GFP/RFP-LC3 (2, 5, 78). It is speculated that related processes could occur between autophagosomes and phagosomes (3, 5, 6, 17) and may take place also in Yersinia-infected cells. These data show that \(\beta_1\) integrin-mediated engulfment of bacteria triggers multiple autophagy-related reactions. The formation of autophagosomes, xenophagy, LC3 mobilization to phagosomes, and the incorporation of autophagic vesicles into phagosomal compartments can concomitantly take place in response to microbial \(\beta_1\) integrin ligation and phagocytosis.

The activation of autophagy pathways has been previously reported for TLR stimulation (16, 21–23). Avirulent, plasmid-cured yersiniae also possess effective capacity to activate TLR signaling (45). However, Yersinia-mediated autophagy differed from autophagy conferred by TLR stimulation. The treatment of macrophages with LPS or with invasin-negative yersiniae or E. coli did not elicit significant autophagic responses compared with invasin-bearing bacteria. Furthermore, LPS desensitization and TLR silencing in macrophages did not reduce Yersinia-triggered autophagy. Similarly, yersiniae induced autophagy also in TLR2- and TLR4-defective macrophages. In addition, \(\beta_1\) integrin-dependent autophagy was observed also in HEK293 and GD25/\(\beta_1\)A cells that are non- or low-responsive to TLR ligands unless transfected with the respective TLRs and coreceptors (45, 79). These results suggest that the coactivation of TLRs does not critically influence Yersinia-induced autophagy. A comparable observation has lately been made in different subsets of primary macrophages, in which TLR signaling neither triggered substantial autophagosome formation nor influenced Salmonella-induced autophagy (80). The activation of autophagy by microbes therefore seems to be largely defined by the receptors and pathways that mediate cell invasion with minor involvement of TLR signaling. Engulfment of Y. pestis by macrophages was recently reported to show features of autophagy (81). However, Y. pestis does not express functional invasin or YadA (24, 29), which suggests the existence of other, possibly \(\beta_1\) integrin-independent pathways that may specifically link uptake Y. pestis with autophagy induction.

Invasin-mediated internalization is important for the initial translocation of enteropathogenic yersiniae through the intestinal mucosa to gain access to the submucosa of the compromised host (25, 26, 82). Once inside the lymphoid tissue of the Peyers’ patches, the bacteria contact phagocytic cells. Yersinia then activate their type III secretion system to counteract phagocyte antimicrobial activities and to establish a competitive advantage (28–31). Type III protein secretion also mediated the suppression of autophagy in infected macrophages. It is meanwhile well established that autophagy promotes the elimination of pathogenic intracellular microorganisms, as previously shown in example for Mycobacterium, Listeria, or Toxoplasma (13, 15, 21, 23, 67, 69). The induction of autophagy by \(\beta_1\) integrins may in the same manner be deleterious for Yersinia. Pharmacological inhibition of autophagy or of autolysosome maturation increased the survival of avirulent invasin-bearing yersiniae which suggested that the activation of autophagy during engulfment of Yersinia enhances bacterial killing. It has been shown that \(\beta_1\) integrins support phagosome maturation and bactericidal activities of macrophages (82). Our data indicate that this proceeds at least partially through the activation of autophagic pathways. The subversion of autophagy in macrophages may therefore be a means of Yersinia-induced immunomodulation that promotes bacterial survival. The inhibition of autophagy by wild-type Yersinia correlated with impaired bacterial uptake which results from the cooperative action of Yops (28–31). However, prevention of autophagy was not merely consequence of phagocytosis resistance. Wild-type Yersinia also repressed the autophagic responses triggered by rapamycin and chloroquine. This suggests that Yop activities may specifically target autophagy in macrophages to provoke global perturbation of the autophagic networks.

The detailed mechanisms by which \(\beta_1\) integrin-dependent phagocytosis is coupled to autophagy activation and how the Yops could interfere with these signaling pathways are, however, not yet fully understood. The stimulation of \(\beta_1\) integrins by Yersinia activates several signaling intermediates that could also function in autophagy. Invasin-mediated cell invasion involves the recruitment of focal adhesion complex components that link integrin signaling to actin cytoskeleton reorganization.
Some focal adhesion-related proteins have recently been shown to be important for autophagosome formation. The focal adhesion kinase family interacting 200 kDa protein (FIP200) regulates cell size and migration but is required also for autophagosome generation (83). FIP200 interacts with the mammalian Atg1 homolog UNC-51-like kinase 1 and may fulfill scaffold functions like Atg17 in yeast. Another focal adhesion protein essential for autophagy is Paxillin. Paxillin interacts with Atg1 in Drosophila (84). Paxillin is a target of YopH which dephosphorylates invasin-induced paxillin to disrupt focal adhesion complexes (85, 86). Invasin-mediated actin polymerization furthermore involves the Arp2/3 complex (87–89). The remodeling of actin structures by Arp2/3 promotes movement of Atg9 and regulates specific functions of autophagic membranes (90). The activation of Arp2/3 is conversely controlled by Rho-GTPases (91) which are targeted by YopE, YopT, and YopO/YpkA. RhoA may also directly be implicated in autophagy regulation via interaction with Atg17 (92). These findings suggest several critical signaling branches at which Yersinia-mediated cell invasion and deregulation by Yops could overlap with autophagy induction. Our results showed that a Yersinia mutant deficient for YopE and YopP was constrained in its ability to suppress autophagy. On the contrary, a Yersinia strain overproducing YopE as only effector Yop substantially counteracted autophagy. These data suggest critical implication of YopE in the inhibition of autophagy by Yersinia. YopE exerts a GTPase-activating protein activity that impairs several Rho-GTPases including Rac (28, 31). The application of clostridial toxins that display inhibitory activity on Rac also prevented Yersinia-conferred autophagy. These findings point out a critical role of Rac in the regulation of β1 integrin-dependent autophagy that is suppressed by wild-type Yersinia. NADPH oxidases and the production of reactive oxygen species have recently been shown to participate in the activation of autophagy upon engagement of TLRs or FcγRs in phagocytic processes (93). Rac molecules are essential subunits of NADPH oxidase complexes (94). The repression of autophagy by Yersinia YopE may therefore be accomplished by hindering functional NADPH oxidase assembly through the targeting of Rac. Several Yops affect Rho-GTPase and NADPH oxidase activities in Yersinia-infected cells and may contribute besides YopE to the blockade of autophagy by the wild-type strain. More detailed future studies will help to specify the β1 integrin-induced autophagic pathways and how they are compromised by individual Yersinia type III effectors.

In conclusion, Yersinia gives a new example for a bacterial pathogen that modulates cellular pathways of autophagy. Our results show that β1 integrin receptors are decisively involved in the activation of Yersinia-dependent autophagy. β1 integrin-mediated phagocytosis was directly linked to autophagy induction. This opens up new perspectives in studying the functions and consequences of integrin stimulation. Many bacteria and viruses activate host cells via integrin receptors (75–77). The sensing of microbes by β1 integrins may therefore be a general principle that serves to induce autophagy and to promote the elimination of extracellular pathogens. This supports the concept that integrins may fulfill functions akin to pattern recognition receptors in providing protective immunity after having detected a pathogen (95–97). Y. enterocolitica could possibly benefit from the suppression of autophagy by the Yop arsenal which may indicate an important new aspect of Yersinia-induced immunomodulation.

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References


