**Staphylococcus aureus** Subvert Autophagy for Induction of Caspase-independent Host Cell Death

**The Journal of Biological Chemistry**

Received for publication, October 17, 2006, and in revised form, November 14, 2006 Published, JBC Papers in Press, November 29, 2006, DOI 10.1074/jbc.M609784200

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**Staphylococcus aureus** is a common bacterial etiology of serious infectious diseases. *S. aureus* can invade various types of non-professional phagocytes to produce host cell death. We show here that shortly after invasion of HeLa cells *S. aureus* transit to autophagosomes was characterized by double membranes and co-localization with LC3. *S. aureus* were not able to replicate and produce cell death in autophagy-deficient *atg5*–/– mouse embryonic fibroblasts. *S. aureus*-containing autophagosomes do not acidify nor do they acquire lysosome-associated membrane protein-2, indicating that *S. aureus* inhibits autophagosome maturation and fusion with lysosomes. Eventually, *S. aureus* escape from autophagosomes into the cytoplasm, which results in caspase-independent host cell death. *S. aureus* strains deficient for *agr*, a global regulator of *S. aureus* virulence, were not targeted by autophagy and did not produce host-cell death. Autophagy induction by rapamycin restored both replication and cytotoxicity of *agr*-deficient *S. aureus* strains, indicating that an *agr*-regulated factor(s) is required for autophagy-mediated cytotoxicity. The results of this study suggest that rapid induction of autophagy is essential for *S. aureus* replication, escape into the cytoplasm, and host cell killing.

*S. aureus* is the major cause of community-acquired and nosocomial infections such as pneumonia, endocarditis, osteomyelitis, and wound infections (1, 2). An important feature of *S. aureus* is the ability to invade the vascular system from local infection sites (3). Such dissemination includes passing across cellular barriers like the endothelial barrier, which leads to bacteremia and sepsis. Recent studies revealed the consistent ability of *S. aureus* to infect various types of non-professional phagocytic host cells such as keratinocytes, fibroblasts, endothelial cells, and epithelial cells (4–6). Adherence to and invasion of non-professional phagocytic cells by *S. aureus* has been implicated in the pathogenesis of invasive and metastatic infections i.e. during hematogenous dissemination (7).

We have recently shown that some but not all *S. aureus* strains are able to induce host cell death after invasion, which correlates with the virulence of a particular *S. aureus* strain (8). However, the exact molecular mechanisms leading to intracellular survival of *S. aureus* and death of host cells remained unclear. Although some investigators reported induction of caspase-dependent programmed cell death (apoptosis) involving tumor necrosis factor or CD95 signaling pathways, others observed necrosis of *S. aureus*-infected host cells induced by α-toxin (9–11).

In many cases microorganisms internalized by host cells are efficiently eliminated by host defense mechanisms. The microbially phagosome matures by sequential transient fusion events with early and late endosomal compartments, which are controlled by Rab GTPases (12). However, some pathogens like *Mycobacteria tuberculosis*, *Legionella pneumophila*, and *Brucella abortus* have evolved species-specific mechanisms of subverting host vesicle trafficking to prevent fusion of the phagosome with the lysosomes. Other bacteria like *Shigella flexneri*, *Listeria monocytogenes*, and *Rickettsia* spp. can escape from phagosomes into the cytoplasm, multiply, and even disseminate into neighboring cells by eliciting actin polymerization (13). *S. aureus* are able to escape the phagolysosomal pathway, which is prerequisite for intracellular bacterial survival and killing of the eukaryotic host cell (14).

In the case of an inefficient phagosomal degradation pathway, infected host cells are capable of combating intracellular pathogens by a process called autophagy, which involves bulk degradation of cytoplasmic components. Autophagy is a conserved membrane-traffic pathway in eukaryotic cells that sequesters cytoplasmic contents by double membranes and eventually delivers them to lysosomes. Autophagy is crucially involved in physiological processes such as cellular homeostasis, morphogenesis, cellular differentiation, tissue remodeling, and antigen presentation but also in response to various stress conditions such as nutrient or insulin limitation (15). Autophagy can be induced by pharmacological means such as rapamycin, an inhibitor of the serine/threonine kinase Tor (target of rapamycin). On the other hand inhibition of the type III phosphatidylinositol 3-kinase by wortmannin or 3-methyladenine results in the reduction of autophagy (16). At the molecular level, a series of factors conserved from yeast to man is involved in sequential steps of autophagy (17). Some of the best-defined markers of autophagy are the Atg5 protein that is associated with the nascent isolation membrane and the microtubule-associated protein 1 light chain (LC32/Atg8) (18). The presence of Atg5 and its proper conjugation with the ubiquitin-like mole-

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8 This work was supported by the Deutsche Forschungsgemeinschaft Grant SFB 670. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: LC3, light chain; z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethyl ketone; PBS, phosphate-buffered saline; CFU, colony-forming units; LAMP-2, lysosome-associated membrane protein-2; m.o.i., multiplicity of infection; PIPES, 1,4-piperazinediethanesulfonic acid; GFP, green fluorescent protein; EGFP, enhanced GFP; MEF, mouse embryonic fibroblasts.
cule Atg12 are specifically required for the elongation of the autophagic isolation membrane. Disruption of the atg5 gene in mice resulted in the absence of autophagy leading to reduced amino acid concentrations in plasma and tissues, energy depletion, and death within 24 h postpartum (19). LC3/Atg8, the only highly specific marker of the autophagosome, undergoes C-terminal proteolytic processing and conjugation with phosphatidylethanolamine, upon which it translocates from the cytosol to the autophagosomal membrane (20).

Recent studies demonstrated a role for autophagy in host cell defense against Streptococcus pyogenes. Once within the cytosol, autophagy targets this pathogen and effectively restricts its growth (21). Metabolically arrested L. monocytogenes are also trapped by autophagosomes and undergo lysosomal degradation (22). Rickettsia spp. have been observed in double-membrane autophagosome-like structures, correlating with bacterial destruction and decreased replication (23, 24). In interferon-activated macrophages autophagy targets M. tuberculosis-containing phagosomes to overcome the block in phagosomal maturation imposed by this pathogen (25). These studies suggest that autophagy targets bacteria in the cytosol and within vacuoles by yet unclear mechanisms.

Notwithstanding, several Gram-negative pathogens including B. abortus, L. pneumophila, Porphyromonas gingivalis, and Coxiella burnetii in fact benefit from the autophagic pathway. Once within an autophagosome, they modify this compartment to create an environment to survive and replicate (26). The role of autophagy for S. aureus survival and replication in host cells is yet to be resolved. Although the role of autophagy is mainly to support cell survival, excessive autophagy can be detrimental. The so-called “autophagic cell death” or “type 2 cell death” is characterized by the appearance of cytoplasmic vacuoles related to autophagosomes. This type of programmed cell death does not require caspase activation and, thus, differs from apoptosis. However, the signal cascades of both death pathways appear to be intertwined. For example, the major anti-apoptotic protein Bcl-2 physically interacts with the autophagy protein Beclin-1 (Atg6), inhibiting starvation-induced autophagy and cell death (27). Moreover, pro-apoptotic stimuli can activate autophagic death in cells that are protected against apoptosis by either overexpression of anti-apoptotic proteins (Bcl-2 or Bcl-XL), lack of pro-apoptotic proteins (Bax−/− and Bak−/−), or inhibition of caspases by z-VAD-fmk, a pancaspase inhibitor (28).

S. aureus possesses a vast array of specific virulence factors, like adhesins, toxins, and enzymes, whose expression is tightly regulated by a set of global virulence regulators. These systems when activated modulate the expression of many extracellular as well as cell wall-associated proteins. The most important and best-studied system is the accessory gene regulator (agr) system (29). This quorum-sensing regulated system is activated by an autoinducing cyclic peptide. When the bacterial cell density reaches a certain threshold, toxins, proteases, and other extracellular proteins are up-regulated, whereas the production of adhesins and other cell wall-associated proteins is diminished (30). Although S. aureus-derived factors triggering cell death have not been identified so far, it has been recognized that one or more of them are under the control of the agr system. It has been reported that loss-of-function agr mutants invade mammary bovine epithelial cells with a higher internalization rate but fail to induce cell death (31). Haslinger-Loeffler et al. (32) also reported strongly impaired cell death in human umbilical vein endothelial cells infected with S. aureus agr mutants.

In the present study we elucidate the role of autophagy for S. aureus during infection of non-professional phagocytes. We demonstrate that S. aureus diverts from the endosomal pathway to autophagosomes in an agr-dependent manner. S. aureus-induced autophagy is required for S. aureus replication, subsequent escape from autophagosomes into the cytoplasm, and S. aureus-induced host cell death.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-cytochrome c, anti-caspase-3 (BD Pharmingen), anti-human lysosome-associated membrane protein-2 (LAMP-2; Southern Biotech, Birmingham, AL), anti-protein A (Sigma), anti human Beclin-1 (BD Transduction Laboratories), and goat anti-mouse Alexa Fluor 568 (Molecular Probes, Leiden, Netherlands) were purchased from indicated companies.

**Bacterial Strains and Growth Conditions**—S. aureus strains MW2 (NRS123), USA300 (NRS384), NCTC8325 (NRS77), NRS151, the isogenic agr-deficient mutant of NCTC8325, and RN4220 (NRS144), partial agr-deficient mutant of NCTC 8325, were obtained from NARSA (The network on antimicrobial resistance in S. aureus, Herndon, VA). S. aureus strain ATCC29213 was obtained from the DMSZ (Deutscche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

For infection experiments S. aureus were grown on Mueller-Hinton agar plates. Colonies from agar plates were cultured in Luria-Bertani broth overnight at 37 °C, diluted with fresh broth, and cultured until mid-logarithmic phase of growth (A600 = 0.3). Bacteria were harvested by centrifugation, washed with phosphate-buffered saline (PBS, Biochrom, Germany), and adjusted to a concentration of 10⁹ CFU/ml.

To generate GFP-expressing S. aureus, GFP was cloned under control of staphylococcal blaZ promoter in the shuttle vector pMK4 (33). A 150-bp segment containing the blaZ promoter was amplified from S. aureus DNA by PCR using primers 5’-GGAATTCAAGCTTACTATGCTCATTATT-3’ and 5’-CCGATCCAAAAAATACCCCTCCG-3’. The amplified DNA segment was gel-purified, digested with EcoRI and BamHI restriction endonucleases, and cloned into pMK4 vector by standard techniques. The open reading frame of enhanced green fluorescent protein (EGFP) was amplified from pEGFP-N1 (BD Clontech, San Jose, CA) with specific primers containing restriction sites for BamHI and PstI. Amplicon was moter was amplified from pEGFP-N1 (BD Clontech, San Jose, CA) with specific primers containing restriction sites for BamHI and PstI. Amplicon was ligated downstream from blaZ promoter using corresponding endonucleases. The resulting construct designated pS1-GFP was introduced into S. aureus by electroporation (34).

**Eukaryotic Cell Culture**—The human epithelial cell line HeLa was obtained from the ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Biochrom). If not indicated otherwise, 100 units/ml of penicillin G, and 100 µg/ml of streptomycin sulfate (Biochrom) were added to Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Biochrom).
medium. All cultivation steps were performed at 37 °C in humid atmosphere containing 5% CO₂.

HeLa cells stably expressing the EGFP microtubule-associated protein LC3 fusion protein and the Δ20G120A mutant were generated using the ViraPower™ II Lentiviral Gateway® Expression System (Invitrogen). The EGFP tag was amplified by PCR using forward, 5'-CACCAGTTGAGGCAAGGGC-3', and reverse, 5'-TTTAAAGCTTGTACGTCGTCCTAGT-3', primers and pEGFP-N1 as template. LC3 open reading frames were generated using forward, 5'-CACAAGCTTATGCGTCGAGAGGACCTAAGCA-3', and reverse, 5'-TTACAAGTTTCATCCCG-3', primers. The mutant LC3 protein lacking 20 C-terminal amino acids and possessing a glycine to alanine substitution at position 120 (LC3Δ20G120A) was generated using the reverse primer 5'-TTAGGGCAAGCCTCTCTGTTGGGAGGCATAG-3'.

To generate cells overexpressing XIAP proteins or Bcl-2 (HeLa-XIAP or HeLa-Bcl-2), XIAP- or Bcl-2-encoding cDNA were obtained by reverse transcription-PCR using a first-strand cDNA derived from HeLa cells as the template and specific primers containing EcoRI and XhoI digestion sites. The PCR products were digested with EcoRI and XhoI followed by ligation into pcDNA3 containing a N-terminal Myc tag (Invitrogen). HeLa cells were stably transfected with pcDNA or pcDNA-Myc-XIAP followed by selection in medium containing 500 μg/ml geneticin (Invitrogen). Mouse embryonic fibroblasts (MEFs) deficient in atg5 and the corresponding wild type cells were kindly provided by Dr. Noboru Mizushima (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (19).

*S. aureus Infection of Eukaryotic Host Cells*—The *in vitro* infection was performed as previously described (8). At 18 h before infection, 1 × 10⁵ eukaryotic cells were seeded in 6-well plates (Nunc, Wiesbaden, Germany). Cells were then washed with growth medium without antibiotics and kept for 1 h at 37 °C to remove lysostaphin and dead bacteria. Extracellular bacteria were killed by adding 100 μg/ml lysostaphin (Sigma) and incubating cells for 15 min at 37 °C. To remove lysostaphin and dead bacteria, cells were washed with growth medium supplemented with antibiotics and reseeded in a new six-well plate. Viability was monitored by using trypan blue (Biochrom) exclusion (36).

CFU Determination—Infected host cells were washed with PBS to remove antibiotics and lysed in PBS containing 0.05% Triton X-100. Lysates were diluted with PBS and plated on Mueller-Hinton agar by using a spiral plater (EDDY-Jet; IUL Instruments, Koenigswinter, Germany). Colony counting and CFU determination were performed according to the manufacturer's instructions.

Induction and Inhibition of Autophagy and Apoptosis—Autophagy was induced by treatment with rapamycin (Sigma) used in concentrations ranging from 20 to 80 μg/ml for 2 h. Alternatively, cells were incubated in starvation medium composed of Hanks' balanced salt solution (Invitrogen) supplemented with 0.035% NaHCO₃ for 2 h at 37 °C. Autophagy was inhibited by addition of 50–200 nm wortmannin (Sigma) or 5 mM 3-methyladenine (Sigma) to cell culture medium before infection. For induction of apoptosis cells were exposed to UV light (20 mJ/cm²). Caspase activity was inhibited by a pretreatment of cells with 100 μM z-VAD(OMe)-fmk (Alexis, Grunberg, Germany) for 30 min.

Transient Transfection of HeLa Cells—Expression vector pRAB7-GFP (37) was transfected into HeLa cells using Lipofectamine 2000 reagent (Invitrogen).

Fluorescence and Electron Microscopy—For fluorescence microscopy, cells were grown on coverslips and infected with *S. aureus* at m.o.i. 20. At the indicated time points, cells were rinsed with PBS and fixed for 15 min with 4% paraformaldehyde. Before staining with antibodies, cells were permeabilized with 0.1% saponin and 5% bovine serum albumin in PBS. Samples were incubated with the indicated specific antibodies followed by Alexa Fluor 568-labeled secondary antibody. All antibodies were used in a 1:1000 dilution. Specimens were mounted on microscope slides in ProLong Gold Antifade Reagent (Invitrogen). For LysoTracker Red DND-99 (Invitrogen) staining, cells were incubated with 50 nM dye for 30 min under normal growth conditions, washed with PBS, and directly observed under the fluorescence microscope. Images were acquired with an inverted Olympus IX81 microscope (Olympus, Hamburg, Germany) equipped with a F-View II Trigger camera and then analyzed using analySIS® software (Soft Imaging Software, Muenster, Germany) or with a confocal microscope Leica TC SL and analyzed by Leica Confocal Software (Leica Microsystems, Heidelberg, Germany).

For electron microscopy, infected cells were fixed overnight in fresh half-strength Karnovsky's fixation (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3), extensively buffer-rinsed, and osmicated for 1 h with 1% OsO₄ in 0.1 M cacodylate. Samples were dehydrated by rinsing in graded ethanol series including an uranyl acetate en bloc staining step overnight in 70% ethanol. Before infiltration with Araldite Cy212 epoxy resin (Serva, Heidelberg, Germany), propylene oxide was used as intermediate. Tissue blocks were cut for 60 h at 0.6°C. Thin (60 nm) cross-sections were taken on an Ultracut UCT ultramicrotome (Reichert, Heidelberg, Germany). Thin sections were stained with 1% aqueous uranyl acetate for 20 min, and sections were counterstained with Reynold's lead citrate for 7 min (38). Thin sections were mounted on 150 mesh Formvar-coated copper grids and examined with a Zeiss EM 902 electron microscope at 80 kV accel-
Subversion of Autophagy by S. aureus

Isolation of S. aureus-containing Phagosomes—Phagosome isolation was performed as previously described (39). HeLa cells were infected as described above. After the indicated infection time, medium was removed and replaced with ice-cold PBS. Cells were harvested and washed twice with ice-cold PBS, resuspended in homogenization buffer comprised of 250 mM sucrose, 0.5 mM EGTA, 20 mM Heps/KOH (pH 7.2), and lysed in a Dura Grind stainless-steel homogenizer. The resulting cell homogenate was cleared by centrifugation, and organelles were fractionated in a discontinuous sucrose density gradient by ultracentrifugation at 100,000 × g. Phagosomes were recovered from the interface of 55 and 65% sucrose layers, separated through a 15% Ficoll cushion at 18,000 × g, and concentrated in a final centrifugation step at 18,000 × g. Quality of intact isolated phagosomes was controlled by electron microscopy. For immunoblotting, phagosomes were lysed in PBS containing 0.1% Triton X-100 and a protease inhibitor mixture (Complete, Roche Diagnostics). Extracted proteins were separated by 10% SDS-PAGE and analyzed by immunoblotting.

Preparation of Cytosolic Extracts and Immunoblotting—For isolation of cytosolic extracts, 10⁷ cells were washed twice with PBS at 4 °C. Cells were resuspended in 50 ml of buffer A (50 mM PIPES, pH 7.0, 50 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 10 mM cytochalasin B, protease inhibitors (Complete, Roche Diagnostics), and 1 mM dithiothreitol and incubated for 20 min on ice for swelling. Cells were cracked by passing through a 27-gauge needle. Cell breakage was verified microscopically by trypan blue exclusion. Membranes were pelleted at 140,000 × g for 20 min at 4 °C, and the resulting supernatants were recovered (cytosolic extract).

Equal volumes of the cytosolic extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane (Protran 0.2 mm; Schleicher and Schuell) via electroelution. Membranes were stained with the indicated antibodies followed by horseradish peroxidase conjugates of anti-rabbit and anti-mouse IgG (Bio-Rad), used as secondary antibodies. Signals were detected by ECL reagent (GE Healthcare).

RESULTS

Caspase-independent Killing of Host Cells by Intracellular S. aureus—Previous studies revealed that induction of host cell death by S. aureus requires bacterial invasion and expression of multiple gene products regulated by the global regulator agr (31, 32, 40). However, whether S. aureus induces cell death by...
necrosis or apoptosis remained controversial. To disentangle the cellular mechanisms of S. aureus-induced cell death, we used HeLa cells in a system that recapitulates the S. aureus responsive phenotype previously reported for a variety of host cell types. As shown in Fig. 1A, S. aureus strains NCTC8325, ATCC29213, MW2, and USA300 efficiently killed HeLa cells with death rates of 65, 82, 95, and 98%, respectively. In contrast, the agr-deficient S. aureus strains NRS151 and RN4220 did not generate any significant cytotoxicity in HeLa cells. It is important to note that all S. aureus strains were taken up by HeLa cells with similar kinetics and in similar quantities (data not shown).

To investigate the impact of apoptotic machinery in cytotoxicity induced during S. aureus infection, HeLa cells were lysed at distinct times after infection, and cellular lysates were analyzed by Western blotting for processing/activation of executioner caspase-3 and mitochondrial release of cytochrome c. As shown in Fig. 1B, compared with UV irradiation used as positive control, cytotoxic S. aureus strains MW2 and ATCC29213 produced only weak processing of caspase-3 and diminished release of cytochrome c. Preincubation of HeLa cells with the pancaspase inhibitor z-VAD-fmk did not prevent S. aureus-induced cell death (Fig. 1C), indicating that S. aureus-induced cell death is independent of the caspase activation cascade. Activity of z-VAD-fmk was controlled (data not shown). Similarly, overexpression of XIAP, an X-linked inhibitor of apoptosis protein, did not prevent S. aureus-induced killing of HeLa cells (Fig. 1D). XIAP is known to negatively regulate the intrinsic apoptosis pathway by directly binding and interfering with caspases-3, -7, and -9, confirming the idea that caspases do not play an essential role in S. aureus-induced cell death. In contrast, S. aureus-induced cell death was suppressed in Bcl-2 overexpressing HeLa cells. It is important to note that Bcl-2 is not only anti-apoptotic but also exerts anti-autophagic function through binding to Beclin-1/Atg6. This raised the question about the molecular mechanisms of caspase-independent S. aureus-induced cell death and prompted us to investigate in greater detail the traffic of intracellular S. aureus, especially with respect to autophagic compartments.

S. aureus Transits the Autophagosomal Pathway—Previous studies with pulmonary epithelial cells revealed that internalized S. aureus reside within endocytic vacuoles in the absence of lysosomal fusion in a 24-h period (41). To investigate the intracellular traffic of S. aureus, HeLa cells were infected with S. aureus strain MW2 and analyzed by transmission electron microscopy. As shown in Fig. 2, A and B, bacteria were taken up through cup formation to localize within 1.5-h post-infection in a Rab7-positive endophagosome. At this time point of infection, multilamellar structures were found to attach to, and to fuse with the membranes of S. aureus vacuoles (Fig. 2C). These multilamellar membranes eventually sequestered the S. aureus-containing compartment together with the surrounding cytoplasm until double membranes closely enveloped the bacteria within 3 h post-infection, which meets the main morphological criteria of autophagosomes.

**FIGURE 2.** S. aureus transits from the endocytic pathway to the autophagic pathway. A, electron micrographs of HeLa cells infected with S. aureus for 15 min and 1.5 h. B, confocal fluorescence microscopy images were obtained from HeLa cells transfected with pGFP-Rab7 for 24 h and infected with S. aureus (ATCC29213). Cells were fixed 1.5 h after infection, and bacteria were stained with anti-protein A antibody followed by a Alexa 568-labeled secondary antibody. C, electron micrographs of HeLa cells infected with S. aureus (ATCC29213). Cells were fixed at the indicated time points with Karnovsky buffer and further processed after standard electron microscopic methods. Bars, 0.5 μm.
Subversion of Autophagy by S. aureus

A no treatment rapamycin

EGFP-LC3

EGFP-LC3

EGFP-LC3-D26G128A

EGFP-LC3-D26G128A

B S. aureus

MW2

EGFP-LC3

Phase

EGFP-LC3-D26G128A

Phase

S. aureus-Alexa 568

Merge

S. aureus-Alexa 568

Merge

C

Bacteria colocalising with LC3 [%]

Time post infection [h]

D

Bacteria colocalising with LC3 [%]

E

S. aureus MW2 [CFU/host cell]

S. aureus MW2 [CFU/host cell]

F

S. aureus MW2 [CFU/host cell]
The same was observed in two more agr-positive *S. aureus* strains (ATCC29213 and NCTC8324). To analyze whether the host cell autophagic machinery accounts for the sequestration of the *S. aureus* vacuole, we examined the subcellular distribution of Atg8 (LC3). LC3 is recruited to autophagosomes as they mature and is located at the inner and outer membrane of autophagosomes (20). We additionally generated a Δ20G120A mutant of LC3 lacking 20 C-terminal amino acids in which glycine at position 120 was substituted by alanine. This mutant has no ability to conjugate with phosphatidylethanolamine, which is required to associate with the autophagic membrane. As shown in Fig. 3A, rapamycin induced recruitment of LC3 but not the LC3Δ20G120A mutant to autophagosomes. When HeLa cells stably expressing EGFP-LC3 were infected with *S. aureus* (MW2, ATCC29213, USA300, or NCTC8324), the majority of *S. aureus* vacuoles were stained positive for LC3 within 180 min of infection. No co-localization was found in the LC3-Δ20G120A mutant HeLa cells (Fig. 3B). Three hours after infection ~90% of *S. aureus* vacuoles were co-localized with LC3 (Fig. 3C). These data indicate that the autophagic machinery of HeLa cells rapidly responds to infection with *S. aureus*. Co-localization of *S. aureus* with LC3 was almost completely abrogated by pretreatment of the infected cells with the autophagy inhibitors wortmannin or 3-methyladenine (Fig. 3D).

**Autophagosome Formation Increases *S. aureus* Replication**—To address the consequences of autophagosome formation for *S. aureus* replication, we employed rapamycin and wortmannin, respectively, as pharmacological inducers and inhibitors of autophagy. One-hour post-infection of HeLa cells with *S. aureus* (MW2) rapamycin was added at graded concentrations, and after 6 h *S. aureus* replication was determined. As shown in Fig. 3E, incubation with rapamycin at a concentration of 80 μg/ml doubled the intracellular load of *S. aureus*. In contrast, wortmannin drastically impaired the growth of intracellular *S. aureus*, suggesting that autophagosomes provide a niche for *S. aureus* replication. Furthermore, *S. aureus* replication was drastically impaired in atg5-deficient MEFs (Fig. 3F), indicating that *S. aureus* requires proteins of the autophagic pathway for replication. Similar results were obtained using all other agr-positive *S. aureus* strains (data not shown).

**Rapamycin-induced Autophagy Restores Replication of agr-deficient *S. aureus***—As shown in Fig. 1A, the agr-deficient *S. aureus* strains NRS151 and RN4220 proved to be noncytotoxic, that is, did not significantly reduce the viability of HeLa host cells. Rapamycin-induced autophagy restores replication of agr-deficient *S. aureus*—As shown in Fig. 1A, the agr-deficient *S. aureus* strains NRS151 and RN4220 proved to be noncytotoxic, that is, did not significantly reduce the viability of HeLa host cells. Rapamycin-induced autophagy restores replication of agr-deficient *S. aureus*—As shown in Fig. 1A, the agr-deficient *S. aureus* strains NRS151 and RN4220 proved to be noncytotoxic, that is, did not significantly reduce the viability of HeLa host cells. Rapamycin-induced autophagy restores replication of agr-deficient *S. aureus*—As shown in Fig. 1A, the agr-deficient *S. aureus* strains NRS151 and RN4220 proved to be noncytotoxic, that is, did not significantly reduce the viability of HeLa host cells.

**FIGURE 3.** *S. aureus*-specific compartments co-localize with the autophagosomal marker LC3. A, autophagy was induced by rapamycin (80 μg/ml) in EGFP-LC3- and EGFP-LC3-Δ120G120A-expressing HeLa cells. After 2 h formation of EGFP-positive autophagosomes was determined by fluorescence microscopy. B, HeLa cells stably expressing EGFP-LC3 and EGFP-LC3-Δ120G120A were infected with wt *S. aureus* at m.o.i. 20, fixed 3 h after infection, and stained with anti-protein A antibody followed by a Alexa 568-labeled secondary antibody. C, kinetics of co-localization of wt *S. aureus* strain ATCC29213 with EGFP-LC3. Cells were infected and processed as described above. Samples were fixed at the indicated time points. Fluorescence microscopy images were analyzed quantitatively for *S. aureus*/EGFP-LC3 co-localization. Data are representative of at least three independent experiments. In each experiment a minimum of 100 cells was evaluated. D, inhibition of autophagy prevents translocation of *S. aureus* into autophagosomes. HeLa cells were infected with *S. aureus* (m.o.i. 20) for 3 h. Autophagy was induced by treatment with rapamycin (Rapa) 80 μg/ml during infection or by incubation of HeLa cells in starvation medium (Starv) for 2 h. Autophagy was inhibited by 100 nM wortmannin (WM) or 5 mM 3-methyladenine (3-MA) for 3 h during infection. Fluorescence microscopy images were analyzed quantitatively for *S. aureus*/EGFP-LC3 co-localization. Averages and S.D. were calculated from three independent experiments. In each experiment a minimum of 100 cells was evaluated. E, autophagosome formation increases intracellular *S. aureus* replication. HeLa cells were infected with wt *S. aureus* (MW2 Strain) at m.o.i. 200. After 3 h infection bacteria were stained with anti-protein A antibody followed by a Alexa 568-labeled secondary antibody. F, intracellular *S. aureus* replication is suppressed in atg5/− MEFs. wt MEFs or atg5/− MEFs were infected with *S. aureus* MW2 at m.o.i. 200. After 1 h extracellular bacteria were eliminated by lysostaphin, and further incubation was performed in antibiotic containing medium. Autophagy was induced for 2 h, whereas the autophagy inhibitors were added at the time of infection. Cells were lysed 6 h post-infection and plated on blood agar. The number of intracellular bacteria was determined by CFU counting.

**FIGURE 4.** Rapamycin enables agr-deficient *S. aureus* to translocate into autophagosomes and to replicate. A, electron micrographs of HeLa cells infected with agr-deficient *S. aureus* strain RN4220. HeLa cells were infected (m.o.i. 200) with agr-deficient *S. aureus*. 3 h after infection, cells were fixed with Karnovsky buffer and further processed following standard electron microscopic methods. Bar, 0.5 μm. B, lack of autophagosomal localization of agr-deficient *S. aureus*. EGFP-LC3-HeLa cells were infected (m.o.i. 20) with agr-deficient *S. aureus* strain RN4220. 3 h after infection bacteria were stained with anti-protein A antibody followed by a Alexa 568-labeled secondary antibody. C, co-localization of agr-deficient *S. aureus* with EGFP-LC3 during autophagy induction or inhibition. HeLa cells were infected (m.o.i. 20) with agr-deficient *S. aureus* strain RN4220. 3 h after infection bacteria were stained with anti-protein A antibody followed by Alexa 568-labeled secondary antibody. Autophagy was induced by treatment with rapamycin (Rapa) 80 μg/ml during infection or by incubation in starvation medium (Starv) for 2 h. Autophagy was inhibited by 100 nM wortmannin (WM) or 5 mM 3-methyladenine (3-MA) for 3 h during infection. Fluorescence microscopy images were analyzed quantitatively for *S. aureus*/EGFP-LC3 co-localization. Averages and S.D. were calculated from three independent experiments. In each experiment a minimum of 100 cells was evaluated. D, autophagosome formation allows intracellular survival of agr-deficient *S. aureus*. HeLa cells were infected with agr-deficient *S. aureus* strain RN4220 (m.o.i. 200) for 24 h. Cells were lysed 6 h post-infection, and lysates were plated on blood agar. The number of intracellular bacteria was determined by CFU counting.
Subversion of Autophagy by S. aureus

A

wt agr S. aureus strains

agr-deficient S. aureus strains

B

\[ \text{Bacteria colocalizing with Lamp-2 [%]} \]

\[ \text{Time post infection [h]} \]

C

| cell lysate    | isolated phagosome |
|---------------|--------------------|
| S. aureus ATCC 29213 | S. aureus ATCC 29213 |
| S. aureus RN 4220   | S. aureus RN 4220   |

\[ \text{LAMP-2} \]

\[ -120kD \]

D

wt agr S. aureus strains

agr-deficient S. aureus strains

E

S. aureus ATCC 29213

S. aureus RN 4220

12 h infection
cells. Moreover, these strains were eliminated by the host cells within 3 days (8), raising the question about the possible impact of autophagy on the intracellular traffic and replication of agr-deficient *S. aureus* strains. As illustrated in Fig. 4A, vacuoles containing the agr-deficient *S. aureus* RN4220 strain were not surrounded by multilamellar membranes, and unlike *S. aureus* MW2, vacuoles containing RN4220 did not co-localize with LC3 during the whole course of infection (Fig. 4B, kinetics not shown), indicating that this agr-deficient *S. aureus* strain is not able to induce autophagosome formation in host cells. Strikingly, when autophagy was induced by either rapamycin or starvation, ~80% of the agr-deficient *S. aureus* RN4220 bacteria co-localized with the autophagosomal marker LC3 (Fig. 4C), and rapamycin-mediated autophagy enabled *S. aureus* replication in a dose-dependent manner (Fig. 4D). This is also true for agr-null mutant NRS77 (data not shown).

**Autophagy-inducing *S. aureus* Strains Evade Lysosomal Fusion Events**—Autophagy is a major catabolic pathway by which eukaryotic cells sequester organelles and invading bacteria for delivery to lysosomes with subsequent degradation. To test whether the formation of autophagosomal vacuoles propels or rather inhibits lysosomal fusion events, possible co-localization of wt *agr* and agr-deficient *S. aureus* strains and LAMP-2 was analyzed. As shown in Fig. 5A, wt *agr* ATCC29213 *S. aureus* vacuoles rarely stained with LAMP-2. In contrast, agr-deficient RN4220-containing compartments acquired LAMP-2. Maximal co-localization was observed 3–4 h after infection with agr-deficient RN4220 (Fig. 5B). Reduced co-localization of wt *agr* *S. aureus* with LAMP-2 was also revealed by Western blot analysis of isolated phagosomes from HeLa cells infected with ATCC29213 or RN4220 *S. aureus* strains (Fig. 5C). Finally, agr-deficient RN4220 but not wt *agr* MW2 *S. aureus* co-localized with Lysotracker dye, indicating diminished acidification of wt *agr* *S. aureus*-containing vacuoles (Fig. 5D). Thus, *S. aureus* appear to encode an agr-dependent factor(s) that not only induces autophagosome formation but also inhibits autophagolysosomal fusion events. As shown by electron microscopy, 8–12 h after infection *S. aureus* ATCC29213, but not agr-deficient *S. aureus*, were mostly found to be in the cytoplasm (Fig. 5E), suggesting that wt *agr* *S. aureus*-induced inhibition of autophagosomal maturation eventually allows escape of *S. aureus* from the autophagosome into the host cell cytoplasm. Similar results were obtained using other agr-positive or agr-deficient *S. aureus* strains (data not shown).

**S. aureus Induces Autophagic-dependent Cell Death**—Autophagy is sensed as a cellular adaptation to nutrient deprivation and sublethal damage. However, in some instances, in particular when apoptosis is inhibited, high levels of autophagy could cause cell death through excessive self-digestion and degrada-

**FIGURE 5. Early autophagosomes formation prevents maturation of *S. aureus*-containing phagosomes.** A, co-localization of wt *agr* and agr-deficient *S. aureus* with the lysosomal marker LAMP-2. HeLa cells were infected with GFP-expressing *S. aureus* (m.o.i. 20). Cells were fixed 4 h after infection. Lysosomes were stained with anti-LAMP-2 antibody followed by Alexa 568-labeled secondary antibody. B, kinetics of co-localization of wt *agr* or agr-deficient *S. aureus* with LAMP-2. Fluorescence microscopy images were analyzed quantitatively for *S. aureus*/LAMP-2 co-localization. C, phagosomes containing wt and mutant *S. aureus* were isolated from HeLa cells 3 h after infection as described under “Experimental Procedures.” Whole cell lysates served as a control. Lysates were subjected to SDS-PAGE and analyzed by immunoblotting. Protein concentration was determined by the BCA assay. Equal amounts of protein were loaded in every lane. D, acidification of lysosomes of agr-deficient *S. aureus*. HeLa cells were infected with GFP-expressing *S. aureus* strains ATCC29213 (wt *agr*) or RN4220 (agr-deficient) (m.o.i. 20). Acidic subcellular compartments were stained with Lysotracker Red at 4 h after infection. E, subcellular localization of wt *agr* and agr-deficient *S. aureus* at late stages of infection. Electron micrographs of HeLa cells infected with wt *agr* *S. aureus* and agr-deficient *S. aureus* (m.o.i. 200). Cells were fixed with Karnovsky buffer 12 h after infection and further processed following standard electron microscopic methods. Bars, 0.5 μm.

**DISCUSSION**

Autophagy is not only a conserved catabolic pathway that sequesters cytoplasmic constituents into double-membrane autophagosomes to fuse with lysosomes. An emerging body of evidence indicates that autophagy-related pathways are used by eukaryotic cells to degrade intracellular microbial pathogens (for a review, see Ref. 16). Some intracellular bacterial pathogens such as *M. tuberculosis* and *S. flexneri* manage to arrest autophagosome maturation. In contrast, the extracellular pathogen *S. pyogenes*, also known as group A Streptococcus, is vulnerable to autophagic destruction (21). In the present study we show that *S. aureus* are targeted by autophagosomes. Like group A Streptococcus, *S. aureus* are “classical” extracellular Gram-positive bacteria that yet also invade non-phagocytic cells. Here we provide evidence that unlike group A Streptococcus, transition into autophagosomes provides a protective niche for intracellular *S. aureus*, where they survive, replicate, and escape into cytoplasm to eventually kill the eukaryotic host cell.

The *S. aureus* Autophagosome Is Unique—The *S. aureus* autophagosome appears to be unique in terms of both molecular composition and function. The distinctive characteristics of the autophagic pathway of *S. aureus* appear to be determined already at early stages of the internalization process. *S. aureus*-containing phagosomes become Rab7-positive early after inter-

**Subversion of Autophagy by *S. aureus***

Induced cell death, we employed a genetic approach utilizing MEFs from *atg5*−/− mice. Atg5 is required to form a cytosolic complex during autophagosome formation (43). Wild type MEFs infected with wt *agr* *S. aureus* strain (ATCC29213) showed marked signs of cell death (i.e. rounding, detachment from the surface of culture plate) 24 h after infection, whereas *atg5*−/− MEFs appear phenotypically unchanged (data not shown). Staining with trypan blue dye revealed that *S. aureus*-induced cell death was markedly reduced in *atg5*-deficient MEF (Fig. 6C), supporting the notion that transition of *S. aureus* into the autophagosomal pathway is required for *S. aureus* cytotoxicity.

Autophagy-inducing *S. aureus* strains (i.e. *agr* and *atl*) promote autophagic cell death through excessive self-digestion and sublethal damage. However, in some instances, in particular when apoptosis is inhibited, high levels of autophagy could cause cell death through excessive self-digestion and degrada-

Indeed, using electron microscopy, all dying HeLa cells infected with wt *S. aureus* displayed abundant vacuolization (Fig. 6A) and looked like “Swiss cheese,” a well known morphological indicator for autophagic cell death.

The ability of *S. aureus* to kill host cells clearly correlated with the rate of autophagy induced. Rapamycin not only enhanced the *a priori* profound cytotoxicity of wt *agr* *S. aureus* but also “converted” the agr-deficient RN4220 to a wt *agr* phenotype (Fig. 6B). These data suggest that autophagosome formation is necessary for *S. aureus* not only for replication but also for killing of the host cell.

To confirm that autophagy is essential for *S. aureus*-induced cell death, we employed a genetic approach utilizing MEFs from *atg5*−/− mice. Atg5 is required to form a cytosolic complex during autophagosome formation (43). Wild type MEFs infected with wt *agr* *S. aureus* strain (ATCC29213) showed marked signs of cell death (i.e. rounding, detachment from the surface of culture plate) 24 h after infection, whereas *atg5*−/− MEFs appear phenotypically unchanged (data not shown). Staining with trypan blue dye revealed that *S. aureus*-induced cell death was markedly reduced in *atg5*-deficient MEF (Fig. 6C), supporting the notion that transition of *S. aureus* into the autophagosomal pathway is required for *S. aureus* cytotoxicity.

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Subversion of Autophagy by S. aureus

A

![Micrograph of dying HeLa cell infected with wt S. aureus](image)

FIGURE 6. S. aureus-induced autophagic cell death requires Atg5. A, electron micrograph of dying HeLa cell infected with wtagr S. aureus. HeLa cells were infected with wtagr S. aureus strain ATCC29213 at m.o.i. 200. Cells were fixed with Karnovsky buffer 8–24 h after infection and further processed following standard electron microscopic methods. Bar, 0.5 μm. B, autophagy induction increases the cytotoxic effect of S. aureus. HeLa cells were infected (m.o.i. 200) with wtagr S. aureus strain ATCC29213 (black bars) and agr-deficient S. aureus strain RN4220 (hatched bars). Autophagy was induced by treatment with rapamycin (80 μg/ml) during infection. Host cell viability was determined after 24 h by trypan blue exclusion. C, Atg5 deficiency prevents S. aureus-induced cell death. MEF from atg5−/− and wt mice were infected with wtagr S. aureus strain ATCC29213 at m.o.i. 100. Percentage of dead host cells infected with wtagr S. aureus strain ATCC29213 (black bars) or agr-deficient S. aureus strain RN4220 (hatched bars) was determined by trypan blue exclusion.

Unlike the S. aureus autophagosome, group A Streptococcus as well as L. monocytogenes-containing autophagosomes co-stained with LC3 and LAMP-1 2–3 h after infection, suggesting fusion with lysosomes (21, 22). This represents the conventional autophagic pathway where autophagosomes fuse with lysosomes and acquire prominent lysosomal proteins, like LAMP-2, cathepsin D, and vacuolar type H⁺-ATPase, the latter leading to a decreased pH. Compared with Gram-positive bacteria, the autophagic pathways of Gram-negative bacteria, like B. abortus, L. pneumophila, P. gingivalis, and C. burnetii, differ in many ways; autophagosomes all express late endosomal markers yet show delayed acquisition of lysosomal markers such as cathepsin D or LAMP-1 (46–50). They induce the autophagic pathway via their type IV secretion system that is essential for the biogenesis of the autophagosome-like vacuole. Such secretion systems have never been observed in Gram-positive bacteria. Notwithstanding, S. aureus appear to induce an arrest of autophagosomal maturation and fusion with lysosomes and, thus, resemble the autophagosomal pathways of Gram-negative bacteria listed above.

In terms of function, the replication of intracellular S. aureus was greatly reduced in the presence of wortmannin (Fig. 3E). Because wortmannin prevents the initial formation of autophagosomes, this finding indicates that autophagy is subverted by S. aureus for its survival. Bacterial subversion of the autophagic pathway has also been observed with P. gingivalis and B. abortus that replicate within a late autophagosome that has lost lysosomal markers (26).

The comparison of the autophagic pathway with S. aureus and B. abortus seems to be especially instructive; both 3-MA and wortmannin were found to reduce bacterial replication, whereas host-cell starvation increased B. abortus growth (49). Moreover, B. abortus carrying a loss-of-function mutant of the virB operon that codes for members of a type IV secretion pathway, almost immediately localized to cathepsin D positive compartments and were defective in intracellular replication (51, 52). Similarity, S. aureus strains defective for the global regulator agr that drives secretion of virulence factors, co-localize with LAMP-2 but not with LC3 and were unable to survive in host cells (Figs. 4 and 5). These observations suggest that autophagosomes...
Subversion of Autophagy by S. aureus

Atg5 Is Required for S. aureus-induced Cell Death—Many investigators have reported induction of apoptotic cell death after S. aureus internalization. However, the involvement of caspase activation, the main hallmark of apoptosis, was not well documented. Consistent with previous reports, we demonstrate here that S. aureus induces host-cell death independent of caspases. No cleavage of the effector caspase-3 was observed in S. aureus-infected HeLa cells. Moreover, neither the pancaspase inhibitor z-VAD-fmk nor overexpression of the X-linked inhibitor of apoptosis, XIAP, prevented cell death induced by S. aureus infection. Together, these findings clearly argue against caspase-dependent HeLa cell apoptosis caused by S. aureus infection.

The results of this study now provide genetic evidence that S. aureus-induced cell death relies on proteins of the autophagic pathway like Atg5. Atg5 plays an essential role in the isolation membrane development. It is indispensable in the elongation process, binds covalently to Atg12, and also targets Atg8/LC3 to the isolation membrane (43). Thus, our observation that S. aureus did not induce host-cell death in atg5−/− MEFs provides formal proof that autophagy is a prerequisite for S. aureus-induced cell death. Furthermore, it has been shown that overexpression of Bcl-2 functionally neutralizes Beclin-1 (27). Such neutralization of Beclin-1 in HeLa cells overexpressing Bcl-2 disabled S. aureus-induced host-cell death.

Twelve hours after infection, S. aureus were found in the cytoplasm lacking any double-membrane structures (Fig. 5E). It remains to be resolved whether and if so how, S. aureus destroys the autophagosomal compartment from within. Notably, the emergence of cytosolic S. aureus was accompanied by host-cell death.

It is worth emphasizing that S. aureus use the autophagosome not only to multiply but also to escape into the cytosol where the host cell apparently loses its control over S. aureus. As shown in Fig. 3C, after reaching a maximum at 3 h, LC3 co-localization with S. aureus-containing autophagosomes declined within 5 h below basal levels, suggesting a dismantling of autophagosomes. This was obviously not secondary to lysosomal fusion but, rather, due to S. aureus replication and presumably S. aureus-mediated destruction of autophagosomes.

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Atg5 is Required for Cell Death—Many investigators have reported induction of apoptotic cell death after S. aureus internalization. However, the involvement of caspase activation, the main hallmark of apoptosis, was not well documented. Consistent with previous reports, we demonstrate here that S. aureus induces host-cell death independent of S. aureus to kill the host cells (Fig. 1D). Beclin-1, the mammalian homolog of Atg6, is part of the phosphatidylinositol 3-kinase complex, which is crucial for the initiation of autophagy. It mediates the localization of autophagic proteins to the preautophagosomal membrane (35, 53). Thus, besides Atg5, Beclin-1/Atg6 represents a second protein of the autophagic pathway that seems to be required for S. aureus-induced host-cell death.

To date, S. aureus represents the only bacterial species that induces cell death depending on autophagic genes. Unlike S. aureus, other bacteria targeted by autophagy such as B. abortus, L. pneumophila, and C. burnetii are professional intracellular pathogens that have evolved special strategies to survive and replicate in host cells without killing their habitat. These bacterial species subvert autophagy as a niche for their replication but not for killing of their host cells. In light of the view that cell death could be caused by excessive autophagy (27), it is important to emphasize that the requirement for proteins of the autophagic pathway does not necessarily mean that S. aureus-induced cell death is directly mediated by autophagy. Indeed, the following reasoning suggests that the extent of autophagy induced by S. aureus does not suffice to mediate cell death. As shown in Fig. 6B, rapamycin did not reduce the viability of HeLa cells, indicating that the extent of autophagy induced by rapamycin does not suffice to induce cell death. Agr-deficient S. aureus are a priori unable to kill their host cells yet do so when autophagy is coincidentally induced by rapamycin. These find-
ings suggest a synergism between agr-deficient S. aureus and autophagy required for induction of cell death.

The results of this study can be reconciled in a model depicted in Fig. 7. We propose that induction of autophagy prevents the maturation of S. aureus-containing phagosomes and subsequent fusion with lysosomes. Autophagosomes provide a protective niche for S. aureus survival and replication. After 12 h S. aureus becomes cytosol-borne to replicate to large numbers that eventually bring about lysis of the host cell. Agr-deficient S. aureus fail to induce autophagy, which results in maturation of agr-deficient S. aureus-containing phagosomes followed by lysosomal degradation. This is consistent with previous reports showing that intracellular survival and cytotoxicity of S. aureus is agr-dependent (31, 32). Notably, upon induction of autophagy by external stimuli, agr-deficient S. aureus are converted to fully cytotoxic bacteria that replicate within autophagosomes, escape into the cytosol, and eventually kill their host cells. Thus, the results of this study clearly indicate that in terms of cytotoxic action, an agr-regulated factor(s) is solely required for immediate induction of autophagy, which is essential for S. aureus-mediated cytotoxicity.

Acknowledgments—We thank Dr. Noboru Mizushima for atg5−/− MEFs, Daniela Grumme for excellent technical assistance, and Dr. P. G. Higgins for helpful comments on the manuscript.

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