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Autophagy and Toxins: A Matter of Life or Death

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Abstract: Bacterial protein toxins are important virulence factors. A particular class of toxins, the pore-form toxins (PFTs), shares the toxigenic mechanism of forming pores in the membrane of target cells. The relationship between autophagy and bacterial PFTs has been described for several toxin-secreting pathogens and in this review we have recapitulated the more recent findings on this issue. A common outcome is that the target cell, by a yet non-completely defined mechanism, senses the toxin attack and builds up complex responses as a protective mechanism for host survival. However, in some cases, this cellular response is beneficial to the microorganism by supplying an intracellular niche or by promoting host-cell death, which facilitates pathogen spreading.

Keywords: Autophagy, intracellular pathogens, LC3, pore-forming toxins.

AUTOPHAGY INDUCTION: A TARGET CELL REACTION TRIGGERED BY BACTERIAL TOXINS

In recent years cumulative evidence indicates the key role played by autophagy in numerous physiological and pathological processes. Macroautophagy (hereafter called autophagy) is one of the best characterized pathways involved in the degradation of long-lived proteins and aged or superfluous organelles [1]. This pathway is initiated by the formation of a phagophore or isolation membrane that engulfs cytoplasmic components forming a sealed double membrane structure called autophagosome. This structure fuses with endosomes or multivesicular bodies (MVBs) to form the amphisome, which in turn encounter the lysosome generating the autolysosome and degrading the sequestered material by the lysosomal proteases (Fig. **1**). Despite of the advances in understanding the autophagy process, the origin of this isolation membrane is yet not completely defined. Several authors including ourselves have indicated the endoplasmic reticulum as the main source of membrane in the autophagosome biogenesis [2-5], but evidence has been presented that mitochondria [6] and even the plasma membrane [7] might contribute to the autophagosome formation upon different stimulus (for a revision please see [8,9]. In addition, certain microbes engage components of lipid raft domains to enter the host cell and to trigger autophagy [10].

Several components of the molecular machinery involved in autophagy have been identified [11]. To date, over 30 autophagy components have been documented and many of them are known as Atgs (for Autophagy related proteins) [12]. The microtubule-associated protein 1 light chain 3 (LC3) is an important element of this machinery and a key protein used as a marker of autophagosomal compartments. After translation, LC3 is proteolytically processed by Atg4 which cleaves a C-terminal glycine generating the LC3-I form. Subsequently, and upon autophagy activation, LC3-I is covalently linked to the lipid phosphatidylethanolamine to generate the membrane-bound LC3-II form [13]. This processing is accomplished by an ubiquitin-like conjugation system comprised of Atg7 and Atg3 (E1-like and E2-like enzymes, respectively) and the complex Atg5-Atg12-Atg16L1 (E3-like enzyme) [14]. The latter seems to determine the site where LC3 will be attached to the membrane [13].

Numerous studies have demonstrated that autophagy is a tightly regulated process (for recent reviews please see [11,15-17]). A basic function of autophagy is to contribute to cell survival, when cells are subjected to different stress conditions such as starvation. However, when levels of autophagy are excessive, it can lead to autophagic cell death, known as type II programmed cell death to differentiate it from apoptosis, the so-called type I programmed cell death, and from necrosis [18-20]. Additionally, it has been demonstrated that a tight relationship between autophagy and apoptosis exist. The anti-apoptotic protein Bcl-2 is able to inhibit autophagy by binding to Beclin 1 [21,22]. This effect of Bcl-2 depends on its cellular localization. When Bcl-2 is at the endoplasmic reticulum it binds to Beclin 1 hampering the autophagic pathway. This inhibition is regulated by phosphorylation, when Bcl-2 is phosphorylated by JNK-1 (Jun N-terminal kinase 1) it releases Beclin 1 allowing autophagy activation [23]. In contrast, when Bcl-2 is localized at the mitochondria, inhibits pro-apoptotic proteins like Bax, which have to be released to allow the apoptotic process [24]. Another protein that regulates both, the autophagic and apoptotic

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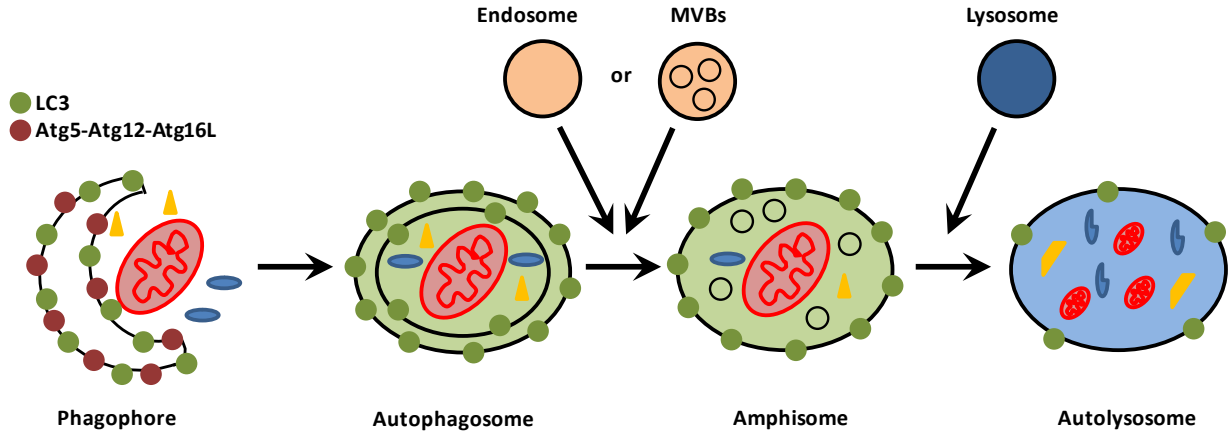

Fig. (1). The autophagic pathway. Different stress situations such as starvation induce the formation of a phagophore or isolation membrane, which sequester portions of cytoplasm. Atg5-Atg12-Atg16L complex and LC3 localize to the phagophore favoring the elongation process. Subsequently, the autophagosome is formed and the Atg5-Atg12-Atg16L complex dissociates from the membrane while LC3-II remains on it. This vesicle is able to fuse with endosomes or MVBs to form the amphisome, which in turn fuses with the lysosome generating the autolysosome to finally degrade the sequestered materials.

processes, is Atg5. As mentioned above, full-length Atg5 participates in autophagosome formation. However, when Atg5 is cleaved by calpain 1 or 2, it is translocated to the mitochondria and causes cytochrome c release. The truncated form of Atg5, but not full-length Atg5, binds to Bcl-xl, and this binding inactivates the Bcl-xl anti-apoptotic activity by releasing Bax [25]. Thus, it is becoming evident that both pathways share common molecules and it is likely that bacterial products interfere with these molecules to switch from a pro-survival to a pro-death mechanism or viceversa.

Autophagy induction is initiated by the activation of the Atg1 complex, which in mammalian cells comprises the kinases ULK1/ULK2, Atg13, Atg101 and FIP200 [26]. This complex is negatively regulated by another kinase, the mTOR (mammalian Target of Rapamycin). Thus, inhibition of mTOR by rapamycin is a potent inducer of autophagy. Likewise, under starvation conditions TOR is inactivated and it dissociates from the ULK complex. In contrast, when nutrients are available TOR engages the Atg1 complex (i.e. ULK complex) and inhibits autophagy by differentially phosphorylating components of this complex [27]. Thus, amino acids levels are a main regulator of TOR and of autophagy [28]. In addition, the lack of amino acids modulates the activity of the phosphatidylinositol 3-kinase complex (PI3K) of class III. Activation of this PI3K/hVps34 complex is an essential step in the initial stages (i.e. vesicle nucleation) of autophagy [29,30].

Autophagy is triggered not only by nutrient conditions, as indicated above, but also by other stress situations. Indeed, cumulative evidence presented during recent years indicates that many intracellular pathogens lead to autophagy activation in the host cell [31-34]. Furthermore, autophagy can also be triggered in the absence of an internalized pathogen. Indeed, we provided the first experimental evidence of a link between this autophagic response and a pore forming exotoxin, the toxin VCC (*Vibrio cholerae* cytotoxin), produced by the extracellular pathogen *Vibrio cholerae* [35]. It is important to take into account that the molecular mechanisms involved in the autophagy triggered by the presence of intracellular pathogens (which may engage signaling *via* surface Toll-like receptors type 4 or intracellular NODs (nucleotide-binding oligomerization domain) receptors, are expected to be different from those involved in the detection of a pore forming toxin. In this latter situation the cell likely senses the presence of damaged membranes and the leaking from a perforated compartment; however, the specific molecular sensors remain to be identified.

BACTERIAL PORE-FORMING TOXINS

Pore-forming toxins (PFT) are important virulence factors produced by numerous pathogenic bacteria as well as other organisms [36,37]. They comprise a large group of bacterial protein toxins that form pores in the membrane of target cells. A remarkable feature of these toxins is that upon secretion by the pathogen as a soluble monomer it binds to a membrane receptor and multimerizes generating an amphipathic structure. Upon a conformational change, ring-like structures insert into the lipid bilayer generating a pore whose size may vary from 2 to 50 nm [38]. This change in size is related to the number of monomers which varies from 7 in the case of aerolysin [39] and staphylococcal α-toxin [40] to 50 in the case of streptolysin O (SLO) and perfringolysin O. The last-mentioned toxins belong to the family of cholesterol-dependent cytolysins (CDC) that use cholesterol as receptors [41]. The CDCs are a large family of pore-forming toxins produced by more than 20 Gram-positive bacterial species from the genera *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus*, and *Arcanobacterium*.

It is important to take into account that the events triggered by pore formation in the plasma membrane of a target cell vary depending on toxin concentration as

well as exposure time to the toxin. Of note, these events would also depend on the pore size generated. One of the first consequences is changes in ionic composition. These toxins are considered potent biological weapons because the formation of the pore leads to cellular ion imbalance. The large pores formed by members of the CDC also allow the efflux of intracellular proteins [42]. Both ion imbalance and efflux of proteins may eventually lead to the death of the host cell. Pore formation can also occur after the pathogen has been internalized allowing the bacteria to escape from the phagosome toward the cytoplasm or egress outside the cell once replication in the target cell has taken place [43].

VIBRIO CHOLERAE CYTOLYSIN (VCC)

The enteropathogenic *Vibrio cholerae* is the agent responsible for causing cholera, a serious human disease characterized by watery diarrhea that may lead to the death of the patients. This extracellular pathogen can produce several toxins including the potent enterotoxin cholera toxin, the zonula occludens toxin and a pore-forming toxin known as *Vibrio cholerae* cytolysin (VCC). VCC, which is encoded by the hlyA gene, is initially secreted as a 79 kDa precursor form [44,45], which is subsequently proteolytically cleaved within its N-terminal domain [46] to generate a 65 kDa form corresponding to the mature toxin.

VCC causes vacuolization or cell lysis (erythrocytes and other mammalian cells) depending on toxin dose and cell type [47-50]. We have demonstrated that the extensive vacuolization caused by VCC was related to autophagy since treatment of cells with either tissue culture supernatants (obtained from a cholera toxin-negative but VCC positive *V. cholerae* strain) or purified VCC toxin increased the punctate distribution of LC3, a feature indicative of autophagosome accumulation [35]. In addition, VCC treatment caused a remarkable increase in the appearance of the form LC3-II visualized by Western blot analysis [35]. Moreover, VCC-generated vacuoles colocalized with LC3 in several cell lines, indicating the interaction of the large vacuoles with autophagic vesicles. Electron microscopy analysis confirmed that numerous vacuoles caused by VCC presented hallmarks of autophagosomes (i.e. double membrane vesicles). Vacuolization was impaired by treatment with classical autophagy inhibitors like 3- methyladenine or in cells deficient for critical autophagy genes (*Atg5*$^{-/-}$ MEFs). Some of the large vacuoles generated in toxin-treated cells also presented features of multivesicular bodies (MVB). Of interest, we have shown by immunofluorescence technique that the toxin itself was present not only on the limiting membrane of the GFP-LC3-labeled vacuoles but also associated to the small internal vesicles. This observation suggests that this could represent a cell defense mechanism to remove the toxin from the membrane since it is likely that the toxin present in the internal structures is subsequently degraded by fusion of these multivesicular/autophagic structures with lysosomes. Fig. (**2A**) depicts a putative intracellular route followed by the toxin. VCC enters to the cell by endocytosis and localizes to MVB, maybe through a microautophagy process. Thereafter, these MVBs likely fuse with autophagosomes generating an amphisome which finally fuses with lysosomes in an attempt to degrade the internalized toxin. Consistent with this possibility the survival capability of Atg5-deficient cells was dramatically impaired [51]. Thus, the loss of a functional autophagic pathway resulted in increased cell death indicating that autophagy was required to protect cells against the toxin cytocidal action.

In summary, our studies with the pore-forming toxin VCC indicate that the toxin triggered an autophagic response in the target cell and, more importantly, demonstrated for the first time that autophagy operates as a cellular defense mechanism against a secreted bacterial toxin.

LISTERIOLYSIN O (LLO) A TOXIN PRODUCED BY *LISTERIA MONOCYTOGENES*

Listeria monocytogenes is a food-borne pathogen that causes serious diseases such as gastroenteritis, encephalitis, and aborts mainly in immunocompromised patients [52,53]. Although infection in humans is not very frequent, it is more common in infants and the elderly and it has predominance among males [54]. This Gram-positive bacterium avoids circulating host defense systems by entering both phagocytic and non-phagocytic cells. Initially the internalized bacterium resides in a phagosomal compartment but subsequently, escapes into the cytoplasm by lysing the phagosomal membrane mainly by the pore-forming protein Listeriolysin O (LLO) [55,56]. Phospholipases also contribute to bacterial escape [57]. This escape is initiated as early as 10 minutes post infection and by 90 min the majority of the bacteria are present in the cytoplasm where the pathogen recruits actin regulatory factors of the host cell *via* the bacterial protein ActA. This allows rapid actin polymerization and the formation of actin “comets” at one of the bacterium poles facilitating pathogen motility and cell-to-cell spread [58-60]. In addition, the bacterial virulence factor InlC perturbs apical cell junctions promoting also cell-to-cell spread of *Listeria* [61].

The interplay between *L. monocytogenes* and the autophagic pathway has been demonstrated in several recent reports. Paul Webster and collaborators have shown that the large majority of chloramphenicol-treated *L. monocytogenes* were surrounded by double-membrane vacuoles in J774 macrophage-like cells, indicating that *L. monocytogenes* treated with this antibiotic can be targeted by autophagy [62]. Subsequently, Py *et al*. [63] showed that *L. monocytogenes* was indeed able to induce an autophagic response as determined by LC3 lipidation and co-localization of intracellular bacteria with this autophagic protein. Another group observed a similar

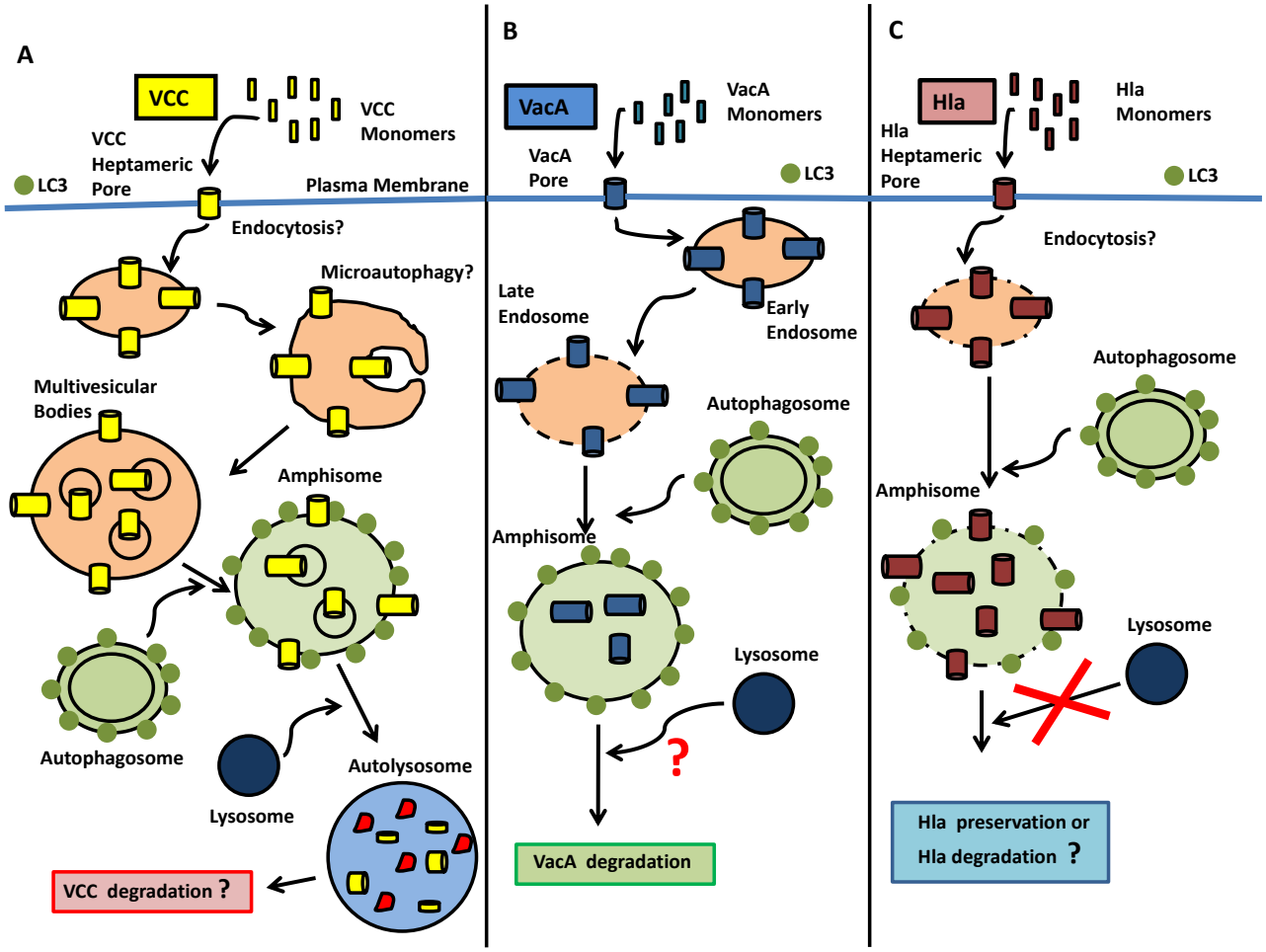

Fig. (2). Toxins: different routes and fates. Left panel: VCC monomers secreted by *V. cholerae* form heptameric pores in the plasma membrane. These pores are internalized probably trough the endocytic pathway. These endosomes acquire a typical Multivesicular bodies (MVB) morphology perhaps by a microautophagy-related mechanism. Then, the MVB fuses with autophagosome to form the amphisome. VCC is detected in the external membrane and in the small vesicles enclosed in both MVB and autophagosomes. Finally, the amphisome fuses with the lysosome which will probably help to VCC degradation because it has been shown that autophagy activation contributes to cell survival. Middle panel: VacA monomers secreted by *H. pylori* forms hexameric pores in the plasma membrane. These pores are internalized by endocytosis. After maturing, the late endosome fuses with the autophagosome, and although it is yet unclear, it is likely that the damage of the endosomal membrane caused by VacA is responsible for autophagy activation. VacA has been localized to the autophagosome, but whether the toxin is present just at the limiting membrane or inside of the autophagosome has not been defined. Autophagy promotes VacA degradation, probably as a mechanism to limit the toxin-mediated cellular damage, but it is unknown if the lysosome actually participates in this VacA degradation. Right panel: Hla monomers secreted by *S. aureus* form heptameric pores in the plasma membrane. In turn, these pores are internalized likely by endocytosis. It is likely that similar to VacA, the damage caused by Hla in the endosomal membrane activates the autophagy pathway, which promotes endosome-autophagosome fusion. Hla has been detected either at the autophagosomal membrane as well inside the autophagosome. It was demonstrated that Hla inhibits autophagosome-lysosome fusion, but it is unknown if Hla remains in the cell or if it is somehow eliminated.

LC3 co-localization with a fraction of untreated bacteria (i.e. 37%) at early times post infection [64], before bacteria escaping from the phagosome. Interestingly, this effect was dependent on the presence of LLO. *L. monocytogenes* lacking LLO presented significantly less co-localization with LC3 than that of the wild type bacteria [63,64]. In contrast, deletion of either of the bacterial phospholipases C (PLCs) did not have a significant effect on the co-localization with LC3-labeled structures although they were required together with ActA and actin polymerization to avoid destruction by the autophagic system. Indeed, under normal infection conditions cytoplasmic wild type bacteria avoid targeting by autophagy in an ActA-dependent manner [64]. In liver granuloma macrophages bacteria were found in large compartments termed spacious Listeria-containing phagosomes (SLAPs) where the bacteria can replicate although at a reduced rate compared to

the cytoplasmic replication. LLO expression was required for SLAP formation and the compartments were also labeled with LC3 [43]. As shown in Fig. (**3A**), the bacteria fate seem to depend on the LLO level [43]. If *L. monocytogenes* express high LLO levels, the bacteria escape from the phagosome before fusing with autophagosomes, form actin comets in the cytoplasm, and finally spread by killing the host cell. If the LLO level is lower, the phagosome fuses with the autophagosome, where bacterial replication occurs slowly. In contrast, if the bacteria do not secrete LLO, the phagosome fuses with the lysosome and *L. monocytogenes* is degraded.

More recently, Portnoy and collaborators [65] have shown that *B. subtilis* engineered to express LLO co-localized with LC3-GFP and induced increased levels of LC3-II indicating that LLO is the factor determinant of this autophagic response. In addition, it was shown that LC3 was also recruited to LLO-containing liposomes internalized by macrophages, in the absence of bacteria, supporting the idea that membrane damage induced by LLO was sufficient for autophagy activation, even in absence of bacterial lipases. Interestingly, in MEF Atg5-/- cells, but not in bone marrow macrophages, a decreased bacterial escape was observed [65] suggesting that under some conditions, autophagy or perhaps just the recruitment of LC3 may favor, rather than prevent, bacterial escape.

STREPTOLYSIN O (SLO): A CYTOLYSIN SECRETED BY GAS

Streptococcus pyogenes (group A streptococcus: GAS) is the agent responsible for a variety of human diseases including acute infections such as pharyngitis and even life-threatening invasive diseases necrotizing fasciitis. This pathogen invades non-phagocytic host cells, and following invasion GAS became enveloped by autophagosome-like compartments decorated by the autophagic protein LC3 [66]. These large compartments were designated as GcAVs for GAS-containing LC3-positive autophagosome-like vacuoles and bacteria were killed upon fusion of these compartments with lysosomes, thus autophagy is likely to contribute to control GAS virulence.

S. pyogenes secretes streptolysin O, a very well characterized cholesterol-dependent cytolysin that has been widely used experimentally to permeabilize biological membranes [41]. Interestingly, an isogenic SLO-deficient mutant was unable to generate the GcAVs [66]. SLO has been involved in the bacterial escape from phagosomes in infected cells but also the toxin is responsible for the induction of autophagy. It has been shown that in cells infected with a wild type strain almost 80% of the cells manifested an autophagic response [67]. In contrast, in cells infected with a SLO-deficient mutant very limited autophagy induction was observed. The authors propose that GAS expressing SLO can escape from and endo/phagosomal compartment but then the cytoplasmic bacteria are sequestered by autophagosome-lysosomal system [67]. In summary, they conclude that the activation of the autophagic pathway is effective for eliminating intracellular GAS and is influenced by SLO-dependent bacterial escape from endosomes.

One point that is puzzling from the studies of Nakagawa and collaborators is that although SLO is one of the major pathogenic factors of GAS, the wild type bacteria were degraded more quickly than the SLO-deficient mutants. The authors try to explain this observation by the fact that the mutant bacteria, due to the lack of SLO, reside within endosomes for long periods of time and apparently are not efficiently eliminated by the phago/lysosomal system. It remains to be determined whether these bacteria-containing compartments have indeed degradative enzymes.

In summary, bacterial cytolysin SLO is known to cause different effects in the host cells such as the induction of inflammatory cytokines, cell death and also the triggering of an autophagic response [68,69]. However, whether these processes are somehow connected or related to each other remains to be fully addressed.

VACA, THE VACUOLATING CYTOTOXIN PRODUCED BY *HELICOBACTER PYLORI*

Helicobacter pylori is a gram-negative spiral bacterium that colonizes the gastric mucosa and has been implicated in the pathogenesis of gastroduodenal disease, including gastritis, peptic ulcer and gastric cancer [70,71]. The infection´s clinical outcome may depend on a combination of several factors including bacterial host and environmental factors. *H. pylori* produce the vacuolating cytotoxin VacA. VacA is a 95 kDa secreted exotoxin which inserts into the cell membrane and causes vacuolization, membrane anion-selective channel and pore formation as well as disruption of the normal endo/lysosomal pathway in the host cell [72-74]. VacA is one of the major virulence factors involved in the pathogenesis of *H. pylori* contributing to the colonization of the gastric mucosa. VacA likely contributes to persistent infection by favoring the generation of intracellular reservoirs [75]. The toxin seems to promote bacterial survival inside both macrophages and gastric epithelial cells by generating vacuoles containing the pathogen [74,76,77]. Interestingly, *H. pylori* infection triggers autophagy in gastric epithelial cells as indicated by the appearance of numerous GFP-LC3 positive structures clearly visible within the cytoplasm [78]. Electron microscopy studies revealed the presence of multilamellar compartments typical of autophagosomes in gastric epithelial cells that were distinguished from the large VacA-induced vacuoles. It is interestingly to mention that in addition to be found in large vacuoles *H. pylori* was also found within autophagosomes [78]. However, it remains to be determined whether residence within these autophagic vacuoles contributes to intracellular *H. pylori* survival.

The vacuolating cytotoxin was necessary and sufficient to induce this autophagic response. Furthermore, the VacA channel-forming activity was

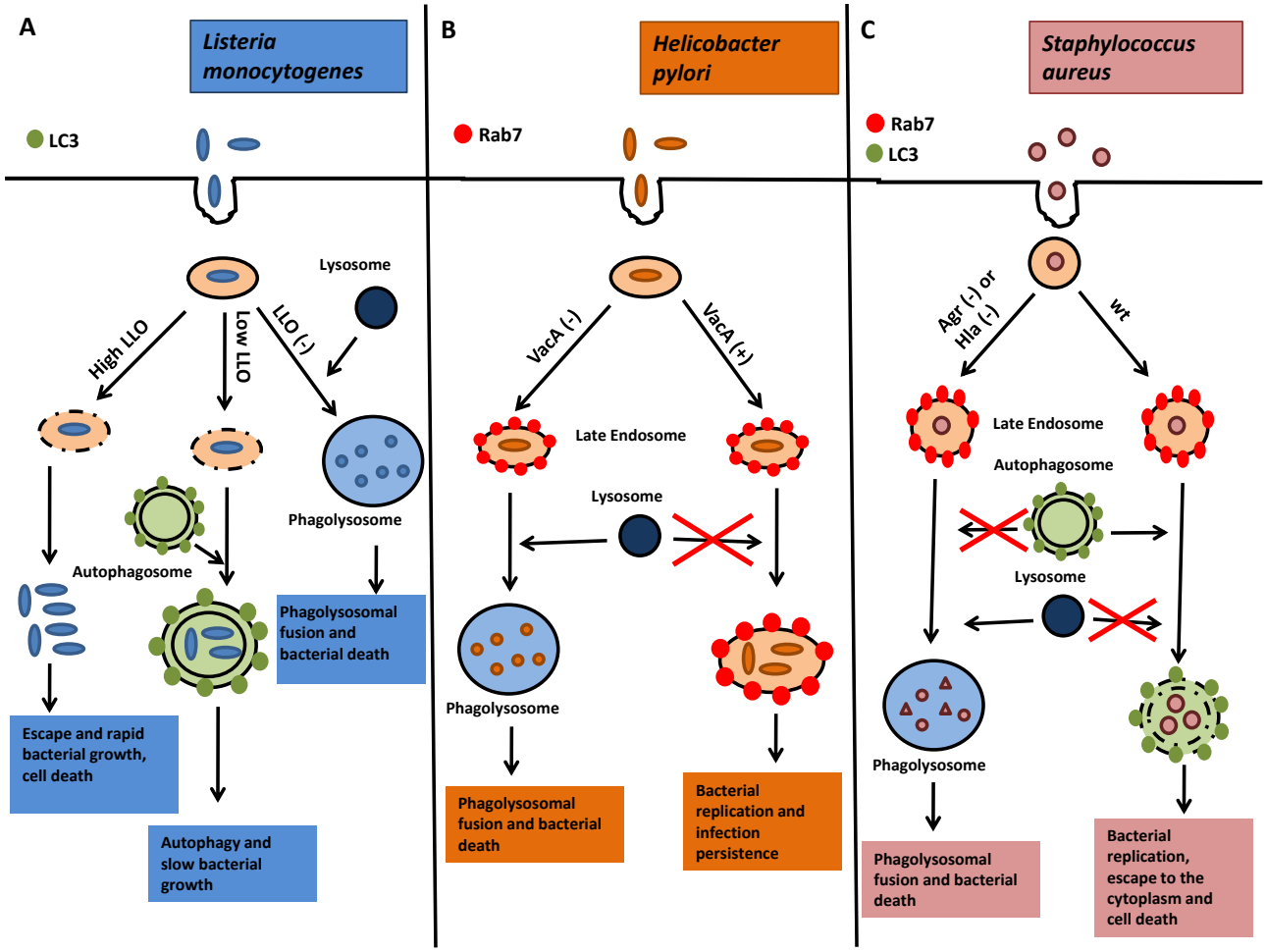

Fig. (3). Bacteria death or bacterial-induced cell death? Left panel: *Listeria monocitogenes* is incorporated to the cell into a phagosome. According to the levels of expressed Listeriolysine O (LLO), the pathogen may have different fates. If the bacteria population is unable to express LLO the pathogen-containing phagosome fuses with the lysosome, where the microorganism is degraded. A second population expresses a low concentration of LLO, which is insufficient to escape from the phagosome but causes membrane damage activating the autophagic pathway. These bacteria may be degraded in autophagolysosomes or may be able to replicate slowly in the autophagosome leading to a persistent infection. The third population lyses the phagosome by means of high LLO amounts. Once in the cytoplasm, the bacterium uses the protein ActA to induce host cell actin polymerization, allowing actin-based motility and cell death. Middle panel: *Helicobacter pylori* is incorporated into an endosome, which then matures acquiring the protein Rab7. *H. pylori* expressing VacA is able to keep Rab7 at the endosomal membrane but avoids endosome-lysosome fusion. In this way, the bacteria are able to replicate in the late endosomes causing a persistent infection. In contrast, *H. pylori* mutants unable to express VacA cannot avoid endosomal maturation and are degraded in the phagolysosome. Right panel: *Staphylococcus aureus* is incorporated into the cell by phagocytosis. In the case of the wild type (wt) strain, which expresses Hla, the damage caused in the endosomal membrane is likely responsible for autophagy activation. These bacteria are able to avoid autophagosome-lysosome fusion, and they likely replicate into the autophagosome. Afterwards, the pathogen escapes to the cytoplasm to finally produce cell death, spreading to neighboring cells. In contrast, strains deficient in Agr or Hla, are unable to activate the autophagic pathway. The phagosomes containing these bacteria fuse with lysosomes and the microorganisms are eliminated.

important for autophagy induction. In addition, the results indicate that toxin-induced cellular injury was somehow controlled by autophagy. Indeed, autophagy induction reduced the stability of VacA and as a consequence there was a decrease in the extent of the large vacuole biogenesis. Thus, the autophagic response seems to be a mechanism employed by the host cell to limit excessive vacuolation and toxin-mediated damage [78]. As shown in Fig. (**2B**), VacA gets into the cell by endocytosis, finally the late endosome fuses with the autophagosome to form the amphisome and to degrade the toxin. It is not clear yet if fusion between autophagosomes and lysosomes is necessary to allow toxin degradation.

Similar to the results obtained in gastric epithelial cells *H. pylori* infection induces autophagosome formation (i.e. LC-3 punctate structures) in macrophages [79]. Interestingly, by 6 h post-infection

dividing *H. pylori* was found in the double-layer vesicle (i.e. autophagosomes) suggesting that these autophagic vesicles were somehow tailored for the multiplication of *H. pylori* in the host cell. Some multiple layer vesicles or onion-like structures became prominent at later infection times. At 24 hrs p.i. less electron-dense *H. pylori* (a sign of bacterial death) were observed in the vacuoles. Interestingly the authors observed an increase in *H. pylori* multiplication in THP-1 cells (a human acute monocytic leukemia cell line) treated with the autophagy inhibitor 3-mehtyladenine, whereas the autophagy inducer, rapamycin, inhibited the bacterium growth, suggesting that autophagy participates in the clearance of *H. pylori*. VacA mutants were internalized by THP-1 cells with a lower efficiency than that of the wild type bacteria. Of note, viable mutant *H. pylori* were barely detectable at 2 hrs p.i.; however, they were recovered from the infected cell at 6 hrs p.i., suggesting that the *H. pylori* mutants were still able to multiply at this p.i. time, but then they were quickly eliminated at later p.i. times [79]. These results are consistent with the idea that VacA is a virulence factor that contributes to the multiplication or survival of the pathogen in macrophages. Fig. (**3B**) depicts that the expression of VacA avoids endosomal maturation and its fusion with the lysosome, allowing bacterial survival and replication. In contrast, if *H. pylori* do not express VacA, the endosome fuses with the lysosome and the bacteria are degraded.

ANTHRAX TOXIN AND AUTOPHAGY

Anthrax toxin is a pore-forming toxin secreted by *Bacillus anthracis.* This toxin consist of three multidomain proteins, Lethal Factor (LF, a Zn^{++}-dependent protease), Edema Factor (ED, a Ca^{++} and calmodulin dependent adenylyl cyclase) and Protective Antigen (PA, a receptor-binding and pore-forming protein). These proteins are secreted by *Bacillus anthracis* as monomers that self-assemble on the cells [80]. EF and LF can be transported to the cytosol by PA and act independently, so they can combine to form ternary complexes as well as binary complexes [80]. In order to interact with LF and EF, PA must be proteolytically activated. This activation generates two fragments, N-terminal 20 kDa (PA_{20}) and C-terminal 63 kDa (PA_{63}) [81]. PA_{63} mediates the biological effects of LF and EF, and it has been shown that PA_{63}, but not native PA, is able to form ion-conductive pores in cellular membranes [82].

The anthrax toxin activity, as well as the proteolytic activation, is dependent on acidic pH within an intracellular compartment. Thus, passage through an acidic intracellular compartment was inferred as a required step for anthrax toxin action [83]. The action of anthrax toxin is initiated by binding of PA to two cellular receptors, ANTXR1/TEM8 (tumor endothelial marker 8) and ANTXR2/CMG2 (capillary morphogenesis factor 2). They are type 1 membrane proteins, share 60% amino acid identity and a metal ion-dependent adhesion site (MIDAS), which is important for the interaction with PA [84].

Aiguo Wu and coworkers [85] have demonstrated that LT was able to activate the autophagy pathway. They have analyzed LC3-II conversion, increased punctate distribution of GFP-LC3 and development of acidic vesicular organelles (AVO). Autophagy activation was observed in LT (PA+LF) treated cells although low induction of autophagy was also observed by PA alone. Since PA may entry into cytosol independently of LF, this moderate induction of autophagy could be attributed to the rapid degradation of PA upon entry into the cytoplasm. In contrast, LF alone was unable to trigger autophagy. Cells pre-treated with the autophagy inhibitor 3-MA showed accelerated cell death compared to control cells, suggesting that autophagy may function as a defense mechanism against LT intoxication [85].

It has been also demonstrated that cathepsin B (CTSB), a lysosomal cysteine protease primarily involved in the degradation or processing of lysosomal proteins, is involved in the delivery of ANTXR2-associated LF into the cytoplasm. Sung Ouk Kim and coworkers [86] showed that LF accumulated in late endosomes in the presence of CA074, a membrane-permeable CTSB inhibitor, or in CTSB-deficient macrophages ($CTSB^{-/-}$). Indeed, in cells treated with the inhibitor, CTSB-containing lysosomes colocalized with LF-containing endosomes, suggesting that CTSB activity was required for the delivery of LF from endolysosomes into the cytoplasm. They also showed that CTSB is required for efficient autophagic flux but not for autophagosome-lysosome fusion, since in $CTSB^{-/-}$ cells or in cells treated with the CA074 inhibitor cells accumulated more LC3-II and the clearance of a fluorescent self-quenched albumin (i.e. DQ-BSA) in autolysosomes was delayed [86].

Jun Ren and coworkers have demonstrated that LT (PA+LF) causes cardiomyocyte contractile dysfunction, in which NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) plays a crucial role due to the production of superoxide anion altering cell survival [87]. That study revealed that this lethal toxin produced subtle although significant upregulation of the expression of the autophagy markers Beclin-1 and LC3-II, suggesting a possible role of autophagy in the anthrax toxin-induced cardiomyocyte mechanical dysfunction [87].

All together, these results indicate that the cells activate the autophagic pathway in response to this lethal toxin, as a defense mechanism to promote cell survival. In addition, knowing that LT is the primary virulence factor for anthrax, we believe that understanding the role of autophagy in response to anthrax may be useful for the development of new therapies to control the anthrax disease.

ALPHA-HEMOLYSIN TRIGGERS AN AUTOPHAGIC RESPONSE THAT FAVORS THE PATHOGEN

α-Hemolysin (Hla) is a β barrel pore-forming toxin lacking cysteine, which is secreted by *Staphylococcus aureus* as a water-soluble monomeric polypeptide of

293 residues. Hla binds to the membrane of a wide variety of eukaryotic cells to form water-filled transmembrane pores, resulting in the release of low-molecular-weight molecules and leading to an eventual osmotic lysis [88]. At high concentrations, Hla forms hexameric ring-shaped structures with an internal diameter of 2 to 3nm [89], but in normal conditions, Hla forms homoheptameric pores on various substrates, including red cell membranes [90]. There is a differential sensitivity of certain cell types toward the attack by Hla, suggesting the existence of a receptor that facilitates pore assembly [91,92]. It has been shown that caveolin might play a role in this pore assembly in some cell types [93-95].

A large number of secreted virulence factors of *S. aureus*, including Hla, are controlled by "the accessory gene regulator, *agr*", and it has been shown that *S. aureus* strains deficient for *agr* were not able to activate the autophagic pathway and did not produce host cell death [96]. Krut and coworkers have demonstrated that *S. aureus* is trapped by autophagosomes after invasion of HeLa cells, as per co-localization with LC3. They have also shown that *S. aureus* was not able to replicate and caused cell death in autophagy-deficient *atg5-/-* mouse embryonic fibroblasts [96], indicating that the autophagic pathway was a key player for bacterial growth and cytotoxicity.

Cheung and coworkers have demonstrated that *S. aureus* wt was able to escape from endocytic vesicles at 2 hours p.i., while the agrA and Hla mutant strains (deficient for agr and Hla, respectively) remained in the endocytic vesicles up to 8 hours p.i., as demonstrated by LAMP-1 and LAMP-2 (Lysosomal-associated membrane protein 1 and 2) co-localization. These data suggest that the lack of Hla is sufficient to avoid the bacterial escape from the endocytic compartment [97]. As shown in Fig. (**3C**), Hla secretion allows *S. aureus* sequestration by the autophagosome, where the bacteria replicate and finally escape to the cytoplasm to kill the host cell. In contrast, if Hla is not present, the *S. aureus*-containing phagosome fuses directly with the lysosome where the bacteria are likely destroyed.

We have recently shown that purified Hla suffice to activate the autophagy in CHO cells (Chinese hamster ovary cells), and that a Hla-deficient *S. aureus* strain was not able to induce autophagy, as demonstrated by co-localization with LC3 and processing of LC3. These results indicate that Hla is the virulence factor secreted by *S. aureus* responsible for triggering the autophagic response [98]. We have also shown that the autophagic pathway induced by the toxin seems to be dysfunctional. By using LysoTraker (a marker for acidic compartments) and DQ-BSA (a degradative compartment marker) we found that the LC3-positive vesicles generated by the toxin are not acidic and non-degradative, suggesting that Hla prevents autophagosome-lysosome fusion altering the normal autophagy flux [98]. Interestingly, our findings indicate that the autophagic response induced by the toxin is independent of the PI3K-Beclin1 complex but required Atg5, indicating that Hla induces autophagy trough a "non-canonical" pathway [98]. As shown in Fig. (**2C**), Hla gets into the cell probably by endocytosis. It is likely that the damage of the endosome membrane leads to the activation of the autophagy pathway and recruitment of LC3. Thus, endosomes fuses with autophagosomes to form the amphisome. However, Hla impairs amphisome maturation. It is not clear if the toxin is degraded or not inside these vacuoles, but it is known that this disrupted autophagic flux is beneficial to bacterial replication.

As for other toxins, it can be concluded that Hla is the factor secreted by *S. aureus* necessary for autophagy activation. Autophagy was required for the generation of the LC3-positive structures that may be used by the bacterium as a replicative niche. Of note, activation of this pathway somehow contributed to bacterial replication and, later on, it was required for host cell death allowing bacterial spread to neighboring cells.

OTHER BACTERIAL TOXINS

The cytotoxic necrotizing factor 1 (CNF1) is a bacterial toxin produced by *Escherichia coli* which allows a non-invasive bacterium to penetrate cells. This toxin is able to activate the Rho GTPases [99] and induces dramatic changes in cell morphology, altering crucial cell functions although without inducing cell death [100]. Apoptosis and mitotic catastrophe seem to be hampered in toxin-treated cells and, indeed, cells show a high survival rate for prolonged periods of time.

Among the dramatic morphological changes observed such as cytoskeleton remodeling, formation of giant multinucleated cells, presence of multipolar mitosis, and aberrant chromosomal aggregations; the cells also showed the appearance of autophagic vacuoles [101]. These autophagic vacuoles often contained cellular debris and residual organelles; thus it has been hypothesized that autophagy activation may contribute to cell survival by removing damaged organelles that could trigger cell death [102].

CONCLUDING REMARKS

Bacterial toxins are powerful weapons used by numerous pathogens to get rid of some of the barriers imposed by the host to eventually avoid bacterial spread. Numerous studies have shed light into the role of autophagy in many biological processes [103]. In this review we have summarized our recent knowledge about the interplay between bacterial toxins and the autophagic pathway. Activation of autophagy seems to be a common protection mechanism of the host cell against the attack of a bacterial toxin. However, further research is required to answer several important questions: Is this autophagic response restricted to a specific group of toxins such as the pore-forming toxins or does it function as a general defense mechanism against any bacterial toxin? How is this interplay between autophagy and a bacterial secreted protein regulated at the molecular level? Which are the signaling pathways involved in autophagy induction in

intoxicated cells? Is the classical autophagy machinery involved in the autophagic response against all bacterial toxins? Certain evidence indicates that this is not the case and that "non-canonical" autophagic pathways may be involved.

Finding the answer to these and other fundamental questions will certainly contribute to our understanding about the strategies used by cells to survive the harmful consequences of bacterial toxins. More importantly, these studies will not only contribute to our knowledge of the pathogen itself but also to the identification of critical cellular factors that govern the scrambling decision between life and death of the host cells.

Finally, a better understanding of how bacterial toxins modulate autophagy is needed to allow its manipulation for therapeutic purposes and for the development of new potential drugs. More potent and specific inhibitors of autophagy are required to target autophagy as a way to prevent its beneficial effects on certain microorganisms. Thus, understanding the molecular mechanisms of bacterial pathogenesis will certainly help to develop new therapies to combat these pathogens.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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