Cellular Microbiology (2010) **12**(1), 10–18
doi:10.1111/j.1462-5822.2009.01403.x
First published online 2 November 2009

Microreview

Bacterial pathogens and the autophagic response

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Summary

The host cell recognition and removal of invading pathogens are crucial for the control of microbial infections. However, several microorganisms have developed mechanisms that allow them to survive and replicate intracellularly. Autophagy is an ubiquitous physiological pathway in eukaryotic cells, which maintains the cellular homeostasis and acts as a cell quality control mechanism to eliminate aged organelles and unnecessary structures. In addition, autophagy has an important role as a housekeeper since cells that have to get rid of invading pathogens use this pathway to assist this eradication. In this review we will summarize some strategies employed by bacterial pathogens to modulate autophagy to their own benefit and, on the other hand, the role of autophagy as a protective process of the host cell. In addition, we will discuss here recent studies that show the association of LC3 to a pathogen-containing compartment without a classical autophagic sequestering process (i.e. formation of a double membrane structure).

General overview

The secret of a good healthy cell lies on a delicate balance between synthesis and degradation processes. Autophagy has a very important role in keeping this homeostasis, since it is a cellular process in charge of degrading long-lived proteins, and removing unwanted or unnecessary material. This ancestral pathway, preserved from yeast to mammals, has captured the attention of many researchers all over the world in the last 40 years. Self-constituents such as misfolded proteins, old/damaged organelles, or simply random parts of the cytoplasm can be targeted by autophagy (Xie and Klionsky, 2007). Upon the appropriate stimuli, a series of membrane rearrangements take place inside the cell, leading to sequestration of parts of the cytoplasm in the so-called 'isolation membrane' or 'phagophore'. Isolation membrane elongates and surrounds the target generating the characteristic double membrane structure termed 'autophagosome', whose size goes from 300 to 900 nm in yeasts and to 0.5–1.5 μm in mammalian cells (Mizushima and Klionsky, 2007). The origin of the membrane supporting autophagosome formation is still under discussion, being the ER and the early secretory pathway the more likely candidates (Hamasaki *et al.*, 2003), as well as the mitochondria in the case of yeasts (Reggiori *et al.*, 2005). Autophagy initiation and completion are highly coordinated by a group of specialized proteins known as Atg (Autophagy related), which were originally described in yeast (Ohsumi, 2001). Two ubiquitin-like conjugation systems, Atg5-Atg12 and Atg8-PE, are crucial for the initiation of autophagosome formation (Cao and Klionsky, 2007). Atg8 has a homologue in mammals, the microtubule-associated protein1 (MAP1) light chain 3, or simply LC3, which is considered one of the most reliable markers of autophagy (Kabeya *et al.*, 2000).

The general function of autophagy involves an adaptation response to starvation conditions. When cells are subjected to nutrient limitations, they use autophagy in an attempt to 'recycle' self-materials generating free amino acids, which can be eventually employed for anabolic processes (Xie and Klionsky, 2007). In addition, this pathway has a housekeeping role, maintaining cells free of unnecessary or potentially toxic material, as well as invading pathogens therefore acting as a surveillance mechanism. Furthermore, autophagy has been also linked to immunity, not only innate but also the adaptive immune response. For instance, thymic selection and MHC class II antigen presentation involves autophagy, which may have a very important role during negative selection of endogenous antigens (Nedjic

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et al., 2008). Regarding innate immunity, a function of autophagy against intracellular pathogens has been described for *Mycobacterium tuberculosis* and Group A *Streptococcus* (Gutierrez *et al*., 2004; Nakagawa *et al*., 2004), among others. Intracellular pathogens can be targeted by the autophagic machinery in a similar way as other cargos. However, the role of autophagy goes further and involves not only the elimination of the 'intruder', but also takes part in the delivery of antigens to support its presentation in the context of MHC class II (Strawbridge and Blum, 2007). Although this is a very limited and general description, autophagic contribution to immunity has been intensively studied (Levine and Deretic, 2007), and is a very attractive goal for future therapeutic applications.

Autophagy: a pathogen monitoring mechanism modulated by several microorganisms

The infection with a pathogenic microorganism can intercept the autophagy pathway in different ways. On one hand, autophagy acts as a defence mechanism taking part of the innate and adaptive immunity. Once pathogens invade the cells they can be recognized by autophagy, and after being engulfed by autophagosomes, they are ultimately degraded in the autolysosomes. On the other hand, several types of pathogens have developed different strategies to subvert the autophagy pathway, escape from lysosomal degradation and exploit this pathway to their own benefit. Therefore, autophagy plays a role in the clearance of certain pathogenic organisms, whereas it supports the intracellular survival of others. These dual functions of autophagy have been widely reviewed in the last few years (Levine, 2005; Levine and Deretic, 2007; Sanjuan and Green, 2008; Campoy and Colombo, 2009), hence a concise summary describing some bacterial pathogens as examples will be developed in this section.

The ability of autophagy to eliminate pathogenic organisms or to provide a niche for their replication depends on the nature of the pathogen. A group of intracellular microbes are targeted by autophagy. These pathogens initially enter into the host cell through phagocytosis, but they can arrest the maturation of the phagosome or turn this organelle into a compartment where they are able to survive and replicate (Alonso and Garcia-del Portillo, 2004). Moreover, other pathogens are able to lyse the phagosomal membrane and escape to the cytoplasm where they can be eventually captured by the autophagy machinery.

Avoidance of phagosome maturation is one of the strategies used by a pathogen to allow its replication and intracellular survival within the host cell. Indeed, one of the most remarkable characteristics of *Mycobacterium tuberculosis* lies on the ability to control the fate of the bacterium-containing phagosome, by blocking its maturation into a degradative compartment. Gutierrez *et al.* (2004) showed that autophagy induction is able to override, at least in part, this blockage. This eventually leads to the maturation of a subset of phagosomes containing the bacillus, partially restraining intracellular bacterial survival. More recently, experiments done by the group of Purdy and Russell demonstrated that upon autophagy activation large quantities of ubiquitinated proteins are transferred to the lysosome (Alonso *et al*., 2007). Degradation within the lysosome generates ubiquitin-derived peptides that have bactericidal activity (Alonso *et al*., 2007). Therefore, one possible mechanism responsible for the killing activity inside the mature phagosome is mediated by the ubiquitin-derived peptides generated when autophagy is activated. It is likely that these ubiquitin-derived peptides may act synergistically with lysosomal enzymes and other components to eradicate the pathogens. Thus, all these results, in agreement with studies of other pathogens, lead to propose autophagy as part of the innate immune system in charge of getting rid of intracellular pathogens (Gutierrez *et al*., 2004; Nakagawa *et al*., 2004).

An interesting point in discussion is how the autophagic cell machinery recognizes the strange element within the cell. It is likely that the secretion of diffusible substances or effector proteins by the pathogen (please see following section) predisposes the cell to awake the autophagic response once the organism invades the host. *Salmonella enterica* serovar Typhimurium lives and replicates in a protective compartment of the host cell called the *Salmonella*-containing vacuole (SCV). The extensively characterized *Salmonella*-type III secretion system (TTSS) injects bacterial effectors into the host cell, which allows the establishment of the SCV. In this way, *Salmonella* controls the fate of its own vacuole and inhibits the maturation of this compartment impeding fusion with the lysosomes. Brumell and collaborators have observed a different behaviour of a population of intracellular bacteria, early after infection. A fraction of the internalized bacteria resides in damaged SCVs, which are targeted by autophagy and this results in the inhibition of bacterial replication (Birmingham and Brumell, 2006). Therefore, autophagy plays a protective role in *Salmonella* infection.

Autophagy has been also proposed as a surveillance system for pathogens that gain access to the cytoplasm after escaping from its membranous compartment. In this way, the autophagic machinery is able to monitor the intracellular milieu, detect and enwrap the invaders in a membranous compartment. Eventually, this latter matures into a degradative autolysosome, which eliminates the pathogen. Some pathogenic bacteria such as *Streptococ-*

cus pyogenes, *Listeria monocytogenes* and *Shigella flexneri*, among others, disrupt the membrane of the pathogen containing-phagosome to escape into the cytoplasm. In these cases autophagy can target these bacteria in the cytoplasm and acts as a defensive response of the host cell.

Streptococcus pyogenes, also known as Group A *Streptococcus* (GAS) invades non-phagocytic cells and induces autophagy (Nakagawa *et al*., 2004). This bacterium secretes a pore-forming toxin termed Streptolysin-O (SLO), which has been suggested to allow the escape of the pathogen into the cytosol. Once the bacterium is free in the cytoplasm, it is targeted by the autophagic pathway and directed into a degradative compartment where it is finally eradicated. Cells with a functional autophagic pathway are able to restrain intracellular bacterial replication and hence GAS infection can be effectively controlled by this intracellular surveillance system. This is another good example of autophagy as an innate immune mechanism.

With regard to *Listeria monocytogenes*, the bacterial toxin Listeriolysin-O (LLO) mediates the microbial escape from the phagosome and is required for autophagy induction (Kayal and Charbit, 2006). Once free in the cytoplasm, *Listeria* forms an actin comet-tail, allowing the movement of the bacterium in the host cell, which may contribute to avoid autophagy detection (Tilney and Portnoy, 1989). Rich *et al.* (2003) have demonstrated that mutant bacteria incapable of polymerizing actin are targeted by autophagy in the cytoplasm. On the other hand, *L. monocytogenes* with deficient LLO activity, although unable to escape to the cytosol, interrupts the maturation of the phagosome and replicates in spacious *Listeria*-containing phagosomes (SLAPs). However, the replication rate is much slower than the wild-type bacteria in the cytosol (Birmingham *et al*., 2008a). SLAP formation depends on a functional autophagy pathway (Birmingham *et al*., 2008a) and probably promotes a persistent infection of *L. monocytogenes* in the host cell (Birmingham *et al*., 2008b).

An interesting way to modulate autophagy by some pathogens is through the utilization of virulence bacterial factors. For example, *Shigella flexneri* is another pathogen that escapes to the cytosol early after infection and develops actin-tail motility by the function of a bacterial membrane protein VirG, which is able to interact with N-WASP. This interaction seems to coordinate actin polymerization and bacterial motility. Studies performed by Sasakawa and collaborators using different bacterial mutants demonstrated that autophagy recognition of *Shigella* in epithelial cells is mediated by the interaction of Atg5 with VirG (Ogawa *et al*., 2005). However, IcsB, a factor secreted by *Shigella* through its TTSS, is also able to interact with VirG with an even higher affinity, therefore inhibiting VirG recognition by Atg5, and thus hiding the bacteria from the autophagic machinery (Ogawa *et al*., 2005). In this way, IcsB not only blocks the detection by Atg5 but also enables the normal function of VirG in polymerizing the host-actin. Therefore, *Shigella* has evolved an efficient mechanism to 'disguise' and get away from autophagy capture once free in the cytoplasm. However, in 2007 Suzuki *et al.* demonstrated that a *Shigella* mutant that lacks VirG (ΔVirG) was able to activate autophagy recognition of the pathogen in a similar manner as the wild-type strain, when using bone marrow-derived macrophages (BMDMs) as host cells (Suzuki *et al*., 2007). This suggests that other factors rather than specifically VirG may be involved in autophagy activation, at least in this cell type. Indeed, a mutant defective in the TTSS was unable to activate autophagy in BMDMs, indicating that components released by the bacterium through TTSS to the cytoplasm could be responsible for this autophagic activation. On the other hand, a recently published article by Dupont *et al.* (2009) further analysed the *Shigella* interplay with autophagy. Once *Shigella* breaks the phagosomal membrane to escape to the cytoplasm, not only the bacterium itself but also the membrane remnants generated upon bacterium escape can be targeted by the autophagy machinery. These vacuolar remnants are first polyubiquitinated to be finally degraded by autophagy.

Another example of pathogens that employ virulence factors to modulate the autophagy response is *Helicobacter pylori*. This bacterium invades epithelial cells in the gastric mucosa and survives inside large vacuoles with characteristics of late endo/lysosomal compartments. The formation of these compartments depends on the presence of the vacuolating toxin VacA (Terebiznik *et al*., 2006). *H. pylori* infection of gastric epithelial cells causes the recruitment of LC3 to a subpopulation of the intracellular compartments where this bacterium resides. The engagement of autophagy is dependent on the presence of the bacterial toxin VacA, since autophagy was not detected when a VacA isogenic mutant bacterium is utilized. The VacA-mediated autophagy does not block the establishment of the vacuole; however, it modulates the levels of the VacA toxin to avoid excessive cellular damage likely induced by the toxin (Terebiznik *et al*., 2009).

As we mentioned before, autophagy stimulation can also be beneficial for the establishment of a pathogen replicative compartment. *Coxiella burnetii*, the etiologic agent of Q fever, is an obligate intracellular bacterium. Once inside the cell, this microorganism multiplies in acidic vacuoles with lysosomal characteristics. Our group has demonstrated that *C. burnetii* survives and replicates in a large replicative vacuole with clear autophagic fea-

tures (Beron *et al*., 2002; Gutierrez *et al*., 2005). *C. burnetii* invades host cells through a classical phagocytosis involving normal components of the endo/phagocytic pathway. However, this pathogen actively interacts with autophagosomes to delay the arrival of hydrolytic enzymes facilitating vacuole development (Romano *et al*., 2007). In addition, *Coxiella* modulates both autophagy and apoptosis to establish a persistent infection in the host cell (Vazquez and Colombo, 2009). Similarly, Niu *et al.* (2008) have showed that the *Anaplasma phagocytophilum*-containing vacuole recruits autophagic proteins. Nevertheless, in this case autophagy induction prevents *A. phagocytophilum* inclusions from fusing with lysosomes, and enables the bacterium to reside in a non-degradative early compartment. Hence, this is another example of how bacteria can benefit from autophagy, and live in a more permissive environment.

Another similar case is that of *Legionella pneumophila*. This bacterium is ingested by macrophages and, instead of following the classical phagocytic pathway, enters in a spacious vacuole, which interacts with early secretory vesicles from the smooth ER. Swanson and collaborators, have postulated that in permissive A/J mouse macrophages, *L. pneumophila* activates autophagy as an immediate response to infection (Amer *et al*., 2005). After few hours, *L. pneumophila*-containing vacuole acquires autolysosomal characteristics, becoming decorated with LC3 and endo/lysosomal proteins. The skill of *L. pneumophila* to replicate in a compartment with clear lysosomal features is possibly due to a delay in the maturation of the vacuole where this bacterium resides (Dubuisson and Swanson, 2006). It is likely that *Legionella* interacts with autophagy in order to delay the maturation of its vacuole, so as to prepare itself for the harsh lysosomal environment. Similarly, *Brucella abortus* resides in an ER-like compartment and subverts autophagy leading to the inhibition of the fusion of its vacuole with lysosomes (Pizarro-Cerda *et al*., 1998).

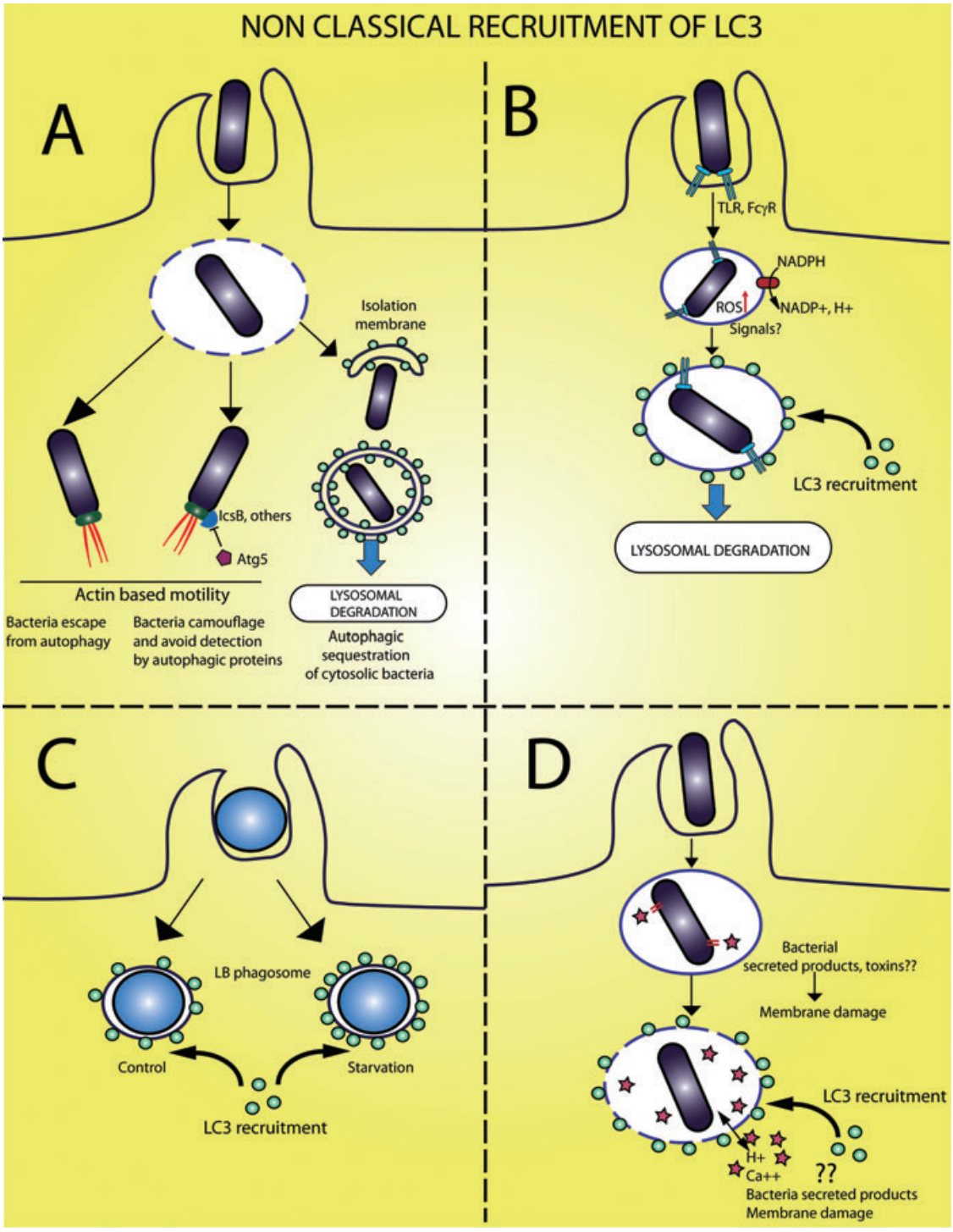
Staphylococcus aureus is an extracellular Gram-positive pathogen that also benefits from autophagy. This bacterium is able to cause intracellular infections in non-professional phagocytes, where it induces cell death after escaping from the phagosome into the host cytosol. In this case, the bacterium transits through autophagic compartments, as it was demonstrated by its colocalization with LC3. Moreover, this interplay is beneficial for the bacteria, since Krut and collaborators (Schnaith *et al*., 2007) demonstrated the failure of the bacteria to replicate in cells deficient for autophagy. It seems that *Staphylococcus*, after staying in early immature autophagosomes, is able to escape to the host cytoplasm to finally induce cell death. All this results in a more permissive environment for bacterial survival and contributes to bacterial spreading.

Autophagosome sequestration of pathogen-containing compartments or direct recruitment of autophagic proteins?

As described in the previous section, among the pleitropic functions of autophagy the sequestration of bacterium-containing compartments by autophagic membranes has become evident upon pathogen invasion of host cells. In general, bacterial pathogens are internalized by a classical phagocytosis mechanism and reside, at least temporarily, in the phagosome, a membrane bound compartment. However, in the last few years cumulative data have emerged from several studies, indicating that upon internalization certain pathogen-containing compartments can be wrapped by isolation membranes (i.e. autophagic double-membrane structures). As indicated above, those pathogens capable of escaping from the phagosomal compartment to replicate in the cytoplasm such as GAS are also trapped by autophagosome-like compartments (Nakagawa *et al*., 2004) (Fig. 1A). This classical form of autophagy, where the invading microorganism is enwrapped by autophagosomal membranes would certainly ensure the elimination of the infectious microbes. It has been shown that metabolically arrested (i.e. treated with choramphenicol) *L. monocytogenes* after escaping into the cytoplasm (6–12 h post infection) becomes enclosed within double membrane vacuoles, and this process can be inhibited by classical autophagy inhibitors (Rich *et al*., 2003). This is a clear example where the autophagic pathway acts as a defence mechanism against an invading pathogen removing the intruder from the cytoplasm to finally deliver it to the endo/lysosomal pathway for degradation. It is important to mention that bacterial protein synthesis must be inhibited in order to be trapped by autophagy, indicating that *Listeria* normally uses an active mechanism for autophagy evasion in the cytoplasm. Likewise, as described in the previous section, *Shigella* has also evolved an efficient mechanism to hide and get away from autophagy sequestration once free in the cytoplasm (Fig. 1A).

Direct recruitment of autophagic molecules to single membrane compartments

Recent evidence suggests that direct recruitment of LC3 to the phagosomal membrane, instead of classical autophagic mechanisms (i.e. formation of double membrane structures labelled with LC3) can be also involved in the autophagic response against invading microorganisms. Green and collaborators (Sanjuan *et al*., 2007) have found that LPS via Toll like receptor (TLR) signalling induces autophagy as well as phagosome maturation. These authors have also shown that a phagocytic particle



engaging TLRs on macrophages triggers the rapid translocation of the autophagic proteins Beclin 1 and LC3 to the phagosome. Interestingly, the recruitment of these proteins was not associated with evident double-membrane structures characteristic of conventional autophagosomes (Sanjuan *et al.*, 2007). In a recent publication it has been shown that not only engagement of TLRs but also Fcγ receptors induced the recruitment of LC3 to the phagosomal limiting membrane (Huang *et al.*, 2009). Interestingly, this process was dependent on the activity of the NOX2 NADPH oxidase, which is activated by both types of receptors generating reactive oxygen species (ROS). The generation of ROS was necessary for LC3 recruitment to the phagosomes (see Fig. 1B).

In addition to LC3, Atg12, a component of the conjugation complex Atg5-Atg12-Atg16, was also recruited to the phagosomes. This latter complex determines the site of the LC3 lipidation (Fujita *et al*., 2008), thus, it is very likely that LC3 is directly lipidated at the phagosomal membrane generating the LC3-II form, without conventional isolation membrane formation. Since it has been shown that LC3 mediates membrane tethering hemifusion (Nakatogawa *et al*., 2007), it is tempting to speculate that the presence of LC3 on the pathogen-vacuole may facilitate the fusion with compartments from the endo/lysosomal pathway leading to microbial killing.

Another interesting observation is that proteomic analysis of highly purified latex beads-containing (LBC) phagosomes has demonstrated the presence of LC3 in the phagosomal membranes (Shui *et al*., 2008) even in unstimulated macrophages. However, the levels of LC3-II in phagosomes increase upon autophagy induction (Fig. 1C). Of note, these LBC phagosomes are known to be surrounded by a unique membrane being devoid of the double-membrane structures typical of autophagosomes. The enrichment of LC3 on LBC phagosomal membranes in starved macrophages was also confirmed by fluorescence microscopy studies. Clear LC3-ring like structures surrounding the latex beads were visualized (Shui *et al*., 2008). Thus, the observation that autophagic proteins seem to be directly recruited to phagosomal membranes supports the link between both pathways autophagy and phagocytosis at a molecular level.

Possible signals involved on LC3 recruitment

The recruitment of LC3 to either phagosomal compartments or autophagic structures seems to be a response to the presence of bacterial products. Indeed, it has been shown that after being engulfed by the host cell, GAS escapes from the phagosome via the secretion of SLO. As indicated previously, it has been proposed that once in the cytoplasm GAS is enwrapped by autophagic membranes (Nakagawa *et al*., 2004). The autophagosomes generated in response to infection with this pathogen diverge from canonical autophagosomes since the vacuoles that surround clusters of GAS are extremely large and they remain for longer periods of time. Interestingly, SLO-deficient GAS does not seem to be trapped by autophagosomal structures (i.e. labelled by LC3). The authors postulate that SLO-deficient GAS cannot escape from the phagosome into the cytoplasm and consequently is not sequestered by autophagosomes. However, an alternative possibility is that LC3 is recruited to those phagosomes damaged by the pore-forming toxin even before escaping from the phagosomal compartment.

LC3-labelling of phagosomal compartments containing a toxin-producing pathogen has also been observed in several other microbial infections. This is the case of *L. monocytogenes*, which is targeted by autophagy in an LLO-dependent manner during the process of escaping from the phagosomal compartment (i.e. at 1 h post infection) (Birmingham *et al*., 2008a). Labelling with LC3 of *L. monocytogenes* compartments is rapidly lost being scarce after 4 h post infection, when the majority of the bacteria are free in the cytoplasm. Indeed, it has been shown that bacterial expression of LLO but not phospholipases is necessary for autophagy induction (Py *et al*., 2007). In addition, autophagy appears to limit the ability of *L. monocytogenes* to escape from the phagosome attenuating intracellular bacterial growth early after internalization, before reaching the cytoplasm where it actively replicates (Py *et al*., 2007).

It is likely that the toxin-damaged vacuole membrane might be the autophagy target (Fig. 1D). Indeed, we have evidence that in the case of *S. aureus*, a pathogen that produces the toxin alpha haemolysin (Hla), their phagosomes are also labelled by LC3. Of note, the LC3-labelled compartments containing bacteria do not accumulate the acidotropic probe LysoTracker (Mestre *et al*., 2009). In

Fig. 1. Non-classical LC3 recruitment to a single membrane compartment. The hallmark of the autophagic protein LC3 has been its association to typical double-membrane autophagosomal structures. However, recent data have set this point under discussion, since it has been found that LC3 can be also recruited to single membrane structures under diverse intracellular conditions.
A. Some bacteria are able to escape from its containing phagosome after damaging the membrane, perhaps through the action of toxins. Once in the cytoplasm, the bacteria may have different fates. Some of them are able to secrete proteins that are used by the pathogen to polymerize host actin, and therefore move inside the cell avoiding autophagy targeting (e.g. *Listeria monocytogenes*). A different strategy is used by *Shigella flexneri*, which hides from autophagy by synthesizing a protein that prevents Atg5 binding to a bacteria structural protein (competitive inhibition). However, other microorganisms once in the cytoplasm might be captured via classical autophagosomal structures upon autophagy stimulation. In this case, LC3 is present on phagophores that will finally sequester and eliminate the intracellular pathogen.
B. Phagocytosis through TLR receptors as well as FcγR has been also related to non-canonical association of LC3 with single membranes. These receptors have been linked to signalling pathways, which leads to LC3 recruitment, phagosomal maturation and accelerated lysosomal degradation.
C. Proteomic studies aimed to identify proteins associated to latex beads phagosomal membranes revealed that LC3 is also targeted to this type of single membrane phagosomes. LC3 amounts on phagosomal membranes respond to typical autophagy regulation; thus its level increases when autophagy is stimulated by starvation.
D. In some cases bacteria harboured within single membrane phagosomes can secrete proteins, i.e. toxins leading to membrane damage, with the consequent egress of ions, bacterial toxins/proteins. Either the damaged membrane itself or the secreted molecules might be signals for LC3 recruitment. This is the case for *Listeria monocytogenes* and *Staphylococcus aureus*.

contrast, *S. aureus* Hla (–) mutant (unable to produce the toxin) localizes in an acidic compartment but unlabelled by LC3. A plausible explanation for these observations is that since Hla is a pore-forming toxin, the generated pores would allow the diffusion of the protons across the membrane. As a consequence, the luminal pH of the bacteria-containing compartment would be neutralized. These results suggest that the LC3 protein is recruited only to those damaged vacuoles (i.e. perforated by the toxin), perhaps as an attempt to defend the cells.

A possible role for permeabilization of the vacuole in the induction of autophagy has been previously proposed during infection with *S. typhimurim* (Birmingham and Brumell, 2006) and *Toxoplama gondii* (Martens *et al.*, 2005). It has been shown that a fraction (20%) of internalized *S. typhimurium* colocalized with LC3 at early times post infection (i.e. 1 h). It was proposed that damaged *Salmonella*-containing vacuoles due to the needle-like TTSS were responsible for autophagy targeting (Birmingham and Brumell, 2006). Whether these compartments were actually surrounded by the typical autophagic double-membrane vacuoles remains to be assessed by a detailed electron microscopy analysis. In the case of *T. gondii*, although the parasite was targeted by autophagy, no autophagic membranes enwrapping the parasitophorous vacuoles were detected (Yap *et al.*, 2007). It was reported that in primed macrophages the parasitophorous vacuole membrane and the parasite plasma membrane become physically disrupted and stripped parasites are exposed to the cytoplasm. This process actually precedes autophagosomal elimination of the parasites (Martens *et al.*, 2005), suggesting that the leakage of internal antigens from the parasite may trigger autophagy. However, work by Andrade *et al.* (2006) shows that activation of a different signalling pathways (i.e. CD40/TNF) leads to autophagosomal digestion of *T. gondii* without apparent disruption of the pathogen-containing compartment.

As indicated above, the damaged-limiting membrane itself can be the target for autophagy. Alternatively, bacteria can be sensed by pathogens recognition receptors (PRRs) that act as sentries for the detection of invading microbes free in the cytoplasm. These receptors, including TLRs, Nods and Nod-like receptors (NLRs), among others; sense the presence of pathogens that have escaped to the cytoplasm. NLRs recognize pathogen through their associated molecular patterns commonly known as PAMPs, and interestingly, they have been recently demonstrated to be involved in autophagy activation (Delgado *et al.*, 2008). Thus, the release and diffusion into the cytoplasm of bacterial factors or vacuolar contents (e.g. ions such as calcium), due to the membrane perforations caused by the toxins, may provide signals for autophagy activation (please see Fig. 1D). Moreover, as indicated above, we have evidence of autophagy induction upon treatment with Hla, the pore-forming toxin from *S. aureus*. This autophagic response is indeed prevented upon calcium chelation (Mestre *et al.*, 2009). This later result supports the idea that certain ions may be, at least in part, responsible for autophagy activation. Cumulative evidence indicates that treatment of cells with other purified bacterial pore-forming toxins, such as the VCC (*Vibrio cholerae* cytolysin) (Gutierrez *et al.*, 2007) or VacA from *H. pylori* (Terebiznik *et al.*, 2009), also triggers an autophagic response. Treatment of cells with these toxins increases the lipidation of LC3 and its association to membranes, an indicative feature of autophagosome formation. At the electron microscopy level the vacuoles generated by VCC present hallmarks of autophagosomes. Interestingly, by immunofluorescence it was observed a marked colocalization between the toxin VCC and LC3 in the same vacuoles (Gutierrez *et al.*, 2007). However, whether LC3 is directly recruited to toxin-damaged vesicles remains to be clarified. In the case of VacA, although *H. pylori* induces autophagy in a toxin-dependent manner, the generated autophagosomes were clearly distinct from VacA-induced vacuoles, which reach a considerably larger size and are labelled by LAMP-1 and Rab7 but completely devoid of LC3. However, as indicated in the previous section GFP-LC3 was detected surrounding intracellular bacteria indicating that autophagosomes target intracellular *H. pylori* (Terebiznik *et al.*, 2009). Therefore, although some common features are observed in cells infected by different toxin-producing pathogens, or in cells directly treated with purified pore-forming toxins, these features need to be analysed case by case.

Challenging outcomes

As an essential homeostatic process, autophagy allows cells to turn over discrete portions of the cytoplasm and to remove damaged organelles and toxic macromolecules (Cao and Klionsky, 2007). In the recent years it has become evident that autophagy is a very important, intracellular defence mechanisms that microorganisms must confront after internalization by the host cell. Recent evidence suggests that proteins involved in the autophagic pathway seem to be recruited directly to phagosomal membranes, leading to an accelerated microbial elimination, in certain cases. In other cases, the presence of autophagic proteins on a damaged membrane may be part of a more general recognition mechanism to protect the host cell from the invading pathogen. However, whether the removal of pathogen-containing compartments requires indeed the presence of autophagosomal proteins but not the classical sequestering process by an isolation membrane needs to be further addressed. In addition, the dissection of the molecular mechanisms

involved in regulating the pathways that leads to the lysosome also warrants further studies. Likewise, the possibility that both pathways autophagy and classical phagocytosis works synergistically against the intruder would be of significant future interest. In summary, all the recent findings described above and the new ones to come will certainly change our vision of how autophagy works as part of the innate immune response.

Acknowledgements

We thank Dr John Brumell and Dr Ju Huang (Cell Biology Program, Hospital for Sick Children, Toronto, Canada) for critical reading of this manuscript and for useful comments. MIC own work is funded by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT2004 # 01-20711 and PICT 2005 #38420), Secyt (Universidad Nacional de Cuyo).

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