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Review

Autophagy in intracellular bacterial infection

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ABSTRACT

Numerous pathogens have developed the capacity to invade host cells to be protected from components of the systemic immune system. However, once in the host cells they utilize sophisticated strategies to avoid the powerful machinery built by the cells to kill invading pathogens. In the last few years cumulative evidence indicates that autophagy is one of the most remarkable tools of the intracellular host cell defense machinery that bacteria must confront upon cell invasion. However, several pathogens subvert the autophagic pathway and, manipulate this process at the molecular level, as a strategy to establish a persistent infection. In this review we have summarized the interaction between autophagy and different bacterial pathogens including those that take advantage of the host cell autophagy, allowing successful colonization, as well as those microorganisms which are controlled by autophagy as part of the innate surveillance mechanism.

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1. Pathogens and the autophagic pathway: subversion vs. innate defense

An important strategy used by numerous pathogens is the invasion of host cells in order to shelter from components of the host immune system, such as the complement system. However, cells have developed powerful means to destroy invading pathogens via generation of reactive oxygen species, modulation of essential cations and nutrients, and degradation by proteolytic enzymes. Thus, once the microorganisms have entered the host cell, these intracellular pathogens have developed sophisticated mechanisms that enable them to overcome host cell defenses and replicate successfully [53,110]. One mechanism employed by bacteria to evade the host innate immune response is escape into the cytoplasm to avoid lysosomal killing [60,112]. Other intracellular microorganisms stay inside the vacuolar phagosome, but hamper their maturation into lysosomes (reviewed in [97]), thus guaranteeing the progression of the infectious process. A third strategy is to divert trafficking from the normal phagosomal pathway towards the autophagic pathway, taking control of this cell defense mechanism to the bacteria's advantage for survival and replication (reviewed in [45,35,34]). For example pathogens such as *Legionella pneumophila*, *Coxiella burnetii* and *Brucella abortus* benefit from autophagy in developing their natural intracellular niches, whereas others escape from an autophagosome-like compartment to replicate in the host cell cytoplasm (see Fig. 1, left). Several of these microbes are expected to manipulate the autophagy pathway at the molecular level as a strategy to establish persistent infection [36,118]. However, transit through the autophagy pathway is not beneficial for most pathogens and autophagic events are critical cell defense mechanisms against invading microorganisms (see Fig. 1, right) (reviewed in [90,2]). Indeed, we have shown that the autophagy machinery has an efficient inhibitory effect on the survival of *Mycobacterium tuberculosis* [63,36]. Autophagy can also effectively eliminate cytoplasmic Group A *Streptococcus* [102] whereas *Shigella flexneri* employs a sophisticated survival strategy to prevent autophagic degradation [107,106]. Thus, autophagy is one of the most remarkable components of the intracellular host cell defense machinery that bacteria must confront upon cell invasion.

2. Autophagy subversion by bacteria

In this section we will focus on those pathogens that not only transit through the autophagy pathway but also seem to benefit in the process (for an overview, please see Fig. 2). Although exploitation of intravesicular niches is a common aspect of the biological life cycle of several intracellular pathogens, in many cases how the bacteria residing within autophagic vacuoles manage to resist lysis is still far from being understood.

2.1. C. burnetii, a pathogen residing in an autophagolysosomal compartment

C. burnetii is the causal agent of Q fever, a human disease that has both acute and chronic phases [68,116]. *C. burnetii* is highly infectious via inhalation of contaminated aerosols and can survive for long periods of time in its natural environment — cattle, goats and sheep being the primary reservoirs for human infection.

C. burnetii is an obligate intracellular microorganism that cannot be grown in axenic medium. Several pathogens exploit specialized Type

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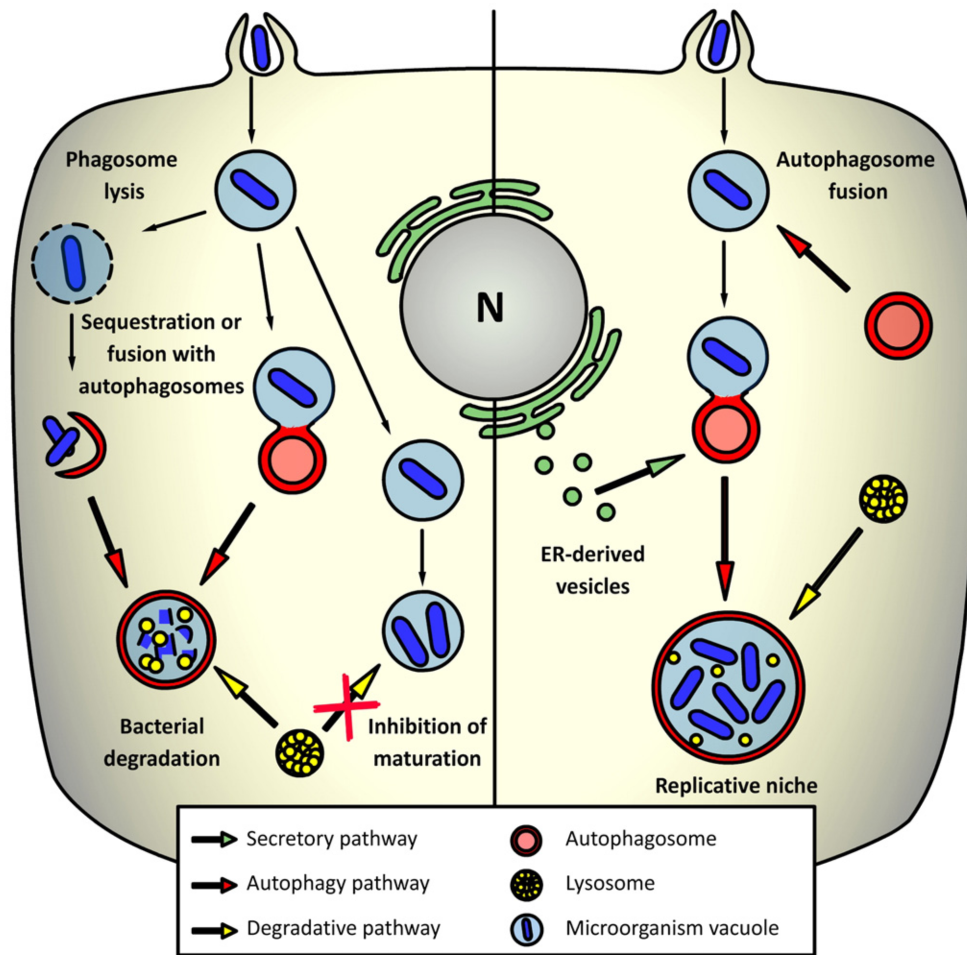

Fig. 1. The two faces of the autophagy/bacteria interplay. Initially, the bacteria are internalized by the host cell and reside in a phagosomal compartment. However, some bacteria (left panel) are able to disrupt the vacuole membrane by secreting certain factors (i.e. pore-forming toxins, phospholipases). Subsequently, the bacterium escapes from the phagosome and can be sequestered by the autophagic pathway and consequently degraded (i.e. *Listeria monocytogenes*; GAS). Alternatively, upon autophagy-activation, the bacterium-containing vacuole is trapped by autophagic membranes or fuses with autophagosomes and this compartment is directed to the degradative pathway (i.e. *Mycobacterium tuberculosis*). In these two cases autophagy plays a role as a defense mechanism against pathogens (left panel). In contrast, some bacteria subvert the autophagic pathway to generate a replication niche where the microorganism survives and actively replicates (i.e. *Coxiella burnetii*; *Legionella pneumophila*). Thus, these bacteria benefit from trafficking via the autophagic pathway (right panel).

IV secretion systems (T4SSs) to achieve intracellular survival [84]. The T4SS, homologous to plasmid transfer systems, has been adapted as a protein export apparatus and is believed to be a key element in determining the intracellular fate and the replication capability of the pathogens via the secretion of effector molecules. *C. burnetii* generates large and spacious vacuoles in which the bacteria replicate. Once *C. burnetii* is internalized by the host cell, it is localized in early phagosomes which fuse with other vesicles, homo- and hetero-typically, to form the large parasitophorous vacuoles (PV) where this pathogen multiplies [68,77]. This bacterium has efficiently adapted to survive and replicate in the harsh environment of the large, acidified, phagolysosome-like vacuoles [10,9], although the mechanism of resistance to acid hydrolases is largely unknown. Interestingly, *C. burnetii* metabolism is activated, at least in part, by the low pH found within the phagolysosome.

Work from our laboratory has demonstrated that the *Coxiella* PV also has the hallmarks of an autophagosomal compartment. The large vacuoles containing *Coxiella* are labeled by the marker monodansylcadaverine (MDC) [19,101] and also by the autophagic protein LC3, confirming the autophagic nature of the PV [17,65]. Recently, we have also demonstrated that the *Coxiella* PV is decorated by the autophagic protein Beclin1 (Vázquez and Colombo, manuscript submitted), a protein that forms a complex with the Class III phosphatidylinositol 3-kinase, VPS34, and which is required for the initial steps in the autophagy pathway.

The protein LC3 is quickly recruited to the *Coxiella* phagosomes post-internalization (i.e. 5 min), indicating that *Coxiella* interacts with the autophagy pathway soon after infection [119]. At later times (60 min post-infection), the majority of the *Coxiella* phagosomes have acquired the lysosomal enzyme cathepsin D, indicating that *C. burnetii* transits the phagocytic pathway and recruits maturation markers. However, the lysosomal enzymes are not acquired immediately after internalization by the *Coxiella*-containing phagosomes, suggesting that there is a delay in the fusion with the lysosomal compartment. Interestingly, this delay is enhanced by starvation but is hampered upon treatment with chloramphenicol, an antibiotic that inhibits bacterial protein synthesis, [119], indicating that the bacterium actively delays lysosomal fusion. Consistent with this, Howe and Mallavia have previously described that viable but not inactivated *C. burnetii* delays phagosome–lysosome fusion early during infection in J774 macrophages [75].

Evidence has been presented that the development of the PVs responds to activation or inhibition of the autophagic pathway. Wortmannin (WM) and 3-methyladenine (3-MA) inhibit autophagy by blocking PI3K activity [109]. Both compounds block *Coxiella* vacuole formation, suggesting that the autophagic pathway has a

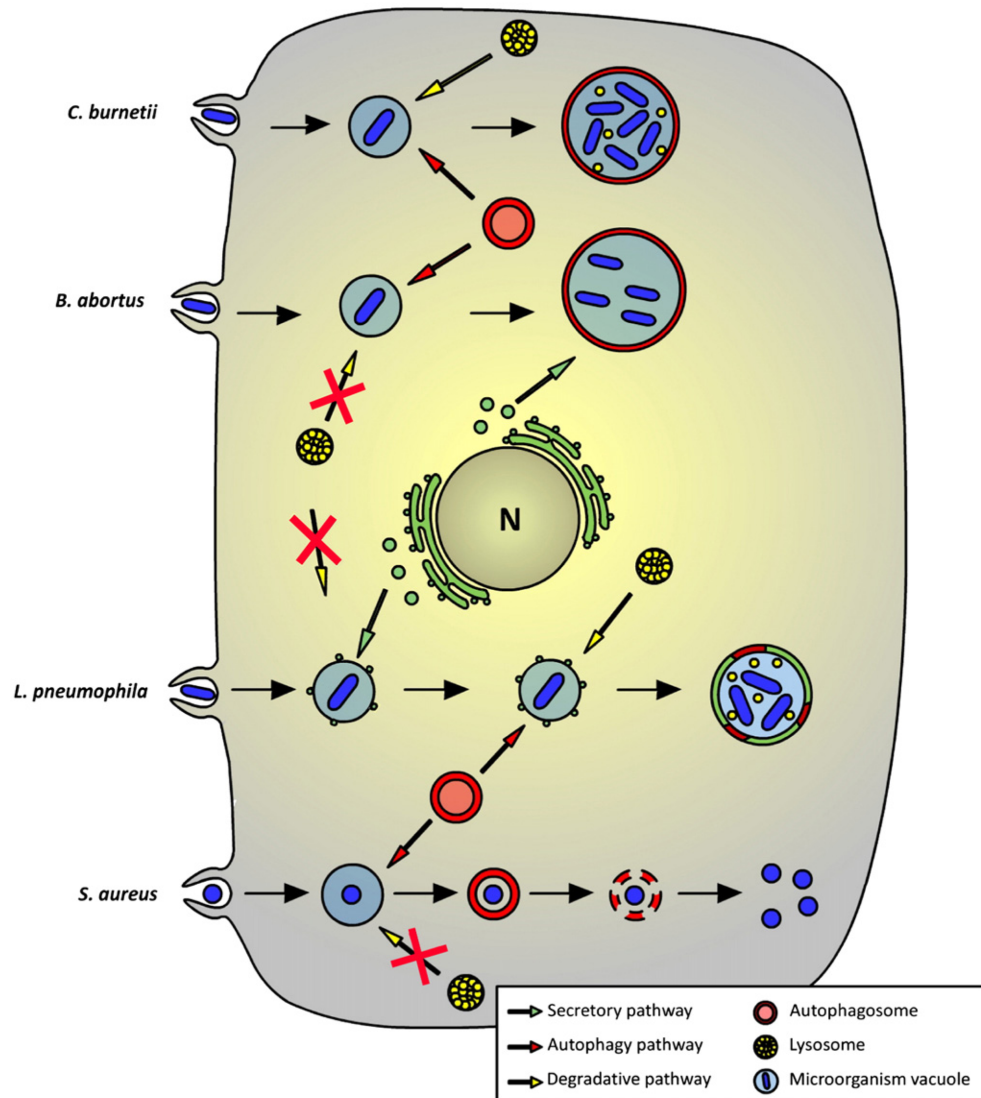

Fig. 2. Autophagy subversion by intracellular pathogenic bacteria. The model depicts several bacteria that use different strategies to survive within the host cell by subverting autophagy. (I) After engulfment, the *Coxiella burnetii*-containing phagosome fuses with compartments from both the autophagic and degradative pathways in order to generate its replicative niche with acidic and degradative characteristics. (II) Early after internalization, *Brucella abortus* impairs fusion with lysosomes and traffics to autophagosome-like compartments, replicating in an ER-derived compartment. (III) Within a few minutes after infection, *Legionella pneumophila* secretes factors that promote interaction with the ER, generating ribosome-lined phagosomes, evading lysosomal fusion and joining the autophagy pathway. Nevertheless, several hours after interacting with vesicles derived from the early secretory pathway, the *Legionella*-containing compartment fuses with lysosomes to establish its replication vacuole. (IV) *Staphylococcus aureus* inhibits fusion with lysosomes and interacts with autophagosomes. After this interaction, *S. aureus* escapes into the cytoplasm by a mechanism dependent on *Staphylococcus*-secreted toxins to finally replicate in the host cell cytoplasm.

critical role in PV development and hence bacterial replication [17]. Incubation of cells under starvation conditions (amino acid- and serum-free medium) or treatment with rapamycin (a pharmacological inducer of autophagy) ([40,137]) increases the percentage of infected cells and the size and development of the PV, as well as *C. burnetii* replication, indicating that autophagy promotes its development. The generation of the *Coxiella* PV is also modified by the overexpression of autophagy-related proteins. In cells overexpressing either wild type (wt) GFP-LC3 or Rab24wt [100], large *Coxiella*-replicative vacuoles are already formed 12 h after infection, whereas in cells transfected with mutants of these proteins both the infection and the size of PV are drastically reduced [65]. It is important to take into account that under normal conditions, during the first 12 h post-infection (p.i.), *Coxiella* resides in small vesicles and that the large PVs are generated only after 48 h. Thus, overexpression of proteins involved in the autophagic pathway remarkably accelerates the development of the parasitophorous compartment where *C. burnetii* replicates, suggesting that components of the cellular machinery of autophagosome formation are subverted to promote *C. burnetii* replication and differentiation. Interestingly, treatment with chloramphenicol hampers LC3 recruitment to the early *Coxiella* phagosome [119]. Furthermore, in cells containing large and spacious PVs, chloramphenicol causes the collapse of the vacuole [76,119]. Thus, it is likely that *C. burnetii* injects effector proteins into the host cells through the T4SS and activates autophagy, generating the large replication niche and creating a more permissive environment for bacterial replication.

In our current model we postulate that *C. burnetii* replication is modulated and favored by conditions that regulate autophagy [65,36]. The nascent *Coxiella*-containing phagosomes formed after *C. burnetii* internalization into host cells fuse rapidly with autophagic compartments rich in nutrients such as amino acids, lipids and other metabolites critical for *Coxiella* growth and reproduction. In addition, a subset of autophagic proteins is actively recruited to the *Coxiella*-containing compartment, having a key role in the generation and maturation of the PV. Hence, *C. burnetii* can be added to the group of

intracellular bacteria that use the autophagic pathway as a mean of surviving, subverting this process for its own benefit.

2.2. *L. pneumophila* and its ER-derived customized niche

L. pneumophila is a Gram-negative, facultative intracellular pathogen and the causative agent of Legionnaires' disease-producing severe pneumonia [50,72,96] it affects both humans and animals, who may become infected after inhaling the microorganisms. *L. pneumophila* infects alveolar macrophages and continues to grow within them when these phagocytic cells are recruited during the subsequent inflammatory response. Infection may then extend to the alveolar epithelial cells that coat the airways, crossing the alveolar wall and finally spreading to multiple organs.

The intracellular multiplication of *L. pneumophila* in alveolar macrophages correlates with its ability to cause the disease [31,55]. Soon after internalization, *L. pneumophila* evades fusion with lysosomes but interacts with other organelles, such as ER-like structures [71]. Indeed, the cytoplasmic face of the *Legionella* phagosomes is studded by ribosomes during the first hours of infection [71,133], reviewed in [120]. The significance of this association is not fully understood; however, association with these organelles appears to be essential for intracellular growth of *L. pneumophila*. Contained in this ER-derived niche, *L. pneumophila* differentiates into the replicative form [99]. At longer times after infection, *Legionella* is indeed diverted to an acidic, lysosomal compartment where it replicates for several hours [130]. Thus, the *Legionella*-containing compartment only merges with the lysosomes after a delay of several hours.

Cumulative evidence indicates that the ER and the autophagic pathway contribute to the development of the *L. pneumophila* replication niche. Swanson and Isberg demonstrated the association between *L. pneumophila* and membranes derived from the ER in infected macrophages by electron microscopy [133]. The hypothesis that *L. pneumophila* exploits host autophagy to favor the development of its replication niche is amply supported by different lines of evidence. One of them is that the ultrastructure of the replication vacuole resembles a nascent autophagosome. Other relevant observations include an increase in the number of intracellular *L. pneumophila* surrounded by ER markers, and the stimulation of bacterial growth in response to autophagy-induction in macrophages [133].

Escape from phagolysosome fusion, intracellular multiplication, and lysis of the host cell are dependent on a T4SS encoded by the *dot*/*icm* gene complex [16,124]. It has been postulated that autophagy would act as a barrier to infection but certain intracellular pathogens can perturb the normal maturation process of the autophagosomes to avoid or delay their delivery to the lysosomes. Indeed, the maturation process of the vacuoles harboring *L. pneumophila* is slower than typical autophagosomes stimulated either by rapamycin or starvation conditions [4]. Additionally, *L. pneumophila* releases soluble factors that activate the autophagy pathway by a mechanism that does not require phagocytosis of the bacteria. Thus, the available data support the idea that *Legionella* delivers virulence factors to slow down the maturation of the autophagosomal vacuole via the T4SS, delaying the arrival of the lysosomal enzymes. Interestingly, the biogenesis of both *Legionella*-replication vacuoles and yeast autophagosomes seems to be promoted by vesicle production from the ER [86,87,66]. Both autophagosomes and *Legionella*-vacuoles acquire lysosomal features (presence of LAMP-1, cathepsin D, and acidic pH) at the terminal stage of development, but during the maturation process a subset of the vacuoles colocalizes sequentially with the specific autophagic marker Atg7 (the first autophagic enzyme involved in ubiquitin-like conjugation), Atg8 (LC3) and the fluorescent dye MDC [98,91,101]. Analysis of the *L. pneumophila* infection process in macrophages supports the contribution of the secretory pathway to autophagosome biogenesis. Indeed, several ER proteins colocalize with the *Legionella*-vacuoles [41,73,94]. Further supporting the connection between *L. pneumophila* and both the secretory and the autophagic pathways, Brefeldin A decreases the colocalization between the autophagic enzyme Atg7 and the *Legionella*-vacuoles [4]. These studies provide additional support for the idea that the ER might be the source for the autophagosome isolation membranes [70,8,125].

Another interesting point is that the interaction of *Legionella* with lipid rafts seems to be required for the localization of this pathogen within autophagic-like structures [3]. It has been proposed that both pathogen internalization and autophagosome formation in response to infection might be stimulated by certain components of the lipids rafts such as the cholesterol content. In contrast, cholesterol depletion does not affect autophagosome formation when autophagy is induced by starvation or rapamycin treatment, indicating that lipid rafts are not critical for autophagosome biogenesis in uninfected cells.

In conclusion, by interacting with the autophagy pathway of the host cell *L. pneumophila* retards the maturation of its vacuole, favoring a replicative, acid-tolerant form [130,99]. However, *L. pneumophila* is able to replicate in several autophagy null mutants of *Dictyostelium discoideum* indicating that a functional autophagy pathway does not seem to be crucial for intracellular bacterial replication in this organism [108]. Thus, it is not clear whether the autophagy machinery is actually required for intracellular multiplication in mammalian cells or if the activation of this pathway just accelerates *Legionella* vacuole development and replication as observed in the case of *C. burnetii.*

2.3. Transit of *Porphyromonas gingivalis* via the autophagy pathway

The Gram-negative anaerobe *P. gingivalis* is an oral pathogen which causes gingivitis, but it is also a risk factor for chronic inflammatory diseases such as atherosclerosis and cardiovascular disease. *P. gingivalis* traffics and replicates in human vascular cells, and the pathogen fimbriae are required for invasion [42]. Both invasion and fimbriae are responsible for activation of endothelial cells, a key event in the development of atherogenesis [134].

It has been demonstrated that, as for *Legionella*, lipid rafts participate in the internalization of *P. gingivalis*. The bacterium colocalizes with the caveolae marker caveolin-1 and the knock down of this protein reduces invasion [135]. Likewise, treatment of the host cell with cyclodextrin, a cholesterol-depleting compound and a caveolae disruptor, results in inhibition of invasion. However, it is still not known whether, as for *Legionella* [3], entry of *P. gingivalis* via lipid rafts is a requirement for its interaction with the autophagy pathway (see below).

Subsequent to its association with the plasma membrane, the bacterium is incorporated into early phagosomes and colocalizes with the small GTPase Rab5 [44], but not with the late endosomal marker mannose-6-phosphate receptor (M6PR), indicating that the pathogen is diverted from the normal phagocytic maturation pathway. In human coronary artery cells (HCAEC) numerous intracellular *P. gingivalis* were located in multimembranous vacuoles resembling autophagosomes [43]. This was the first report suggesting a connection between the intracellular niche of *P. gingivalis* and the autophagy pathway. Later, the same group fully characterized the intracellular trafficking of this pathogen. Shortly after internalization the bacterium is targeted to the autophagy pathway, as shown by the recruitment of the autophagic protein HsGsap (i.e Atg7) [44]. Subsequently, *P. gingivalis* transits to a late autophagosomal compartment that contains both the RER marker-protein BIP and the lysosomal membrane marker Lgp120 (Lamp1) but is devoid of the lysosomal enzyme cathepsin L. This compartment does not acquire hydrolytic enzymes for at least the following 2 h, indicating that *P. gingivalis* hampers the formation of autolysosomes either by postponing the autophagosome–lysosome fusion or by redirecting the normal autophagic transit. At the ultrastructural level, profiles of bacteria in division can be visualized in these double-membrane vacuoles containing non-degraded cytoplasmic components, suggesting that the bacterium replicates in these compartments. In cells

treated with the PI3kinase inhibitors WM or 3-MA, a large fraction of the bacteria are diverted from a Rab5 compartment to a vacuole labeled with M6PR which subsequently acquires cathepsin L. More importantly, in endothelial cells treated with these autophagy inhibitors the survival of the bacteria decreases dramatically, and analysis at the electron microscopy level revealed the presence of degraded bacteria at later infection times. These data indicate that a functional autophagy pathway is required for the generation of the intracellular niche of *P. gingivalis* and for the establishment of a persistent and chronic infection.

An important point to take into account is that the interaction of *P. gingivalis* with the autophagy pathway appears to be cell specific. Whereas this bacterium localizes in autophagic vacuoles in HCAEC cells [43,44], in gingival epithelial cells (GEC) and KB human epithelial cells the pathogen is found free in the cytoplasm and in the perinuclear area [15,74]. Furthermore, in a recent publication it has been shown that *P. gingivalis* may elude the autophagic pathway and instead directly traffic from the phagosomal compartments to lysosomes [85,142]. However, depending upon the strain used, the bacterium colocalizes with LC3 during the first hours p.i. Moreover, in the infected cells the autophagy pathway is indeed activated as demonstrated by the formation of LC3 puncta and accumulation of LC3-II [142]. Nevertheless, the same authors found considerable differences depending upon the strain used. Thus, future experiments may clarify the cell type-specific behaviour of the different *P. gingivalis* strains, and elucidate its significance for the in vivo infection of different tissues.

It is essential to bear in mind that *P. gingivalis* is an asaccharolytic pathogen, thus trafficking through the autophagic pathway is not only a way of acquiring protection from cell defense mechanisms but also a means of acquiring essential nutrients. The replicating autophagic vacuole contains host peptides and amino acids that can be used by the pathogen to survive and replicate within the host cell (reviewed in [14]).

2.4. *Staphylococcus aureus* subverts the autophagy pathway

S. aureus is a Gram-positive bacterium responsible for a variety of serious infections such as endocarditis, pneumonia, septic arthritis and abscess formation. In many cases these infections originate from hospital-derived antibiotic-resistant bacteria. Also in patients with cystic fibrosis this pathogen colonizes the lungs causing recurrent and health-deteriorating infections. *S. aureus* usually causes septic and toxic shock and these severe complications may lead to multiple organ failure [93,95]. As with other bacterial pathogens, *S. aureus* presents small colony variants (SCVs) which grow very slowly and have an unusual colony morphology (i.e. nonpigmented, nonhemolytic colonies when grown on rabbit blood agar). These SCVs are more resistant to antibiotics and thus these strains have been linked to resistant and relapsing infections (reviewed in [114]).

Although *S. aureus* has been classically considered an extracellular pathogen, it can colonize a wide variety of mammalian cells [1,13,81] and the intracellular survival of *S. aureus* is a critical feature in staphylococcal persistence and chronic disease. One of the key features of *S. aureus* infection is the production of a series of pathogenic factors, including secreted enzymes and toxins. The expression of many of the *S. aureus* pathogenic genes is controlled by two regulatory systems, the *Agr* and *Sar* [29,30]. *S. aureus*-induced cytotoxicity is mediated in part by alpha toxin, a secreted protein which causes the formation of pores at the plasma membrane, but also leads to cell death via the activation of the mitochondrial apoptotic machinery [12].

It has been reported that once the bacterium gets into close proximity with the host cell surface, it is engaged via the fibronectin- and collagen-binding proteins present on the cell wall and the pathogen is internalized. *S. aureus* resides within phagosomes that avoid fusion with the lysosomal compartment [88]. In a recent publication [122] the intracellular transport of this bacterium has been analyzed in HeLa cells at the electron microscopy level. At 1.5 h p.i. the bacteria is present in a Rab7-positive phagosomal compartment which is attached to multilamellar membranes. By 3 h post-infection many of the phagosomes are surrounded by these membranes, forming double-membrane autophagosomes enclosing the bacteria. It is important to take into account that Rab7 is present, not only in late endosomal compartments, but also in autophagic vacuoles [65,79]. These compartments are also labeled with the autophagic protein GFP-LC3, as evinced by confocal microscopy. At later times the majority of the *S. aureus* are found free in the cytoplasm indicating that the pathogen escapes from the autophagosomes into the host cell cytoplasm. The appearance of bacteria in the cytoplasm coincides with signs of cell death [13,81].

Preincubation with the autophagy-inducer rapamycin markedly increased the number of intracellular pathogens, indicating that autophagy favors *S. aureus* replication. As expected, the intracellular growth of the bacterium is dramatically reduced by treatment with WM. Likewise, *S. aureus* replication is notably impaired in cells deficient for the key autophagy protein Atg5. Notably, an *agr*-deficient strain was not surrounded by multilamellar membranes and did not colocalized with LC3 at any post-infection time studied. This indicates that the *agr*-mutant *S. aureus* is not capable of inducing an autophagic response in the host cell [122]. In addition, phagosomes containing wt *S. aureus* rarely colocalize with the lysosomal protein LAMP-2, whereas the *agr*-deficient mutant clearly acquires this marker, indicating that one or more *agr*-dependent factors inhibit fusion between the autophagosomal compartments and the lysosomes. In contrast to the vacuoles containing wt *S. aureus*, the *agr*-deficient bacterium is labeled by Lysotracker, indicating that the wt bacteria also avoid acidification. Taken together these results indicate that *S. aureus* prevents the maturation of the autophagosomal compartment, avoiding both acidification and fusion with the lysosomes. In summary, infection with *S. aureus*, despite some differences, has several points in common with the intracellular pathogens *L. pneumophila*, *P. gingivalis* and *C. burnetii* in the sense that all seem to subvert the autophagy pathway, actively delaying fusion with a degradative protease-containing compartment.

One of the key questions is how *S. aureus* escapes from the phagosomal compartment. An interesting observation is that, in the presence of the antibiotic rifampin, an inhibitor of the bacterial RNA polymerase, bacterial escape from the phagosomal compartment was hampered; indicating that de-novo transcription of a gene product was required. The group of Cheung and collaborators has recently demonstrated that an AgrA mutant was incapable of escaping from the endosomal compartment [82]. The AgrCA controls the expression of a number of pathogenic genes, including several toxins (for a review see [104]. One of the major toxins up-regulated by the *agr* system is alpha-hemolysin, a pore-forming toxin [61]. Overexpression of alpha-hemolysin partly recovered the escaping phenotype of a *S. aureus agr* mutant, indicating that this toxin, perhaps in conjunction with another factor, is required for the lysis of the phagosomal membrane to allow the bacteria escape into the cytosol.

2.5. *B. abortus* develops a compartment segregated from lysosomes

B. abortus, an aerobic, slow growing Gram-negative intracellular bacterium is the causative agent of brucellosis, a zoonosis that affects a wide variety of mammals, including man [57,62] The microorganisms reach animals or humans via small injuries or aerosols, or via the ingestion of contaminated dairy products. In humans, brucellosis is a chronic disease characterized by fever and debility, which leads to endocarditis, meningitis, osteoarthritis, and even some neurological disorders.

One of the first targets during *Brucella* infection are the macrophages and other professional phagocytes, where the bacteria can survive and replicate efficiently with minimal disturbance by the

immune system [83]; reviewed in [126,59]. This bacterium can also infect HeLa or Vero cell lines. The bacteria are taken up via receptor molecules and activate small GTPases of the Rho subfamily leading to recruitment of actin filaments. Upon internalization, a virulent *Brucella* strain reaches a privileged intracellular niche, an early phagosome, which avoids fusion with late endosomes and lysosomes (see below; reviewed by [59]). Ultrastructural studies have demonstrated that at 24 h p.i. some bacteria were localized in typical single membrane phagosomes whereas others were found in a compartment containing ribosomes and surrounded by multiple membranes, consistent with it being an autophagic compartment [113]. These ribosomes are likely trapped during the process of autophagosome formation. When cells were incubated in the presence of the autophagy inhibitors 3-MA or WM, lower bacterial yields were recovered at 24 h p.i. In contrast, in cells subjected to autophagy-induction (i.e. starvation) an increase in the number of CFU was observed, indicating that transit via the autophagic pathway plays a key role in the intracellular replication of *Brucella*. In a more detailed study the same authors showed that both a virulent and an attenuated strain were localized to an early phagocytic compartment labeled by EEA1 at 5–15 min post inoculation. However, at later infection times (i.e. 30 min) the compartments containing the virulent *B. abortus* were labeled neither by Rab7 nor by M6PR, markers of late endosomes. Similarly, very little colocalization was observed when the non-virulent strain was used. In contrast, both *Brucella* strains were targeted to a LAMP1-positive compartment devoid of the lysosomal enzyme cathepsin D.

Interestingly the *B. abortus*-containing compartment in HeLa cells is labeled with the protein sec61β, a subunit of the ER translocon; however neither the ER protein ribophorin nor the intraluminal protein BiP were detected, indicating that the *Brucella*-containing compartment is derived from a specialized region of the ER. Furthermore, this compartment is also labeled with the autophagic marker MDC, confirming the localization of *B. abortus* in an autophagic-like vacuole as visualized by electron microscopy. A two-component regulatory system known as BvrS-BvrR (virulence-related sensory and regulatory proteins) has been described in *Brucella* [127]. Interestingly, the *bvrS* and *bvrR* mutants, which invade HeLa cells very poorly, do not localize to an autophagic compartment and are rapidly directed to a cathepsin D-positive compartment. In contrast, a mutant harboring a bvrR plasmid recovered the virulent phenotype and the targeting to MDC-labeled autophagosomes. However, it is important to mention that, in contrast to what has been observed in HeLa cells, in macrophages only a small percentage of the wt bacteria were found in compartments labeled with MDC or with an ER marker [6], suggesting that the fate of the pathogen may vary according to the host cell.

Although, during the first 2 h p.i., both virulent and attenuated *Brucella* strains seem to be found in the same intracellular compartments, at 8 h post inoculation the fates of both strains diverge drastically. Whereas the virulent *B. abortus* comes to reside in a compartment no longer labeled by LAMP1 and avoids lysosomal fusion, the attenuated pathogens are found in LAMP1- and cathepsin D-positive compartments with evident signs of bacterial degradation [113]. However, the autophagosome does not seem to be the replication niche for virulent *B. abortus* since at prolonged times after infection it is no longer labeled by MDC. Thus, the *Brucella*-containing vacuole is transiently labeled by LAMP1 and MDC. Additional experiments have demonstrated that the compartment where this pathogen replicates presents features of a specialized ER compartment located in the perinuclear region [5,113]. In more recent publications it has been shown that the biogenesis of this replication compartment depends on the *Brucella* T4SS VirB [37,27]. Furthermore, the *Brucella*-containing vacuole interacts with ER exit sites (ERES) and disruption of these ERES by inhibition of the small GTPase Sar1 hampers *Brucella* replication [28].

It is known that the early endocytic pathway converges with the nascent autophagic vacuoles [92,47]. Thus, it is likely that shortly after internalization *Brucella*-containing compartments fuse with autophagic vacuoles. Alternatively, the phagosome can be trapped by newly formed autophagosomes. Although further experiments are required to differentiate between these possibilities, cumulative evidence indicates that autophagy is a key player in the biogenesis of the *Brucella*-replication niche.

2.6. Anaplasma phagocytophilum replicates in an early autophagosome compartment which does not mature into autolysosomes

A. phagocytophilum, the etiologic agent of human granulocytic anaplasmosis, is a Gram-positive obligate intracellular pathogen (reviewed in [26]). This disease is an emerging tick-borne zoonosis in Europe and United States characterized by fever, headache, and myalgia. The typical signs are leucopenia, thrombocytopenia and elevated hepatic transaminases. *A. phagocytophilum* has a particular tropism for neutrophils and the pathogen has developed different strategies for evading and neutralizing the microbicidal activities of the polymorphonuclear leucocytes [11,46].

After entering into host cells, this pathogen avoids being degraded by the endo/lysosomal machinery of the cell, and remains in a non-acidic vacuole that does not stain for endosomal or lysosomal markers [141]. Interestingly, a recent publication reports that the *Anaplasma*-containing compartment presents features of an autophagic compartment, including a double-lipid bilayer membrane and colocalization with the autophagic proteins LC3 and Beclin1 at 48 h p.i. [103] These LC3-decorated vacuoles do not colocalize with the lysosomal protein LAMP3, indicating that autolysosome formation is hampered. Furthermore, evidence has been presented that this bacterium induces autophagosome formation as indicated by the increase in LC3-processing. In addition, *A. phagocytophilum* infection is favored by the autophagy activator rapamycin, whereas the inhibitor 3-MA arrests its growth, indicating that autophagy-induction enhances bacteria replication. Thus, this pathogen subverts the autophagy pathway leading to the biogenesis of an early autophagosome-like compartment which avoids fusion with lysosomes. It is likely that this autophagosomal compartment allows the bacterium access to host cytosolic nutrients needed for its growth.

3. Autophagy as a defense mechanism against pathogens

As discussed above, the autophagy pathway is a 'microorganism-friendly process' that favors the intracellular survival of certain microorganisms. Indeed, several studies indicate that many pathogens have evolved strategies to protect themselves against autophagy or to control the components of autophagy to their own benefit. However, autophagy is an important host defense mechanism in the elimination of intracellular bacteria and protozoans, indicating that this degradative pathway does have a role in innate immunity against intracellular bacteria (for an overview, please see Fig. 3).

3.1. Effective killing of M. tuberculosis by autophagy

M. tuberculosis is a facultative intracellular pathogen that colonizes and multiplies in host macrophages. The success of *Mycobacterium* as a pathogen relies on its ability to modulate the intracellular environment. It has been shown that, after internalization, *M. tuberculosis* is capable of persisting within the phagosomal compartment, interfering with the stereotypical phagosomal maturation process inside the host phagocytic cell [138]. This process is believed to occur by means of inhibition of phagosome–lysosome fusion. Indeed, the mycobacterial vacuole is characterized by retention of early endosomal markers, allowing it access to incorporated transferrin [32]. As a hallmark of the maturation block imposed by *M. tuberculosis*, Rab5 is retained on the

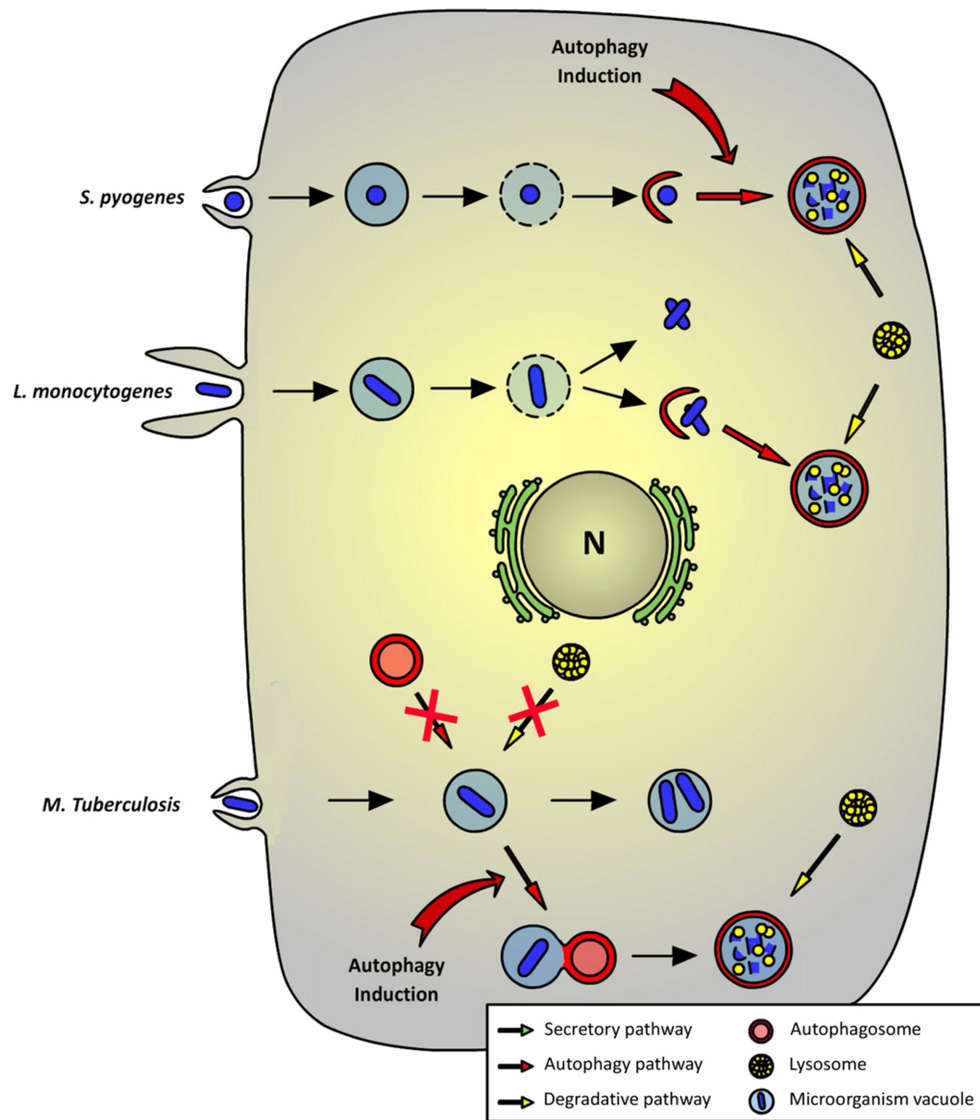

Fig. 3. Autophagy as a defense mechanism against pathogen infections. The figure shows the autophagic pathway acting as an innate immune response to different bacterial infection processes. (I) After internalization, *S. pyogenes* escapes from the phagosome and, upon autophagy-induction, the bacteria are engulfed and degraded in an autophagosome-like compartment. (II) *Listeria monocytogenes* uses a mechanism governed by *Listeria*-secreted factors to escape from the phagosome and replicate in the host cytoplasm. After replication the bacteria disseminate to neighboring cells. However, a subset of the bacteria present in the cytoplasm is sequestered by autophagy and degraded inside an autolysosome-like vacuole. (III) *Mycobacterium tuberculosis* resides in an early phagocytic compartment which avoids maturation and fusion with the lysosome. In contrast, when autophagy is induced by physiological or pharmacological means, the mycobacterium-containing phagosome is directed to the autophagic pathway and degraded by interaction with the lysosomal compartment.

phagosomes and is not exchanged by activated Rab7 [139,131]. In addition, evidence indicates that the *Mycobacterium*-containing phagosomal compartment shows diminished acidification due to the lack of the H^+-ATPase [129]. *M. tuberculosis* seems to disrupt the delivery of V_0 H^+-ATPase subunits and lysosomal hydrolases which occurs via a TGN-to-phagosome pathway dependent on the PI3K hVPS34 [51]. Mycobacterial phagosomes also exclude the proteins Hrs and EEA1 (*E*arly *E*ndosome *A*ntigen *1*) involved in tethering of endocytic vesicles [51], which are commonly recruited to the endosomal membranes via binding to PI3P, the product of the PI3K VPS34. It is likely that the deficient recruitment of the protein Hrs may hamper the association of ESCRT (*E*ndosomal *S*orting *C*omplex *Re*quired for *T*ransport) which has been recently shown to restrict the growth of the non-pathogenic *Mycobacterium smegmatis* [111].

In Gutierrez et al., we demonstrated that several markers of late endosomal and lysosomal compartments, including lysosomal-associated membrane protein 1 (LAMP-1), lysobisphosphatidic acid (LBPA) and cathepsin D, as well as the autophagosomal markers, LC3 and Beclin-1, colocalize with the *Mycobacterium*-containing vacuole after stimulation of autophagy [63]. As indicated, under starvation conditions a marked increase in the colocalization of GFP-LC3 with *M. tuberculosis* phagosomes is observed. Furthermore, since these compartments are also labeled by cathepsin D the data suggest that the pathogen is targeted to an autolysosome. Thus, autophagy-induction counteracts the maturation block imposed by mycobacterium. Furthermore, Raw macrophages infected with *M. tuberculosis* and exposed to starvation conditions or rapamycin treatment, which render mycobacterial phagosomes susceptible to acidification, demonstrate that the acidification defects of the mycobacterial vacuoles are also overridden in cells incubated under conditions that activate autophagy [63].

In addition, at the ultrastructural level it has been shown that an autophagosome-like membrane (i. e. double-membrane or onion-like structure) surrounds the *Mycobacterium*-containing compartment and that the bacterium shows signs of undergoing degradation. These compartments are likely formed by the fusion of *Myco-*

bacterium-containing vacuoles and autophagosomes. Consistent with this observation, physiological or pharmacological (i.e. rapamycin) induction of autophagy suppresses mycobacterial survival, indicating that the bacteria sequestered by autophagy become susceptible to this host killing-activity. Autophagy inhibitors, such as 3-MA or WM, abrogate the effect of autophagy-induction on *M. tuberculosis* survival. Thus, induction of the autophagic pathway is transduced into an acidification and maturation of the *M. tuberculosis* phagosome, and this increased maturation coincides with a decrease in the intracellular survival of the bacilli.

It is known that interferon gamma (IFN-γ), is a cytokine associated with protective immunity against *M. tuberculosis* [49]. Activation of macrophages with IFN-γ results in increased proteolysis of long-lived proteins, which is a hallmark of stimulated autophagy. Thus, IFN-γ-induced autophagy in phagocytic cells is consistent with previous reports in HeLa cells [78]. Activation of cells with this cytokine results in a characteristic increase in MDC-staining of autophagosomes and also induces the translocation of the autophagic marker LC3 from the cytosol to vesicular structures, consistent with the formation of autophagosomes. Furthermore, there is a partial colocalization of LC3 with mycobacteria in IFN-γ activated cells, indicating that the mycobacterium is targeted to autophagic structures. Thus we have demonstrated a physiological role for IFN-γ activated macrophage autophagy in the innate immune response to eradicate *M. tuberculosis*, establishing a key relationship between immune mediators and protection against intracellular pathogens.

In macrophages the p47 GTPases are strongly inducible by IFN-γ [136]. Within this family of GTP-binding proteins, LRG-47 has been implicated as being specifically active against *M. tuberculosis*. We presented evidence that the small GTPase LRG-47 participates in the IFN-γ-dependent induction of autophagy [63]. This initial evidence was supported by demonstrating that LRG-47 stimulates early stages of autophagy including LC3-I to LC3-II conversion [145]. Moreover, LRG-47 controls *M. tuberculosis* infection by a process that leads to enhanced mycobacterial phagosome maturation; consequently this factor plays a crucial role in conferring resistance against mycobacterial infections.

3.2. Group A Streptococcus is efficiently killed by autophagy

Streptococcus pyogenes, also known as Group A streptococcus (GAS) (reviewed in [24]), is one of the most common human pathogens and causes numerous diseases with only minor consequences, in addition to serious diseases that can even lead to death. This is a typical extracellular Gram-positive pathogen responsible for pharyngitis, rheumatic fever, and acute glomerulonephritis, as reviewed in [39].

GAS is capable of invading non-phagocytic cells by the generation of large host membrane invaginations which engulf bacteria. The microbe first localizes in phagosomal structures and then a substantial part of the bacterial population reaches the cytoplasm. Tamotsu Yoshimori and collaborators established that, early after internalization GAS, localizes in compartments decorated by the autophagy protein LC3 in HeLa cells [102]. About 80% of intracellular GAS are eventually trapped by these large (5–10 μm) LC3-labeled compartments. Also, *Streptococcus* chains closely surrounded by LC3 are often observed in the host cells.

Evidence indicates that GAS induces the autophagic response in the host cell. Indeed, in cells deficient for the critical autophagy gene Atg5, required for autophagosome formation, no LC3-decorated GAS containing vacuoles are observed, indicating that their formation requires molecular components of the normal autophagic pathway [102]. However, the autophagosomes generated in response to bacterial infection differ from canonical autophagosomes since the vacuoles that engulf clusters of GAS are extremely large and they remain for longer periods of time.

It is believed that after being internalized GAS escapes from the phagosome via the secretion of streptolysin O (SLO), a cholesterol-dependent pore-forming toxin. In the cytoplasm GAS is enwrapped by autophagic structures. More importantly, these trapped bacteria are killed by fusion of these autophagosome-like compartments with lysosomes, which carry degrading enzymes. The role of autophagy in bacteria viability was demonstrated by comparing the colony-forming units in cells wild type and KO for Atg5. The experimental data obtained indicate that in autophagy-competent cells most of the GAS are killed, preventing the dissemination of the infection, reviewed in [143]. Thus, autophagy efficiently kills a pathogen in non-phagocytic cells, contributing to the innate immunity of the host cell against invading microorganisms.

3.3. Shigella camouflages itself in order to evade autophagy-recognition

S. flexneri is a highly adapted pathogen that affects humans causing serious bacillary dysentery, also known as shigellosis, manifested by a severe mucous and bloody diarrhea that kills a million children per year, mainly in developing countries. The pathogen is ingested via the fecal-oral route, invading and replicating within the human colonic epithelial cells and leading to ulcerative lesions.

Shigella is a Gram-negative bacterium that secretes factors by means of the type III secretion system (TTSS) [123;105]. Among these factors, IcsB and IcsA (VirG) are essential molecules that play a crucial role in the bacterial escape from autophagy. *Shigella* belongs to the group of bacteria that can escape from the phagosome into the cytoplasm where they can multiply and induce local actin polymerization at one pole of the bacteria. These actin tails are used by the bacteria to actively move and to invade neighboring cells. The actin-dependent motility of *Shigella* is regulated by VirG, an outer membrane protein. VirG interacts with N-WASP which, in conjunction with the Arp2/3 complex, is required for actin polymerization. VirG is a key molecule in the Atg5-dependent autophagy targeting of *Shigella* (see below).

A bacterial mutant deleted for IcsB, although capable of invading and escaping from the vacuoles, is defective in multiplying within host cells [106,105]. In contrast to wt bacteria, at 4 h p.i. this mutant was found to colocalize with markers for autophagosomes (MDC and LC3) and for acidic lysosomes (Lysotracker), implying that the *icsB* mutant is targeted by autophagy. Furthermore, observation by electron microscopy shows that the mutant bacteria are wrapped in multi-lamellar membranous structures, while wt *Shigella* present long actin tails and are free in the cytoplasm. The failure of this mutant to evade autophagy has also been demonstrated in MEF cells knocked out for Atg5 and overexpressing GFP-LC3. As expected, in the Atg5−/− cells the *icsB* mutant is not targeted to LC3-labeled compartments and its intracellular replication rate is restored to the level of the wt bacterium, indicating that autophagy is effectively restricting intracellular growth of the *icsB Shigella* mutant.

Interestingly, Sasakawa and collaborators [107,105] observed that the signals for LC3 or Atg5 are often distributed asymmetrically, sometimes accumulating in one pole of the bacterium, reminiscent to the distribution of the VirG protein — the secreted bacterial protein involved in actin tail formation. Indeed, they demonstrated that VirG interacts with the autophagic protein Atg5 by pull down assay. Furthermore, IcsB also binds to Atg5, and Atg5 binding to VirG was competitively inhibited by IcsB, suggesting that IcsB prevents the interaction between the protein VirG and Atg5. Thus, they propose a model in which binding of IcsB to VirG allows the formation of the bacterial actin tails, protecting VirG from being recognized by components of the autophagy machinery (i.e. Atg5). In contrast, in *Shigella* mutants lacking IcsB, VirG binds to Atg5, allowing the pathogen to act as a nucleation site for the formation of isolation membranes, the precursor of autophagosome formation. In summary, this is a clear example of a pathogen that, via the secretion of key

factors, avoids recognition by autophagy, as part of the innate immune response.

It has been previously shown that *Shigella* infection causes caspase-1 activation and leads to cell death (pyroptosis) in macrophages. In a recent study [132] it was demonstrated that caspase-1 activation is mediated via Ipaf, a member of the NOD-like receptor (NLR) family, a cytosolic pattern-recognition receptor of the nucleotide-binding oligomerization domain. These receptors sense the presence of unique microbial components called PAMPs (pathogen-associated molecular patterns) in the cytosol [80]. Caspase-1 activation also requires ASC, the adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain. In the absence of caspase-1 or Ipaf, but not ASC, autophagy is also markedly enhanced in *Shigella*-infected macrophages. In contrast to infected epithelial cells, the bacterial factor VirG was not required for autophagy-induction in macrophages. Interestingly, autophagy inhibition by 3-MA promotes cell death, suggesting that autophagy protects infected macrophages from *Shigella*-induced pyroptosis. Therefore, this study provides evidence that autophagy is inhibited by Ipaf and caspase-1 in *Shigella*-infected macrophages, providing a novel role for NLR proteins in the interplay between pathogens and the host cell.

3.4. *Listeria monocytogenes* evades killing by autophagy

L. monocytogenes, the etiologic agent of listeriosis, is a Gram-positive facultative anaerobe. This disease affects mainly the immune-compromised, infants, the elderly and pregnant women. In the immune-compromised population, listeriosis is very severe and has a high fatality rate.

L. monocytogenes like *Shigella* is an intracellular pathogen that replicates in the cytoplasm after escaping from the phagosome. This phagosomal escape is accomplished by a secreted pore-forming toxin, listeriolysin O (LLO), and it is enhanced by two bacterial phospholipase Cs (reviewed in [67]). Once in the cytoplasm, *Listeria* recruits the host actin polymerization machinery to generate actin tails, and then invades neighboring cells.

In the initial studies by Paul Webster and collaborators [117] it was shown that a *Listeria* mutant defective in the protein ActA, required for the assembly of the actin tail, and metabolically arrested with chloramphenicol, is targeted by autophagy in the host cell cytoplasm. When infected macrophages are treated with chloramphenicol after the bacteria lysed the phagosome, *L. monocytogenes* is trapped in the cytoplasm in double-membrane autophagic vacuoles. This process is inhibited by the autophagy inhibitors WM and 3-MA. Furthermore, the vacuoles are labeled with the protein LAMP1, indicating fusion with endo-lysosomal compartments. Taken together these results suggest that metabolically arrested bacteria get caught in the autophagic pathway, are removed from the cytoplasm, and then delivered to the lysosomal pathway for degradation.

It has been shown that in fibroblasts and epithelial cells infection by *L. monocytogenes* induces an autophagic response [115]. Bacterial production of LLO, but not phospholipases, is necessary for autophagy-induction. Although it has been suggested that permeabilization of the vacuole may have a role in the induction of autophagy, it is likely that the toxin *per se* is responsible for activating autophagy (please see Section 4). Autophagy-induction is observed by 2 h after infection, as determined by an increase in the ratio of LC3-II/LC3-I, which corresponds to an early stage of the bacteria infection [115]. After internalization of *L. monocytogenes* by macrophages, a significant fraction of intracellular bacteria colocalize with the autophagy marker LC3 at early times after infection, indicating that these bacteria are targeted by autophagy [115]. *L. monocytogenes* infected equally both MEF Atg5 deficient (MEF Atg5−/−) or wt cells; however, in the wt cells intracellular replication of the bacterium is delayed, indicating that cells incompetent for autophagy are less restrictive for bacterial growth. Indeed, after several hours of infection the number of bacteria per infected cell was much higher in Atg5 knock out (KO) cells [115]. Interestingly, the replication capacity of a *L. monocytogenes* strain deficient in bacterial phospholipases is hampered in autophagy-competent cells (MEF Atg5 wt), but in cells knocked out for Atg5 (MEF Atg5−/−) the bacterial growth is almost normal, suggesting that the phospholipases produced by the bacterium may help the pathogen to avoid the bactericidal effects of autophagy. In summary, *Listeria* is targeted for degradation by autophagy at early stages of the intracellular cycle, i.e. immediately after escaping from the phagosome but before actively multiplying in the cytoplasm.

In a more recent publication John Brumell and collaborators [21,23] have characterized the relationship between *L. monocytogenes* and autophagy in macrophages. Similar to the results obtained in fibroblasts (i.e. MEFs), there is formation of the LC3-II profile as early as 30 min p.i., with maximal levels at 8 h p.i., confirming that in RAW 264.7 macrophages autophagy is induced upon *L. monocytogenes* infection. Also, at 1 h p.i., a subset of the bacteria localize to compartments decorated by GFP-LC3, a colocalization that is blocked by treatment with autophagy inhibitors. Furthermore, these compartments are also labeled by the lysosomal membrane protein-1 (LAMP-1). However, the colocalization with LC3 is rapidly lost with time, suggesting that *L. monocytogenes* seems to evade targeting by autophagy. In contrast to wt bacterium a mutant deficient for LLO does not colocalize with LC3, which is consistent with previous findings indicating that LLO is required for autophagy-induction. Evidence has been presented indicating that certain virulence factors regulated by the bacterial transcription factor PrfA are critical to avoid autophagy. However, ActA, a protein regulated by PrfA and responsible for the formation of the actin comets, does not seem to be absolutely required for bacterial evasion, although it may participate in the escape mechanism in conjunction with other PrfA-regulated virulence factors [21]. Although the mechanism remains to be elucidated, bacterial phospholipases also contribute to autophagy-evasion in infected macrophages, as demonstrated in fibroblasts. Thus, *L. monocytogenes* utilizes multiple PrfA-regulated mechanisms to avoid killing by the autophagy pathway. Interestingly, a population of *L. monocytogenes* with a low production of LLO was able to multiply slowly in large non-degradative autophagosome-like compartments named SLAPS for *S*pacious *L*isteri*a*-containing *P*hagosomes [22]. One of the key points of these findings is that, even though the host cell seems to control rapid bacterial replication, it is unable to eradicate these pathogens. Thus, the SLAPs may represent a mechanism by which persistent infection is established.

3.5. Intracellular survival of *Burkholderia pseudomallei* is suppressed by autophagy.

Melioidosis, a serious disease of humans and animals endemic in tropical areas, is caused by the facultative intracellular pathogen *B. pseudomallei*. This bacterium invades epithelial cells and also survives and proliferates inside phagocytes. After phagocytosis *B. pseudomallei* escapes from phagocytic vesicles into the cytoplasm where it replicates (reviewed in [128]). In the cytoplasm this pathogen induces actin polymerization, forming actin comets [89]. *B. pseudomallei* also generates actin-based membrane protrusions which allow cell-to-cell spreading.

In a recent publication it was shown that, upon infection of macrophages, autophagy is induced and a subset of the bacteria colocalize with the autophagy protein LC3 [38]. As expected, this colocalization is abrogated by inhibitors of the autophagy pathway. Infection of macrophages with *B. pseudomallei* leads to an increase in the levels of LC3-II, indicating that autophagy is activated in response to cell infection. On the other hand, induction of autophagy by pharmacological means enhances the colocalization of this pathogen with autophagosomes, decreasing its intracellular survival. However,

no effect in survival is observed in cells treated with autophagy inhibitors, suggesting that *B. pseudomallei* has developed strategies to avoid killing by the autophagy pathway. Consistent with this premise, the number of bacteria colocalizing with autophagic vacuoles is clearly enhanced by treatment with chloramphenicol, indicating that bacterial evasion is an active process that depends on protein synthesis. Interestingly, a factor secreted by the *B. pseudomallei* TTSS, the protein BopA, was shown to play a role in autophagy-evasion. Indeed, a *bopA* deletion mutant displayed reduced intracellular survival and increased colocalization with the autophagic marker LC3 [38]. Of note, the protein BopA shows 23% homology to IcsB, the factor secreted by *S. flexneri*, which allows *Shigella* to avoid cellular autophagy (see above). However, the molecular mechanism by which BopA actually permits *B. pseudomallei* to escape from autophagy remains to be elucidated.

In conclusion, this is another example where the autophagic pathway is part of the innate defense system against invading pathogens. Thus, novel therapeutic strategies could be directed at bacterial proteins like BopA, which prevent autophagic destruction of this microorganism.

3.6. Damaged Salmonella Typhimurium-containing compartments are targeted by autophagy

The facultative intracellular pathogen *Salmonella enterica*, serovar *Typhimurium*, causes salmonellosis, a food poisoning disease, and typhoid fever. *S. Typhimurium* actively invades non-phagocytic cells by inducing actin rearrangement and membrane ruffling. This pathogen localizes within a membrane-bound compartment called the *Salmonella*-containing vacuole (SCV) where the bacterium replicates, protected from the immune system. Both bacterial invasion and the generation of its customized intracellular niche (SCV) are accomplished by two needle-like TTSS, one encoded within pathogenicity island 1 (SPI-1) and the other encoded within pathogenicity island 2. Invasion is achieved by the activity of bacterial proteins injected via the SPI-1 TTSS whereas the generation of the SCV requires initially the activity of the SPI-1 TTSS and after few hours, the activity of the second TTSS (reviewed in [140,54]).

The damage to the SCV membranes caused by the SPI-1 TTSS seems to result in recognition by the autophagy pathway. Although typically the bacteria is located in a membrane-bound compartment, a proportion of *S. Typhimurium* escapes into the cytoplasm early after infection [18,25]. Once in the cytoplasm the bacteria are recognized by the ubiquitin system. It was shown that at 1 h p.i. approximately 20% of intracellular *S. Typhimurium* colocalize with the protein LC3. This colocalization is blocked by WM, suggesting an autophagy-dependent process. The colocalization is lost after 2 h p.i., although the bacteria remain labeled by ubiquitinated proteins. Thus, it has been proposed that, as soon as the SCV is damaged, autophagy targets *S. Typhimurium* early after internalization and prior to maximal escape into the cytoplasm. It is likely that this recognition by the autophagy pathway restricts *Salmonella*, protecting the host cell cytoplasm [20]. Using Atg5−/− MEFs cells, an increase in the number of cytosolic bacteria and enhanced replication was demonstrated, suggesting a role for autophagy in preventing the escape of the bacterium into the cytoplasm and restricting its replication. Treating infected cells with the antibiotic chloramphenicol prevents the escape of *S. Typhimurium* from the SCVs, indicating that this process requires bacterial protein synthesis. However, SPI-2 TTSS-deficient bacteria show no major differences in autophagy-recognition compared to wild type bacteria. More specifically, a mutant lacking SifA, a protein secreted via the SPI-2 TTSS system and required to maintain the integrity of the SCV, is not recognized by the autophagy pathway even though this mutant bacterium, similarly to the wt bacterium, is released into the cytoplasm at 6–8 h after infection. These results confirm that, in the case of *Salmonella*, it is not the bacterium already present in the cytoplasm which induces autophagy, but more likely that the damage to the SCV triggers the autophagic pathway in order to prevent bacterial escape. To date, the actual target for autophagy-recognition of *S. Typhimurium* is not known, although the bacterial protein SipB, an SPI-1 effector protein, has been implicated in activating autophagy and causing cell death in infected macrophages [69]. In summary, autophagy seems to play a protective role against *Salmonella* infection at very early stages of the pathogen intracellular cycle, preventing the colonization of the cytoplasm of mammalian cells.

4. Autophagy as a response mechanism against bacterial toxins

In the last few years, accumulating evidence has highlighted the importance of autophagy in numerous biological processes. Although autophagy has been described as a cellular response mainly triggered by whole intracellular pathogens, we provided the first experimental evidence for a link between the autophagic pathway and a specific molecule secreted by an extracellular pathogen, *Vibrio cholerae* [64].

The secretion of factors essential for virulence is a common feature of many bacterial pathogens, whether Gram-negative or -positive. Among the factors produced by bacterial microorganisms, one third of the toxins correspond to the so called pore-forming toxins (for a review see [58,52,7]). These toxins are secreted by the bacteria as monomeric molecules. Once in contact with the target membrane the monomers polymerize forming ring-like arrangements of variable stoichiometry (hexameric, heptameric or larger structures) which behave as membrane pores. The binding to the host cell membrane takes place by specific interactions with lipids, as in the cases of *L. monocytogenes* listeriolysin (LLO) or of *Aeromonas hydrophila* aerolysin (reviewed by [56]). In general, depending on the pore size, the toxin causes alterations in the ionic or protein composition of the cytoplasm due to the leaking of ions or proteins, respectively. A common outcome is that the target cell senses the presence of the toxin and builds up sophisticated mechanisms to respond and protect itself against it. Whatever the protective mechanisms, it is predicted that the defensive actions would be critical for host survival upon the attack by pathogenic microorganisms.

4.1. VCC from V. cholerae

V. cholerae is the etiologic agent of cholera in humans, a potentially fatal disease characterized by watery diarrhea. This pathogen is an extracellular microorganism that, apart from the potent enterotoxin cholera toxin, produces a pore-forming toxin known as *V. cholerae* cytolysin (VCC). This toxin causes cell vacuolization or lysis depending on toxin dose and cell type [144,33,48].

We have analyzed the autophagic cell response upon intoxication with VCC and determined that the extensive vacuolization induced by this exotoxin is indeed related to autophagy [64,121]. This was shown in CHO cells overexpressing the autophagy protein GFP-LC3, which changes to a typical punctate distribution in cells treated with culture supernatants obtained from a cholera toxin-negative (but VCC positive) *V. cholerae* strain but not from a VCC deficient mutant (isogenic hlyA null mutant). This was confirmed using purified VCC, which also causes the appearance of LC3 puncta in non-transfected cells, such as human colonic-derived Caco-2 and C2BBe1 (brush border-expressing Caco-2 clone). In accordance with this observation, a remarkable increase in the appearance of the processed LC3-II isoform is also produced by VCC treatment. Interestingly, the accumulation of LC3-II was further increased when lysosomal degradation was blocked, indicating that the autophagy flux is normal in VCC intoxicated cells.

The autophagic nature of the VCC-induced vacuoles was also confirmed at the ultrastructural level. Numerous double-membrane vesicles and structures with typical features of autophagic vesicles are

clearly present in toxin-treated cells. Furthermore, our studies demonstrate that a functional autophagy pathway is required for the VCC-dependent vacuolization, since the process is markedly reduced by classical autophagy inhibitors or in cells deficient for critical autophagy genes.

Interestingly, the toxin itself localizes not only to the limiting membrane of the GFP-LC3-labeled vacuoles generated by the toxin but also to the small internal vesicles contained in the large vacuoles [121], suggesting that VCC may be trapped in these structures a part of a cell defense mechanism against the toxin attack. It can be postulated that the toxin sequestered in the lumen of these multivesicular-like structures is degraded by subsequent fusion of the autophagic vesicles with lysosomes. In line with this, cell survival is seriously compromised in Atg5-deficient cells unable to undergo autophagy, which are more susceptible to the cytocidal effect of VCC [64].

In summary, the studies with the pore-forming toxin VCC indicate that the toxin triggers an autophagic response in the target cell and, more importantly, demonstrate for the first time that autophagy acts as a cellular defense pathway against a secreted bacterial toxin.

It is important to take into account that this autophagic response upon intoxication with VCC does not seem to be unique to this toxin. Indeed, the laboratory of Dr. Nicola Jones, with whom we are collaborating, has established that VacA, the vacuolating cytotoxin produced by *Helicobacter pylori*, is responsible for inducing autophagy. Furthermore, this autophagic response seems to control toxin-induced cellular injury [146]. Furthermore, we have evidence that the α-hemolysin of *S. aureus* also stimulates autophagy in CHO cells treated with this toxin (Mestre and Colombo, manuscript in preparation). Thus, activation of autophagy seems to be a common defense mechanism of the host cell against the attack of a bacterial toxin.

5. Future perspectives

Molecular studies have shed new light on the pathogenesis of several of the intracellular bacterial parasites that colonize host cells causing chronic and persistent diseases. One point in common is that several of these pathogens subvert the autophagic pathway to generate a replication niche. In this review we have summarized current knowledge of the pathogenic mechanisms employed by a number of bacteria to take advantage of host cell autophagy, allowing the colonization and successful replication of a number of intracellular parasites. On the other hand, we have described how autophagy acts as an innate cytoplasmic surveillance mechanism which enables the host cell to remove pathogens, and to subsequently deliver them to the degradative lysosomal compartment. However, pathogens have developed different strategies to avoid this pathway; indeed, some of them have created genuine molecular shields for their protection. Many key points remain to be solved, mainly concerning the identity of the bacterial factors that govern individual transport steps of the target cell. The search for virulence factors and the elucidation of how they control the autophagy machinery will be the basis of novel therapeutic intervention against intracellular pathogens.

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References

[1] R.A. Almeida, K.R. Matthews, E. Cifrian, A.J. Guidry, S.P. Oliver, *Staphylococcus aureus* invasion of bovine mammary epithelial cells, J. Dairy Sci. 79 (1996) 1021.
[2] A. Amano, I. Nakagawa, T. Yoshimori, Autophagy in innate immunity against intracellular bacteria, J. Biochem. 140 (2006) 161.
[3] A.O. Amer, B.G. Byrne, M.S. Swanson, Macrophages rapidly transfer pathogens from lipid raft vacuoles to autophagosomes, Autophagy 1 (2005) 53.
[4] A.O. Amer, M.S. Swanson, Autophagy is an immediate macrophage response to *Legionella pneumophila*, Cell. Microbiol. 7 (2005) 765.
[5] T.D. Anderson, N.F. Cheville, Ultrastructural morphometric analysis of *Brucella abortus*-infected trophoblasts in experimental placentitis. Bacterial replication occurs in rough endoplasmic reticulum, Am. J. Pathol. 124 (1986) 226.
[6] G.N. Arenas, A.S. Staskevich, A. Aballay, L.S. Mayorga, Intracellular trafficking of *Brucella abortus* in J774 macrophages, Infect. Immun. 68 (2000) 4255.
[7] R. Aroian, F.G. van der Goot, Pore-forming toxins and cellular non-immune defenses (CNIDs), Curr. Opin. Microbiol. 10 (2007) 57.
[8] E.L. Axe, S.A. Walker, M. Manifava, P. Chandra, H.L. Roderick, A. Habermann, G. Griffiths, N.T. Ktistakis, Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum, J. Cell Biol. 182 (2008) 685.
[9] O.G. Baca, Y.P. Li, H. Kumar, Survival of the Q fever agent *Coxiella burnetii* in the phagolysosome, Trends Microbiol. 2 (1994) 476.
[10] O.G. Baca, D. Paretsky, Q fever and *Coxiella burnetii*: a model for host–parasite interactions, Microbiol. Rev. 47 (1983) 127.
[11] J.S. Bakken, S. Dumler, Human granulocytic anaplasmosis, Infect. Dis. Clin. North Am. 22 (2008) 433 viii.
[12] H. Bantel, B. Sinha, W. Domschke, G. Peters, K. Schulze-Osthoff, R.U. Janicke, alpha-Toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling, J. Cell Biol. 155 (2001) 637.
[13] K.W. Bayles, C.A. Wesson, L.E. Liou, L.K. Fox, G.A. Bohach, W.R. Trumble, Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells, Infect. Immun. 66 (1998) 336.
[14] M. Belanger, P.H. Rodrigues, W.A. Dunn Jr., A. Progulske-Fox, Autophagy: a highway for *Porphyromonas gingivalis* in endothelial cells, Autophagy 2 (2006) 165.
[15] C.M. Belton, K.T. Izutsu, P.C. Goodwin, Y. Park, R.J. Lamont, Fluorescence image analysis of the association between *Porphyromonas gingivalis* and gingival epithelial cells, Cell. Microbiol. 1 (1999) 215.
[16] K.H. Berger, R.R. Isberg, Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*, Mol. Microbiol. 7 (1993) 7.
[17] W. Beron, M.G. Gutierrez, M. Rabinovitch, M.I. Colombo, *Coxiella burnetii* localizes in a Rab7-labeled compartment with autophagic characteristics, Infect. Immun. 70 (2002) 5816.
[18] C.R. Beuzon, S. Meresse, K.E. Unsworth, J. Ruiz-Albert, S. Garvis, S.R. Waterman, T.A. Ryder, E. Boucrot, D.W. Holden, *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA, EMBO J. 19 (2000) 3235.
[19] A. Biederbick, H.F. Kern, H.P. Elsasser, Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles, Eur. J. Cell Biol. 66 (1995) 3.
[20] C.L. Birmingham, A.C. Smith, M.A. Bakowski, T. Yoshimori, J.H. Brumell, Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole, J. Biol Chem. 281 (2006) 11374.
[21] C.L. Birmingham, V. Canadien, E. Gouin, E.B. Troy, T. Yoshimori, P. Cossart, D.E. Higgins, J.H. Brumell, *Listeria monocytogenes* evades killing by autophagy during colonization of host cells, Autophagy 3 (2007) 442.
[22] C.L. Birmingham, V. Canadien, N.A. Kaniuk, B.E. Steinberg, D.E. Higgins, J.H. Brumell, Listeriolysin O allows *Listeria monocytogenes* replication in macrophage vacuoles, Nature 451 (2008) 350.
[23] C.L. Birmingham, D.E. Higgins, J.H. Brumell, Avoiding death by autophagy: interactions of *Listeria monocytogenes* with the macrophage autophagy system, Autophagy 4 (2008) 368.
[24] A.L. Bisno, M.O. Brito, C.M. Collins, Molecular basis of group A streptococcal virulence, Lancet Infect. Dis. 3 (2003) 191.
[25] J.H. Brumell, P. Tang, M.L. Zaharik, B.B. Finlay, Disruption of the *Salmonella*-containing vacuole leads to increased replication of *Salmonella enterica* serovar *Typhimurium* in the cytosol of epithelial cells, Infect. Immun. 70 (2002) 3264.
[26] J.A. Carlyon, E. Fikrig, Invasion and survival strategies of *Anaplasma phagocytophilum*, Cell. Microbiol. 5 (2003) 743.
[27] J. Celli, C.C. de, D.M. Franchini, J. Pizarro-Cerda, E. Moreno, J.P. Gorvel, *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum, J. Exp. Med. 198 (2003) 545.
[28] J. Celli, J.P. Gorvel, Organelle robbery: *Brucella* interactions with the endoplasmic reticulum, Curr. Opin. Microbiol. 7 (2004) 93.
[29] A.L. Cheung, J.M. Koomey, C.A. Butler, S.J. Projan, V.A. Fischetti, Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (sar) distinct from agr, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 6462.
[30] A.L. Cheung, S.J. Projan, Cloning and sequencing of sarA of *Staphylococcus aureus*, a gene required for the expression of agr, J. Bacteriol. 176 (1994) 4168.
[31] N.P. Cianciotto, B.I. Eisenstein, C.H. Mody, G.B. Toews, N.C. Engleberg, A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection, Infect. Immun. 57 (1989) 1255.
[32] D.L. Clemens, M.A. Horwitz, The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin, J. Exp. Med. 184 (1996) 1349.
[33] A. Coelho, J.R. Andrade, A.C. Vicente, V.J. Dirita, Cytotoxic cell vacuolating activity from *Vibrio cholerae* hemolysin, Infect. Immun. 68 (2000) 1700.
[34] M.I. Colombo, Autophagy: a pathogen driven process, IUBMB. Life 59 (2007) 238.
[35] M.I. Colombo, Pathogens and autophagy: subverting to survive, Cell Death Differ. 12 (Suppl. 2) (2005) 1481.

[36] M.I. Colombo, M.G. Gutierrez, P.S. Romano, The two faces of autophagy: *Coxiella* and *Mycobacterium*, Autophagy 2 (2006) 162.

[37] D.J. Comerci, M.J. Martinez-Lorenzo, R. Sieira, J.P. Gorvel, R.A. Ugalde, Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole, Cell. Microbiol. 3 (2001) 159.

[38] M. Cullinane, L. Gong, X. Li, N. Lazar-Adler, T. Tra, E. Wolvetang, M. Prescott, J.D. Boyce, R.J. Devenish, B. Adler, Stimulation of autophagy suppresses the intracellular survival of *Burkholderia pseudomallei* in mammalian cell lines, Autophagy 4 (2008) 744.

[39] M.W. Cunningham, Pathogenesis of group A streptococcal infections, Clin. Microbiol. Rev. 13 (2000) 470.

[40] N.S. Cutler, J. Heitman, M.E. Cardenas, TOR kinase homologs function in a signal transduction pathway that is conserved from yeast to mammals, Mol. Cell Endocrinol. 155 (1999) 135.

[41] I. Derre, R.R. Isberg, *Legionella pneumophila* replication vacuole formation involves rapid recruitment of proteins of the early secretory system, Infect. Immun. 72 (2004) 3048.

[42] R.G. Deshpande, M.B. Khan, C.A. Genco, Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*, Infect. Immun. 66 (1998) 5337.

[43] B.R. Dorn, W.A. Dunn Jr., A. Progulske-Fox, Invasion of human coronary artery cells by periodontal pathogens, Infect. Immun. 67 (1999) 5792.

[44] B.R. Dorn, W.A. Dunn Jr., A. Progulske-Fox, *Porphyromonas gingivalis* traffics to autophagosomes in human coronary artery endothelial cells, Infect. Immun. 69 (2001) 5698.

[45] B.R. Dorn, W.A. Dunn Jr., A. Progulske-Fox, Bacterial interactions with the autophagic pathway, Cell. Microbiol. 4 (2002) 1.

[46] J.S. Dumler, K.S. Choi, J.C. Garcia-Garcia, N.S. Barat, D.G. Scorpio, J.W. Garyu, D.J. Grab, J.S. Bakken, Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*, Emerg. Infect. Dis. 11 (2005) 1828.

[47] C.M. Fader, M.I. Colombo, Autophagy and multivesicular bodies: two closely related partners, Cell Death Differ. 16 (2009) 70.

[48] P. Figueroa-Arredondo, J.E. Heuser, N.S. Akopyants, J.H. Morisaki, S. Giono-Cerezo, F. Enriquez-Rincon, D.E. Berg, Cell vacuolation caused by *Vibrio cholerae* hemolysin, Infect. Immun. 69 (2001) 1613.

[49] J.L. Flynn, J. Chan, Immunology of tuberculosis, Annu. Rev. Immunol. 19 (2001) 93.

[50] D.W. Fraser, T.R. Tsai, W. Orenstein, W.E. Parkin, H.J. Beecham, R.G. Sharrar, J. Harris, G.F. Mallison, S.M. Martin, J.E. McDade, C.C. Shepard, P.S. Brachman, Legionnaires' disease: description of an epidemic of pneumonia, N. Engl. J. Med. 297 (1977) 1189.

[51] R.A. Fratti, J.M. Backer, J. Gruenberg, S. Corvera, V. Deretic, Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest, J. Cell Biol. 154 (2001) 631.

[52] B. Freche, N. Reig, F.G. van der Goot, The role of the inflammasome in cellular responses to toxins and bacterial effectors, Semin. Immunopathol. 29 (2007) 249.

[53] J.E. Galan, P. Cossart, Host–pathogen interactions: a diversity of themes, a variety of molecular machines, Curr. Opin. Microbiol. 8 (2005) 1.

[54] J.E. Galan, H. Wolf-Watz, Protein delivery into eukaryotic cells by type III secretion machines, Nature 444 (2006) 567.

[55] L.Y. Gao, O.S. Harb, K.Y. Abu, Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa, Infect. Immun. 65 (1997) 4738.

[56] B. Geny, M.R. Popoff, Bacterial protein toxins and lipids: pore formation or toxin entry into cells, Biol. Cell 98 (2006) 667.

[57] M.K. Glynn, T.V. Lynn, Brucellosis, J. Am. Vet. Med. Assoc. 233 (2008) 900.

[58] M.R. Gonzalez, M. Bischofberger, L. Pernot, F.G. van der Goot, B. Freche, Bacterial pore-forming toxins: the (w)hole story? Cell Mol. Life Sci. 65 (2008) 493.

[59] J.P. Gorvel, E. Moreno, *Brucella* intracellular life: from invasion to intracellular replication, Vet. Microbiol. 90 (2002) 281.

[60] E. Gouin, M.D. Welch, P. Cossart, Actin-based motility of intracellular pathogens, Curr. Opin. Microbiol. 8 (2005) 35.

[61] G.S. Gray, M. Kehoe, Primary sequence of the alpha-toxin gene from *Staphylococcus aureus* wood 46, Infect. Immun. 46 (1984) 615.

[62] H. Guerra, The brucellae and their success as pathogens, Crit Rev. Microbiol. 33 (2007) 325.

[63] M.G. Gutierrez, S.S. Master, S.B. Singh, G.A. Taylor, M.I. Colombo, V. Deretic, Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages, Cell 119 (2004) 753.

[64] M.G. Gutierrez, H.A. Saka, I. Chinen, F.C. Zoppino, T. Yoshimori, J.L. Bocco, M.I. Colombo, Protective role of autophagy against *Vibrio cholerae* cytolysin, a pore-forming toxin from *V. cholerae*, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 1829.

[65] M.G. Gutierrez, C.L. Vazquez, D.B. Munafo, F.C. Zoppino, W. Beron, M. Rabinovitch, M.I. Colombo, Autophagy induction favours the generation and maturation of the *Coxiella*-replicative vacuoles, Cell. Microbiol. 7 (2005) 981.

[66] M. Hamasaki, T. Noda, Y. Ohsumi, The early secretory pathway contributes to autophagy in yeast, Cell Struct. Funct. 28 (2003) 49.

[67] M. Hamon, H. Bierne, P. Cossart, *Listeria monocytogenes*: a multifaceted model, Nat. Rev. Microbiol. 4 (2006) 423.

[68] R.A. Heinzen, T. Hackstadt, J.E. Samuel, Developmental biology of *Coxiella burnetii*, Trends Microbiol. 7 (1999) 149.

[69] L.D. Hernandez, M. Pypaert, R.A. Flavell, J.E. Galan, A *Salmonella* protein causes macrophage cell death by inducing autophagy, J. Cell Biol. 163 (2003) 1123.

[70] P. Hirsimaki, H. Reunanen, Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase, Histochemistry 67 (1980) 139.

[71] M.A. Horwitz, The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome–lysosome fusion in human monocytes, J. Exp. Med. 158 (1983) 2108.

[72] M.A. Horwitz, Characterization of avirulent mutant *Legionella pneumophila* that survive but do not multiply within human monocytes, J. Exp. Med. 166 (1987) 1310.

[73] M.A. Horwitz, B.W. Lee, B.J. Dillon, G. Harth, Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1530.

[74] S. Houalet-Jeanne, P. Pellen-Mussi, S. Tricot-Doleux, J. Apiou, M. Bonnaure-Mallet, Assessment of internalization and viability of *Porphyromonas gingivalis* in KB epithelial cells by confocal microscopy, Infect. Immun. 69 (2001) 7146.

[75] D. Howe, L.P. Mallavia, *Coxiella burnetii* exhibits morphological change and delays phagolysosomal fusion after internalization by J774A.1 cells, Infect. Immun. 68 (2000) 3815.

[76] D. Howe, J. Melnicakova, I. Barak, R.A. Heinzen, Maturation of the *Coxiella burnetii* parasitophorous vacuole requires bacterial protein synthesis but not replication, Cell. Microbiol. 5 (2003) 469.

[77] D. Howe, J. Melnicakova, I. Barak, R.A. Heinzen, Fusogenicity of the *Coxiella burnetii* parasitophorous vacuole, Ann. N. Y. Acad. Sci. 990 (2003) 556.

[78] B. Inbal, S. Bialik, I. Sabanay, G. Shani, A. Kimchi, DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death, J. Cell Biol. 157 (2002) 455.

[79] S. Jager, C. Bucci, I. Tanida, T. Ueno, E. Kominami, P. Saftig, E.L. Eskelinen, Role for Rab7 in maturation of late autophagic vacuoles, J. Cell Sci. 117 (2004) 4837.

[80] C.A. Janeway Jr., R. Medzhitov, Innate immune recognition, Annu. Rev. Immunol. 20 (2002) 197.

[81] T.M. Jarry, A.L. Cheung, *Staphylococcus aureus* escapes more efficiently from the phagosome of a cystic fibrosis bronchial epithelial cell line than from its normal counterpart, Infect. Immun. 74 (2006) 2568.

[82] T.M. Jarry, G. Memmi, A.L. Cheung, The expression of alpha-haemolysin is required for *Staphylococcus aureus* phagosomal escape after internalization in CFT-1 cells, Cell. Microbiol. 10 (2008) 1801.

[83] S.M. Jones, A.J. Winter, Survival of virulent and attenuated strains of *Brucella abortus* in normal and gamma interferon-activated murine peritoneal macrophages, Infect. Immun. 60 (1992) 3011.

[84] M. Juhas, D.W. Crook, D.W. Hood, Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence, Cell. Microbiol. 10 (2008) 2377.

[85] T. Kadowaki, R. Takii, K. Yamatake, T. Kawakubo, T. Tsukuba, K. Yamamoto, A role for gingipains in cellular responses and bacterial survival in *Porphyromonas gingivalis*-infected cells, Front Biosci. 12 (2007) 4800.

[86] J.C. Kagan, C.R. Roy, *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites, Nat. Cell Biol. 4 (2002) 945.

[87] J.C. Kagan, M.P. Stein, M. Pypaert, C.R. Roy, *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle, J. Exp. Med. 199 (2004) 1201.

[88] B.C. Kahl, M. Goulian, W.W. van, M. Herrmann, S.M. Simon, G. Kaplan, G. Peters, A.L. Cheung, *Staphylococcus aureus* RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line, Infect. Immun. 68 (2000) 5385.

[89] W. Kespichayawattana, S. Rattanachetkul, T. Wanun, P. Utaisincharoen, S. Sirisinha, *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading, Infect. Immun. 68 (2000) 5377.

[90] K. Kirkegaard, M.P. Taylor, W.T. Jackson, Cellular autophagy: surrender, avoidance and subversion by microorganisms, Nat. Rev. Microbiol. 2 (2004) 301.

[91] M. Komatsu, I. Tanida, T. Ueno, M. Ohsumi, Y. Ohsumi, E. Kominami, The C-terminal region of an Apg7p/Cvt2p is required for homodimerization and is essential for its E1 activity and E1-E2 complex formation, J. Biol. Chem. 276 (2001) 9846.

[92] W. Liou, H.J. Geuze, M.J. Geelen, J.W. Slot, The autophagic and endocytic pathways converge at the nascent autophagic vacuoles, J. Cell Biol. 136 (1997) 61.

[93] F.D. Lowy, *Staphylococcus aureus* infections, N. Engl. J. Med. 339 (1998) 520.

[94] M.P. Machner, R.R. Isberg, Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*, Dev. Cell 11 (2006) 47.

[95] P. Marrack, J. Kappler, The staphylococcal enterotoxins and their relatives, Science 248 (1990) 1066.

[96] J.E. McDade, C.C. Shepard, D.W. Fraser, T.R. Tsai, M.A. Redus, W.R. Dowdle, Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease, N. Engl. J. Med. 297 (1977) 1197.

[97] S. Meresse, O. Steele-Mortimer, E. Moreno, M. Desjardins, B. Finlay, J.P. Gorvel, Controlling the maturation of pathogen-containing vacuoles: a matter of life and death, Nat. Cell Biol. 1 (1999) E183–E188.

[98] N. Mizushima, T. Noda, T. Yoshimori, Y. Tanaka, T. Ishii, M.D. George, D.J. Klionsky, M. Ohsumi, Y. Ohsumi, A protein conjugation system essential for autophagy, Nature 395 (1998) 395.

[99] A.B. Molofsky, M.S. Swanson, Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle, Mol. Microbiol. 53 (2004) 29.

[100] D.B. Munafo, M.I. Colombo, Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24, Traffic 3 (2002) 472.

[101] D.B. Munafo, M.I. Colombo, A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation, J. Cell Sci. 114 (2001) 3619.

[102] I. Nakagawa, A. Amano, N. Mizushima, A. Yamamoto, H. Yamaguchi, T. Kamimoto, A. Nara, J. Funao, M. Nakata, K. Tsuda, S. Hamada, T. Yoshimori, Autophagy defends cells against invading group A *Streptococcus*, Science 306 (2004) 1037.

[103] H. Niu, M. Yamaguchi, Y. Rikihisa, Subversion of cellular autophagy by *Anaplasma phagocytophilum*, Cell. Microbiol. 10 (2008) 593.

[104] R.P. Novick, Autoinduction and signal transduction in the regulation of staphylococcal virulence, Mol. Microbiol. 48 (2003) 1429.
[105] M. Ogawa, Y. Handa, H. Ashida, M. Suzuki, C. Sasakawa, The versatility of *Shigella* effectors, Nat. Rev. Microbiol. 6 (2008) 11.
[106] M. Ogawa, C. Sasakawa, *Shigella* and autophagy, Autophagy 2 (2006) 171.
[107] M. Ogawa, T. Yoshimori, T. Suzuki, H. Sagara, N. Mizushima, C. Sasakawa, Escape of intracellular *Shigella* from autophagy, Science 307 (2005) 727.
[108] G.P. Otto, M.Y. Wu, M. Clarke, H. Lu, O.R. Anderson, H. Hilbi, H.A. Shuman, R.H. Kessin, Macroautophagy is dispensable for intracellular replication of *Legionella pneumophila* in *Dictyostelium discoideum*, Mol. Microbiol. 51 (2004) 63.
[109] A. Petiot, E. Ogier-Denis, E.F. Blommaart, A.J. Meijer, P. Codogno, Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells, J. Biol. Chem. 275 (2000) 992.
[110] A. Phalipon, P.J. Sansonetti, *Shigella*'s ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival? Immunol. Cell Biol. 85 (2007) 119.
[111] J.A. Philips, Mycobacterial manipulation of vacuolar sorting, Cell. Microbiol. 10 (2008) 2408.
[112] J. Pizarro-Cerda, P. Cossart, Bacterial adhesion and entry into host cells, Cell 124 (2006) 715.
[113] J. Pizarro-Cerda, E. Moreno, V. Sanguedolce, J.L. Mege, J.P. Gorvel, Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments, Infect Immun. 66 (1998) 2387.
[114] R.A. Proctor, J.M. Balwit, O. Vesga, Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections, Infect. Agents Dis. 3 (1994) 302.
[115] B.F. Py, M.M. Lipinski, J. Yuan, Autophagy limits *Listeria monocytogenes* intracellular growth in the early phase of primary infection, Autophagy 3 (2007) 117.
[116] D. Raoult, T. Marrie, J. Mege, Natural history and pathophysiology of Q fever, Lancet Infect. Dis. 5 (2005) 219.
[117] K.A. Rich, C. Burkett, P. Webster, Cytoplasmic bacteria can be targets for autophagy, Cell Microbiol. 5 (2003) 455.
[118] P.H. Rodrigues, M. Belanger, W. Dunn Jr., A. Progulske-Fox, *Porphyromonas gingivalis* and the autophagic pathway: an innate immune interaction? Front Biosci. 13 (2008) 178.
[119] P.S. Romano, M.G. Gutierrez, W. Beron, M. Rabinovitch, M.I. Colombo, The autophagic pathway is actively modulated by phase II *Coxiella burnetii* to efficiently replicate in the host cell, Cell. Microbiol. 9 (2007) 891.
[120] C.R. Roy, L.G. Tilney, The road less traveled: transport of *Legionella* to the endoplasmic reticulum, J. Cell Biol. 158 (2002) 415.
[121] H.A. Saka, M.G. Gutierrez, J.L. Bocco, M.I. Colombo, The autophagic pathway: a cell survival strategy against the bacterial pore-forming toxin *Vibrio cholerae* cytolysin, Autophagy 3 (2007) 363.
[122] A. Schnaith, H. Kashkar, S.A. Leggio, K. Addicks, M. Kronke, O. Krut, *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death, J. Biol. Chem. 282 (2007) 2695.
[123] G.N. Schroeder, H. Hilbi, Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion, Clin. Microbiol. Rev. 21 (2008) 134.
[124] G. Segal, M. Purcell, H.A. Shuman, Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 1669.
[125] A. Simonsen, H. Stenmark, Self-eating from an ER-associated cup, J. Cell Biol. 182 (2008) 621.
[126] H. Smith, Mechanisms of microbial pathogenicity, Sci. Basis Med. Annu. Rev. (1968) 53.
[127] A. Sola-Landa, J. Pizarro-Cerda, M.J. Grillo, E. Moreno, I. Moriyon, J.M. Blasco, J.P. Gorvel, I. Lopez-Goni, A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence, Mol. Microbiol. 29 (1998) 125.
[128] M.P. Stevens, A. Haque, T. Atkins, J. Hill, M.W. Wood, A. Easton, M. Nelson, C. Underwood-Fowler, R.W. Titball, G.J. Bancroft, E.E. Galyov, Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei* bsa type III secretion mutant in murine models of melioidosis, Microbiology 150 (2004) 2669.
[129] S. Sturgill-Koszycki, P.H. Schlesinger, P. Chakraborty, P.L. Haddix, H.L. Collins, A.K. Fok, R.D. Allen, S.L. Gluck, J. Heuser, D.G. Russell, Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase, Science 263 (1994) 678.
[130] S. Sturgill-Koszycki, M.S. Swanson, *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles, J. Exp. Med. 192 (2000) 1261.
[131] J. Sun, A.E. Deghmane, H. Soualhine, T. Hong, C. Bucci, A. Solodkin, Z. Hmama, *Mycobacterium bovis* BCG disrupts the interaction of Rab7 with RILP contributing to inhibition of phagosome maturation, J. Leukoc. Biol. 82 (2007) 1437.
[132] T. Suzuki, G. Nunez, A role for Nod-like receptors in autophagy induced by *Shigella* infection, Autophagy 4 (2008) 73.
[133] M.S. Swanson, R.R. Isberg, Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum, Infect. Immun. 63 (1995) 3609.
[134] Y. Takahashi, M. Davey, H. Yumoto, F.C. Gibson III, C.A. Genco, Fimbria-dependent activation of pro-inflammatory molecules in *Porphyromonas gingivalis* infected human aortic endothelial cells, Cell Microbiol. 8 (2006) 738.
[135] R. Tamai, Y. Asai, T. Ogawa, Requirement for intercellular adhesion molecule 1 and caveolae in invasion of human oral epithelial cells by *Porphyromonas gingivalis*, Infect. Immun. 73 (2005) 6290.
[136] G.A. Taylor, C.G. Feng, Sher, p47 GTPases: regulators of immunity to intracellular pathogens, Nat. Rev. Immunol. 4 (2004) 100.
[137] D.A. van Sluijters, P.F. Dubbelhuis, E.F. Blommaart, A.J. Meijer, Amino-acid-dependent signal transduction, Biochem. J. 351 (Pt. 3) (2000) 545.
[138] I. Vergne, J. Chua, S.B. Singh, V. Deretic, Cell biology of *Mycobacterium tuberculosis* phagosome, Annu. Rev. Cell Dev. Biol. 20 (2004) 367.
[139] L.E. Via, D. Deretic, R.J. Ulmer, N.S. Hibler, L.A. Huber, V. Deretic, Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7, J. Biol. Chem. 272 (1997) 13326.
[140] S.R. Waterman, D.W. Holden, Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system, Cell. Microbiol. 5 (2003) 501.
[141] P. Webster, J.W. IJdo, L.M. Chicoine, E. Fikrig, The agent of human granulocytic ehrlichiosis resides in an endosomal compartment, J. Clin. Invest. 101 (1998) 1932.
[142] K. Yamatake, M. Maeda, T. Kadowaki, R. Takii, T. Tsukuba, T. Ueno, E. Kominami, S. Yokota, K. Yamamoto, Role for gingipains in *Porphyromonas gingivalis* traffic to phagolysosomes and survival in human aortic endothelial cells, Infect. Immun. 75 (2007) 2090.
[143] T. Yoshimori, Autophagy vs. Group A *Streptococcus*, Autophagy 2 (2006) 154.
[144] A. Zitzer, M. Palmer, U. Weller, T. Wassenaar, C. Biermann, J. Tranum-Jensen, S. Bhakdi, Mode of primary binding to target membranes and pore formation induced by *Vibrio cholerae* cytolysin (hemolysin), Eur. J. Biochem. 247 (1997) 209.
[145] S.B. Singh, A.S. Davis, G.A. Taylor, V. Deretic, Human IRGM induces autophagy to eliminate intracellular mycobacteria, Science 313 (2006) 1438.
[146] M.R. Terebiznik, C.L. Vázquez, D. Raju, K. Torbricki, R. Kulkarni, S. Blanke, T. Yoshimori, N. Mizushima, M.I. Colombo, N.L. Jones, Helicobacter pylori's vacuolating cytotoxin, VacA disrupts the autophagy pathway in gastric epithelial cells, Autophagy 5 (2009) 370.