FEBS Letters

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REVIEW

Autophagy as an innate immunity response against pathogens: a *Tango* dance

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Intracellular infections as well as changes in the cell nutritional environment are main events that trigger cellular stress responses. One crucial cell response to stress conditions is autophagy. During the last 30 years, several scenarios involving autophagy induction or inhibition over the course of an intracellular invasion by pathogens have been uncovered. In this review, we will present how this knowledge was gained by studying different microorganisms. We intend to discuss how the cell, via autophagy, tries to repel these attacks with the objective of destroying the intruder, but also how some pathogens have developed strategies to subvert this. These two fates can be compared with a *Tango*, a dance originated in Buenos Aires, Argentina, in which the partner dancers are in close connection. One of them is the leader, embracing and involving the partner, but the follower may respond escaping from the leader. This joint dance is indeed highly synchronized and controlled, perfectly reflecting the interaction between autophagy and microorganism.

Keywords: ATG; autophagy; intracellular microorganisms; invading bacteria; LAP; LC3; parasites; viruses

Abbreviations
4E-BP1, *eukaryotic translation initiation factor* 4E (eIF4E)-binding protein 1; ActA, actin assembly-inducing protein; AIM/LIR, Atg8/LC3-interacting motif/region; AMPK1, AMP-activated kinase 1; ATG, autophagy-related proteins; BECN1, Beclin1, Bcl-2-interacting protein; CMA, chaperone-mediated autophagy; CTSL, cathepsin L; FIP200, FAK family kinase interacting protein of 200 kDa (FIP200 or RB1CC1); GABARAP, Gamma-aminobutyric acid receptor-associated protein; IRGM1/LRG47, immunity-related GTPase M 1; LAMP2A, lysosomal-associated membrane protein 2A; LAP, LC3-associated phagocytosis.; LC3, microtubule-associated proteins light chain 3; M6PR, mannose-6-phosphate receptor; mTORC1, mechanistic target of rapamycin kinase complex 1; NDP52/CALCOCO2, nuclear dot protein 52 - kDa/Calcium-binding and coiled-coil domain-containing protein 2; OPTN, optineurin; PtdIns3K, class III phosphatidylinositol 3-kinase complex I; S6K, ribosomal S6 Kinase; SARs, selective autophagy receptors; SQSTM1/p62, sequestosome 1; T3SS, type 3 secretion system; UIM, ubiquitin-interacting motif; ULK, unc-51 like kinase complex; WIPI4, WD repeat domain phosphoinositide-interacting protein 4.

Autophagy is a highly dynamic process classically involved in the delivery of unwanted intracellular cytoplasmic components, and aged, nonfunctional or excess organelles, to lysosomal degradation and subsequent recycling of their basic components. Three main types of autophagy have been described in mammalian cells: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. Each one of these processes has a different mechanism to deliver the cargo to lysosomal vesicles for breaking up and recycling of the generated molecules. In microautophagy, the cytoplasmic components are directly captured by lysosomes via the invagination of their limiting membrane. In CMA, proteins with a specific target sequence are recognized by a chaperone machinery and the lysosomal-associated membrane protein 2A (LAMP2A) and transported through the lysosomal membrane. During macroautophagy, cytoplasmic material is trapped by double membrane vesicles known as autophagosomes, which finally fuses and deliver their cargo into lysosomes.

Macroautophagy and in particular autophagosome generation, is regulated by the autophagy-related (ATG) proteins, which are organized in 6 functional groups: the unc-51 like (ULK) kinase complex, the ATG9A-positive vesicles, the autophagy-specific class III phosphatidylinositol 3-kinase (PtdIns3K) complex I, the ATG2-WIPI4 complexes, and the ubiquitin-like ATG12 and LC3 conjugation systems [1-3]. The ULK kinase complex (composed by ULK1 or ULK2, ATG13, FIP200, and ATG101) plays a central role in regulating the initiation of autophagosome formation and its activity, and thus autophagy, is inhibited by active mechanistic target of rapamycin kinase complex 1 (mTORC1), a nutrient sensor [4-7], and activated by AMP-activated kinase 1 (AMPK1), a sensor of the cellular levels of ATP [8]. The ULK kinase complex, together with the ATG9A-positive vesicles and the class III PtdIns3K complex I [formed by ATG14L, BECLIN1 (BECN1), phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3/VPS34), phosphoinositide-3-kinase regulatory subunit 4 (PIK3R4/VPS15) and autophagy and BECN1 regulator 1 (AMBRA1)], cooperates in the generation of the phagophore, the precursor structure of autophagosomes [1]. The class III PtdIns3K complex II is important for the generation of phosphatidylinositol 3-phosphate (PtdIns3P), a lipid that is essential for the elongation and closure of the phagophore into an autophagosome [1]. While the ATG2-WIPI4 complex (composed by ATG2A or ATG2B and WIPI4) appears to be central in the transfer of lipids from the endoplasmic reticulum necessary for the expansion of the phagophore, the two ubiquitin-like conjugation systems are more critical for the phagophore closure and cargo selection [1]. Those two systems are interconnected. In the first, ubiquitin-like

ATG12 is activated by E1-like ATG7 and then transferred to the E2-like ATG10, which covalently conjugates it to ATG5. The ATG12–ATG5 conjugate binds the pool of ATG16L1 recruited to the phagophore membrane by interacting with both the PtdIns3P-binding protein WIPI2 and the ULK kinase complex via FIP200. In the second system, members of the Atg8 protein family (LC3A, LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2) are conjugated to either phosphatidylethanolamine (PE) [9-11] or phosphatidylserine (PS) [11] via the consecutive action of ATG7, the E2-like ATG3 and the ATG5–ATG12-ATG16L1 complex, which acts as an E3 ligase [1,12].

Autophagy can be both a non-selective and selective degradation mechanisms [13]. The so-called selective autophagy receptors (SARs) play a central role in selective types of autophagy since they bind on the one hand the cargo and the other hand components of ATG machinery, thereby mediating the specific cargo sequestration within autophagosomes [14]. In particular, all the SARs binds to members of the LC3 protein family via an Atg8/LC3-interacting motifs/regions (AIM/LIR) and/or the ubiquitin-interacting motif (UIM)-like sequence [14]. While some SARs are embedded in protein complex and organelles, others, such as sequestosome 1 (SQSTM1/p62), nuclear dot protein 52 kDa (NDP52/CALCOCO2) and optineurin (OPTN), are soluble and bind to ubiquitinylated cargoes, to specifically target them to lysosomal degradation.

For a long time, autophagy was merely considered a degradative mechanism enhanced during nutrient scarcity to provide energy for cell survival. However, our knowledge about this pathway has grown enormously over the past decades, and now we know that autophagy is involved in a large number of physiological processes in eukaryotic organisms. One of these processes is the complex interplay with intracellular pathogens, which has become a critical component of both innate and adaptive immunity [15-17]. In this review, we will focus on innate immunity. A large number of researchers have focused on the mechanism of selective autophagy delivering pathogens into the lysosomes by the host for elimination, a process also known as xenophagy. However, cumulative evidence indicates that pathogens have evolved strategies to manipulate or subvert xenophagy, reducing the efficacy of this defense mechanism and allowing the intruder to survive (for comprehensive reviews on the topic, see [17-21]). Thus, like dancing a *Tango*, some pathogens are efficiently restrained and "hugged" by autophagosomes, whereas others manage to escape from their dancer escort to survive and replicate in the cytoplasm or other intracellular compartments.

Bacteria and autophagy

Bacteria that modulate autophagy to their advantage

Very early studies on the pathogen–autophagy interaction highlighted the modulation of the autophagy by pathogens for their own advantage. This was the case for the virulent *Brucella abortus, a facultative intracellular Gram-negative bacterium*, in HeLa cells, which, in contrast to attenuated *B. abortus*, distributed within autophagosome-like compartments that avoided fusion with lysosomes [22]. Likewise, *Porphyromonas gingivalis*, a Gram-negative oral anaerobe, localized in vacuoles resembling autophagosomes, which seemed to include cytoplasmic components and also eluded fusion with cathepsin L (CTSL)-positive lysosomal compartments in human coronary artery endothelial cells [23]. In these pioneering studies, it was postulated that these bacteria might manipulate autophagy to establish a favorable shelter niche. Indeed, when autophagy was inhibited with 3-methyladenine or wortmannin, internalized *P. gingivalis* was delivered and eliminated in a compartment containing the mannose-6-phosphate receptor (M6PR) and CTSL [23].

When the conserved molecular components of the autophagy-related (ATG) machinery were uncovered [1], the members of a family of homologous key proteins, the ATG8/microtubule-associated protein 1A/1B-light chain 3 (LC3) proteins, became the tool of choice to label autophagic intermediates [24]. In our laboratory, we demonstrated that the large replicative acidic vacuole developed in non-professional phagocytes by the intracellular bacterium *Coxiella burnetii*, a Gram-negative intracellular bacterium, was pronouncedly labeled by LC3 but also RAB7 and RAB24, two small GTPases that have been involved in the maturation of autophagosomes [25,26]. Moreover, we demonstrated for the first time that autophagy induction by either starvation or overexpression of proteins involved in the autophagy such as LC3, BECN1, or RAB24 positively regulates the replication of this bacterium, resulting in a persistent infection [26,27]. Indeed, *C. burnetii* controls both autophagy and apoptotic pathways by inhibiting the BECN1-BCL2 (B-cell lymphoma 2) interaction, preventing host cell apoptosis to establish a successful and long-lasting infection [28]. It was demonstrated that *this bacterium* exploits cAMP-dependent protein kinase (PKA) signaling in host cells to inhibit macrophage apoptosis [29]. Several *Coxiella* effector proteins negatively regulate cell death, both apoptosis and pyroptosis [30]. However, there is no evidence so far if *Coxiella* also

modulates autophagic cell death. Interestingly, Winchell and co-workers demonstrated that *C. burnetii* type IV-mediated secretion is key for the recruitment of autophagosomes in macrophages [31], which is critical to provide both nutrients and membranes necessary for the expansion of the *Coxiella* vacuoles [32]. Likewise, *Francisella tularensis*, an intracellular Gram-negative bacterium, takes advantage of autophagy since it requires autophagy-generated nutrients such as amino acids, for intracellular growth and consistent inhibition of host autophagy results in diminished bacterial replication. However, autophagy induced by *F. tularensis* is ATG5-independent [33].

Staphylococcus aureus, a Gram-positive coccus, also co-opts autophagy for survival. Early after internalization in non-professional phagocytic cells, *S. aureus* transits to LC3-labeled autophagic-like compartments, some of which have double or multilamellar membrane characteristic of autophagosomes [34]. These vacuoles neither acidify nor acquire lysosomal-associated membrane protein 2 (LAMP2), indicating that fusion with lysosomes is prevented. Of note, *S. aureus* is not able to replicate in $atg5^{-/-}$ knockout (KO) mouse embryonic fibroblasts (MEFs), underlying the notion that this bacterium requires an intact autophagy for replication [34]. Indeed, as determined by electron microscopy studies, *S. aureus* replicates in these autophagosome-like compartments before escaping into the cytoplasm. This transit is governed by one or more bacterial gene products that depend on the global regulator *agr*. In our laboratory, we demonstrated that one of the factors secreted by *S. aureus that is* required to stimulate autophagy is the toxin alfa-hemolysin [35]. The induction of autophagy by this toxin is independent of PIK3C3 activation and is negatively modulated by both cAMP and the exchange *protein* activated by 3′-5′-cAMP (EPAC) [36]. In addition, alfa-hemolysin triggers the formation of LC3-positive tubules emerging from the *S. aureus*-containing vacuoles, and these tubules appear to be critical for pathogen survival, although the reason for this beneficial effect is presently unknown [37]. More recently, we have shown that the *S. aureus*-containing phagosomes recruit protein kinase C-alpha (PKCα), a specific member of the protein kinase C family. Interestingly, overexpression of this kinase interferes with bacterial replication by inhibiting autophagy, suggesting that its activity, when associated to the bacteria-containing phagosomes, may be critical for bacterial survival. The molecular mechanism involved in this process, however, is currently unknown [38]. Intriguingly, autophagy is induced upon infection likely to defend cells against pathogen infection, but the generated autophagic compartments seem to serve as a shelter for bacterial

infection. Amano and co-workers found that autophagic degradation was effective against methicillin-sensitive S. aureus but not against a methicillin-resistant strain, indicating that these strains produce factors that may differentially affect host autophagy [39]. Interestingly, Liu and collaborators have revealed that the virulence factor IsaB is elevated in a methicillin-resistant strain, and it inhibits the autophagic flux, allowing bacterial survival and the transmission of the bacterium in both macrophage-like cells and in vivo [40]. In another report, it was shown that S. aureus is trapped in autophagosomes positive for WIPI1, also a protein involved in autophagy. In cells treated with the lysosomal proton pump inhibitor Bafilomycin A1, the number of WIPI1-positive autophagosome-like vesicles containing S. aureus markedly increase, letting the authors inferring that part of these vesicles are destined for lysosomal degradation [41]. Despite all these observations, the relationship and the specific mechanism behind the interaction between S. aureus and autophagy are far from being fully understood.

The opportunistic human pathogen Serratia marcescens, a rod-shaped Gram-negative bacterium, proliferates in large vacuoles that are positive for autophagy marker proteins, including LC3 and RAB7, upon internalization. These vacuoles do not fuse with lysosomes since they are non-acidic and non-degradative. In addition, functional autophagy is required for S. marcescens to grow inside host cells as its proliferation is abrogated in $Atg5^{-/-}$ KO MEFs, indicating that ATG5-dependent autophagy is required for S. marcescens to multiply intracellularly [42].

Taken together, all these reports and other ones show that specific intracellular pathogens, by secreting bacterial effectors, manipulate autophagy for their own benefit (Table 1). In several cases, these processes also involve the regulation of host survival to avoid premature cell death and ensure intracellular pathogen propagation.

The other side of the coin: the bacterium uses disguising strategies to avoid elimination by autophagy

Virtually at the same time of our pioneering investigations on the interaction between C. burnetii and autophagy, the study of Mycobacterium tuberculosis, an acid-fast weakly Gram-positive (due to their lack of an outer cell membrane) bacterium, uncovered a completely different scenario. That is, autophagy does not favor M. tuberculosis survival, but rather the activation of this degradative pathway results in increased killing of the bacterium [43]. Upon autophagy induction

by starvation or treatment with rapamycin, a mTORC1 inhibitor, M. tuberculosis variant bovis BCG was localized in acidic compartments positive for LC3, the lysosomal proteins lysosomal associated membrane protein 1 (LAMP1) and cathepsin D (CTSD), in RAW 264.7 macrophages, indicating that autophagy induction circumvents the phagosome maturation halt imposed by M. tuberculosis. Almost at the same time, it was reported that during invasion of epithelial cells, Streptococcus pyogenes (Group A Streptococcus; GAS), a Gram positive coccus, escapes from phagosomes but when in the cytoplasm, this bacterium is recognized and sequestered by autophagomes before being degraded in lysosomes [44]. These two seminal papers revealed that autophagy is a central player in the autonomous cellular innate immunity by defending cells against invading microorganisms, through xenophagy [45,46]. In the case of M. tuberculosis, it was shown that the interferon gamma (IFNγ), a critical cytokine involved in immune defense against pathogens [47], was able to stimulate autophagy in uninfected cells and increase the localization of pathogens within autophagy-related compartments similarly to rapamycin or starvation treatments [43]. Autophagy induction under these conditions appears to be mediated by the immunity-related GTPase M (IRGM1/LRG47), which participates in intracellular mycobacteria elimination [48].

It is interesting to mention that in monocytes obtained from patients affected by tuberculosis, T cells produced significant IFNγ when monocytes were exposed to M. tuberculosis antigens, leading to autophagy induction [49]. A positive correlation between IFNγ and LC3-II levels was observed as well. However, IFNγ is not the only cytokine required to ensure bacterial eradication by autophagy. Interleukin 17 (IL17A), another cytokine, augmented autophagy in infected monocytes from patients with strong immune responses to M. tuberculosis, leading to the killing of the bacteria [50]. These two publications as well as others, highlight the importance of cytokines in modulating autophagy, which is integrated and participates to the general host response against pathogens. For a complete overview on the immune response against bacteria, please see other reviews about this central issue in the interplay between bacteria and host cells [51-54].

Study of M. marinum, a close relative of M. tuberculosis, revealed that LC3 recruitment to the M. marinum-containing phagosomes in RAW 264.7 macrophages depends on the functional ESX-1 type VII secretion system [55]. These LC3-positive M. marinum-containing phagosomes, however, do not present the characteristics of late endolysosomal compartments since CTSD is not present in their interior and they do

Table 1. Autophagy and bacteria interactions described in the review.

Bacteria & autophagy						
Pathogen	Subversion	References	Destruction	References	Evasion	References
B. abortus	Autophagosomal components involved in the establishment of intracellular bacteria-containing vacuoles	[22]				
P. gingivalis	Autophagosomal components involved in the establishment of intracellular bacteria-containing vacuoles	[23]			Eluded fusion with CTSL-positive lysosomal compartments	[23]
C. burnetii	Autophagosome-mediated supply of nutrients. Type IV-mediated secretion is key for the recruitment of autophagosomes	[25–27,31]				
F. tularensis	Autophagosome-mediated supply of nutrients. Autophagy is induced in a ATG5-independent	[33]				
S. aureus	Autophagosomal components involved in the establishment of intracellular bacteria-containing vacuoles. This is governed by the expression of one or more bacterial effectors that depends on the global regulator agr.	[34–37]			Fusion with lysosomes is prevented. Vacuoles neither acidify nor acquire LAMP2. The virulence factor IsaB is elevated in a methicillin-resistant strain inhibiting the autophagic flux	[34,35,40]
S. marcescens	Autophagosomal components involved in the establishment of intracellular bacteria-containing vacuoles	[42]			Fusion with lysosomes is inhibited	[42]
M. tuberculosis, M. bovis (BCG), M. marinum			If induced, autophagy eliminates the bacterium IFNγ increases the localization of mycobacteria within autophagy-related compartments Formation of M. marinum-containing phagosomes depends on the I ESX-1 type VII secretion system	[43,49,55]	M. marinum and M. tuberculosis inhibits autophagic flux in an ESX-1-dependent manner	[55–58]
S. pyogenes (GAS)			Cytoplasmic GAS is targeted and eliminated by autophagy	[44]	SpeB destroys SARs while SpyCEP stimulate the inactivation of BECN1 and ATG5	[73,74]
L. monocytogenes					ActA mediates bacterial motility to efficiently elude autophagy and also avoids being recognized by	[67–70,81]

Table 1. (Continued).

Bacteria & autophagy

Pathogen	Subversion	References	Destruction	References	Evasion	References
					preventing ubiquitination. InlK allows the establishment of a cage that shields the bacterium	
S. flexneri					IscB avoids being recognized by the ATG machinery	[71]
S. thypimurium					SseL removed ubiquitination making the bacterium not recognizable	[72]
L. pneumophila					RavZ inactivates LC3 proteins, while Lpg1137 degrades STX17	[75,76,78]

not display degradative activity. However, those are not classical autophagosomes since their ultrastructural analysis showed that most of the bacteria-containing phagosomes have a single membrane (see the next section about LAP). Of note, this maturation block was bypassed by rapamycin treatment but not by starvation, suggesting the involvement of a different molecular mechanism than in the case of *M. tuberculosis*. Nonetheless, *M. marinum* infection induces an autophagic response although the autophagic flux seems to be negatively regulated [55]. This inhibition in the autophagic flux was clearly demonstrated by investigating the virulent *M. tuberculosis* strain H37Rv in dendritic cells, which also depend on the ESX-1 system for successful infection [56]. In contrast, attenuated strains such as the avirulent *M. tuberculosis* strain H37Ra or *M. bovis* BCG, which both lack the ESX-1 secretion system, were incapable of hampering autophagosome maturation [56]. The inhibited autophagic flux induced by *M. tuberculosis* was also circumvented by rapamycin treatment, suggesting the participation of mTORC1 in this process [56]. In another study, it was shown that the EXS-1 system is required to prevent *M. marinum* ubiquitinylation in the cytoplasm and subsequent targeting into LAMP1-positive compartments [57]. Interestingly, *M. marinum* infection in the ameba *Dictiostelium discoideum* initially activates autophagy by upregulating the transcription of *ATG* genes in an ESX-1-dependent manner, but the autophagic flux is then inhibited by modulating mTORC1 [58].

It has also been reported that *M. tuberculosis* survival in cultured cells is increased when typical proteins involved in autophagy such as ATG5, ATG7, ULK1, and SQSTM1 are depleted [2,59,60,61]. Indeed, SQSTM1 is required for the bactericidal activity of the *M. tuberculosis*-containing phagosome [62]. This SAR appears to mediate the delivery of ubiquitinated cytosolic proteins to the phagosomes, where they are cleaved and converted into bactericidal peptides that contribute to eliminate the pathogen. *In vivo*, it was found that Atg5 has a specific antibacterial and anti-inflammatory role [63]. Thus, it was suggested that autophagy has a protective role against active tuberculosis [64]. However, it has been revealed that the specific deletion of critical ATG proteins such as Atg14, Atg12, Atg16L1, Atg7, and Atg3 in the myeloid compartment did not affect the outcome or severity of tuberculosis, suggesting that autophagy is not critically involved in the progression of this disease [65]. In agreement with previous reports, however, the same study showed that mice specifically lacking *Atg5* in myeloid-derived cells, develop a more severe disease and die earlier when infected with *M. tuberculosis* [65].

Table 2. LAP and pathogen interactions described in the review.

Pathogens & LAP

Pathogen	Subversion	References	Destruction	References	Evasion	References
S. enterocolitica			LAP restricts intracellular replication	[84,86]		
C. trachomatis	LC3A and LC3B knockdown diminished chlamydial infectivity	[87]				
B. pseudomallei					BopA is required for LAP evasion	[88]
L. monocitogenes	ROS production is necessary for the formation of spacious Listeria-containing phagosomes	[89]				
A. fumigatus					p22phox subunit is excluded from phagosomes	[105]
L. major					LAP and VAMP8 downmodulation	[107]
M. tuberculosis					CpsA impairs NOX recruitment to phagosomes	[108]
H. capsulatum	Replication in LAP structures	[109,110]				
Y. pseudotuberculosis	Replication in LAP structures	[111]				

Altogether, these results suggest that ATG5 has a specific autophagy-independent function in controlling tuberculosis *in vivo* [16]. Indeed, a very recent publication has shown that the absence of Atg5 in a murine experimental model of tuberculosis, leads to exocytosis of lysosomes and secretion of extracellular vesicles in various cell types, as well as a marked degranulation in neutrophils [66]. This increased exocytosis of vesicles could contribute to seal plasma membrane damages by adding new membrane.

Infection with *Listeria monocytogenes*, a facultative, intracellular, Gram-positive rod, also leads to autophagy induction in macrophages [67]. The intracellular bacterium initially localizes to LC3-positive compartments before escaping into the cytoplasm and uses multiple factors to avoid destruction by autophagy. *L. monocytogenes* nucleates actin filaments via the bacterial protein ActA to mediate bacterial motility in the cytoplasm and efficiently elude autophagy [68,69]. Interestingly, independently of its actin nucleating activity, ActA allows bacteria to avoid recognition by autophagy, by preventing its ubiquitinylation and the subsequent binding to SQSTM1 and LC3, indicating that this is a specific camouflage adopted by this bacterium to avoid autophagy degradation [70].

Other bacteria have devised other strategies to avoid destruction by autophagy. For example, *Shigella flexneri*, a Gram-negative nonmotile rod, secretes the factor IcsB through the type 3 secretion system (T3SS) to elude autophagic degradation by interfering with the binding of the bacterial surface protein VirG to ATG5 [71]. Likewise, *Salmonella thypimurium*, a Gram-negative flagellated bacillus, secretes the virulence factor SseL, a deubiquitinase, which decreases bacterium's ubiquitination, impairing the binding of SARs such as SQSTM1 to escape killing by autophagy [72]. Similarly, group A GAS degrades the SARs SQSTM1 and NDP52 using its virulence factor SpeB, a cysteine protease, to avoid lysosomal turnover by autophagy [73]. In a very recent publication about GAS, it has been revealed that the bacterial interleukin-8 protease SpyCEP stimulates the activation of calpains, which in turn also repress autophagy by cleaving proteins like BECN1 and ATG5 leading to a significant decrease in the bacterium being captured by autophagosomes [74].

Another pathogen that disrupts autophagy is *Legionella pneumophila*, a Gram-negative bacillary and aerobic bacterium, which secretes the virulence factor RavZ via its type IV secretion system Dot/Icm [75]. The RavZ protease irreversibly processes LC3 on its terminus, making it not conjugable to PE or PS anymore and consequently unable to associate to autophagosomal membranes, allowing *L. pneumophila* to avoid autophagic degradation [75,76]. Moreover, *L. pneumophila* can prevent xenophagy by inhibiting the recruitment of

SARs to the bacteria-containing vacuoles in a RavZ-independent manner through a strategy that is not clearly defined yet [77]. Finally, *L. pneumophila* via its effector Lpg1137 inhibits autophagy by degrading syntaxin 17 (STX17), a SNARE involved in autophagosome fusion with endo-lysosomal compartments [78]. Thus, *L. pneumophila* appears to have developed a series of stratagems to counteract autophagy.

Interestingly, several bacteria cause amino acid depletion in the host cells, which in some cases is transient over the course of *S. typhimurium* infection [15,79]. *In* the case of *S. flexneri*, however, a persistent decrease is observed, which likely leads to autophagy activation via a starvation-dependent mechanism [79]. It is known that mTORC1 is translocated from the cytosol to the membranes of late endosomes/lysosomal compartments upon activation by amino acids. A marked redistribution of mTORC1, which loses its association with lysosomes and becomes dispersed in the cytoplasm, has been observed in *S. typhimurium*-infected cells [80]. This change in mTORC1 localization is accompanied by a reduction in the phosphorylation and thus inactivation of its targets such as the ribosomal S6 Kinase (S6K1) and the *eukaryotic translation initiation factor* 4E (eIF4E)-binding protein 1 (4E-BP1). Thus, modulation of mTORC1 subcellular distribution is another mechanism used by certain bacteria.

The number of strategies and the diversity of the effectors produced by different pathogens to modulate autophagy and survive intracellularly that have been identified, has increased enormously in the past years (Table 1). In some cases, those were unpredicted. For example, InlK is a virulence factor anchored on the *L. monocytogenes* surface, produced mainly *in vivo*, which interacts with the major component of the cytoplasmic ribonucleoproteic particles called vaults. The major vault protein (MVP) is recruited to the bacteria via InlK, acting like a cage that shields the bacterium from autophagy recognition allowing it to survive in the host cells [81]. It is expected that many other unanticipated molecular components from the host cell, governed and usurped by different bacteria to replicate and grow successfully, will be uncovered in the future.

LC3-associated phagocytosis, an alternative process for killing microorganisms

As mentioned above, members of the LC3 protein play a key role in the formation, elongation, and closure of phagophores. Their discovery in 2000 was a significant advance in the study of autophagy in mammalian cells and tissues [24], and paved the way for additional research into the physiological functions of this pathway. Later, however, LC3 was observed in autophagy-unrelated structures, including cytoplasmic LC3-decorated vesicles with ultrastructural characteristics distinct from the classical double membrane autophagosomes. The findings revealed that, in addition to autophagy, LC3 proteins participated in other cellular processes. Because of its ability to bind membranes following lipidation, it has been theorized that Atg8-like proteins covalently alter membranes similarly as ubiquitin through a process known as atg8ylation [82]. This novel perspective has the potential to elucidate Atg8-like proteins involvement in a variety of processes, of which conventional autophagy represents just one of them. A work published in 2007 demonstrated that opsonized latex beads and zymosan, which are phagocytosed via interactions with toll-like receptors (TLRs), end up inside vesicles decorated with LC3 that have a single membrane [83]. The anchoring of LC3 still depends on the ATG5–ATG12-ATG16L1 complex and PIK3C3 activity, and it appears to be essential for vesicle acidification, suggesting a critical role of the LC3 modification in phagosome maturation. Soon after, it was also found that phagocytosis of IgG-opsonized latex beads via binding to the Fc receptor in macrophages also resulted in the recruitment of LC3 to the particle-containing vesicles [84]. This recruitment required the production of reactive oxygen species (ROS) [84] (see below). Since this observation, it has been documented that several bacteria transit intracellularly in a single membrane vesicle that harbor LC3 [85], including *Escherichia coli* [83], *Salmonella enterocolitica* [84,86], *Chlamydia trachomatis* [87], *Burkholderia pseudomallei* [88], *M. marinum* [55], and *L. monocitogenes* [89].

To differentiate this process from conventional autophagy, the term LC3-associated phagocytosis (LAP) was coined, and the vesicles involved became known as LAPosomes [90]. In addition, this pathway was also linked to the internalization of non-pathogenic cargos such as dead cells [91]. LAP is distinguished from the conventional autophagy by the lack of requirement on the ULK kinase complex, WIPI proteins and possibly a few other ATG proteins [91-93]. Consistently, mTORC1 and AMPK, which regulate the initial steps of autophagy, do not seems to modulate LAP [94]. ROS production requirement is another key feature of LAP of for LAPosome formation [95]. ROS generation in the phagosome is mediated by nicotamide adenine dinucleotide phosphate (NADPH) oxidase-2 (NOX2) [95]. The multiprotein complex NOX2 associates with the phagosome membrane and is composed by p67, p22, Rac family small

GTPase 1 (RAC1) and p40, the last with the capacity to bind PtdIns3P-containing membranes [92,96,97]. Therefore, the function of class III PtdIns3K complex II is necessary prior to both ROS generation and LC3 recruitment. The class III PtdIns3K complex II participating in LAP is formed by BECN1, PIK3C3/VPS34, PIK3R4/VPS15, UVRAG and rubicon autophagy regulator (RUBCN) [95,98]. Moreover, in contrast to its inhibitory role in autophagy [99], RBCN promotes the maturation of LAPosomes by stabilizing the enzyme NOX2 and thereby increasing ROS production [100,101], which in turn enhances LC3 recruitment [84,95]. Since the ULK kinase complex and WIPI2 are not involved in LAP, it remains unknown the mechanism of recruitment of the LC3 conjugation machinery in this process, although the ATG16L1 determinants essential for LAP are different than those required for autophagy [94,95]. LC3 plays a role in LAP's downstream events, including stimulating both phagosome-endosome and phagosome-lysosome fusion [95,102]. This notion is supported by the observation that recruitment of LC3 to phagosomes enhance its maturation and microbial clearance capacity [83,84,91,103]. Moreover, the presence of LC3 is required for the interaction of TLR-phagocytosed particles with lysosomes [83].

Another aspect still unclear is the signal that initiates the LAP. Pattern recognition receptors (PRRs) such as toll-like receptors (TLR1–TLR2, the TLR2–TLR6 and TLR4), immunoglobulin (Ig) receptors, and receptors mediating the clearance of cell corpses such as T cell immunoglobulin- and mucin domain-containing molecule-4 (TIM4), are involved in the recognition of LAPosome cargos [83,91,100,102,104,105]. However, it is still unknown how the binding to these receptors results in the recruitment of LAP regulators to the phagosome.

In recent years, the list of microorganisms targeted by LAP has expanded, including bacteria, viruses, fungi, and protozoa [106]. In addition, evasion mechanisms of LAP by microorganisms such as *Aspergillus fumigatus*, *Leishmania major*, *L. pneumophila*, *M. tuberculosis*, *B. pseudomallei*, *Histoplasma capsulatum*, and *Yersinia pseudotuberculosis* have been described. One of the main microorganism strategies to prevent LAP degradation is the inhibition of NOX2 activity. The p22phox subunit is excluded from the phagosome as a consequence of melanin expression by *A. fumigatus* [105], while *L. major* trough the metalloprotease GP63 activity down modulates VAMP8, a protein required to NOX2 assembly [107], and the protein CpsA from *M. tuberculosis* has the capacity to impair NOX2 recruitment to phagosomes [108]. In other instances, blocking the acidification of

LAPosomes through a not defined mechanism allows the replication of microorganisms such as *H. capsulatum* [109,110] and *Y. pseudotuberculosis* [111].

In the interplay between autophagy and microorganisms, the primary function of LC3 proteins is the recognition of the pathogen trough their direct interaction with the LIR domains of SARs such as p62, CALCOCO2 (calcium binding and coiled-coil domain 2, also known as NDP52), OPTN (optineurin), and specific galectins [106,112]. While SARs recognize the ubiquitin chains on bacteria appended in the cytoplasm, galectins bind to carbohydrates that are exposed from the interior of damaged phagosomes ruptured by bacteria [106,113]. Some examples of these phenomena have been reported during the infection of *M. tuberculosis* [114] or *S. typhimurium* [112], and even some viruses [115-117]. In the case of LAP, however, microorganisms are not exposed to the cytoplasm because the LAPosome membrane appears to be intact, and ubiquitination and SARs are not implicated [118-120].

Recently, an alternative pathway to LAP has been described. This new pathway, termed pore-forming toxin-induced non-canonical autophagy (PINCA), is analogous to LAP in that LC3 is recruited to the bacteria-containing phagosome in a way independent of the ULK kinase complex. However, unlike LAP, in this pathway ROS production by NOX2 is not required [121]. In PINCA, which was described during *L. monocytogenes* infection, the mechanism of LC3 recruitment is triggered by the damage of the phagosome membrane caused by the pore forming toxin secreted by the bacterium [121]. Similar to LAP, PINCA promotes the fusion between lysosomes and *L. monocytogenes*-containing phagosomes, but this did not significantly contribute to the anti-listeria activity of bone marrow derived macrophages (BMDM). Interestingly, in PINCA LC3-positive phagosomes generated by PINCA pathway were less frequently damaged than LC3-negative phagosomes [121]. This suggests that the targeting of phagosomes by PINCA may be associated with a membrane damage repair program. Notably, injury caused by *S. flexneri* or *S. typhimurium's* needle-like T3SS did not result in PINCA in macrophages [121], suggesting that this mechanism is not universal. Perhaps the expression of additional bacterial virulence factors is necessary to induce PINCA [122,123].

Parasites and autophagy

Intracellular protists are a group of pathogens that require the interaction with host cells for the completion of their biological cycle. They are responsible for

causing many of the most widespread infectious diseases in the world. Paludism, toxoplasmosis, leishmaniasis, and Chagas disease are caused by *Plasmodium* spp., *Toxoplasma gondii*, *Leishmania* spp. and *Trypanosoma cruzi*, respectively, and are severe, life-threatening illnesses with limited treatments, which is why they are considered orphan or neglected diseases. In contrast to bacteria, protists are eukaryotic cells that possess many processes like mammalian cells, including autophagy in some cases (parasite autophagy will not be covered in this review; see [124]). Nevertheless, mammalian cells represent an excellent niche for the nutrition, replication, and immune system evasion of these parasites. These advantages have induced some protists to develop mechanisms to gain access and propagate inside host cells. Thus, they actively interact with several host pathways including host autophagy.

A brief introduction to the intracellular cycle of pathogenic protists

Trypanosoma cruzi can infect both professional and non-professional phagocytic cells under both trypomastigote and amastigote forms. After a transitory stage in a membrane-bound compartment known as the *T. cruzi* parasitophorous vacuole (TcPV), *T. cruzi* accesses the host cell cytosol, where it replicates in the amastigote form. Amastigotes can either egress via accidental cell rupture or transform back into trypomastigotes that also exit the cell by inducing cell lysis and start a new infective cycle. Heart, esophagus and colon are the main target organs of *T. cruzi* [125].

Leishmania spp. only infects phagocytic cells, and into the phagolysosome the promastigote form evolves into amastigote form. To survive intracellularly, the parasite has developed a few mechanisms to block the maturation of phagolysosome. *Leishmania* spp. amastigotes disseminate after his multiplication to other macrophages in the bone marrows and reticuloendothelial system [126].

Toxoplasma gondii actively invades many types of nucleated mammalian cells, including non-professional and professional phagocytes. Invasion occurs with the formation of a special structure known as the moving junction, a tight apposition between the invading parasite and the host plasma membrane trough a receptor-ligand-mediated binding [127]. This junction starts at the apical pole and progressively moves to the posterior end of the parasite as it forcibly enters the cell through the plasma membrane invagination of the host [128]. The resulting parasitophorous vacuole (PV) is a specialized non-fusogenic intracellular compartment where the parasite avoids degradation by lysosome fusion, in the early steps [129]. Thereafter, the PV is modified by the parasite secretion of lipids and proteins and its intracellular replication is promoted.

In the case of *Plasmodium* spp., the productive infection is produced when sporozoites, the infective form of these parasites, induce their invagination at the host cell plasma membrane to form a specialized and stable compartment, also called PV, in which the parasite reproduces [130]. Like *T. gondii*, the PV membrane integrity is crucial for the successful development of *Plasmodium*, promoting the uptake of nutrients and the release of waste products, and generating a tubular vesicular network that functions as a reproductive niche for the parasite [131].

Through the course of these 30 years, the perception about the interplay between host autophagy and pathogenic protists evolved from earlier works describing the beneficial effect of autophagy for the parasite life cycle to an opposite view, 10 years later, in which autophagy is seen more as a component of the innate immune response against the parasites. This apparent discrepancy is due to different reasons, including the pathogen virulence, the host cell background, and even the stage of the infection that is analyzed. As a sort of general rule, for parasites that can infect several different types of cells, it has been observed that autophagy is responsible for the parasite death in professional phagocytes, while this process is subverted or evaded in non-immune cells. The following sections present the experimental evidence for each protist that has led to this general rule.

Parasite subversion of host autophagy

One of the first observations about the interplay between host autophagy and intracellular protists was about the interaction of the parasites with ATG proteins, mainly LC3, and the utilization of the autophagic response for their own benefit. During *T. cruzi* invasion of CHO cells, an epithelial-derived cell line, GFP-LC3, was found to be recruited onto the membrane that both envelops the internalizing parasites and surrounds the TcPV. Since induction of autophagy prior to infection increased the percentage of infected cells and the localization of lysosomal markers to the TcPV, these findings were associated with a lysosomal-dependent mechanism for *T. cruzi* entry into host cells [132]. During invasion, trypomastigotes of *T. cruzi* damage the host cell plasma membrane, triggering the calcium-dependent exocytosis of lysosomes to repair the plasma membrane and thus promoting *T. cruzi* infection [133,134]. Therefore, the increment in the

number of degradative compartments, i.e., lysosomes and autolysosomes, in the host cell upon autophagy induction explained the major rate of *T. cruzi* infection observed under these conditions [135]. Increased parasite burden upon autophagy induction was also found in macrophages from Balb/c mice infected with *Leishmania amazonensis*. This autophagy-related beneficial effect correlated with an enhancement in lipid body and prostaglandin E2 production, and a decrease in nitric oxide (NO) production by the infected macrophages [136]. Subsequent studies performed *in vitro* and *in vivo* indicated that upon cell entry, infected cells display an activation of autophagy. *L. amazonensis*-infected macrophages have an increased generation of LC3-II accompanied by a major uptake of the lysosome-specific dye lysotracker, and the formation of myelin-like structures [137]. Importantly, leishmaniasis skin samples were found positive for LC3-II by immunocytochemistry analysis. These findings, which may indicate an autophagy induction, suggested a beneficial role of this process for the infection, since the autophagy inhibitor 3-methyladenine (3MA) reduced the infection index [137]. A major expression of the genes *Lc3B* and *Atg5* was also observed in BMDMs after *Leishmania major* infection [138] and a greater survival of this parasite was detected in macrophages from CBA mice after exogenous stimulation of autophagy [139]. Other studies also revealed an induction of autophagy during *Leishmania* spp. infection, although the effect of this process yields contradictory results, i.e., beneficial versus detrimental, and the autophagy role for the infection outcome remains unclear [140-143].

To support its growth, *T. gondii* very efficiently intercepts and subverts host organelles shortly after invasion, during PV establishment. A few hours after invasion, HeLa cells and primary fibroblasts showed a significant recruitment of LC3-positive vesicles and a localization of BECN1 around the TgPV [144]. A subsequent study showed that *T. gondii* infection triggers lipophagy to provide the free fatty acids required for the parasite development [145]. These observations suggested that in the absence of IFNγ host cell autophagy is exploited by *T. gondii* to acquire nutrients to sustain its growth and propagation. Autophagy was also highlighted as a potential important source of nutrients for the *Plasmodium* spp. in the liver. Like *T. gondii*, LC3-positive vesicles surrounded the parasites from early time points after invasion and throughout infection, and colocalized with its PV membrane. Moreover, genetic inhibition of autophagy by LC3B, BECN1, VPS34 or ATG5 depletion, led to a reduction in the size of parasites [146]. This growth defect could be partly compensated by supplementing

extra amino acids to infected $Atg5^{-/-}$ KO MEFs [147]. These data were also supported by *in vivo* experiments showing that parasite liver loads were significantly reduced in *Atg5*-deficient mice [146]. Moreover, pharmacological and physiological activation of autophagy resulted in an extraordinary increased parasite loads *in vivo*, characterized by a significant enhancement in the parasite size and survival [147]. However, it is important to note that the increased growth of parasites in rapamycin-treated mice could be due to the immunosuppressive effect of this compound [148].

Parasiticidal functions of host autophagy

In contrast to non-professional phagocytic cells in which autophagy does significantly modify *T. cruzi* load at the advanced infection stages [132,149], see also below), this pathway plays an important role in controlling *T. cruzi* infection in macrophages. That is, *T. cruzi*-infected macrophages showed formation of autophagosomes and autolysosomes, and the NLR family pyrin domain-containing 3 (NLRP3) inflammasome protein is required for autophagy induction and elimination of the parasites [150]. The function of autophagy as a defense against *T. cruzi* was also demonstrated in *in vivo* experiments with $Becn1^{+/-}$ heterozygous KO mice [151]. These animals displayed higher parasitemia and early mortality compared to the wild-type animals. Additionally, higher levels of infection were found in peritoneal cells obtained from both $Becn1^{+/-}$ or $Becn1^{+/+}$ mice treated with the autophagy inhibitor chloroquine [151] or diphuoromethyl ornithine (DFMO) [152], confirming the function of autophagy in the inhibition of parasite growth. Interestingly, recent findings revealed an increment in the clearance of amastigotes by xenophagy in RAW 264.7 macrophages and H9C2 cardiac cell line when treated with the autophagy inducer ursolic acid, supporting the role of autophagy in containing *T. cruzi* intracellular infection [153]. Like for *T. cruzi*, autophagy can also be involved in the intracellular elimination of *Leishmania* spp. Infection of *L. major* increased the autophagic compartments (e.g. autophagosomes and myelin-like structures), concurrently with the elimination of amastigotes in macrophages from Balb/c mice [138]. Macrophages from C57BL/6 mice infected with *L. major* also display higher autophagy, which probably accounted for the restriction of the parasite replication. Signaling by endosomal TLRs is required for this effect because macrophages lacking Tlr3, Tlr7 and Tlr9 did not exhibit *L. major*-induced autophagy [142]. Similarly, shRNA-mediated suppression of Atg5 impaired the restriction of *L. major* replication. Collectively, these

observations led to the conclusion that autophagy operates downstream of TLR signaling and is a relevant immune response against *L. major* infection in macrophages [142].

In the case of *T. gondii*, members of the LC3 protein family were found in the parasitophorous vacuole membrane in macrophages, showing the recognition of the invader by the host autophagy. However, this mechanism was described as a LAP-like process due to lipidated LC3 was directly anchored in the vacuole membrane in the absence of fusion of LC3-positive autophagosomes [154]. Downstream of LC3, two mechanisms, both connected to autophagy or the ATG proteins, mediate the parasiticidal response against *T. gondii* in macrophages. In the first one, the CD40 receptor of macrophages, and its main ligand, CD154, lead to ULK1 activation and upregulation of BECN1, promoting the arrival and fusion of host lysosomes to the PVs that lead their destruction [155-157]. When expose to *T. gondii*, $Cd40^{-/-}$ or $Cd154^{-/-}$ KO mice showed a higher susceptibility to toxoplasmosis than the controls confirming *in vivo* that CD40- restricts infection of *T. gondii* [158,159]. Consistently, the $Becn1^{+/-}$ mice as well as those lacking *Atg7* in myeloid cells are more sensitive to cerebral and ocular toxoplasmosis in comparison to the WT animals [158]. The other mechanism is carried out by IFNγ. In mouse-derived cells; IFNγ induces the recruitment of IFN-regulated GTPases (IRGs) and guanylate-binding proteins (GBPs) to TgPVs [160,161]. These proteins disrupt the TgPV membrane thereby exposing the denuded *T. gondii* to the capture by autophagosomes [162]. In human epithelial cells, IFNγ induces the association of ubiquitin, NDP52, SQSTM1, and LC3, which promote the envelopment of TgPVs in a multilayer structure, which appears to be bacteriostatic, restricting parasite growth [163]. In human endothelial cells, in contrast, ubiquitin, NDP52 and SQSTM1 recruitment onto TgPVs is followed by the one of RAB7, which induced the parasite killing by fusion of TgPV with lysosomes [164].

The autophagy-related pathways against *Plasmodium* spp. can be divided into three different responses: the *Plasmodium*-associated autophagy-related (PAAR) response; the LAP-like response; and the PtdIns3P-associated sporozoite elimination (PASE). Within the PAAR response, LC3 proteins are rapidly conjugated to the parasite's PV membrane through a mechanism that does not require the ULK kinase and PtdIns3K complexes. Although the PAAR response can target almost all the liver-stage *P. berghei*, only 50% gets eliminated [147,165]. The degree of parasite elimination by the PAAR response was the same in $fip200^{-/-}$ and wild-type cells, indicating that this process does not involve

canonical autophagy [147,165]. The PAAR response is principally triggered by *P. berghei* and *P. yoelii* invasion. In *P. vivax*-infected cells, LC3 association to the PVs resembles to the LAP process and depends on the formation of PtdIns3P by the class III PtdIns3K complex II. This LAP-like response occurs after IFNγ stimulation and is effective in eliminating 30% of liver-stage *P. vivax* [166].

Finally, PASE occurred against the transient vacuoles formed by transmigrating sporozoites during the non-productive invasion of *P. berghei*. When sporozoites transmigrate the cells without forming a moving junction, they can disrupt the vacuole membrane and escape into the cytosol. Parasites deficient in perforins like protein-1 (PLP-1/SPECT2) are not able to exit their transient vacuoles, which are thus successfully acidified by lysosomal fusion and the parasites are destroyed [167].

Parasite evasion from the autophagy response

A third class of interaction between host autophagy and pathogenic protists comprises cases in which parasite actively evade the parasiticidal actions of autophagy and related processes. Although still unclarified, these mechanisms are probably key in allowing the parasites to survive intracellularly and establish persistent infections, at least in some host cell types.

The inhibitory action of *T. cruzi* on the formation of autolysosomes to avoid the destruction of its amastigotes is an example of such evasion mechanism. In the non-professional phagocytic fibrosarcoma cell line HT1080, despite *T. cruzi* activating autophagosome formation, autolysosomes were not observed in the infected cells, suggesting that this parasite probably blocks the fusion of autophagosomes with the degradative compartments [149].

In the case of *T. gondii*, it was shown that < 10% of PVs, are decorated by LC3. One process to avoid host autophagy involves the stimulation of epidermal growth factor receptor (EGFR)/PtdIns3K/RAC (Rho family)-alpha serine/threonine-protein kinase (Akt) signaling axis. Extracellular *T. gondii* releases proteins, known as microneme proteins (MICs), from the apical secretory organelles, which are called micronemes. Gliding motility is supported by these proteins facilitating cell invasion. Several MICs contain domains with homology to the epidermal growth factor (EGF) and therefore that also act as EGFR ligands [168]. MICs binding to EGFR triggers the downstream activation of the kinase Akt, which in turn stimulates mTORC1 to suppress host autophagy [169]. The rhoptries (ROPs), secreted from the rhoptry organelles, are

Table 3. Autophagy and parasite interactions described in the review.

Parasites & autophagy						
Pathogen	Subversion	References	Destruction	References	Evasion	References
T. cruzi	Increment of infection at early times in non-professional phagocytic cells	[132,135]	Clearance of amastigotes at later times of infection in phagocytic cells	[150–153]	Autophagy inhibition at later times of infection in non-professional phagocytic cells	[149]
Leishmania sp.	Increase in parasite burden in *L. amazonensis*- and *L. major*-infected macrophages from Balbc and CBA mice respectively	[136,139]	Clearance of *L. major* amastigotes in macrophages from Balb/c and C57BL/6 mice	[138,142]		
T. gondii	Source of nutrients at early times of infection in the absence of INFγ	[144,145]	LAP-like process and vacuole degradation in CD40- and INFγ- stimulated cells	[154–164]	Autophagy inhibition by *Toxoplasma* derived MICs and ROPs proteins	[169,171]
Plasmodium sp.	Increase in parasite size *in vitro* and parasite load *in vivo*	[146–148]	PAAR response against *P. berghei* and *P. yoelii*, LAP-like response in *P. vivax* and PASE during non-productive *P. berghei* invasion	[147,165, 166,167]		

other *Toxoplasma*'s proteins that modulate host autophagy. For instance, ROP17 binds to BCL2 and inhibits the interaction between BCL2 and BECN1, and augments LC3B and SQSTM1 expression [170]. Moreover, ROP16 enhances the focal adhesion kinase (FAK)/-signal transducer and activator of transcription 3 (STAT3) signaling cascade, which promotes expression of key molecules that reduce autophagy response, impairing the clearance of intracellular *Toxoplasma* by autophagy [171].

In conclusion, subversion, inhibition or destruction are three actions that can be found in the pathogenic protist-host autophagy interplay (Table 3). The outcome of the infection, however, seems to depend on different factors, including pathogen virulence, host cell background, infection stage, etc. A better knowledge of the interaction between autophagy and parasites will help to uncover the different interplay mechanisms and possibly find new therapeutic interventions against the various parasites.

Virus and autophagy

Viruses are symbionts that have co-evolved with nearly all forms of cellular life and have different types of genomes, i.e., single-stranded positive-sense, negative-sense, or double-stranded RNA (+sRNA, −sRNA, and dsRNA, respectively) or single or double-stranded DNA (ssDNA, and dsDNA, respectively), which determine the pathway of viral replication and protein expression. Numerous RNA and some DNA viruses replicate and transcribe their genomes in cytoplasmic membranous or non-membranous organelle-like compartments, where they also establish a molecular shield from the host defenses to favor efficient genome replication. Autophagy represents a threat to virus survival and consequently multiple viruses have evolved strategies to subvert autophagy. The first evidence of an interaction between viruses and autophagy was published almost 23 years ago by the group of Karla Kirkegaard [172]. Based on the double-membraned morphology, the cytoplasmic content, the labeling with LC3 and the apparent ER origin, they proposed that the membranous-replication niches of poliovirus (PV), an enveloped +sRNA virus belonging to the *Picornaviridae* family have an autophagy-related origin [172]. Since then, there have been a multitude of reports showing the interplay between viruses from practically all virus families and autophagy.

Viral subversion of the protective function of autophagy

Upon virus infection, PRRs, including the retinoic acid-inducible I (RIG-I)-like receptors (RLRs), recognize pathogen-associated molecular patterns (PAMPs) and

active downstream signal cascades that lead to the production of interferons (IFNs) and cytokines, which are part of the first line of innate defense against viruses [173-176]. In recent years, autophagy has emerged as a mechanism to downregulate RLRs-mediated antiviral signaling [177]. In this context, acceleration of autophagy appears to be a beneficial strategy for viruses to evade antiviral defenses. Indeed, the role of autophagy in the negative regulation of RLRs-mediated antiviral immune response was initially revealed by measuring an enhancement of the RLRs-mediated IFN response in $Atg5^{-/-}$ KO MEFs infected with vesicular stomatitis virus (VSV), a −sRNA virus belonging to the *Rhabdoviridae* family [178]. PRRs are sensing molecules localized in the cytosol and on the surface of membranous compartments [179]. Thus, mitochondria and the associated ER serve as a platform for the assembly and signal transduction of specific players of RLRs-mediated antiviral immunity [180-182]. Tal *et al.* [183] were the first to reveal that inhibition of autophagy increases the type I IFN response that is characterized by an accumulation of mitochondria, inferring that autophagy negatively regulates RLRs-mediated antiviral immunity by selectively removing ROS-producing dysfunctional mitochondria. ROS induce the overproduction of the mitochondrial antiviral-signaling protein (MAVS) and the hyperactivation of RLRs signaling. Enhanced autophagy also inhibits hepatitis C virus (HCV), an enveloped +sRNA virus belonging to the *Flaviviridae* family. HCV, however, subverts autophagosomal membranes to favor various aspects of its viral life cycle. This virus induces autophagy through ER stress and more in particular the unfolded protein response (UPR) [184]. Autophagosomal membranes are used by HCV as platforms for RNA replication [13,14,185]. HCV also uses autophagosomal membranes to promote the interaction between its E2 envelope protein and apolipoprotein E (ApoE), which is necessary for the assembly of infectious HCV particles [186]. Finally, HCV-triggered autophagy impair the innate immune response through the turnover of the tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), a key signal transducer that activates the nuclear factor kappa B (NF-kB) signaling pathway and the downstream expression of proinflammatory cytokines [184,187].

Mitophagy is likely to be either promoted by viruses to either suppress antiviral immunity or inhibited to accumulate mitochondria, which results in a robust immune response and severe injury of the host. Indeed, human immunodeficiency virus 1 (HIV-1), an enveloped +sRNA virus belonging to the *Retroviridae* family [188-190], herpes simplex virus-1 (HSV-1), a dsDNA virus belonging to the *Herpesviridae* family

[191], influenza virus (IAV), an enveloped -sRNA virus belonging to the *Orthomyxoviridae* family [192,193], Epstein–Barr virus (EBV), another member of the *Herpesviridae* family [194], human parainfluenza virus type 3 (HPIV3), an enveloped -sRNA virus belonging to the *Paramyxoviridae* family [195], senecavirus A (SVA), a non-enveloped +sRNA virus belonging to the *Picornaviridae* family [196] and SARS-CoV-2, an enveloped +sRNA virus belonging to the *Coronaviridae* family [21-23], all appear to have evolved strategies targeting mitochondria. In the case of EBV for example, the viral BHRF1 protein inhibits IFN-response induction by stimulating autophagy and mitochondrial fission via dynamin 1 (DNM1) and/or dynamin-related protein 1 (DRP1) [194]. In particular, BHRF1 promotes the reorganization of the mitochondrial network to form juxtanuclear mitochondrial aggregates, while numerous mitochondria are also found inside the autophagosomes and acidic compartments. Thus, BHRF1 can counteract the activation of innate immunity by inducing mitochondrial fission to facilitate their sequestration into mitophagosomes and degradation [194].

A study on HSV-1 was the first to report the inhibition of autophagy as a result of a viral infection [197]. Since then, and based on the notion that autophagy is a component of the intracellular innate defense against viruses, a number of strategies adopted by viruses to directly stop this pathway have been uncovered. Autophagy initiation and autophagosome-lysosome fusion are the two steps in the pathway that are mainly targeted by viruses. HSV-1 blocks autophagy upregulation with its Us11 protein, which directly interacts and inhibits *eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2)*, but also disrupts the tripartite motif containing 23 (TRIM23), which is a key regulator of autophagy-mediated antiviral defense mediated by TANK binding kinase 1 (TBK1). Us11 disrupts the TRIM23-TBK1 complex by binding to the ADP-ribosylation factor (ARF) domain in TRIM23, spatially excluding TBK1 from the TRIM23 complex [198,199]. HSV-1 also inhibits the early steps of autophagosome formation through the binding of viral ICP34.5 to BECN1. As in the case of cellular BCL2, ICP34.5 association to BECN1 impair the assembly of the class III PtdIns3K complex I and PtdIns3P biosynthesis [200]. Interestingly, the genome of herpesviruses belonging to the *Gammaherpesvirinae* subfamily, including Kaposi's sarcoma-associated virus (KSHV) and murine gammaherpesvirus-68 (MHV68), encode for BCL2 homologs that also downregulation through an inhibitory binding to BECN1 [201-203]. Human cytomegalovirus (HCMV), a herpesvirus

belonging to the *Betaherpesvirinae* subfamily, carries two homologs of HSV-1 ICP34.5, called TRS1 and IRS1, which affect the formation of both class III PtdIns3K complex I and II [98,204,205]. In fact, the targeting of BECN1 by viral BCL2 homologs is a mechanism acquired by several viruses to subvert autophagic pressure, highlighting the strong threat that autophagy is for virus survival [98]. The human papillomavirus type 16 (HPV16), a double-stranded DNA virus belonging to the *Papillomaviridae* family, employs a two-arm strategy to block autophagy. On the one hand, this virus stimulates the cell surface EGFR, while on the other hand, it inhibits phosphatase and tensin homolog (PTEN), resulting in the induction of the class I PtdIns3K-AKT-mTORC1 signaling cascadethat leads to an inhibition of autophagy [206,207]. Finally, the foot and mouth disease virus (FMDV), a +sRNA virus belonging to *Picornaviridae* family, downregulates autophagy by processing and thus inactivating the ATG5-ATG12 complex using its viral protease 3C^{PRO} [208].

The block of fusion between autophagosome and lysosome, in contrast, prevents the degradation of viruses or viral components within lysosomes. For example, HIV-1 Nef protein impairs this step of autophagy by interfering with the assembly of the UV radiation resistance associated (UVRAG)-containing class III PtdIns3K complex II and the RUBCN-positive class III PtdIns3K complex II, which are required for autophagosome maturation [98,209,210]. Similarly, IAV blocks autophagy maturation using its matrix protein M2 [211], while HPIV3 utilizes its phosphoprotein (P) that competitively binds to synaptosome-associated protein 29 (SNAP29), to prevent the formation of the SNARE complex with STX17 that mediates autophagosome fusion with lysosomes [212]. Consistently, knock down of SNAP29 increases the yield of extracellular HPIV3 viral particles [212]. Thus, a plausible scenario is that by hampering fusion, the concomitant autophagosome accumulation might serve as a carrier for viral egression. This is an important concept since similar observations have been made for other viruses such as coxsackievirus B3 (CVB3), a +sRNA virus belonging to the *Picornaviridae* family [213,214]. In a parallel line of evidence, EBV, which is known to inhibit the autophagic flux [215], subverts the ATG machinery or at least part of it, to generate the viral envelope, which, as a result, contains lipidated LC3 [216]. HCMV, which initially induces the formation of autophagosomes and then prevents their fusion with lysosomes, was found to exhibit a similar pattern as EBV [204,217]. Thus, the

inhibition of autophagosome-lysosome fusion may offer the double advantage of avoiding degradation concomitantly with the generation of a network of membranes necessary for the progression of the viral cycle.

Viral hijacking of the autophagosomal membranes

Within the virosphere, +sRNA viruses replicate their genomes in the cytosol of host cells. Current evidence indicates that the optimal production of several +sRNA viruses depends on the stimulation of autophagy, a quite astonishing notion given that autophagy is specifically dedicated to degrade cytosolic components, including viral material. The majority of +sRNA viruses also induce massive membrane remodeling in infected host cells, resulting in the formation of membranous structures often referred to as replication factories (RFs). According to their ultrastructural morphology, these RFs are generally classified as spherules or double membrane vesicles (DMVs) [218]. Since the original studies indicating a possible autophagosomal contribution to the generation of DMVs induced by PV [172,219,220], this type of membrane rearrangements has been connected to the ATG machinery only in a few viral infections. Indeed, autophagosomes and DMVs are both double-membrane structures, but differ in the membrane remodeling processes. A shared characteristic of these DMVs is the recruitment of LC3, but not necessarily its autophagy-competent form conjugated to PE. For instance, it has been observed the presence of DMVs in CVB3-infected cells, which increase in number as the infection progresses [221]. However, autophagy is not required as the membrane source of these DMVs is variable and autophagosomal membrane may be only one of these sources [222].

Infections with coronaviruses (CoVs) such as the mouse hepatitis virus (MHV) [223], the Middle East respiratory syndrome coronavirus (MERS-CoV) [224], the severe acute respiratory syndrome coronavirus (SARS-CoV) [225] and SARSCoV-2 [226,227], leads to the formation of a reticulovesicular network consisting of DMVs connected to a complex of convoluted membranes (CMs), which are derived from the ER. Newly synthesized viral RNAs and the dsRNA intermediate generated by the replication of the genomic RNA are mostly localized within these DMVs, indicating that DMVs represent the main center of viral RNA synthesis but also a way to both protect viral RNA from destruction and avoid activation of an immune response. Despite an important interplay between

Table 4. Autophagy and virus interactions described in the review.

Viruses & autophagy						
Pathogen	Subversion	References	Destruction	References	Evasion	References
VSV	Autophagy enhancement to augment the downregulation of RLRs-mediated antiviral signaling	[178]				
HCV	Autophagy enhancement and inhibition of the autophagic flux to hijack autophagic membranes and downregulate antiviral signaling	[184,186,187]				
EBV	Mitophagy induction to avoid innate immunity activation	[194]				
HSV-1					Block of autophagy upregulation	[197-200]
KSHV					Block of autophagy upregulation	[201,203]
MHV68					Block of autophagy upregulation	[202,203]
HCMV					Block of autophagy upregulation	[98,204,205]
HPV16					Blockof autophagy upregulation	[206,207]
FMDV					Blocks of autophagy upregulation	[208]
HIV-1					Block of the autophagosome-lysosome fusion	[98,209,210]
IAV					Block of the autophagosome-lysosome fusion	[211]
HPIV3					Block of the autophagosome-lysosome fusion	[212]
PV	Hijack of the autophagosomal membranes	[172,219,220]				
CVB3	Hijack of the autophagosomal membranes	[221,222]				
CoVs	Hijack of the autophagosomal membranes	[223-230]				

CoVs and the ATG machinery, DMVs in cells infected with CoVs, such as MHV, SARS-CoV and SARS-CoV-2, are not generated by the canonical ATG machinery [223,228,229]. The current idea about the relationship between CoVs and autophagy, mostly coming from recent studies on SARSCoV-2, is that autophagy is induced, but CoVs subvert this pathway by avoiding the autophagosome-lysosome fusion, hence lysosomal degradation [230].

The afore mentioned cases are just mere examples (Table 4); for exhaustive recent revisions on the topic, including plant viruses, please refer to [224-239].

Concluding remarks

Autophagy is an important degradative pathway to clear intracellular microorganisms, including bacteria, viruses, and parasites. However, several of them have successfully developed molecular strategies to avoid and/or subvert autophagy to their own advantage, promoting their intracellular survival and propagation. This may represent a xenophagy *Tango* dance, in which both partners fight persistently with the final purpose of gaining the competition (Fig. 1). However, all three classes of pathogens have intrinsically unique capacities to react (or take the lead in the dance) when the autophagy is activated. For example, bacteria manipulate autophagy by secreting effector proteins whereas viruses relay on their own proteins that often are multifunctional. Although the host cell possesses an effective and multilayered defense, the dance is not always dominated by the host, and the partner sometimes manages to successfully evade it. While not comprehensive, we have described in this review multiple examples of autophagy–pathogen
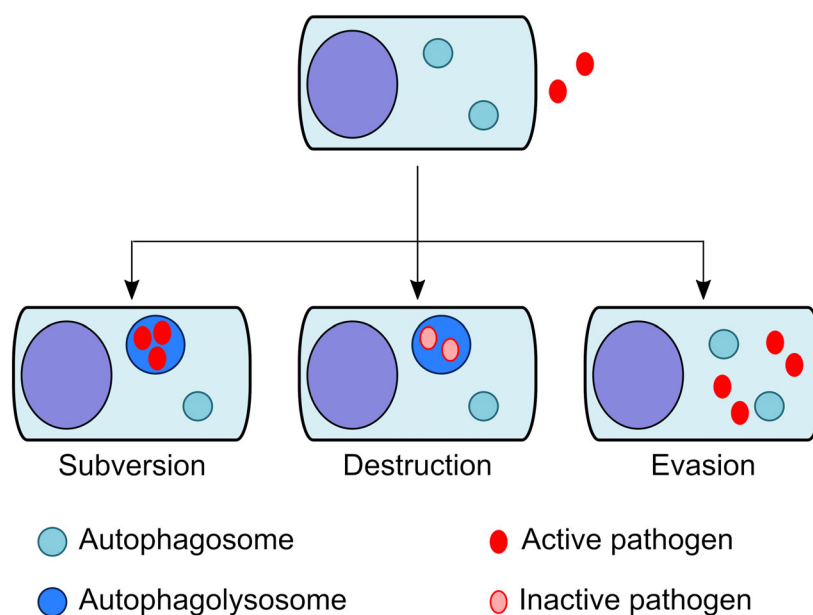
Fig. 1. Schematic representation showing the possible interactions (subversion, inhibition, or destruction) between various intracellular microorganisms and autophagy. In the article, we cover the wide range of intricate interactions that take place between those players.

interactions, to emphasize their complexity (Tables 1–4). Nevertheless, it is important to consider that even though the specific molecular details have perhaps been identified for only one defined event, pro- and anti-pathogen pathways are probably active concurrently in each host–pathogen interaction situation.

In conclusion, we have gained enormous knowledge about the interplay between pathogens and autophagy over the last decades, uncovering multiple subversion strategies through the identification of several pathogens' effectors and ATG machinery targets. Importantly, this area of investigation has also contributed to the discovery of alternative autophagic mechanisms, but also non-conventional types of autophagy and novel functions of the classical molecules involved in autophagy. Although the knowledge about the interaction between autophagy and pathogens has grown exponentially, this probably represents just the tip of the iceberg and future investigations will uncover new interplay mechanisms. This will not only characterize better the life cycle of the studied microorganisms but also possibly provide new therapeutic targets to fight some of the devastating disease caused by them.

Acknowledgements

Data accessibility

Data sharing is not applicable to this manuscript as no new data were created in this article.

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