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Chapter 13

Infectious Agents and Autophagy: Sometimes You Win, Sometimes You Lose

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1. Introduction

Successful microorganisms are those that can evade the immune responses of the host. To reach this purpose, many pathogens have evolved as intracellular organisms, acquiring the capacity to live and to develop inside cells. This cellular parasitism has many benefits for pathogens such as protection from circulating antibodies, free access to nutrients and to specialized compartments that microorganisms use to establish their replicative niches. According to their particular lifestyles and requirements, many pathogens such as *L.monocytogenes, Shigella* or *T. cruzi*; reproduce in cell cytosol, while others target specific vesicles to generate particular compartments called parasitophorous vacuoles (PVs). This class of parasitism is utilized by *M. tuberculosis, C. burnetii* or *T. gondii*. On the other hand, in response to this level of adaptation, mammalian cells have developed different processes for eliminating intracellular microorganisms or for keeping them under strict control. These mechanisms are part of the innate immune responses, being phagocytosis (and the related processes) the best characterized. Innate cellular immunity also encompasses the autophagic process, a well conserved eukaryotic pathway that interacts with intracellular pathogens under certain circumstances to produce the destruction of the foreign organism. Autophagy comprises the inclusion of pathogens in autophagic-derived compartments and delivers them in lysosomes for digestion. Some pathogens, however, have acquired the capacity to subvert autophagy for their own benefit. This chapter will describe the interaction between intracellular microorganisms and the defense mechanisms of host cells, with special focus on the dual involvement of autophagy against pathogens, and the net outcome of this interaction.

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2. The phagocytosis process

Phagocytosis is a form of endocytosis mainly present in specialized types of cells: the professional phagocytes that include the neutrophil, monocytes/macrophages, and dendritic cells. In the initial stage of phagocytosis, the cells change shape by sending out membrane projections (pseudopodia) that contact and surround the particle (bacteria, apoptotic bodies, etc) in a receptor-mediated and actin-dependent process (Figure 1). When the tips of the pseudopodia meet each other, membrane fusion occurs and the particle is enveloped in a vesicular compartment called phagosome. According to the type of internalized particle and the class of phagocyte involved, a different set of processes are activated that end in the destruction of the enclosed material. These mechanisms involve:

2.1. The respiratory or oxidative burst

The respiratory or oxidative burst is produced by the activity of specialized enzymes such as NADPH oxidase, which generates superoxide that recombines with other molecules as NO to form peroxynitrite, a potent oxidant agent against bacteria and parasites. Neutrophils and monocytes also utilize myeloperoxidase to further combine H_2O_2 with Cl^- to produce hypochlorite, a harmful component of phagosomes [1]

2.2. The production of microbicidal substances

Lysozime and defensins attack cell walls and membranes of certain bacteria.

2.3. Phagosome maturation

Phagosome maturation is a process that confers to nascent phagosomes the ability to kill pathogens or to degrade the ingested materials. Phagosomal maturation involves a complex sequence of reactions that result in the drastic remodeling of the phagosomal membrane and contents, produced as a consequence of vesicular fusion and fission events between the nascent vacuole and other cellular compartments mainly belonging to the endocytic pathway [2]. Rab and SNARES proteins are the main molecular components that regulate these events. Rabs are small GTP-binding proteins that control intracellular trafficking and supervise the maintenance of specific organellar identity, whereas SNARES are transmembrane proteins that, associating with their specific partners, form complexes that are the final executioners of the fusion processes between membranes. Interactions between phagosomes and endosomes commence soon after phagosome sealing, in a fashion that recapitulates the endocytic sequence: nascent (early) phagosomes (Eph) seemingly fuse initially with sorting (early) endosomes (EE), followed by late endosomes (LE) and ultimately lysosomes (Ly). Therefore, the membrane of Eph initially acquires components present in early endosomes such as Rab 5, phosphatidylinositol 3-phosphate (PI3P), Early Endosomal Antigen 1 (EEA-1) and Vamp-3. In contrast, late phagosomes (Lph) present Rab 7, Mannose-6-phosphate receptor, Vamp-7 and Lysosomal associated membrane proteins 1 and 2 (LAMPS 1 and 2) [2]. Furthermore, the luminal environment of phagosomes turned progressively more acidic due to the accumula-

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tion of H+ ATPase complexes in the phagosome membrane [3]. Phagolysosomes (PLy), the hybrid compartment generated by fusion between Lph and lysosomes, reach a pH of around 4.5, favoring the maturation of acidic hydrolases that will finally digest the materials (Figure 1, red compartments). Lysosomes contain several proteases, including Cathepsin D and Elastase, which are essential for killing various bacteria.

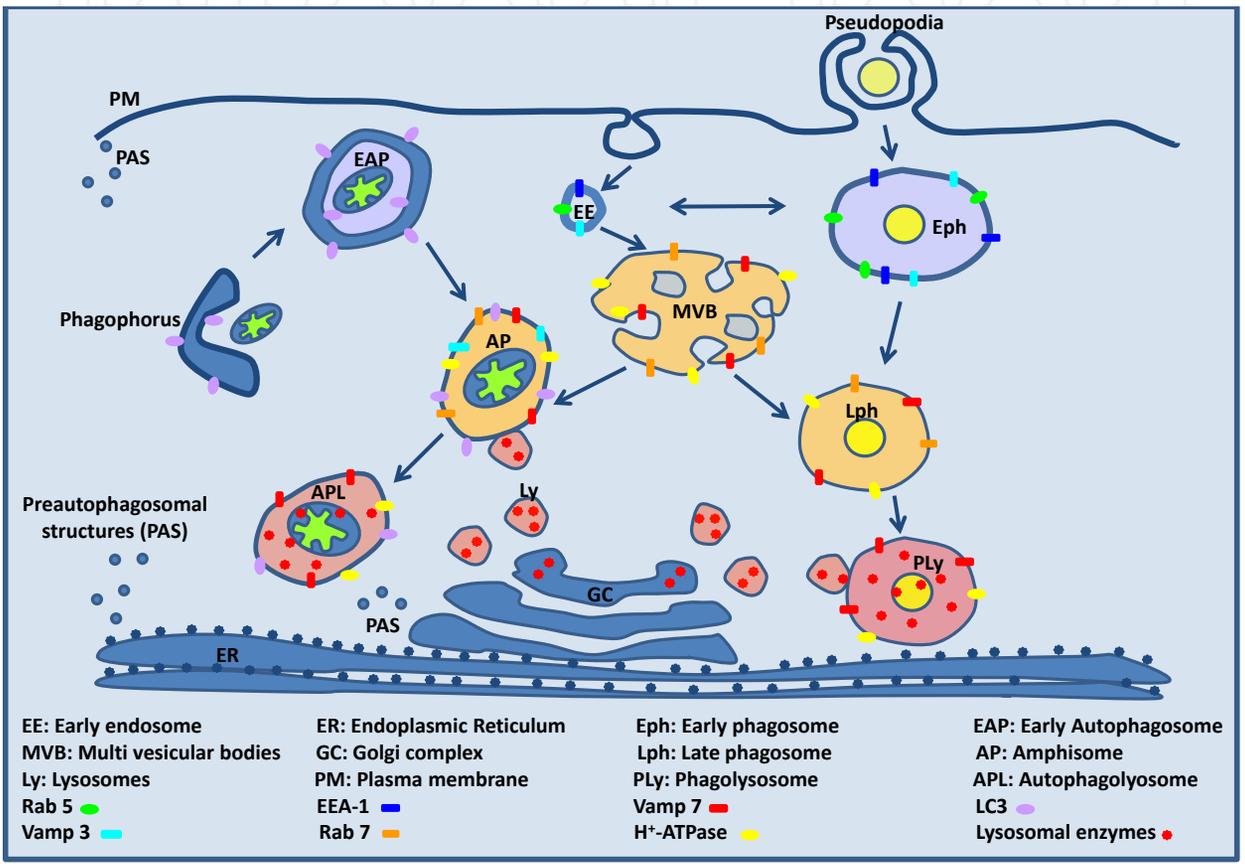

Figure 1. Phagocytosis and autophagy are the main cellular processes involved in the innate immune responses against pathogens. The scheme depicts the vesicular compartments that belong to each process and the main molecules that characterize them. Note that earlier, non-degradative compartments are colored in blue whereas acidic, lysosomal derived compartments are showed in red.

All these mechanisms generate a high level of protection against a wide range of pathogens. Paradoxically, phagocytosis can also have deleterious effects for the host: certain pathogens, exemplified by *Mycobacteria*, take advantage of the phagocytic machinery to gain access to the cell interior where, by subverting the maturation process, become intracellular pathogens [4–6].

Phagocytosis can also be produced in a class of cells different from immune cells. These "non -professional phagocytes" are cells with low phagocytic competence. Pathogens that can colonize these cells avoid the harmful ambient of the phagocyte-derived phagosome because

many of the killing processes described for phagocytes are low or absent in non-professional phagocytes, keeping lysosomal degradation as the main defense system. In this way, the autophagic pathway which delivers cytoplasmic materials to lysosomes constitutes an important mechanism for eliminating pathogens, especially in these non-immune cells.

3. The autophagic pathway

Autophagy is a catabolic process that involves the degradation of cell components through the lysosomal machinery. Macroautophagy, the most studied type of autophagy, is important in many physiological situations such as development, cell growth, and cell differentiation. As a constitutive process, autophagy functions at basal levels in the turnover of long lived proteins and old organelles for maintaining cellular homeostasis. It can also be stimulated under different stress situations such as nutrient starvation, oxidative stress and intracellular infections [7].

The autophagic process involves specific compartments inside the cell. The initial preautophagosomal structures (PAS) are recruited to the cellular sites where autophagy is initiated [8]. A large number of studies have shown that specialized regions of the endoplasmic reticulum (ER) are involved in the formation of PAS [9,10]. However, more recent data indicate that besides ER other compartments like mitochondria, Golgi complex (GC) or plasma membrane (PM) may participate in this process [11–13]. The phagophorus, or isolation membrane, generated by fusion of PAS, is a curved membrane that in a way similar to that of the pseudopodia of macrophages, wraps the materials to be trapped which, in this case, consist of soluble or membranous content from cytoplasm (Figure 1, left). This membrane finally closes in a structure called autophagosome that transports the cargo for degradation. Immature (early) autophagosomes (EAP) are double-membrane vesicles easily recognized by electron microscopy [14]. Autophagosomes fuse with endocytic compartments (LE or multivesicular bodies, MVB) to form amphisomes (AP) that, in turn, fuse with lysosomes; forming autophagolysosomes (APL) where the materials are degraded (Figure 1, red compartments).

At the molecular level, a large number of proteins engage in autophagy. The specific autophagy related proteins (Atgs) are a large family of proteins that regulate the nucleation of PAS, and the formation and elongation of phagophorus and autophagosomes. At least 16 genes were found to be important for autophagy in yeasts, especially in the PAS nucleation [15]. Two protein conjugation reactions, both catalyzed by the action of Atg7, (an E3-like ubiquitin ligase activity), are mainly required for autophagosome formation in mammals. The mammalian Atg5-Atg12-Atg16L complex is recruited to the isolation membranes, favoring the elongation of the precursor membrane. The second conjugation system yields LC3-II which inserts into the autophagosomal membrane and contributes to vesicle elongation [16]. Pro-LC3 is initially cleaved by Atg4 to produce LC3-I. This molecule is a soluble protein distributed in the cytoplasm. After autophagic induction, LC3-I is conjugated with phosphatidylethanolamine (PE), allowing the insertion in the membrane of autophagic vesicles [16]. Two key signaling nodes converge to correlate autophagy with

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cell nutrient or stress conditions. The Tor-Atg1 signaling cascade transduces the response from growth factors, via class I PI3K, Akt/PKB, and so forth, to negatively regulate autophagy [17]. The second system, formed by Beclin1 (Atg6) and hVps34, is a lipid kinase that produces PI3P, which plays a pivotal role in early autophagosome formation, LC3 lipidation and the maturation of autophagosomes into autolysosomes [18].

Proteins that regulate transport and fusion events between vesicles are also important in autophagosome formation and maturation. Rab 7, a protein involved in transport to late endosomes and in the biogenesis of the perinuclear lysosome compartment is required for the normal progression of autophagosomes to autophagolysosomes [19]. The N-ethylmaleimide-sensitive factor attachment protein receptors (SNARES) Vamp3 and Vamp7 are important during the first steps of autophagy [20,21], whereas Vamp7 and Vamp8 also participate in the autophagosome-lysosome fusion [20,22]. Furthermore, it has been recently shown that actin has a role in the very early stages of autophagosome formation, linked to the PI3P generation step [23]. The description above shows that autophagosome formation and maturation engage similar molecular transport components and fusion machinery than that required for progression in endocytic and phagocytic pathways (Figure 1).

The process of autophagy can be monitored intracellularly by utilizing LC3 fused to a fluorescent protein (GFP-LC3 or mCherry-LC3). As the fluorescent LC3 is incorporated into autophagosomes, they can be seen as small puncta within the cell. As autophagy is a highly dynamic process, the number of puncta seen in a cell is a function of initiation as well as clearance (lysosomal fusion and subsequent degradation) [24]. Autophagosome initiation can be inhibited by blocking Class III PI3 kinases (Vps34) with 3-methyladenine or wortmannin, or by knockdown of essential factors such as Atg5 or Beclin-1, a component of Vps34 kinase complex. Autophagosome clearance can be prevented by interfering with lysosomal fusion by Bafilomycin A1, chloroquine, and other agents that tend to alkalinize the lysosome (e.g. NH_4Cl). Rapamycin inhibits the TOR signaling pathway, leading to induction of autophagy. Spermidine and resveratrol have been recently characterized as autophagy inducers. Genetic and functional studies indicate that spermidine inhibits histone acetylases, while resveratrol activates the histone deacetylase Sirtuin 1. Although it remains elusive whether the same histones (or perhaps other nuclear or cytoplasmic proteins) act as the downstream targets of spermidine and resveratrol, these results point to an essential role of protein hypoacetylation in autophagy control [25]. This hipoacetylated protein status leads to upregulation of several *atg* genes, including *atg7*, *atg11* and *atg15* in several organisms such as mammals, yeasts, nematodes and flies [26].

As explained above, host autophagy is a component of the innate responses against intracellular pathogens that generally functions as a second barrier when phagocytic or other defense mechanisms are exceeded. However, some pathogens have the capacity to evade autophagic responses or to subvert the autophagic pathway and to live and replicate inside autophagosomal compartments. The following sections describe the opposite effects of autophagic response against microorganisms.

3.1. Autophagy as a component of the innate immune responses

In order to internalize host cells, many pathogens induce their own ingestion in phagocytic cells. After entry, pathogenic microorganisms manipulate the normal (or "canonical") phagocytic pathway to evade lysosomal degradation and to achieve the maximal benefits (protection, nutrition, survival, and replication) from cells. These actions include inhibition of phagosome maturation, escape from phagosome to cytoplasm and development in a vacuole with particular characteristics. As a component of immune responses, autophagy hampers these mechanisms enclosing viruses, bacteria or parasites in compartments which share characteristics and molecular machinery with canonical autophagosomes, a process usually named as xenophagy [27]. For a better comprehension of the autophagic action, each pathogenic strategy will be described, and the most characteristic pathogen of each group will be exemplified in the following paragraphs.

- *The Mycobacteria case*: A marquee feature of the powerful human pathogen *Mycobacterium tuberculosis* is its macrophage parasitism. The intracellular survival of this microorganism rests upon its ability to arrest phagolysosome biogenesis, avoid direct cidal mechanisms in macrophages, and block efficient antigen processing and presentation. Lipoarabinomannan (LAM) and phoshatidylinositol mannoside (PIM) are two toxins elaborated by *Mycobacterium tuberculosis* that stimulate fusion between phagosomes and early endosomes and prevent Rab conversion on phagosomes by interference with Rab effectors, especially the type III PI3K (hVps34). LAM abolishes the normal recruitment of PI3K to mycobacterium phagosomal membrane decreasing the levels of PI3P. The final result is the reduction of the recruitment of EEA-1 and other effectors and the inhibition of the normal progression from early to late phagosomes [28]. Thus, a critical feature of the *M. tuberculosis* phagosome is its lack of the vacuolar H+ ATPase [29] and mature lysosomal hydrolases, such as Cathepsin D. In stark contrast, the induction of autophagy by physiological, pharmacological or immunological signals, including the major antituberculosis Th1 cytokine IFN-gamma, can overcome mycobacterial phagosome maturation block. Almost ten years ago, Gutierrez and colleagues demonstrated that when infected macrophages were treated in conditions that induce autophagy, mycobacterium-containing phagosomes become more acidic and also acquire markers of maturation, including the vacuolar H+ ATPase, LAMP-1, LBPA and Cathepsin D. Additionally, starvation promotes recruitment to mycobacterial phagosomes of critical autophagy components such as LC3, indicating that these phagosomes are redirected to a compartment with autophagic characteristics that finally fuses with lysosomes [30]. The most remarkable finding of this work was the demonstration that autophagy induction hampered the survival of this intracellular pathogen, recognizing to autophagy as an effector of innate immunity (see Figure 2A).

- *The g*roup A *Streptococcus case:* The second case belongs to pathogens that escape from phagosomes and turn into cytosolic invaders. The Group A of *Streptococcus* (GAS) is often internalized into nonphagocytic epithelial cells via the endocytic pathway. At early times after infection, GAS secrets its major virulence factor: the cytolysin streptolysin O (SLO) that supports the escape of GAS into the cytoplasm from endosomes [31]. After escaping, GAS became enveloped by autophagosome-like compartments and were killed upon fusion of

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these compartments with lysosomes. In contrast, in autophagy-deficient Atg5-/- cells, GAS survived, multiplied, and were released from the cells [32,33]. Additionally a SLO-deficient mutant of GAS was viable for a longer time than the wild-type strain, although it failed to escape the endosomes [31]. Both results highlight the crucial role of autophagy in the suppression of intracellular survival of this pathogen. A similar conclusion was recently obtained with *Staphylococcus aureus*. After invasion of non-phagocytic cells, virulent strains of this gram positive bacterium stimulate autophagy and become entrapped in intracellular PI3P-enriched vesicles and its effector WIPI-1, a protein present in the membrane of both phagophores and autophagosomes. This interaction seems to be deleterious for bacteria, given that these autophagosome-like WIPI-1 positive vesicles that envelope *S. aureus* are finally targeted for lysosomal degradation [34].

- *The Toxoplasma case*: The third strategy used by pathogenic organisms is to create a specialized compartment that remains isolated from the host endocytic or phagocytic networks. *Toxoplasma gondii* relies on this mechanism; the membrane of its PV is nonfusogenic due to its unique composition lacking host proteins. Nonetheless, macrophages infected with *Toxoplasma* can reroute the pathogen-containing compartment to lysosomes. Autophagy

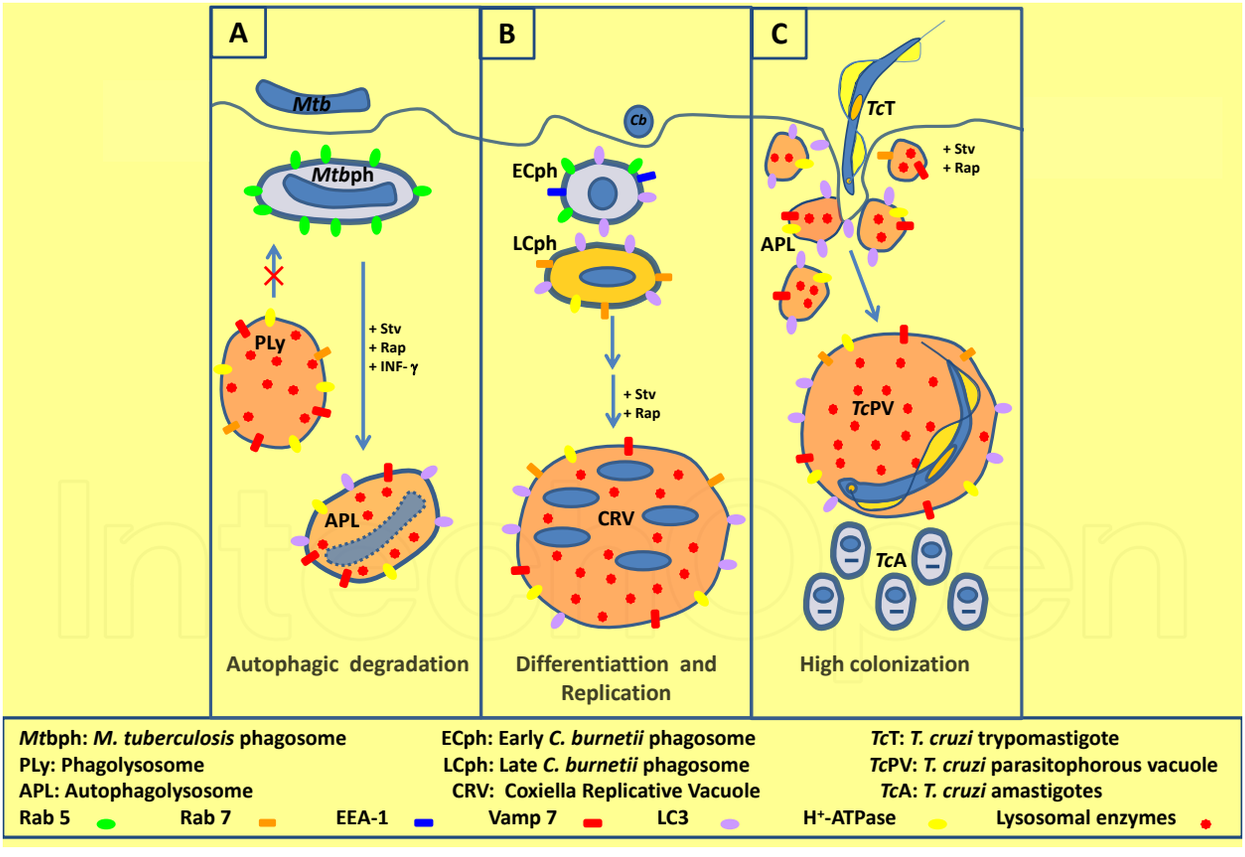

Figure 2. Mechanisms of pathogen-host autophagy interactions. As a component of innate immune responses against intracellular pathogens, autophagy can effectively eliminate some pathogens, re-routing them to lysosomes (autophagolysosomes). That is the case of *Mycobacterium tuberculosis* (A). In contrast, some microorganisms such as *Coxiella burnetii* utilize autophagic compartments to delay lysosomal fusion until differentiating into more resistant forms (B). On the other hand, *Trypanosoma cruzi* exploits the autophagic pathway to efficiently colonize host cells (C).

plays a key role in this process [35,36]. When CD40 of human or mouse macrophages infected with *T. gondii* is stimulated with (CD154+) CD4+ T cells or exposed to anti-CD4 antibodies, the nonfusogenic nature of the PV is reversed and the vacuole fuses with late endosomes and lysosomes. This fusion is dependent on autophagy, as indicated by the inhibition of this mechanism in cells knockdown for Beclin 1 or treated with 3-MA, an inhibitor of phagophore formation. CD40 activation also stimulates expression of LC3 that localizes to the PV [36].

3.2. Evasion of autophagic responses

Despite the potent effect of autophagy in killing intracellular pathogens, some microbial pathogens have the capacity to control cellular autophagy and successfully parasitize eukaryotic cells. These highly evolved microorganisms have developed specific virulence factors to protect themselves from autophagic elimination by producing:

- *Prevention of autophagy induction:* Several viruses direct their products to essential autophagic proteins, causing them to be functionally inhibited. The herpes virus family can produce autophagy blockage through different mechanisms. The HSV-1 ICP34.5 viral protein encoded by herpes simplex virus type 1 blocks Beclin1 function and confers neurovirulence in mice [37]. A similar mechanism was recently shown for human cytomegalovirus; the virus protein TRS1 interacts with Beclin 1 to inhibit autophagy [38]. Gamma-herpesviruses, including important human pathogens such as Epstein Barr virus or Kaposi's sarcoma-associated HIV, displayed a different type of inhibition. They encode homologs of the antiapoptotic, host Bcl-2 protein to promote viral replication and pathogenesis. Cellular Bcl-2 and their viral homologs have the property to bind and inhibit Beclin1, suppressing both apoptotic and autophagic responses [39]. It is not yet clear whether other intracellular pathogens besides viruses also actively suppress initiation of the autophagy pathway. In contrast, many bacteria display the following actions.

- *Suppression of autophagosome maturation into autolysosome*: Similar to mycobacterium phagosome maturation arrest, other pathogens have the ability to suppress autophagosome maturation. They specifically reside in vacuoles with autophagosomal characteristics in order to survive and replicate, but avoid transient or permanent fusion with lysosomes. *Porphyromonas gingivalis*, a bacterial periodontal pathogen that can localize to atherosclerotic plaques, traffics to autophagosomes as a way of evading the conventional endocytic trafficking to lysosomes [40], After intracellular uptake, *P. gingivalis* transits from early autophagosomes to late autophagosomes and prevents the formation of autolysosomes, a mechanism not yet well elucidated [41]. On the other hand, a delay in the delivery of lysosomal enzymes to phagosomes was initially described for dimorphic bacteria and named “the pregnant pause”. The dimorphic life cycles of these pathogens have dramatic consequences for phagosome traffic. In the transmissible state, *C. burnetii, L. pneumophila* and others, such as *Leishmania sp*., block phagosome maturation; after a pregnant pause that includes the bacterial differentiation process, replicative forms emerge and thrive in lysosomes [42]. Autophagy is one of the mechanisms activated by these intracellular pathogens for delaying lysosomal fusion. At late stages of cellular infection, both *Coxiella*

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burnetii and *Legionella pneumophila* develop vacuoles that have characteristics of phagolysosomes and are also decorated with LC3 [43,44]. In the case of *C. burnetii,* acquisition of LC3 is even an early event in the transit of phagosomes containing bacterium (Cph) and depends on bacterial protein synthesis because chloramphenicol avoid this LC3 recruitment [45]. Interactions with autophagic and also late endocytic compartments are maintained during the transit of Cph and in the development of the Coxiella replicative vacuoles (CRV) [46]. Indeed, autophagy induction or the overexpression of autophagic proteins LC3 and Rab 24 favor the generation and maturation of this CRV [47]. Taking together, these results demonstrated that *C. burnetii* transits through the normal endo/phagocytic pathway but actively interacts with autophagosomes at early times after infection. This intersection delays fusion with the lysosomal compartment, possibly favoring the intracellular differentiation and survival of the bacteria. In this period, *C. burnetii* differentiates from the transmissible forms (named small cell variant) to the replicative forms (large cell variant) (Figure 2B). In the case of *L. pneumophila*, it was recently demonstrated that this bacterium produced several Type IV effector proteins that control the timing of bacteria during intracellular transport. The early secretion of DrrA/SidM, LidA, and RalF factors, prolong association with the ER and permit the persistence of the bacteria in immature autophagosomal vacuoles for a period sufficient to differentiate into an acid-resistant, replicative form. Subsequent secretion of LepB releases the block of autophagosome maturation, and the adapted progeny continue to replicate within autophagolysosomes [48].

- *Evasion of pathogen recognition by the autophagic machinery*: This strategy is especially important in intracytoplasmic pathogens such as *Shigella flexneri*, *L. monocytogenes*, and *Burkholderia pseudomallei*. Shigella VirG, a protein required for intracellular actin-based motility, induced autophagy and favored the microorganism trap by autophagosomes, after binding between VirG and Atg5. However, *Shigella,* encoding Type III secretion effector, IcsB, competitively binds to Atg5, thereby camouflaging its own bacterial target molecule VirG from autophagic capture [49]. Furthermore, BopA, the counterpart of IcsB in Burkholderia pseudomallei, have similar autophagy-evading properties [50]. *Listeria monocytogenes* is a classic example of a "cytosol-adapted pathogen"; it can rapidly escape from the phagosome of macrophages and other non-phagocytic cells and replicate rapidly in the cytosol. Phagosome escape also enables cell-to-cell spread by the bacteria through a bacterial driven actin-based motility mechanism. Besides Act A, that as was shown plays a critical role in autophagic escape by polymerizing actin which favors bacteria movements [51], another virulence factor of *L. monocytogenes,* InlK, was recently shown to counteract the autophagic process. InlK interacts with the Major Vault Protein (MVP), the main component of cytoplasmic ribonucleoproteic particules named vaults. The recruitment of MVP to bacterial surface disguises intracytosolic bacteria from the autophagic recognition system leading to an increased survival rate [52].

3.3. Autophagy as a survival mechanism

A different type of host-microbial interaction belongs to the group of organisms that harness cell autophagy. Independently of the final localization within the cell, these particular

organisms improve their intracellular cycle when interacting with autophagic compartments. Postulated benefits of host autophagy for microbes include the promotion of viral replication or morphogenesis via utilization of the autophagic machinery. Polyovirus localized in double-membrane autophagosome-like structures positive for LC3 serve as lipid membrane-scaffolds that enhance viral replication [53]. In a similar way, the rotavirus NSP4 protein colocalizes with LC3-positive vesicular compartments and is postulated to play a role in the formation of viroplasms and/or the packaging or transcription of the rotavirus genome [54].

Another mechanism is the utilization of autophagosomes as a protective intracellular niche to enhance the survival and growth of bacteria. As described above, dimorphic bacteria such as *C. burnetii* or *L. pneumophila* follow this method. In contrast, *Francisella tularensis,* enters LC3-positive compartments to allow cytoplasmic bacteria to regain access to the endocytic compartment to finally promote bacteria egress through exocytosis [55]. Autophagy could also favor intracellular pathogen survival by providing nutrients to pathogens, particularly those that reside in sequestered vacuoles that lack access to cytoplasmic nutrients. That is the case of *T. gondii,* which establishes its vacuole in the vicinity of autophagic compartments and that displays an impaired growth in *Atg5*-deficient MEF cells, leading to the conclusion that host cell autophagy plays a role in promoting parasite growth through nutrient recovery [56].

A special type of pathogen-autophagy interaction is produced by the protozoan parasite *Trypanosoma cruzi.* This pathogen exploits the autophagic pathway to efficiently colonize host cells, as will be described in detail in the next section.

3.4. Autophagy as an invasion strategy: The *Trypanosoma cruzi* case

The protozoan parasite *Trypanosoma cruzi* can invade a wide range of phagocytic and non-phagocytic cells in the infective, non-proliferative trypomastigote form. Inside the cell, trypomastigotes are temporarily contained in a membrane vesicle, the parasitophorous vacuole. Subsequently, the parasites escape to the cytosol, differentiate into the amastigote form, and replicate by binary division [57,58]. This replication process culminates after 9 cycles, followed by a new differentiation period where the parasites undergo a transition back into trypomastigotes. After that the parasites are released from the cell and infect the neighboring cells, maintaining the infection process.

The characteristics of the *T. cruzi* parasitophorous vacuole (TcPV) are directly related to the parasite invasion mechanism. Previously published data showed that two main invasion processes involving different signaling pathways are participating: the calcium dependent fusion of lysosomes with the host plasma membrane [59,60], and the activation of class I PI3K that produces a plasma membrane-derived vacuole initially devoid of lysosomal markers [61]. Although both pathways require the disruption of the host cell actin cytoskeleton, the lysosomal independent *T. cruzi* entry model appears to be more significant early after internalization (50% *versus* 20% lysosome-dependent entry process). However, lysosomal fusion is essential for the establishment of a productive infection [62] and for the progression and completion of the *T. cruzi* intracellular cycle [63,64], since the parasite Tc-Tox is activated in the acid environment provided by the lysosomes [57,65]. These strategies of cell invasion indicate that *T. cruzi* entry is a complex process that employs

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different components from the plasma membrane and the endocytic pathway to finally produce the intracellular infection. More recently, studies provided by our laboratory demonstrated that the *T. cruzi* parasitophorous vacuole (TcPV) is decorated with LC3 protein and that the autophagic inhibitors wortmannin, 3-methyladenine or vinblastine suppress this recruitment and also significantly reduce the intracellular infection. In contrast, induction of autophagy before infection by starvation or other means significantly increased the percentage of infected cells. Interestingly, infection was diminished in the absence of the specific autophagy genes Beclin1 or Atg5, which are required for initiation of autophagy, indicating that autophagic-derived compartments are required for efficient entry of *T. cruzi* into the host cell [66]. Live imaging using confocal microscopy showed that GFP-LC3 positive vesicles move towards plasma membrane and contact the sites where trypomastigotes, the *T. cruzi* invasive forms, bind the membrane [66,67] (Figure 2C). The pro-pathogen effects of autophagy on *T. cruzi* infection were observed in different classes of cells and *T. cruzi* strains, demonstrating that this interaction is a wide-spread phenomenon [68]. The autophagy modulation of host cells during the following stages of the *T. cruzi* intracellular cycle -trypomastigote to amastigote differentiation, amastigote replication and amastigote differentiation back to trypomastigote- seems to suffer no mdification compared to cells maintained in control conditions [66]. Since *T. cruzi* is an unicellular eukaryotic organism that also has its own autophagic pathway [69], other experimental procedures will be necessary to decipher the possible dual action of autophagy modulation on *T. cruzi* infected cells. Indeed, unpublished data from our laboratory show that classical inducers and inhibitors of mammalian autophagy have similar effects on *T. cruzi* and that protozoan autophagy is activated during *T. cruzi* metacyclogenesis, a process that renders metacyclic trypomastigotes from epimastigotes and that takes place in the digestive apparatus of the triatomine vectors.

3.5. Autophagy in action: *in vivo* infections studies

To date the outcome of the pathogen/autophagy relationship on *in vivo* infections models, with the exception of a few cases, remains little understood. Actions of autophagy as an innate immune component are easier to understand, particularly with the use of knockout mice. In this way, studies on *in vivo M. tuberculosis* murine infections showed that the most susceptible mice are those deficient in either IFN-γ or IFN-γ receptors [70]. These results clearly demonstrate that macrophage activation, and the macrophage autophagic pathway [30], are required as a critical components for controlling infection.

The main concerns with the *in vivo* models arise from the cases of pathogens that *in vitro* studies show to be favored by autophagy induction. At the moment, no current evidence demonstrates that autophagy gene deletion in the host attenuates microbial disease. Therefore, the physiological significance of microbial utilization of autophagy for "promicrobial" effects remains to be established [71]. The discrepant conclusions between *in vitro* and *in vivo* studies in *T. gondii* infection models exemplify this concept. Although *T. gondii* has impaired growth in *Atg5*-deficient cells, leading to the conclusion that host cell autophagy plays a role in promoting parasite growth through nutrient recovery [56], this parasite has increased virulence in mice

with macrophage-specific deletion of Atg5 [72]. In agreement with these results, unpublished data from our laboratory show that autophagy-impaired mice are more susceptible to *T. cruzi* infection, while our previously results on cell cultures clearly demonstrate that decreased autophagic levels in Atg5 KO cells or in Beclin 1 KD cells significantly decreased *T. cruzi* infection [66].

One possible explanation for these discrepancies is the different effects of autophagy (or autophagic proteins) on phagocytic and non-phagocytic cells. When *T. gondii* infected macrophages were stimulated with CD40 receptor agonists, the parasite vacuole fuses with endosomes and lysosomes in an autophagy-dependent process leading to parasite destruction [35,36]. In contrast, in the non-phagocytic HeLa cells, Wang and colleagues reported the beneficial effects of autophagic induction for parasite survival and growth [56]. Considering this possibility, the comparative analysis of autophagic modulation on the course of a specific pathogen infection in phagocytic and non-phagocytic cells *in vitro* prior to mice infection would be productive in the future. However, this simplistic point of view will never replace the conclusions obtained from mice experiments, especially when immune responses are implicated.

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