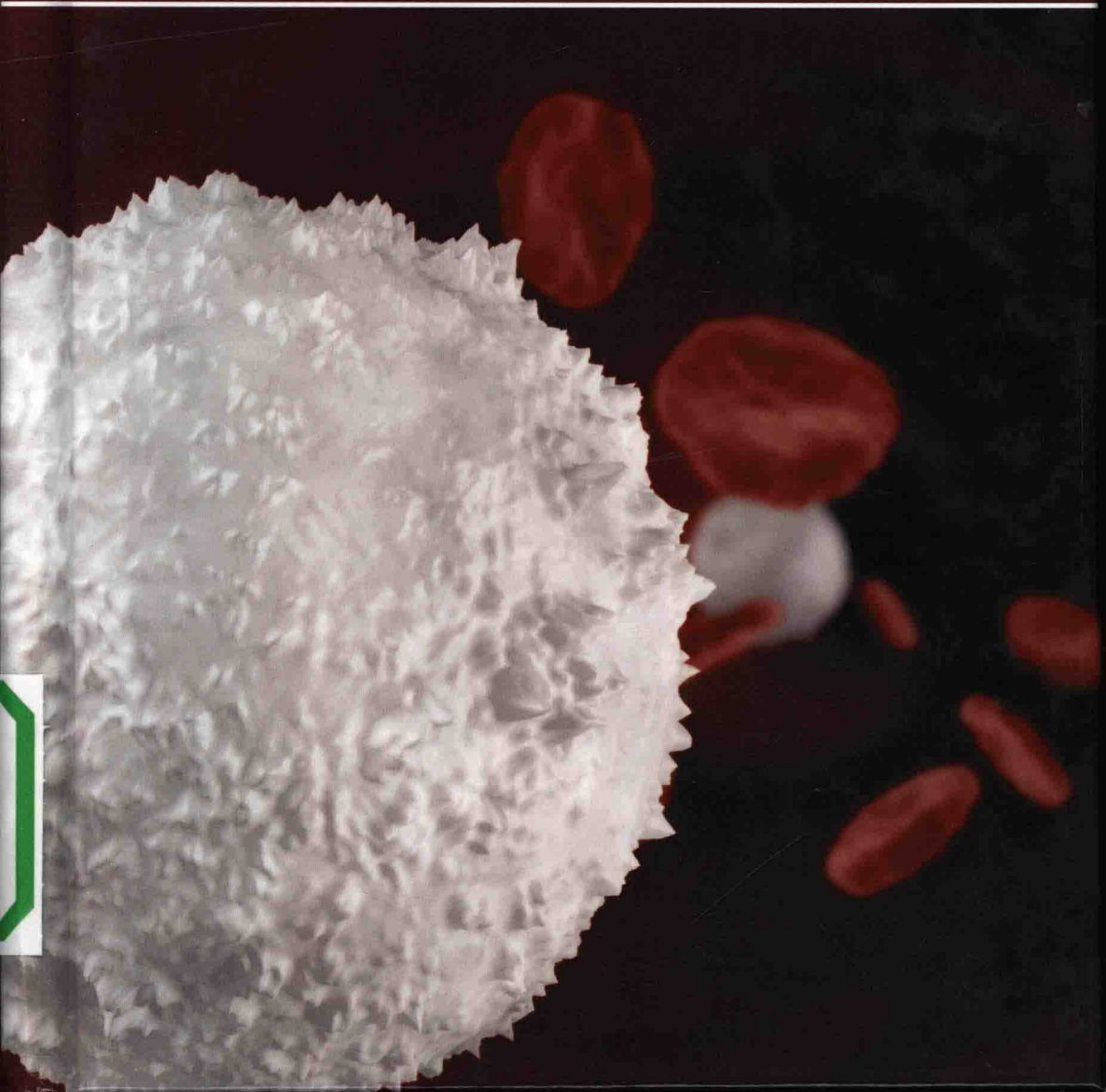
AUTOPHAGY

Beyond the Basics

Alexandra Hewitt



Autophagy: Beyond the Basics

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Preface

This book highlights some of the demanding research topics related to autophagy and also analyzes the recent developments in its molecular mechanisms. The emphasis is on the role this cell defense mechanism plays in studying various diseases which include liver diseases, cancers, myopathies and infectious diseases. The contradictory role of autophagy i.e. cell survival versus cell death; draws the focus on the importance of keeping in mind this double-edged nature in future developments of the currently promising autophagy-modulating therapies. This book provides new ground breaking researches and expands the base for further study in the field of autophagy.

The researches compiled throughout the book are authentic and of high quality, combining several disciplines and from very diverse regions from around the world. Drawing on the contributions of many researchers from diverse countries, the book's objective is to provide the readers with the latest achievements in the area of research. This book will surely be a source of knowledge to all interested and researching the field.

In the end, I would like to express my deep sense of gratitude to all the authors for meeting the set deadlines in completing and submitting their research chapters. I would also like to thank the publisher for the support offered to us throughout the course of the book. Finally, I extend my sincere thanks to my family for being a constant source of inspiration and encouragement.

Editor

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1. Introduction

Protozoa are unicellular eukaryotes that are able to live as parasites or as free-living organisms and interact with a great variety of environments and organisms, from bacteria to man; in addition, they represent one of most important sources of parasitic diseases. Every year, more than one million people die from complications from protozoal infections worldwide [1-5]. Of the medically relevant protozoa, Trypanosomatidae and Apicomplexa constitute a substantial group including the causative agents of several human diseases such as Chagas disease, sleeping sickness, leishmaniasis, malaria and toxoplasmosis [1,5,6]. The life cycles of these parasites are highly complex, involving different hosts and different specific interactions with a variety of cells and tissues [7- 11]. Some of these parasites live in the extracellular matrix or blood of host mammals, but the majority of them infect host cells to complete their cycle. Despite the high infection and mortality rates of these protozoa, especially in low-income populations of developing regions such as Africa, Asia and the Americas, current therapies for these parasitic diseases are very limited and unsatisfactory. The development of efficient drugs is urgently necessary, as are serious public health initiatives to improve patients' quality of life [12-16].

The Trypanosomatidae family belongs to the order Kinetoplastida and is comprised of flagellated protists characterised by the presence of the kinetoplast, a DNA-enriched portion of the mitochondrion localised close to the flagellar pocket. The most studied pathogenic trypanosomatids are the following: (a) *Trypanosoma brucei*, which is responsible for sleeping sickness in Africa; (b) *T. cruzi*, which is the causative agent of Chagas disease in Latin America; and (c) a variety of *Leishmania* species that cause leishmaniasis in tropical and subtropical areas worldwide. These illnesses have been classified by the World Health Organization as neglected diseases, which affect people living in poverty in developing countries and for which no efficient therapy is available [17-19].

The Apicomplexa family encompasses a large group of protists, including approximately 5,000 known parasitic species, which are characterised by the presence of an apical complex containing a set of organelles involved in the infection process. Apicomplexan parasites infect invertebrate and vertebrate hosts, including humans and other mammals. The most serious parasitic disorder is caused by apicomplexan *Plasmodium* species, the etiological agent of malaria, which causes more than one million deaths annually [1]. Toxoplasmosis is another important disease caused by the apicomplexan parasite *Toxoplasma gondii;* it has been estimated that almost half of the human population worldwide is infected with this protozoa [20]. The life cycle of the apicomplexan parasites generally consists of complex asexual and sexual reproduction, but some differences are observable among distinct genera. Malaria transmission occurs during the blood feeding of the *Anopheles* mosquito, whereas toxoplasmosis is mainly transmitted by the ingestion of raw meat or contaminated cat feces.

Autophagy is a physiological self-degradative pathway essential for the maintenance of the metabolic balance in eukaryotes, leading to the turnover of cellular structures during both the normal cell cycle and during conditions of stress, such as starvation [21,22]. This process depends on double-membrane vesicles known as autophagosomes, which are responsible for the engulfment of macromolecules and organelles and the recycling of their components without an inflammatory response [23]. In eukaryotic cells, proteins known as Atgs contribute to the formation of autophagosomes and their targeting to lysosomes [24]. The autophagic machinery interfaces with many cellular pathways, such as that of the immune response and the inflammatory process, and acts as an inductor or suppressor of these processes [25]. Some molecules and organelles can undergo autophagy by specific proteins, such as in the selective pathway known as xenophagy, which is also observed in the degradation of intracellular pathogens [26,27]. The involvement of autophagy in this process has been demonstrated in the interactions of different pathogens with the host cells [28-30]. In protozoan infections, the role of autophagy has been debated in light of conflicting evidence presented in the literature, which tends to vary with the experimental model. Some studies suggest that parasites evade host cell defences using autophagy, while others suggest that the host uses autophagy to eliminate the pathogen [31-35]. However, there is no doubt that the autophagic machinery decisively influences the pathogenesis and virulence of protozoan infections; this machinery may therefore represent a promising target for drug discovery [36]. The autophagic process also occurs in the protozoa [37,38] and could occur in parallel to the host cell pathway, thus increasing the complexity of the phenomena. In the following sub-sections, the biology of

Trypanosomatidae and Apicomplexa protozoa will be reviewed in relation to the role of autophagy during the infection of the host cells.

2. Trypanosomatids and autophagy

As previously mentioned, the transmission of neglected diseases caused by trypanosomatids (sleeping sickness, Chagas disease and leishmaniasis) depends on an insect vector, and the environmental change from one host to another is a drastic event for the protozoa. To complete its life cycle, many metabolic and morphological changes must occur for the parasite to survive in a new host [39-42]. In addition to the kinetoplast, other characteristic ultrastructural structures are present in these parasites, including a single mitochondrion, unique flagella, sub-pellicular microtubules, glycosomes, acidocalcisomes and reservosomes (the last one is present exclusively in *T. cruzi*) [8]. In the context of the remodelling of sub-cellular structures, autophagy is greatly involved in eukaryotic homeostasis (including in that of trypanosomatids). However, the deregulation of this pathway, which is induced by conditions of stress, also leads to the parasite's death (Table 1). The sequencing of the complete genome of trypanosomatids has enabled the identification of parasitic genes [43-45]. Blast analysis comparing the trypanosome genome with yeast and mammalian genomes, with a particular emphasis on genes encoding autophagic machinery, has indicated the presence of some *ATG* genes in trypanosomatids [46,47]. However, the partial lack of a ubiquitin-like system, which is crucial for autophagosome formation, and the absence of cytoplasm-to-vacuole-targeting pathway orthologs suggest that these parasites have alternative autophagic features.

3. *T. brucei*

T. brucei is the etiological agent of sleeping sickness (or African trypanosomiasis) and is transmitted by the infected tsetse fly (*Glossina* sp.). After a blood feeding, procyclic trypomastigotes migrate from the insect midgut to the salivary gland where they undergo differentiation to infective metacyclic forms. Subsequently, these metacyclic trypomastigotes are inoculated into the mammalian host during the blood meal of the fly and differentiate into a proliferative bloodstream slender form. Interestingly, after a new differentiation, adapted short-stumpy forms evade the host immune system and disseminate the infection to the whole body; these forms are also able to cross the blood-brain barrier, which causes severe behavioural abnormalities, such as somnolence during daytime [48] (Figure 1). Unlike all other pathogenic trypanosomatids, which have an intracellular life-stage, *T. brucei* remains in the bloodstream of the mammalian host throughout the process of infection and, as such, is exposed to different environmental conditions that can trigger autophagy.

3.1. Role of autophagy in *T. brucei*

The first report on this parasite and autophagy was published in the 1970s by Vickerman and colleagues. These authors described the presence of myelin-like structures in different forms

of the parasite observed by transmission electron microscopy [49, 50]. Many years later, it was suggested that the autophagic pathway is involved in the turnover of glycosomes during protozoan differentiation [51]. Glycosomes are peroxysome-like organelles that perform early glycolytic steps and are also involved in lipid metabolism. It was demonstrated that glycosome contents are altered depending on the form of the parasite, with many of these organelles being close to glysosomes during the differentiation process. A similar phenomenon was observed after nutrient deprivation of the parasite, reinforcing the fact that differentiation may cause the degradation of glycosomes by pexophagy.

Further genomic and bioinformatic analyses were performed that identified in *T. brucei* many *ATG* orthologs to those of yeasts and mammals [47,52]. These genes are involved in different steps of the autophagic pathway, such as induction (*ATG24*, *PEX14*, *TOR1* and *TOR2*, *VAC8*), vesicle nucleation (*ATG6*, *VPS15* and *VPS34*) and vesicle expansion and completion (*ATG3*, *ATG7*, *ATG9*, two isoforms of *ATG4* and *ATG8*). Two isoforms of Atg4 and two of Atg8 were recently characterised structurally [53], and it was postulated that Atg8.2 is essential for autophagosome formation and that Atg8 depletion is associated with delayed cell death [54].

It is thought that many drugs may trigger autophagy in African trypanosomes. Dihydroxyacetone (DHA), spermine (snake venom) and vasoactive intestinal peptide (VIP – a neuropeptide secreted by the immune system) induce the appearance of morphological features of autophagy in *T. brucei* [55-58]. DHA is an interesting compound to be used in therapy for sleeping sickness because its phosphorylation is DHA kinase-dependent, and DHA kinase is present in mammals and other eukaryotes but not in trypanosomes. After DHA uptake, this compound is not eliminated, leading to typical morphological characteristics of autophagy similar to those found in rapamycin treatment. In another report [59], the authors showed that hydrogen peroxide can produce the appearance of autophagic profiles, suggesting that the release of reactive oxygen species acts as a signal in the autophagic pathway in *T. brucei*, as it does in other eukaryotic cells [60-62].

4. *T. cruzi*

T. cruzi is the causative agent of Chagas disease. It is mainly transmitted by triatomine bugs, which are commonly known as "kissing bugs". In the insect midgut, proliferative forms of the parasite called epimastigotes differentiate to metacyclic trypomastigotes after migration to the posterior intestine. During the blood meal, triatomines eliminate urine and feces with infective trypomastigotes that then gain access to the vertebrate bloodstream. After internalisation in the host cell, trypomastigotes remain in parasitophorous vacuoles (PV) that fuse with lysosomes, allowing an acidification of this compartment, which is an essential step towards differentiation into proliferative amastigotes. In the cytosol, successive parasite cycles occur until a new intracellular differentiation to trypomastigotes occurs; it is these forms that are responsible for the infection and dissemination to other cells and tissues [8] (Figure 2).

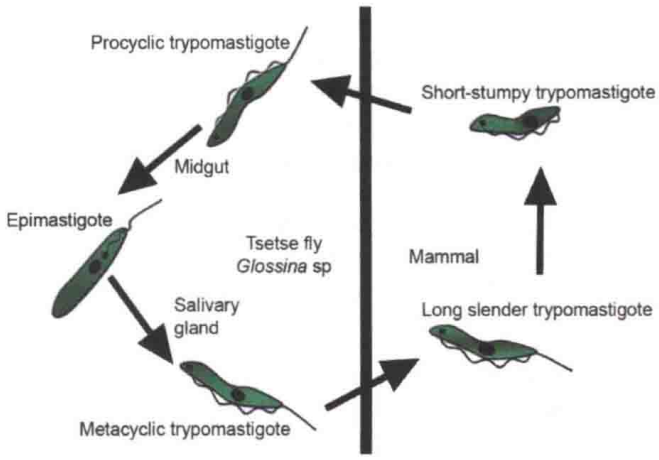

Figure 1. *T. brucei* life cycle.

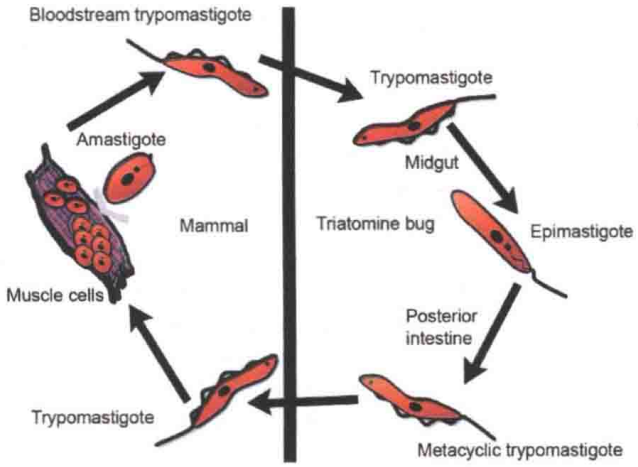

Figure 2. *T. cruzi* life cycle.

4.1. Role of autophagy in *T. cruzi*

Ultrastructural evidence of autophagy in *T. cruzi* was observed after the treatment of epimastigotes and bloodstream trypomastigotes with drugs; the appearance of myelin-like figures was the most recurrent feature detected [63-67]. Recently, the synergistic combination of amiodarone and posoconazole was able to trigger autophagy in replicative amastigotes [68].

In this way, different classes of therapeutic agents are able to induce the formation of autophagosomes, an event associated with parasite-related autophagic cell death, being the interplay between other programmed cell death as apoptosis or necrosis not discarded [69]. Due to the limitations of cell models, previous studies of different parasite forms have employed alternative techniques, such as monodansylcadaverine (MDC) staining and ATG gene expression, to demonstrate autophagy in the parasite [66,67]. Unfortunately, *T. cruzi* molecular machinery does not allow the use of double-stranded RNA to knock down target RNAs [70]; in addition, the lack of recognition of protozoan proteins by anti-Atg commercial antibodies hampers the evaluation of autophagy in this parasite. In spite of the advances in molecular and cellular biology, transmission electron microscopy remains a gold standard for autophagy analysis [71,72].

Aside from the description of autophagosomes in all *T. cruzi* life stages, description of the Atg cascade involved in autophagosome formation is not complete. Almost all *T. brucei ATG* genes have ortholog genes in *T. cruzi* [37,47]. In this parasite, two isoforms of Atg8 were described, with only Atg8.1 localised in autophagosomes as expected. These data suggest that there is only partially shared autophagic machinery, as is observed in human Atg8 orthologs [37]. In another study [37], the authors described the participation of *T. cruzi* Atg4 and Atg8 isoforms under conditions of nutritional stress and in the differentiation process from epimastigotes to metacyclic trypomastigotes, a process known as metacyclogenesis. The authors observed a remarkable expression of Atg8.1 by immunofluorescence microscopy, which was suggestive of intense autophagy in differentiating epimastigotes. Moreover, Atg8 co-localised with reservosomes, which are pre-lysosomal compartments related to energy supply that are present only in epimastigotes [73,74]. The reservosomal content consumed during metacyclogenesis and the presence of Atg8 in this organelle strongly suggest that there is crosstalk between autophagy and reservosomes [75,76]. Transmission electron microscopy studies have produced images from endoplasmic reticulum profiles surrounding reservosomes that indicate the possible origin of preautophagosomal structures [66]. It is well known that PI3K inhibitors, such as 3-methyladenine and wortmannin, prevent autophagy in different experimental models [54,66]; however, these data are controversial due to a previous report demonstrating that treatment with kinase inhibitors staurosporine, genistein, 3-methyladenine and wortmannin led to the formation of autophagosomes [77]. The data indicate the necessity of careful use of PI3K inhibitors to block autophagy and the urgent need for the development of new specific autophagic inhibitors [78].

4.2. Host cell autophagy and *T. cruzi* infection

Though thought to be essential for parasite success, lysosomal fusion could be involved in autophagy during host cell interaction and might contribute to the process of degradation and elimination of *T. cruzi*. In 2009, the role of autophagy in parasite entry and co-localisation with the PV was described, resulting in increased infection of Chinese hamster ovary cells; this observation was subsequently confirmed in macrophage and heart cell lineages [34,79]. Starvation conditions and the addition of rapamycin led to an increase in the scale of the infection; this increase was partially reversed by 3-methyladenine, wortmannin and vinblas-

tine, suggesting that autophagy favours the parasite during *T. cruzi*-host cell interactions. However, other groups demonstrated that classical autophagic stimuli (nutritional stress and rapamycin) did not produce an increase in parasite proliferation or even in the number of infected cells [33]. Recently, studies have emphasised role of autophagy in the control of *T. cruzi* infection using different cells and parasite strains (Figure 3) [80,81]. Once more, the conflicting data presented in the literature need to be further debated in light of the complexity of the protozoal strains and host cell models employed.

5. *Leishmania* species

The other medically important trypanosomatids are *Leishmania* species. Leishmaniasis is transmitted to mammals by sandflies, mainly of the *Phlebotomus* and *Lutzomia* genuses. Amastigotes differentiate into replicative procyclic promastigotes in the digestive tract of these sandflies, proliferate in the Phlebotominae gut, and then migrate to the proboscis where a new differentiation occurs to metacyclic promastigotes, the infective forms of the parasite. During the sandflies' blood meals, metacyclic promastigotes are inoculated into mammalian tissue and are phagocytised by macrophages. Inside the host cells, promastigotes differentiate into amastigotes that replicate and are responsible for cell lysis and dissemination in the organism (Figure 4). Currently, more than 20 species of *Leishmania* are known, each causing different clinical manifestations of the disease, including cutaneous leishmaniasis and visceral leishmaniasis (or Kala-azar). The pathogenicity depends on the *Leishmania* species and the host's immune response [8].

5.1. Role of autophagy in *Leishmania* sp.

Many groups have investigated autophagy cell death induced by drugs or antimicrobial peptides in various *Leishmania* species using electron microscopy and MDC staining [82-89]. Bioinformatics analysis has been a crucial checkpoint in the characterisation of *ATG* and *TOR* pathways in trypanosomatids [38,47,90]. In 2006, the role of autophagy in the differentiation process of *L. major* and *L. mexicana* was first evaluated [38,90]. The authors developed *L. major* VPS4, a mutant that could not complete the differentiation to the infective forms due to interference in autophagosome formation during conditions of starvation. The increase in Atg8 expression in differentiating forms supports the hypothesis that autophagy plays a pivotal role in metacyclogenesis [38,91]. In *L. mexicana*, the lack of cysteine peptidases CPA and CPB impairs autophagosomes formation and parasite differentiation; this finding is corroborated by the results of wortmaninn treatment and *ATG* deletion [90].

Recently, a subunit of protein kinase A in *L. donovani* that interferes with autophagy and protozoa differentiation was identified [92]. As observed in other trypanosomatids, the presence of Atg8-like proteins and their association with Atg4 in *Leishmania* species indicates that these proteins play a role in vesicle expansion [93]. Interestingly, the Atg5-Atg12 complex involved in autophagosome elongation was not previously detected [47], but recent studies have demonstrated its existence. It has also been shown that Atg5 deletion severally affects

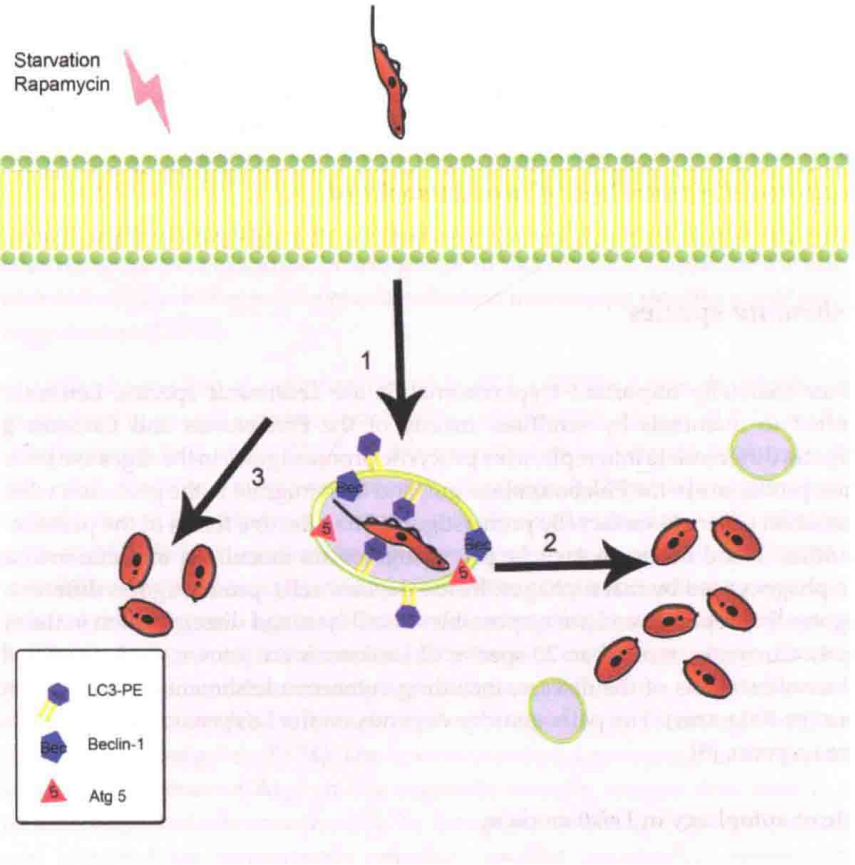

Figure 3. Autophagy in *T.cruzi*–host cell interaction. Romano et al [34] showed the co-localization of parasite vacuole with Atg proteins in the beginning of infection (1). Moreover, the replication of amastigotes is the same with or without autophagy induction (2) [33,34]. Rapamycin and starvation control infection reducing the number of amastigotes per cell (3) [80,81].

parasite homeostasis, producing a phenotype characterised by mitochondrial disruption, phospolipid accumulation and abnormal promastigote morphology [93,94]. Table 1 summarises the autophagic events in the three pathogenic trypanosomatids described in this chapter.

5.2. Host cell autophagy and *L. amazonensis* infection

The connection between the endosomal/lysosomal pathway and the PV results in macromolecules being taken up by the parasite, as demonstrated in *T. cruzi* infection [96]. In this context, a notable increase in the proliferation of *L. amazonensis* amastigotes was observed after autophagic induction by nutritional deprivation, rapamycin treatment or interferon-gamma. This mechanism was partially reversed by the autophagic inhibitors wortmaninn or 3-methyladenine, which significantly reduced amastigote replication (Figure 5) [33]. However,