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Between Armour and Weapons — Cell Death Mechanisms in Trypanosomatid Parasites

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Abstract

Among the pathogenic protozoa, trypanosomatids stand out due to their medical and economic impact, especially for low-income populations in tropical countries. Together, sleeping sickness, Chagas disease and leishmaniasis affect millions of humans and animals worldwide, yet are neglected by the pharmaceutical industry. The current drugs for trypanosomatid infections are limited and unsatisfactory, with severe side effects leading to reduced quality of life and, in several instances, to the abandonment of treatment. An intense search for alternative compounds has been performed, aiming at specific parasite targets by cellular, molecular and biochemical approaches. One interesting strategy could be interference with the protozoan cell death pathways. However, these pathways are poorly understood in unicellular eukaryotes, with the controversial existence and uncertain biological relevance of programmed cell death (PCD). This chapter will discuss apoptosis-like and autophagic cell death and necrosis in *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* sp. and the possible implications of these pathways for the parasite life cycle and infection persistence. It will also revisit the genomic and proteomic metadata of these trypanosomatids in the literature to rebuild the map of cell death proteins expressed under different conditions. The interaction of leading candidates with parasite-specific molecules, especially with enzymes that regulate key steps in the cell death process, is a rational and attractive alternative for drug development for these neglected diseases.

Keywords: Cell death, apoptosis-like, autophagy, necrosis, *Leishmania* sp, *T. cruzi*, *T. brucei*

1. Introduction

Neglected tropical diseases (NTDs) are a group of the seventeen mostly life-threatening infections, which affect more than a billion people worldwide. They affect poor populations,

often in underdeveloped and developing countries (low-income countries) [1]. Among NTDs, infections caused by the so-called "protozoan" parasites, such as African trypanosomiasis, Chagas disease and leishmaniasis, are responsible for a high annual death toll among the poor populations of tropical countries. New safe and affordable medicines are urgently needed. These diseases all present therapeutic difficulties by developing resistance to existing therapies and/or by toxic side effects.

1.1. Neglected tropical diseases and trypanosomatids

1.1.1. Sleeping sickness

Human African trypanosomiasis (HAT), or sleeping sickness, is caused by extracellular protozoa belonging to the genus *Trypanosoma* and the species *T. brucei*. Two subspecies of *T. brucei* cause diseases with different epidemiological and clinical patterns: *T. b. gambiense*, a chronic disease present in western and central Africa accounting for 98% of the cases, and *T. b. rhodesiense*, an acute zoonosis located in eastern and southern Africa that occasionally infects humans. In 2001, WHO launched a major initiative to reinforce disease control and surveillance. After 10 years, the number of new cases of HAT decreased by 73.4%. Presently, the estimated the number of cases is 30, 000, and 70 million people are at risk [2, 3]. HAT clinically evolves in two stages. In the first stage, parasites are found in the lymphatic system and bloodstream. After a variable period of time, which is much shorter for the rhodesiense form, the second stage begins, with the parasites penetrating the blood-brain barrier and invading the central nervous system, leading to progressive neurological damage [4]. HAT is usually fatal if left untreated. Rhodesiense HAT usually progresses to death within six months, while gambiense HAT has a more chronic progressive course with an average duration of almost three years [5].

T. brucei is transmitted by the tsetse fly Glossina spp when it takes a blood meal. Non-dividing metacyclic forms enter the bloodstream of the mammalian host and differentiate into a rapidly dividing slender form able to evade antibody responses through antigenic variation [6]. Most of these forms undergo cell cycle arrest and develop into short-stumpy forms. When the tsetse fly bites an infected host, only the short-stumpy parasites survive in the insect's midgut and develop into a procyclic form, which undergoes multiple developmental phases on its way to the salivary gland, finally culminating in the infective metacyclic form [7, 8].

The drug of choice for treatment depends on the infecting species and the stage of infection. In early stages, *T. b. gambiense* and *T. b. rhodesiense* infections can be treated with pentamidine and suramin, respectively [9]. If the disease has progressed, treatment relies on melarsoprol or eflornithine. Melarsoprol, an arsenical drug, is extremely toxic. Eflornithine is less toxic, but is expensive, and has a difficult administration than melarsoprol and lacks efficacy against *T. b. rhodesiense* [2]. Since 2001, this drug has been combined with nifurtimox (NECT) for first-line treatment for CNS-stage *T. b. gambiense* HAT. It is the most recent breakthrough in anti-trypanosomiasis drug research and was added to the World Health Organisation's list of essential medicines in 2009. A major problem related to the treatment of HAT is the development of resistance to melarsoprol and the other drugs [10].

1.1.2. Chagas disease

Chagas disease is caused by the intracellular obligatory parasite *Trypanosoma cruzi* and affects approximately eight million individuals in Latin America [11]. The transmission of this disease occurs through the faeces of sucking triatominae insects, blood transfusions, organ transplantation, oral contamination, laboratory accidents and congenital routes [12, 13]. Current major concerns are the outbreaks of acute Chagas disease associated with the ingestion of contaminated food and its emergence in non-endemic areas, such as North America and Europe, due to the immigration of infected individuals [14-16]. This disease is characterised by two clinical phases. The acute phase appears shortly after infection and is defined by patent parasitaemia. If left untreated, symptomatic chronic disease develops in about one-third of individuals after a long latent period (10-30 years), which is known as the indeterminate form. The main clinical manifestations of Chagas disease include digestive and/or cardiac alterations. The chronic cardiac form of the disease is the most significant clinical manifestation. Consequences include dilated cardiomyopathy, congestive heart failure, arrhythmias, cardioembolism and stroke [17].

The life cycle of *T. cruzi* involves four major developmental stages during its passage through vertebrate and invertebrate hosts [18]. The infective stage of the parasite, the metacyclic trypomastigote, enters the mammalian host from insect faeces through wound openings or mucous membranes. In the mammalian host, the metacyclic trypomastigote differentiates into the amastigote form. After several rounds of replication in the host cells, the amastigote differentiates into the bloodstream trypomastigote, which can enter new cells and perpetuate the infection. When the insect bites an infected host, the bloodstream trypomastigote differentiates into the replicative epimastigote that lives in the insect's gut. Finally, in the rectum of the insect, the epimastigote differentiates into the infective metacyclic trypomastigote, which is ready to infect its host again.

The available chemotherapy for this illness includes two nitroheterocyclic agents, nifurtimox and benznidazole, which are effective against acute infections, but show poor activity in the late chronic phase, with severe collateral effects and limited efficacy against different parasitic isolates. These drawbacks justify the urgent need to identify better drugs to treat chagasic patients, and several new compounds are currently in preclinical development involving *in vitro* parasite phenotype screens and target-based drug discovery [19-21]. Recently, clinical trials with the azoles posaconazole and E1224 (ravuconazole prodrug) led to higher percentages of treatment failure in chronic patients than benznidazole [22, 23], suggesting their potential use in combination therapy [24].

1.1.3. Leishmaniasis

Leishmaniasis, which is caused by different species of *Leishmania,* is a vector-borne disease, with an estimated 12 million cases worldwide. Infection is caused by the bite of infected female sand flies of the genera *Phlebotomus* (Europe, Asia, Africa) and *Lutzomyia* (America) [25]. *Leishmania* parasites live a digenetic life cycle as either a promastigote flagellar or an amastigote form. The type of clinical manifestation depends on the infecting species and host factors, such as general health and genetic and immune constitution [26]. It is a disease complex with three

clinical manifestations, visceral (VL, kala-azar), cutaneous (CL) and muco-cutaneous (MCL), which arise from parasite replication in the mononuclear phagocyte system, dermis and naso-oropharyngeal mucosa, respectively [27]. Some post-treated *L. donovani*-infected patients develop the diffuse cutaneous form named post-kala-azar dermal leishmaniasis (PKDL) [28, 29]. VL, after initial skin lesions, takes 2-8 months to develop gross inflammatory reactions within the viscera (liver and spleen in particular) and is usually fatal unless treated. CL manifests as an open sore at the site of the insect bite and will frequently self-heal, leaving a scar. The diffuse form of CL is more problematic, causing lepromatous type lesions disseminated across the skin that can be difficult to heal. The MCL form, endemic in parts of Latin America, starts with skin sores that spread to the mucosal membranes of the face. Profound inflammatory damage can lead to the erosion of the nostrils and mouth in particular [29].

In the *Leishmania* life cycle, there are two principal parasite forms: amastigotes and motile promastigotes. In the alimentary tract of the insect vector, the parasite exists as multiplicative, non-infective procyclic promastigotes and non-multiplicative, infective metacyclic promastigotes [30]. Upon injection into the mammalian host, promastigotes are taken up by macrophages where the metacyclic forms differentiate into small multiplicative, non-motile amastigotes that live in a lysosomal compartment known as the parasitophorous vacuole [31]. These developmental forms are distinguished by their nutritional requirements, their growth rate and ability to divide, the regulated expression of their surface molecules, and their morphology. Metacyclic promastigotes are pre-adapted for survival in the mammalian host, as they are complement-resistant. Amastigotes are intracellular, non-motile forms that have adapted to the low pH of this compartment and have an adapted energy metabolism.

The current drugs are highly toxic, resistance is common and compliance of patients to treatment is low, as the treatment is long and the drug price is high. Although recent initiatives have improved the antileishmanial drug arsenal by combining current medicines or using new formulations of old ones, none are ideal for treatment due to their high toxicity, resistance issues, prohibitive prices, long treatment length and need of intravenous administration [32-34]. Pentavalent antimonials (glucantime and pentostan) are first-line drugs for both VL and CL. However, they present several limitations, including variable efficacy, need for daily injectable administration for approximately one month, and severe side effects. Many patients are unable to complete the treatment, increasing the risk of drug resistance development. Amphotericin B is a systemic antifungal that is used as a second-line drug for VL. It is highly toxic, requiring careful and slow intravenous administration. Lipid formulations of amphotericin B have been developed to improve its bioavailability and pharmacokinetic properties, reducing toxicity [35]. Miltefosine is the most recent antileishmanial drug on the market and the first effective oral treatment against VL [36]. However, it has common gastrointestinal side effects and is also limited by its relatively high cost [34], potential teratogenicity and growing concerns in relation to increases in clinical isolate susceptibility [37]. Paromomycin is an aminoglycoside antibiotic that is used in topical treatment for CL and as a parenteral drug for VL. Pentamidine was used as a second-line drug in antimony-resistant VL treatment. However, its high toxicity combined with decreased efficacy led to the abandonment of this drug to treat VL in India, but it is valuable for combined therapies [38].

2. Cell death: State of art

As used for whole organisms, the term death is employed to describe a sequence of events culminating in the breakdown of all biological functions. However, more than one century after the first citation [39], cell death still represents a crucial gap in our understanding of cellular physiology. It can be triggered by natural processes or induced by extrinsic factors (exposure to chemicals or physical stresses). The consequent tissue injury usually leads to a state of disease [40]. On the other hand, many studies pointed to cell death playing a fundamental role in the physiology of multicellular organisms, especially in processes such as metamorphosis and embryogenesis [41]. In this context, in 1964, the term programmed cell death (PCD) was created, proposing a sequence of well-controlled steps regulating a non-accidental cell death process in the absence of an inflammatory response [42]. Currently, it is known that distinct death mechanisms and phenotypes participate in PCD, with apoptosis and autophagy being the most prominent [43].

2.1. Apoptosis

The apoptotic pathway was first described in the early 1970s as a fundamental step for proper embryo development [44]. This process is crucial during tissue development, especially in immune response regulation and removal of infected or damaged cells [45, 46]. Apoptosis is involved not only in growth regulation in multicellular organisms [47, 48] but also in their defence against viral, bacterial or parasitic infections [49-53] and even against cancer development [54-57]. The removal of non-functional cells by the apoptotic pathway is efficient and prevents the inflammatory response [58].

During apoptosis in multicellular organisms, the cell activates death machinery that culminates in chromosomal condensation and nuclear DNA fragmentation [59, 60]. Biochemically, apoptosis is orchestrated by the activation of a family of cysteine proteases, named caspases, that are activated by extrinsic and intrinsic factors [45, 46]. The extrinsic pathway is activated by the interaction of death ligands with their respective cell surface receptor (i.e., FasL/Fas, TNF-α/TNFR) [61-63]. Such binding triggers the cleavage of procaspase 8 into active caspase 8, which cleaves procaspase 3. Executioner caspase 3 activates endonuclease G (EndoG), starting the characteristic DNA fragmentation, a distinctive marker of apoptosis [63-65]. On the other hand, the intrinsic pathway can be triggered by two distinct mechanisms with mitochondrion or endoplasmic reticulum (ER) dependency. In the mitochondrial pathway, activation occurs by membrane permeabilization, releasing cytochrome c, apoptosis induction factor (AIF), EndoG and regulators of the B-cell lymphoma 2 (Bcl2) protein family into the cytosol. In the cytosol, the apoptosome is formed by the interaction of released cytochrome c with apoptotic protease activating factor 1 (APAF-1) and procaspase 9, activating caspase 9, which subsequently activates the effector caspase 3 [66-70]. The ER pathway is mainly caspase 12-dependent and occurs in this organelle during stress conditions. Because this pathway was described in the mouse and humans lack functional caspase 12, the relevance of ER-mediated apoptosis is still debatable [71-73].

Undoubtedly, the caspase cascade represents a central point in the apoptotic process. Its regulation is well-controlled by pro- and anti-apoptotic molecules from the Bcl-2 family [74]. The apoptotic morphological and biochemical phenotypes include cell shrinkage, membrane blebbing (formation of apoptotic bodies), chromatin condensation and typical internucleosomal DNA fragmentation, externalization of phosphatidylserine (PS), loss of mitochondrial membrane potential ($\Delta\Psi$m), and target protein degradation by caspase activation [75-79]. The characterization of apoptosis is experimentally based on the detection of apoptotic markers. The loss of $\Delta\Psi$m (labelling with rhodamine 123 derivatives, such as TMRE), PS exposure (binding to labelled annexin V), chromatin condensation (DAPI labelling) and DNA fragmentation (TUNEL technique) are usually quantified by fluorescence microscopy or flow cytometry. DNA fragmentation can also be assessed by agarose gel electrophoresis, presenting a laddering pattern that represents internucleosomal cleavage. Analysis of caspase activity using labelled specific substrates and/or inhibitors can be performed by immunotechniques such as ELISA [80].

2.2. Autophagy

In the 1950s, acidic organelles involved in the intracellular degradation of macromolecules were described and termed lysosomes by Dr. Christian de Duve. In a subsequent study [81], he proposed the term autophagy for a self-degrading process [82]. Currently, the autophagic pathway is considered to be the main cellular mechanism for the degradation of non-functional organelles and/or macromolecules and is fundamental for homeostasis in eukaryotic cells [83]. In other words, autophagy is a housekeeping self-digestion mechanism that is crucial for cellular turnover and recycling and occurs by the engulfment of cytosolic portions containing material that should be degraded. Degradation starts immediately after the fusion of autophagosomes to lysosomes in an organelle named the autophagolysosome [84, 85].

In multicellular organisms, autophagy is involved in many physiological situations, including development, cell growth and cell differentiation. Autophagy sustains cell survival under 'extracellular stress', such as nutrient starvation, hypoxia, acidic pH and high temperature. It acts as a housekeeping device under 'intracellular stress' by removing damaged or redundant cytoplasmic components, including organelles [86]. Increased autophagic activity is observed in pathological states and in host defences against pathogens [87-92]. Despite the relevant role of autophagy for the maintenance of the regular cell cycle, prolonged starvation periods or other strong autophagic stimuli induce a cellular misbalance and promote autophagic cell death [93, 94].

The autophagic molecular machinery was first assessed in the yeast model *Saccharomyces cerevisiae*, and 30 proteins, called Atgs (AuTophaGy-related), were described and associated with different steps of the pathway [95]. Atg orthologues were identified in all eukaryotes, with Atg8 (LC3 in mammals) being one of the most studied [82]. Autophagy can be a selective or non-selective process, degrading specific or random cellular components. Examples of selective routes are mitophagy, pexophagy or reticulophagy, in which mitochondria (or part of the organelle), peroxisomes and ER are degraded, respectively [82].

Additionally, there are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). The most common is macroautophagy, a process that involves the engulfment of cytosolic portions by a double membrane structure called the phagophore. The double-membrane vesicle formed from phagophore engulfment is named the autophagosome and is directed to lysosomes for degradation by lysosomal hydrolases. These steps are regulated by Atgs [92, 96-98]. The chronological events related to macroautophagy are (a) autophagic induction; (b) cargo selection; (c) phagophore elongation; (d) autophagosome formation; (e) fusion to lysosomes; and (f) cargo degradation [99]. The early steps in this process depend on the serine/threonine protein kinase TOR (target of rapamycin), which is essential for autophagic regulation. TOR complexes 1 and 2 work as sensors of nutritional availability (especially amino acids). The autophagic enzyme Atg6 (Beclin 1 in mammals) is a phosphatidylinositol 3-kinase (PI-3K) and shares its signalling function with other cellular pathways. For autophagy, these kinases present a critical role for autophagosome formation [82].

In contrast, there are no autophagosomes in the microautophagic pathway. Invagination of the lysosomal membrane occurs, resulting in a single-membrane small vesicle inside the lysosomes that will be degraded. Interestingly, both macro- and microautophagy could be selective or non-selective processes. Indeed, CMA appears to be the most selective type of autophagy. The proteins that will be degraded contain pentapeptide motifs (KFERQ, QREFK or VDKFQ), the binding sites of a cytosolic chaperone. Such a chaperone-substrate complex binds to a LAMP-2A receptor in the lysosomal membrane, promoting receptor dimerization. A membrane channel is formed, and the specific protein reaches the lysosomal lumen to be degraded [82, 100].

For many years, electron microscopy was the only tool available for the identification of autophagic morphological features, especially the presence of double-membrane vesicles (autophagosomes). In the last 20 years, advances in the molecular description of autophagy allowed the detection, localization and quantification of Atgs by molecular, biochemical and morphological approaches. Currently, the gold-standard method to monitor autophagy is Atg8/LC3 detection by different techniques: (a) Western blotting (presence of two isoforms); (b) confocal or fluorescence microscopy (identification of LC3 puncta); (c) knock down or knock out (deletion and analysis of the phenotype); and (d) pharmacological induction/inhibition (rapamycin and/or PI-3K inhibitors). These techniques can also be employed *in vitro* or *in vivo* for other Atgs, indicating autophagic activity [101].

2.3. Necrosis

Necrosis is a term that is extensively employed as synonymous with cell death. In the Greek aetiology, it signifies the "stage of dying". In this death type, strong cellular damage occurs caused by external stimuli (drugs, infection, mechanical trauma), promoting the random degradation of the whole cell, with plasma membrane disruption. Necrosis is defined as an accidental cell death process, differing from PCD (especially apoptosis) [102]. One of the main differences between apoptosis and necrosis is the induction of the inflammatory response in the latter. The release of intracellular material into the extracellular environment during

necrotic cell death triggers intense inflammation in the surrounding cells and tissues [103]. Classical necrotic features are the loss of plasma membrane integrity, cytosolic vacuolization, disruption of calcium homeostasis, general degradation by lysosomal hydrolases and induction of the inflammatory response.

Necrosis can also be a regulated process. Necroptosis is a programmed and non-accidental death pathway. Surprisingly, the activation of this pathway can occur by TNF-α or FasL, classical apoptotic ligands. Necroptosis depends on the participation of the receptor-interacting protein kinases 1 and 3 (RIPK1 and RIPK3), which are kinases that regulate this pathway. RIPK1 is pharmacologically inhibited by a small molecule named necrostatin-1 (Nec-1) [104-106].

2.4. Others

In addition to apoptosis, autophagy and necrosis (accidental or not), other non-canonical death styles can take place in eukaryotic cells. In an inflammatory context, pyroptosis and NETosis are prominent. Pyroptosis, primarily observed in macrophages after bacterial infection, is caspase 1-dependent. This caspase promotes an increase in the inflammatory cytokine levels (IL-1β and IL-18) and the formation of plasma membrane pores, leading to the release of cellular material to the extracellular matrix. The main difference between pyroptosis and apoptosis is the participation of caspase 1, which is only involved in the pyroptotic death pathway, a proinflammatory PCD [106-108]. Another type of cell death that plays a crucial role in the innate immune response is the neutrophil extracellular trap (NETosis), where neutrophilic death leads to the release of a neutrophil DNA network coated with histones and elastase to the extracellular environment to capture pathogens. However, the direct antimicrobial effect of the NETs is still controversial [109, 110]. Currently, DNA release has also been described in other immune cells, such as eosinophils, basophils, macrophages and mast cells, but its precise role deserves further analysis [110-114].

Other cell death types not involved in inflammation have been characterized. Ferroptosis is iron-dependent cell death that has been identified in some mammalian cells and involves oxidative stress induced by a small molecule named erastin, which is inhibited by ferrostatin 1. Despite that lack of complete understanding of the erastin mechanism, the X_C–Cys/Glu antiporter system is inhibited in ferroptosis, leading to a misbalance of these amino acids inside the cell [106, 115]. Additionally, there is another non-canonical cell death pathway in cancer cells (*in vitro* and *in vivo* models) called autoschizis, which involves oxidative stress induced by treatment with ascorbate and menadione. Autoschizic cell death presents remarkable morphological evidence, with electron microscopy as the best technique for its identification. Among the autoschizic features are cell shrinkage, extrusion of large portions of the cytosol (without any organelles), random DNA fragmentation and the subsequent deterioration of all cellular structures [116, 117]. Interestingly, annexin V (AV) and propidium iodide (PI) assays (gold standards for apoptosis detection in mammals) of cells treated with ascorbate and menadione demonstrate high percentages of AV-/PI+ cells [117], which are not discussed in almost all apoptotic studies, suggesting that these membrane shedding events could occur in a large variety of cell models. Table 1 summarizes the main types of cell death discussed herein.

Cell death	Features	References
apoptosis	cell shrinkage	[44, 76, 78]
	membrane blebbing	
	DNA fragmentation	
	externalization of PS	
	activity of caspases	
	regulation by Bcl-2 family proteins	
	loss of $\Delta\Psi$	
	release of cytochrome c	
	no inflammatory response	
autophagy	presence of autophagosomes	[82, 101]
	participation of Atgs	
	regulation by PI-3K and TORC	
	degradation by lysosomes	
	presence of KFERQ, QREFK or VDKFQ motifs in the protein to be degraded (only in CMA)	
necrosis	disruption of plasma membrane	[102, 103]
	cytoplasmic vacuolization	
	imbalance of Ca^{2+} homeostasis	
	release of lysosomal enzymes	
	induction of inflammatory response	
necroptosis	participation of RIP1 and RIP3	[104, 106]
	inhibition by Nec-1	
pyroptosis	participation of caspase 1	[106-108]
	increase in IL-1β and IL-18 levels	
	induction of inflammatory response	
NETosis	formation of NETs	[109, 110]
	participation of elastase and histones	
	occurrence in neutrophils, macrophages, mast cells, eosinophils and basophils	
ferroptosis	participation of iron	[106, 115]
	presence of oxidative stress	
	induction by erastin	
	blockage of X_C–Cys/Glu antiporter system	
	inhibition by ferrostatin 1	
autoschizis	cell shrinkage	[116, 117]
	random DNA fragmentation	
	extrusion of large cytosolic portions (without organelles)	
	degradation of cellular components	
	increase in the AV-/PI+ population	

Table 1. Types of cell death

3. Cell death in trypanosomatids: An overview

The term PCD was employed for decades to exclusively describe cell death in metazoans and its involvement in embryogenesis and maintenance of homeostasis. Indeed, the relevance of PCD for lower eukaryotes is unclear. In an evolutionary scenario, these regulated processes could allow clonal selection in the parasite population, guaranteeing the propagation of identical genetic information even in adverse environmental conditions. However, differences in the cell death mechanisms observed between metazoans and protozoans must be considered [78, 118]. In the following sections, we will discuss the role of different death styles described in pathogenic trypanosomatids.

3.1. Apoptosis-like

In trypanosomatids, the first PCD report was published in 1995 by Ameisen and coworkers describing apoptotic characteristics (DNA fragmentation and cytoplasmic and nuclear morphological alterations) in *T. cruzi* epimastigotes during differentiation to trypomastigotes [119]. In the last two decades, a variety of stimuli were reported to induce the appearance of the apoptotic phenotype in this parasite, including exposure to fresh human serum (FHS), heat shock and drugs [76, 119-128]. Curiously, the apoptosis-like phenotype was also associated with the regulation of the *T. cruzi* life cycle [129]. These cell death phenotypes in pathogenic trypanosomatids have been characterized by the use of classical apoptotic markers (see item 2.1) [76, 79, 118, 123, 130-133]. Among the apoptotic hallmarks identified, we found (a) loss of $\Delta\Psi m$, (b) cytochrome c release, (c) PS externalization, and (d) abnormal DNA condensation and fragmentation [76, 119, 129, 130, 134] (Table 3, Figure 1).

In *Leishmania* sp., apoptotic features (nuclear condensation, DNA fragmentation, cell shrinkage, loss of $\Delta\Psi m$, and release of cytochrome c) were also observed in stress conditions induced by heat, starvation, oxidative agents and drugs [118, 134, 136-139, 135]. *L. donovani, L. major* and *L. mexicana* stationary phase promastigotes and axenic amastigotes exhibited DNA fragmentation with a laddering electrophoretic profile, suggesting oligonucleosomal cleavage. These data were corroborated by the description of a non-canonical, Ca^{2+}- and Mg^{2+}-independent 45-59 kDa endonuclease [76, 136, 140].

As in other pathogenic trypanosomatids, apoptotic features were also identified in *T. brucei* under non-physiological conditions, such as incubation with drugs, cytokines or ROS [129, 133, 141-143]. Interestingly, the gene for prohibitin and the receptor for activated protein kinase C have been correlated with the apoptotic process, suggesting convergence between these pathways in protozoa and mammals (Table 2) [129]. Despite several reports about caspase-like activity in trypanosomatids [75, 134, 136, 144], the exact role of these proteases in protozoa is not clear. Metacaspases are structurally similar to mammalian orthologues, but their catalytic activity on caspase substrates is quite controversial [145-147]. Despite their presence in *T. cruzi, T. brucei* and *Leishmania* sp., only *L. major* metacaspase shows *in vitro* self-proteolytic activity (Table 2) [146]. In fact, the participation of metacaspases cleaving vital substrates in the cell death cascade has not yet been described [148, 149]. Surprisingly, experimental
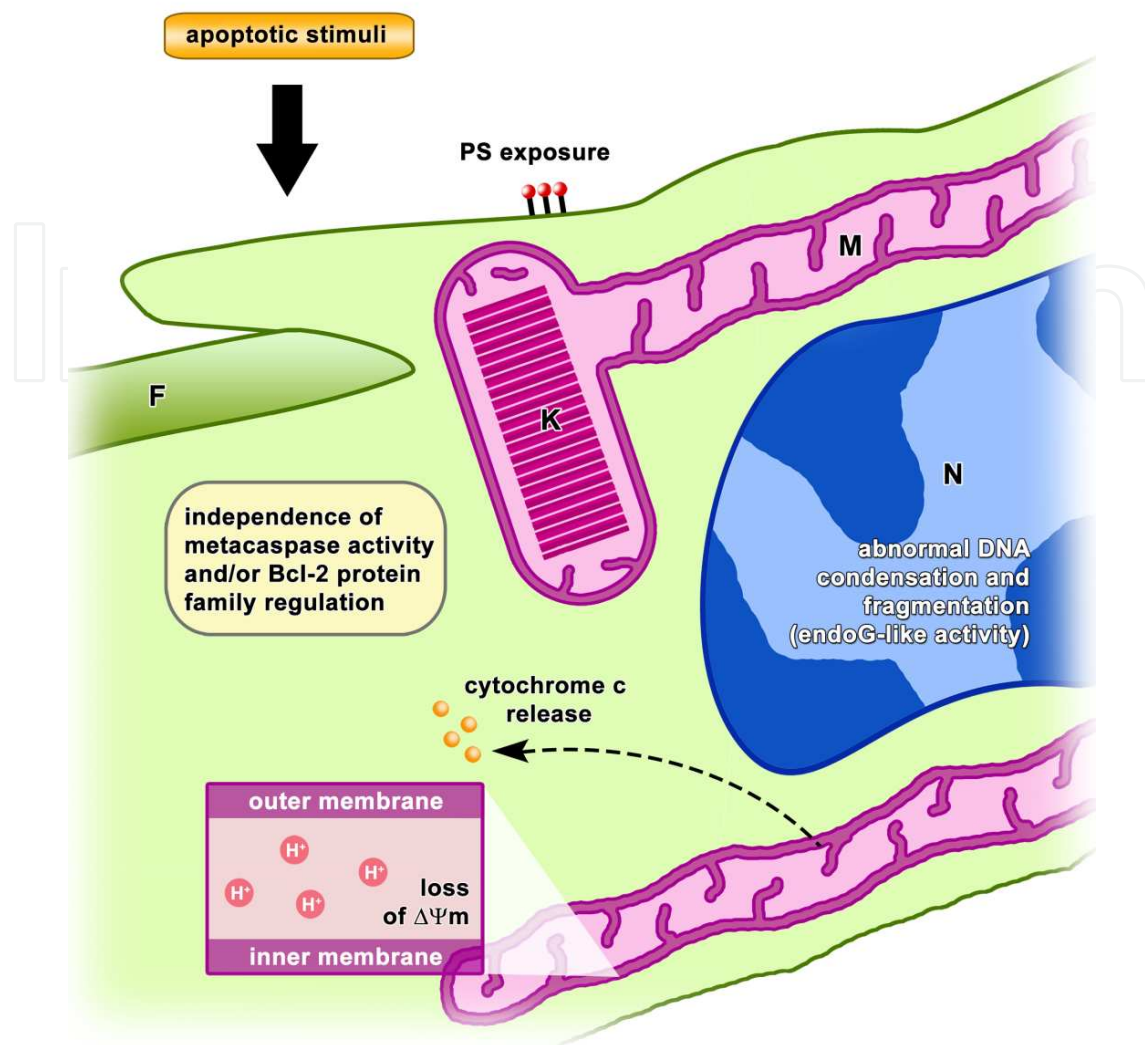
Figure 1. Schematic representation of apoptosis-like PCD in pathogenic trypanosomatids. Apoptotic stimuli induce loss of $\Delta\Psi$m, release of mitochondrial cytochrome c to the cytosol, PS externalization and DNA fragmentation by En-doG activity. Apoptotic regulators from the Bcl-2 family were not found until now, and the role of metacaspases is controversial, suggesting that apoptosis-like PCD in trypanosomatids is a caspase-like- and Bcl-2-independent pathway. N: nucleus; M: mitochondrion; K: kinetoplast; F: flagellum.

evidence pointed to the involvement of these proteases in cell cycle control and metacyclo-genesis, not in death [145, 150-153].

In unicellular organisms, the mitochondrion is a central organelle in cell death pathways, leading to ROS production [125]. In *T. brucei* procyclic forms, mitochondrial Ca^{2+} influx misbalance culminates in ROS generation [154]. Additionally, prostaglandin D2-induced ROS production in both the bloodstream and procyclic forms led to the labelling of different apoptotic markers, with the death phenotype reverted by oxidative scavengers, such as N-acetyl cysteine [130, 155, 156]. In *L. donovani*, hydrogen peroxide induced classical apoptotic features (DNA fragmentation, loss of $\Delta\Psi$m and caspase-like activity). This phenotype was partially reverted by caspase inhibitors [134, 137]. Oxidative stress plays a crucial role not only in apoptosis-like PCD but also in autophagy and necrosis, as we will discuss later [78, 157].

Molecule	Organism	References
Prohibitin RACK	*T. brucei*	[129]
Elongation factor 1 ∝	*T. cruzi*	[161]
Metacaspases 1	*L. donovani* *T. brucei*	[147, 162, 163]
Metacaspases 2	*L. donovani* *T. brucei*	[145, 147, 162]
Metacaspases 3	*T. cruzi* *T. brucei*	[145, 150, 153, 162]
Metacaspases 4	*T. brucei*	[162, 164]
Metacaspases 5	*T. cruzi* *T. brucei* *L. major*	[145, 150, 162, 165]
Metacaspase Z-DEVD-FMK -sensitive	*T. cruzi* *L. donovani*	[124, 134, 136]
Endonuclease G	*L. major* *T. brucei* *L. infantum* *L. donovani*	[132, 158, 166]
LdFEN-1 LdTatD-like nuclease	*L. donovani*	[158]

Table 2. Apoptotic molecules described in pathogenic trypanosomatids

The participation of EndoG-like in mitochondrial-mediated cell death has been reported, but the process is metacaspase-independent (Table 2) [132, 158, 159]. *L. infantum* submitted to heat stress also presents an apoptotic pattern, but without caspase-like activity, which was partially reversed by the expression of the anti-apoptotic mammalian gene Bcl-XL [160]. On the other hand, the overexpression of mammalian anti-apoptotic Bcl-2 in *T. brucei* caused no reversion of the mitochondrial damage induced by ROS [154]. However, members of the Bcl-2 protein family have not been described in trypanosomatids [129]. More studies regarding the regulation steps of apoptosis-like processes in trypanosomatids need to be performed.

3.2. Autophagy

Almost forty years ago, the first morphological autophagic evidence was described in trypanosomatids by electron microscopy of *T. brucei* [170]. In the last four decades, many studies have described recurrent autophagosome formation (initially named autophagic vacuoles), multivesicular bodies as well as myelin-like structures in pathogenic trypanosomatids treated

with different classes of drugs (Figure 2) [169, 168, 171-178]. Such autophagosomes showed distinct levels of degradation depending on the degree of cellular structure damage inside the organelle. Myelin-like structures are one of the most frequent ultrastructural alterations detected in drug-treated parasites and are suggestive of the cellular recycling of damaged structures. Currently, it is postulated that myelin-like structures are phagophores (or pre-autophagosomal structures, PAS), an early step in the formation of doubled-membrane autophagosomes (Table 3). In *T. cruzi*, ER profiles were reported as the main origin of phago-phores (Figure 2). These profiles usually surround a pre-lysosomal compartment, named the reservosome, suggesting the participation of this organelle in autophagolysosome formation in epimastigote forms [82, 178].
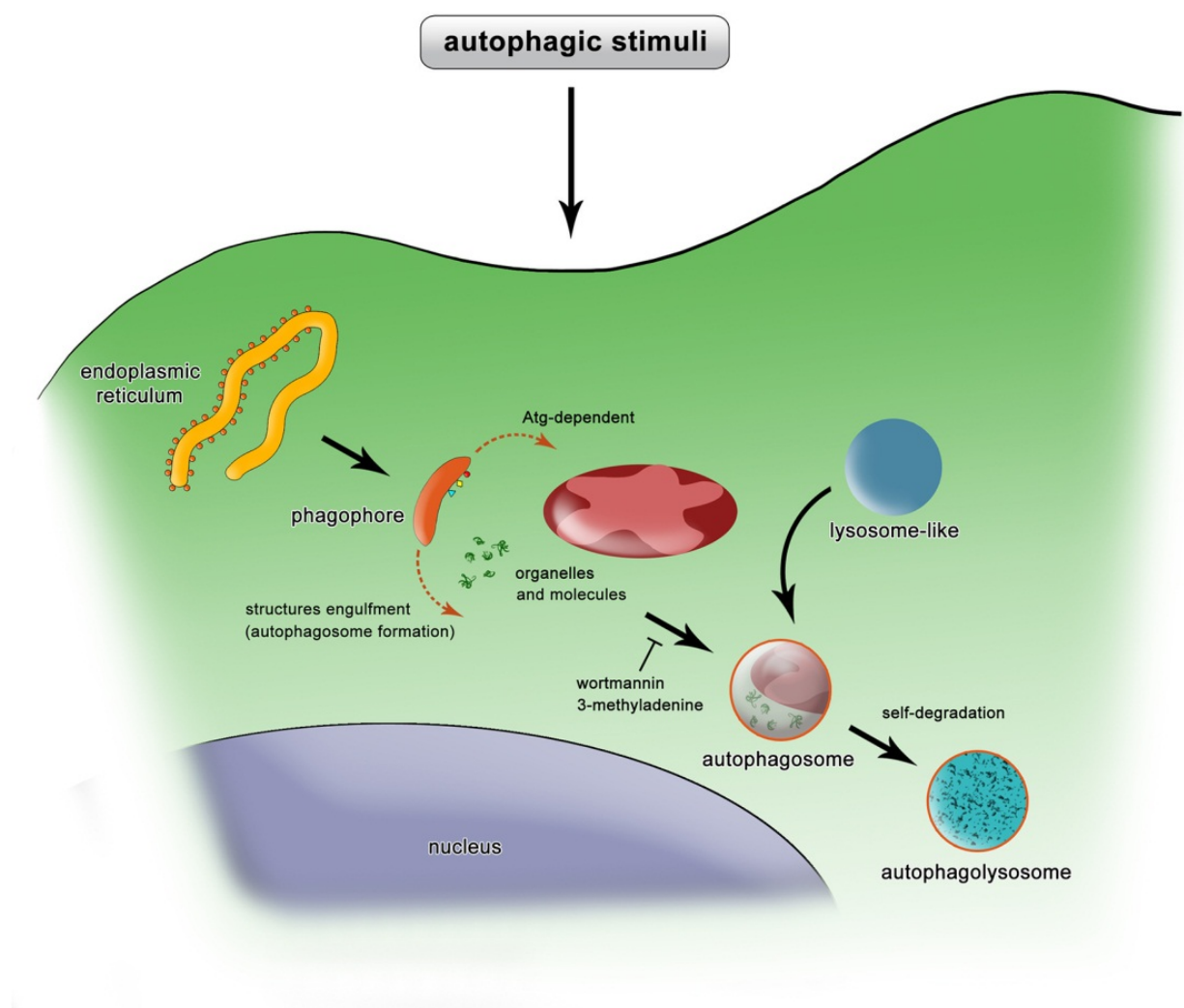

Figure 2. Schematic representation of autophagy in pathogenic trypanosomatids. Autophagic stimuli induce the for-mation of phagophores from ER profiles. The phagophore engulfs organelles and molecules, generating autophago-somes. Targeting and engulfment are Atg-dependent processes. These autophagosomes fused with lysosomes generate autophagolysosomes. Continuous autophagic stimuli lead to autophagic cell death, which is inhibited by the pre-treat-ment of the parasite with autophagic inhibitors (wortmannin or 3-methyladenine).

Cell death	Features	Organism	References
apoptosis-like	cell shrinkage	*T. cruzi*	[76, 118, 119, 130]
	membrane blebbing	*T. brucei*	
	DNA fragmentation	*Leishmania* sp.	
	PS exposure		
	loss of the $\Delta\Psi m$		
	release of cytochrome c		
autophagy	presence of autophagosomes-like	*T. cruzi*	[82, 92, 167]
	Golgi and/or ER profiles surrounding	*T. brucei*	
	organelles	*Leishmania* sp.	
	detection of Atg8 and Atg4		
necrosis	cytosolic vacuolization	*T. cruzi*	[149, 168, 169]
	plasma membrane disruption	*T. brucei*	
		Leishmania sp.	

Table 3. Types of cell death described in pathogenic trypanosomatids

In the last few years, a functional autophagic pathway was characterized in trypanosomatids and ATG homologues were identified. However, almost half of the yeast Atgs are lacking in these protozoa [167, 179-181]. Currently, in trypanosomatids, twenty autophagic genes have been found to be involved in all of the steps, from vesicle expansion and completion to degradation (Figure 2) [167]. Bioinformatic approaches revealed all four genes of the Atg8 conjugation system (Atg3, Atg4, Atg7 and Atg8). Atg8 is well-characterized in *T. cruzi*, *T. brucei* and *Leishmania* sp. and is located in autophagosomes, as observed in yeast and mammals. Atg8 has four isoforms (Atg8, Atg8A, Atg8B and Atg8C) that are processed by two isoforms of Atg4 (Atg4.1 and Atg4.2) [92, 181-185] (Table 3). On the other hand, the Atg12 conjugation system has poor sequence similarity in trypanosomatids, and Atg5, Atg10 and Atg12 sequences are lacking [167, 180]. Pathogenic trypanosomatids have two TOR kinases (TOR1 and TOR2) that form their respective complexes, TORC1 and TORC2 (Table 4). These two complexes show a distinct molecular behaviour, subcellular localization and susceptibility to rapamycin [186, 187]. The treatment of *T. brucei* bloodstream forms with rapamycin led to cell cycle arrest and an increase in the number of autophagosomes due to TORC2 inhibition. However, rapamycin had no effect on the parasite TORC1, suggesting another function for this complex [186, 188].

In addition to the recycling function, autophagy plays a fundamental role in parasite differentiation and survival, mitochondrial function and homeostasis of phospholipids [92, 182, 189, 190]. In metacyclogenesis, the autophagic pathway is triggered by nutritional deprivation, playing an important function in both the infectivity and virulence to the vertebrate host [182]. During the *T. cruzi* life cycle, epimastigotes are submitted to starvation in the insect rectum, a crucial event for protozoa differentiation. Starved epimastigotes express Atg8.1, but such expression is decreased in metacyclic forms [82, 92]. Reservosomes disappeared during

Molecules	Organism	References
Atg4.1, Atg4.2	*L. major*	[197]
Atg5	*L. major*	[190]
Atg5, Atg10, Atg12	*L. major*	[183]
Atg8A, Atg8B, Atg8.2	*T. brucei*	[183]
Atg8.1, Atg8.2	*T. cruzi*	[92]
Atg8, Atg8A, Atg8B, Atg8C	*L. major*	[183]
TOR1 TOR2	*T. brucei* *L. major*	[186, 198, 199]
TOR3	*L. major*	[199]
Vps34	*T. cruzi* *T. brucei*	[200, 201]

Table 4. Autophagic molecules described in pathogenic trypanosomatids

differentiation, most likely due to the cysteine proteinase activity, in particular, cruzipain [159, 191, 192].

In *Leishmania* sp., autophagy is essential for metacyclogenesis, with several observed auto-phagosomes during the process [193, 194]. The deletion of Atg4.2 led to an accumulation of Atg8 lipidated isoforms, compromising the autophagic activity. Subsequently, a reduction in the number of differentiating promastigotes was observed [194]. Interestingly, autophagy also participates in the differentiation of *L. mexicana* metacyclic promastigotes to amastigotes [193]. In the sandfly, the exposure of promastigotes to different stress stimuli, including higher temperature, low pH, and nutritional deprivation, acts as a crucial event for the success of the metacyclogenesis [189, 192]. *L. mexicana* shows that lysosome-like structures, called mega-somes, are involved in parasite differentiation, with the activity of two megasomal cysteine peptidases (CPA and CPB) associated with autophagy. The deletion of these proteases strongly impaired its differentiation into amastigotes, leading to an accumulation of autophagosomes containing multi-vesicular tubules (structures related to endocytosis) [180, 193].

A peculiar role for autophagy was observed in *T. brucei*. In a selective pathway, glycosomes are degraded during differentiation from bloodstream to procyclic forms. This organelle is a peroxisome-like structure that is also involved in the glycolytic pathway, and its degradation via autophagy led to important changes in the protozoa bioenergetics [195]. This evidence supported the existence of pexophagy in trypanosomes, an essential event for energy balance during the parasite life cycle. Depending on the environmental conditions (distinct hosts), the sources of energetic substrates vary, as does the ATP demand [180]. Recently, it was also reported that *T. brucei* acidocalcisomes (an acidic compartment that stores ions responsible for polyphosphate metabolism) regulate autophagy by the acidification of this organelle. More-over, the blockage of acidocalcisome biogenesis also inhibited the autophagic pathway without

the impairment of lysosomal biogenesis or function, suggesting the relevance of acidocalci-somes as an autophagic regulator [196].

Autophagic cell death occurs when the homeostatic balance is broken [40]. To evaluate whether autophagy participates in the cell death process, the use of the PI-3K inhibitors wortmannin and 3-methyladenine (3-MA) before the autophagic stimulus is provided is an interesting experimental approach. Pre-treatment with these inhibitors totally abolished the trypanocidal activity of naphthoimidazoles in *T. cruzi* epimastigotes and trypomastigotes. Although the involvement of components of the Atg8 conjugation system was also demonstrated, the molecular mechanisms of cell death regulation in this parasite deserve further examination [82, 178].

3.3. Necrosis

As described for higher eukaryotes, necrosis is poorly studied in protozoa, especially due to its conception as an accidental and uncontrolled process. The most typical necrotic feature is the plasma membrane rupture that leads to the loss of cellular homeostasis and consequent cell lysis as the consequence of a mechanical or chemical stimulus [103]. Necrosis is always the cell death endpoint, culminating in the generation of cellular debris. Thus, independent of the cell death mechanism that is induced, all parasites will lyse in a system without phagocytic cells to clean the microenvironment. In this context, a high percentage of anti-trypanosomatid natural or synthetic drugs present a mechanism of action with a lytic effect [29, 149, 202-205] (Table 3).

Another crucial stress condition that induces trypanosomatid disruption is the activation of the complement pathway. This cascade can be triggered by the binding of lectins to lipophos-phoglycans presented on the surface of *Leishmania* sp. promastigotes and of glycosylated molecules in the *T. cruzi* metacyclic form [206-209]. Indeed, pathogenic trypanosomatids show different mechanisms to evade the complement pathway. For example, *T. brucei* expresses a vast number of variant surface glycoproteins (VSG) that change the parasite coat to escape from the host immune system [210]. In relation to programmed necrosis, RIPK-like molecules have not yet been identified in unicellular organisms, and the direct effect of Nec-1 has not been evaluated, suggesting that an orchestrated pathway similar to necroptosis is absent in trypanosomatids.

3.4. Others

Curiously, no studies have been reported about non-canonical PCD pathways in trypanoso-matids. Pyroptosis and NETosis are processes that are characterized exclusively in mammalian cells, specifically during an inflammatory response. Such pathways involve the death of immune cells to block the progression of any infection by a well-regulated mechanism [106, 110]. The absence of these PCD types in unicellular organisms is not strange. On the other hand, the existence of specific oxidative stress-related cell death types in trypanosomatids would be reasonable. Continuous exposure of these parasites to ROS under distinct environ-mental conditions during their life cycles indicates the important role of oxidative stress in the

control of protozoa populations. ROS involvement in trypanosomatid apoptosis-like processes and autophagy has been described in different experimental conditions [130, 155, 156, 211, 212], but ferroptosis has not yet been investigated. Further studies about the effect of erastin as well as the inhibition by ferrostatin 1 should be performed in these parasites. Autoschizis was only observed in cancer cells under very specific conditions, but interestingly, an auto-schizic phenotype (high percentages of AV-/PI+ cells) was detected in *T. cruzi* treated with naphthoimidazoles [178]. The AV-/PI+ population is ignored in the majority of the studies, including in pathogenic trypanosomatids [213-215]. A better characterization of this parasite population must be performed to exclude the existence of autoschizis in protozoa.

3.5. Cell death and evasion of host immune response

Trypanosomatids presented a highly sophisticated repertoire to evade mammalian immune systems, including the capacity to prevent the cell death pathways of the infected host cells [188]. This efficient strategy allows host PCD modulation by the parasites to establish the infection. Depending on the protozoan species and the host cell type, PCD exacerbation or inhibition fluctuates. For example, the induction of apoptosis in immune cells increases the parasite persistence and survival in immunocompetent hosts [78]. In *T. cruzi* infection, apoptosis of lymphocytes and macrophages is essential for the parasite to escape, promoting inflammation reduction by anti-inflammatory cytokines and also amastigote proliferation [78, 216, 217]. The *Leishmania* strategy is quite different. Promastigotes externalize PS to be recognized by phagocytic cells. The binding of PS to its receptor on the phagocyte surface triggers a signalling cascade that guides TGF-β production and the subsequent anti-inflammatory response. This phenomenon, called apoptotic mimicry, facilitates parasite internalization and increases the success of the infection [218, 219]. Additionally, the intracellular cycle of *Leishmania* sp. also depends on the impairment of host cell apoptosis. This event is necessary to stop or delay the elimination of infected cells. For example, *L. major* uses the infected apoptotic granulocytes as "Trojan horses" to invade macrophages, the definitive host cells, avoiding the direct activation of phagocytes via the interaction between host receptors and protozoa [220].

Host autophagy also represents a valuable mechanism for both innate and adaptive responses to stop the infection. Its blockage is a crucial tactic for pathogenic trypanosomatids to evade host defences. Autophagy uses a process to eliminate pathogens, called xenophagy, directing microorganisms to be digested in lysosomes. This strategy is usually employed by protozoa living inside parasitophorous vacuoles to use the autophagic machinery to provide nutrients [82]. However, protozoa, such as *Leishmania* sp., change the autophagosomal pH and impair vesicular traffic, compromising the fusion to lysosomes. *L. amazonensis* amastigotes proliferate in starvation or even after treatment with rapamycin, but the proliferation is inhibited by incubation with the autophagic inhibitors wortmannin or 3-methyladenine [221]. The importance of autophagy for the *Leishmania* infection was corroborated by the observation that this pathway is exacerbated in *L. amazonensis*-infected mice and in a natural *L. donovani* infection in humans [222, 223]. Similar data were observed in the *in vitro T. cruzi* infection, suggesting that the autophagic pathway favours the parasite during its interaction with the host cell [224,

225]. However, the role of host autophagy in this trypanosomatid is still controversial due to the autophagic participation in the control of *T. cruzi* infection [226-228]. Furthermore, differences among strains and host cells must be considered to clarify whether host autophagy kills *T. cruzi* or provides nutrients for its survival.

4. Concluding remarks

In spite of the variety of studies about cell death in protozoans, including trypanosomatids, and the evidence of PCD, the detailed aspects of the molecular mechanisms and regulation remain unclear. The absence of key molecules together with the lack of an obvious role for this process in unicellular organisms makes the existence of PCD in these cells a debatable point, and the term "apoptosis-like" is more convenient [130, 172, 229]. In this context, the lack of a strong correlation between the proteolytic properties of caspases and their role in PCD should be highlighted. Currently, there is no description of the participation of trypanosomatid metacaspases in cell death processes, but these proteases have been postulated to function in proliferation and differentiation, which are important events for parasite survival [145, 148, 149, 153, 230]. In the post-genomic era, a rigorous search should be performed in proteomic databases of pathogenic trypanosomatids to correct misannotations in cell death proteins, validating the real role of these molecules for PCD processes.

Nevertheless, PCD was conserved during evolution, suggesting its essential function for the survival and maintenance of these species. However, it has been proposed that these pathways appeared in the phylogenetic tree in the multicellular organism branches, suggesting that the death molecular mechanisms identified in unicellular parasites came from a divergent evolutionary event [48]. This idea is supported by the replacement or complete absence of some PCD molecules, justifying the differences observed in protozoa mechanisms [79]. In addition to being an interesting evolutionary model for PCD, its physiological relevance for protozoa is still the most attractive question.

An altruistic hypothesis has been raised for unicellular organisms, especially for pathogenic trypanosomatids [130]. It was associated with the control of parasite populations, including protozoa density regulation, clonal selection and immune host system evasion, events related to the success of the infection [7, 76, 82, 136, 231]. Trypanosomatid cell death limits parasite colonization in insects in response to scarce resources of nutrients, avoiding invertebrate death [118, 130, 134]. On the other hand, PCD of *T. cruzi* or *L. amazonensis* insect forms under mammalian temperatures could evade host immune response derived from parasite lysis, facilitating the infection [76, 119, 135].

Autophagic cell death has been proposed as a PCD pathway, suggesting an active role of autophagy in death processes, but the precise mechanisms of regulation are not yet clear [174, 178, 232]. The majority of the autophagic studies were performed in yeast and mammal models. However, little is known about protozoan pathways. Autophagy is a regulated process that is directly involved in the preservation of cellular homeostasis and survival. Several hypotheses have been raised about the participation of this pathway in cell death in dying cells. The selective autophagic degradation of essential cellular factors, such as cell death regulators,

triggers death events, including caspase activation [232, 233]. Another hypothesis suggested that autophagy is not a specific and regulated cell death process but is a consequence of extensive injury. Once such an injury compromises cellular physiology, the damaged structure needs to be degraded for cell survival. This hypothesis also explains the presence of similar phenotypes in parasites after treatment with different compounds with distinct mechanisms of action. Such autophagic phenotypes, detected independent of the stimuli, reinforced this pathway as a desperate attempt of the cells to stay alive [168, 212, 232]. The determination of the connection between the autophagic cell death of pathogens, such as trypanosomatids, could have crucial implications for human health, but further mechanistic studies should be addressed in this field.
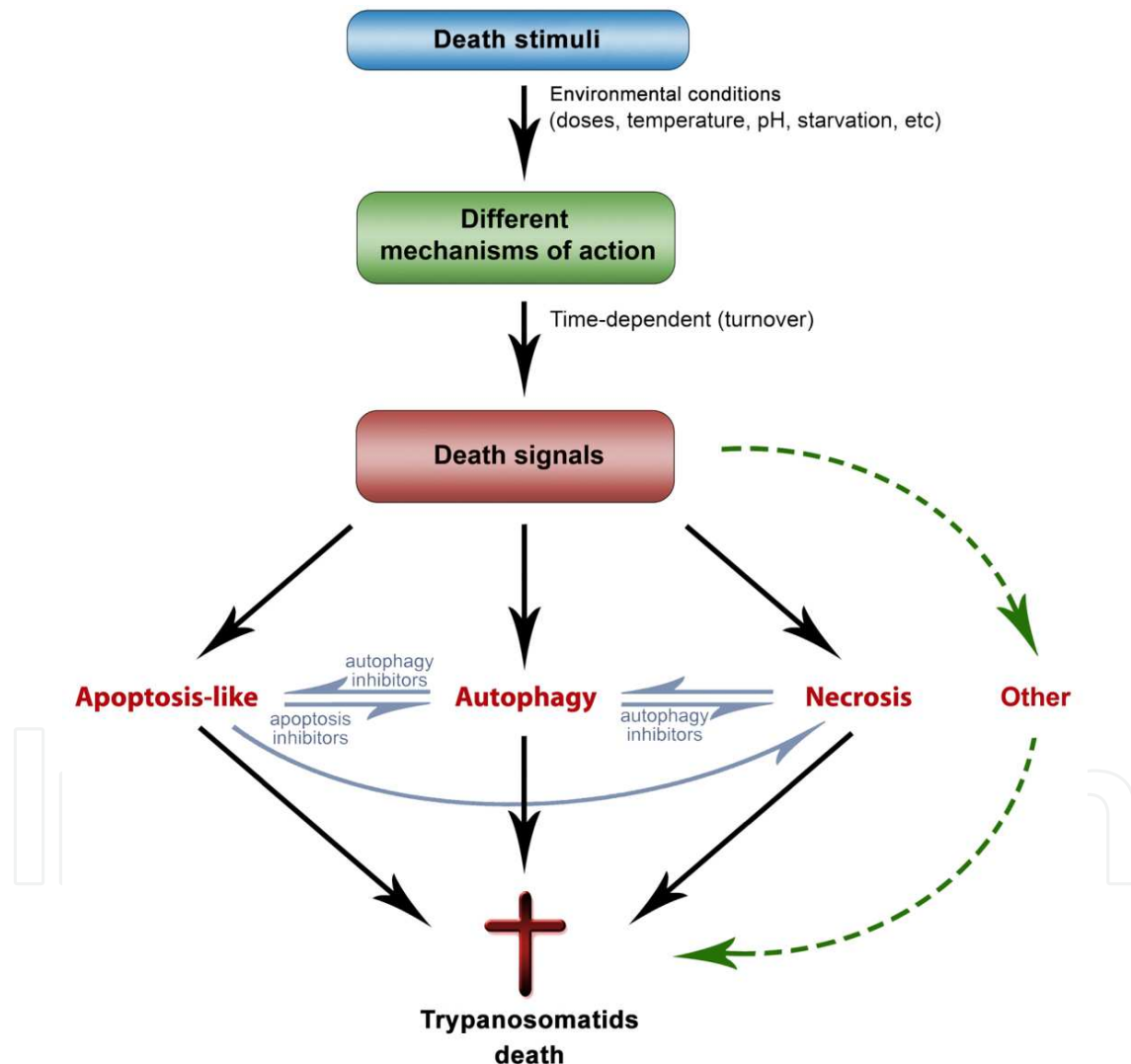

Figure 3. Different pathways of trypanosomatid death. The death stimulus triggers specific mechanisms of action depending on the environmental conditions, time of treatment and dose. Death signals lead to distinct well-known phenotypes from each pathway. Cross-talk could also be observed between apoptosis-like processes, autophagy and necrosis, culminating in protozoa death. The existence of an alternative unknown process cannot be discarded (dashed arrow).

The existence of cross-talk among different cell death pathways, especially autophagy and apoptosis, has been proposed (Figure 3) [93, 234]. In unicellular parasites, different cell death types have been described to be induced by physical and/or chemical stress conditions (drugs, heat shock, and nutritional deprivation, among others), resulting in a non-classical cell death phenotype. The total absence of commercial typical PCD markers, such as antibodies and enzyme activity kits, for protozoa and of key autophagic and apoptotic-like molecules reinforce the hypothesis of an interplay of distinct death mechanisms, suggesting their convergence, leading to necrosis. Likewise, the possibility of the occurrence of other PCD forms cannot be excluded [74, 78, 168, 178]. A better molecular characterization of cell death in pathogenic trypanosomatids is essential for advances in novel alternatives for therapeutic intervention.

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