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PERSPECTIVES

OPINION

Cell death in parasitic protozoa: regulated or incidental?

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Abstract | Apoptosis and other types of regulated cell death have been defined as fundamental processes in plant and animal development, but the occurrence of, and possible roles for, regulated cell death in parasitic protozoa remain controversial. A key problem has been the difficulty in reconciling the presence of apparent morphological markers of apoptosis and the notable absence of some of the key executioners functioning in higher eukaryotes. Here, we review the evidence for regulated cell death pathways in selected parasitic protozoa and propose that cell death in these organisms be classified into just two primary types: necrosis and incidental death. It is our opinion that dedicated molecular machinery required for the initiation and execution of regulated cell death has yet to be convincingly identified.

The undisputed finality of life belies the inherent complexity behind the processes of cellular demise. Metazoan cell death functions in wide-ranging physiological processes and can occur by distinct, well-defined mechanisms[1] (FIG. 1). Such mechanistic and functional diversity necessitates a clear system of classification, and, at the most fundamental level, metazoan cell death can be broadly classified into regulated and unregulated processes. Regulated cell death is controlled and executed by dedicated molecular mechanisms, and functions in metazoan development, homeostasis and immunity.

Apoptosis is perhaps the best characterized regulated cell death subtype. It is initiated by the transduction of stress signals originating from extracellular (extrinsic) or intracellular (intrinsic) sources. When pro-death signals predominate, different apoptosis subtypes use distinct biochemical pathways to carry out the destruction of a cell in a manner that minimizes potential harm to its surroundings[1,2]. A prominent, albeit not universal, feature of apoptosis is mitochondrial dysregulation; following stimulation, pro-apoptotic proteins from the BCL-2 family carry out mitochondrial outer-membrane permeabilization (MOMP), which ultimately drives a collapse in the mitochondrial membrane potential ($\Delta\Psi_m$) and allows the release of toxic intermembrane space (IMS) proteins[3]. This, in turn, stimulates downstream executioner mechanisms, including the activation of caspases, that directly effect cell death through proteolysis of key cellular substrates[4]. The biochemical events of apoptosis culminate in a cell death phenotype with characteristic features. Typically, the cell volume decreases, and chromatin is condensed and fragmented by nucleases. The plasma membrane remains largely intact, although localized perturbations cause membrane blebbing, and a loss of membrane asymmetry results in the exposure of phosphatidylserine (PS) on the outer-membrane leaflet. Finally, the cell breaks down into vesicles known as apoptotic bodies, which are rapidly taken up by phagocytes owing to the presence of PS on the cell surface, thereby avoiding activation of an inflammatory response[2].

Another form of regulated cell death is autophagic cell death. Macroautophagy (generally known as autophagy[5]) is an intracellular catabolic mechanism for the degradation of long-lived proteins and organelles and the recycling of their constituents. It is characterized by the formation of a double-membrane-bound structure, the autophagosome, around cargo destined for lysosomal degradation. Autophagy is widely conserved in the eukaryotic lineage and typically represents a cytoprotective force, functioning as a vital homeostatic mechanism in response to cellular stress. In this context, autophagy has been implicated in many physiological processes ranging from the starvation response to cellular growth, development and differentiation[6]. Despite the plethora of pro-survival roles for autophagy, under certain circumstances it seems to also execute a specific regulated cell death subtype known as autophagic cell death.

By contrast, unregulated cell death events occur without the concerted action of a specific cellular apparatus and are commonly associated with senescence and death caused by abnormal conditions that are unfavourable for life. Unregulated necrosis (hereafter referred to simply as necrosis) is a widely reported cell death subtype that is classically defined as premature cell death occurring without molecular and morphological markers of apoptosis or autophagy[7]. It is thought to occur in all cell types and involves terminal morphological alterations, including cell enlargement (oncosis), organelle swelling and plasma membrane rupture[7]. The apparent absence of molecular regulation underlies the long-held view that such processes are passive and can be regarded, to some extent, as purely uncontrolled or accidental (thus, the term accidental necrosis has been used as an alternative to unregulated necrosis in some instances). However, it should be noted that regulated necrosis has been found to occur and involves death receptor signalling and caspase inhibition, culminating in cell death with necrotic morphological features[8].

In some instances, harsh non-physiological treatments lead to phenotypes with inconsistent or overlapping features that are apparently typical of multiple death subtypes[9]. Therefore, these cases do not wholly conform to any of the currently established cell death subtypes, and they can be broadly referred to as accidental or incidental death. We suggest that incidental cell death is the

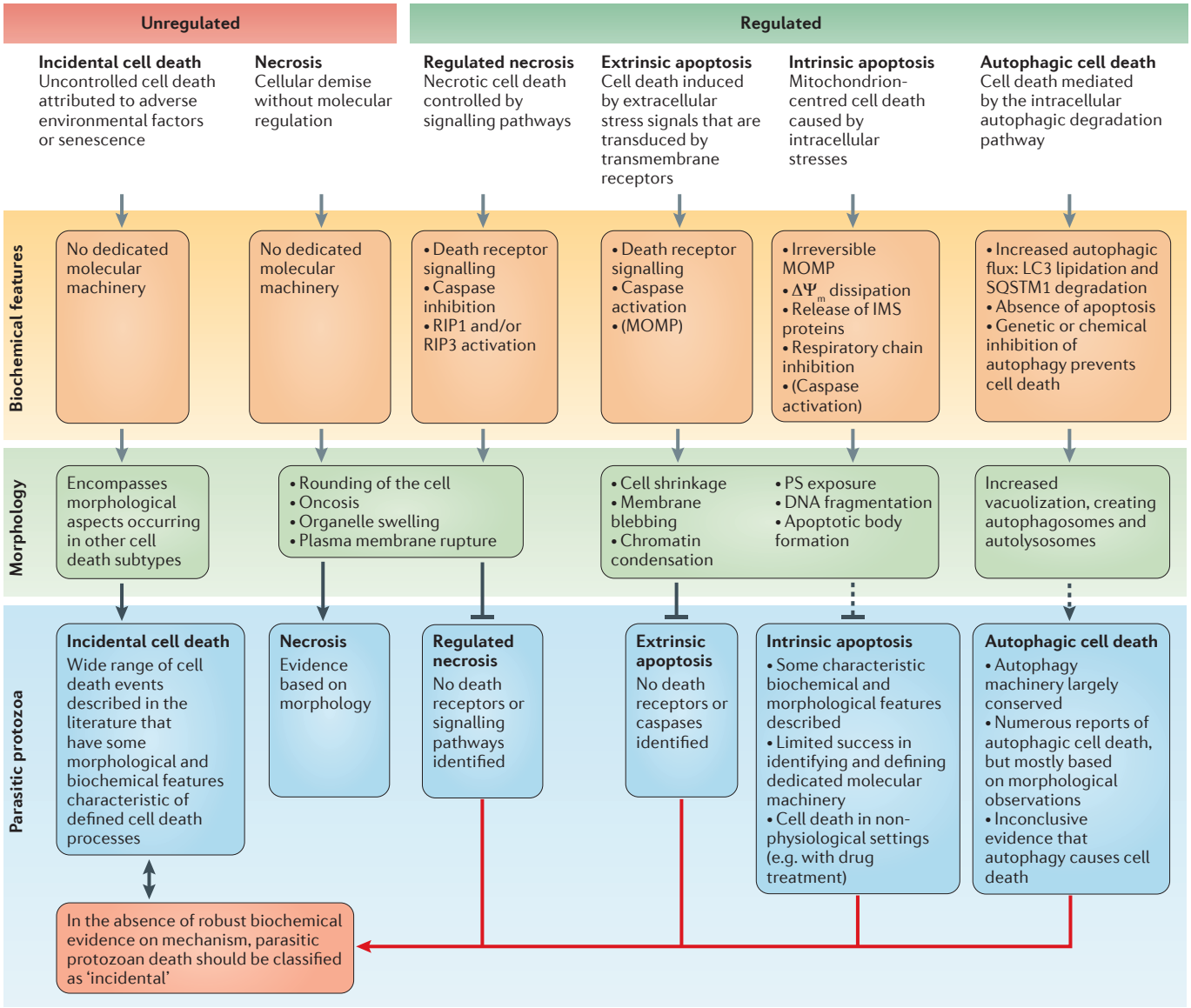


Figure 1 | **Major cell death modalities in eukaryotes.** Different cell death subtypes are defined according to characteristic biochemical and morphological features[1] (biochemical features in brackets are not always required). Applying this classification framework to parasitic protozoa highlights notable disparities in these organisms owing to their lack of genes encoding the key molecular regulators and executioners that are known in other organisms. Regulated necrosis and extrinsic apoptosis are precluded in these protozoa (blocking arrow) owing to the absence of established death receptors and caspases. Intrinsic apoptosis is unlikely to occur (dashed blocking arrow) despite the observation of apoptotic morphologies in protozoa, as no molecular pathways have yet been identified that specifically regulate the process. Protozoan autophagic cell death is possible (dashed arrow), as functional autophagy machinery is present, but definitive proof of an involvement for this machinery in cell death is currently lacking. Until dedicated molecular pathways are confirmed as mediating cell death in parasitic protozoa, we propose that cell death in these organisms should be classified as either incidental cell death or necrosis. $\Delta\Psi_m$, mitochondrial membrane potential; IMS, intermembrane space; LC3, microtubule-associated protein 1 light chain 3; MOMP, mitochondrial outer-membrane permeabilization; PS, phosphatidylserine; RIP, receptor-interacting serine/threonine protein kinase; SQSTM1, sequestome 1.

better term with regard to parasitic protozoa, as this avoids any anthropomorphic connotations and provides clear distinction from the term accidental necrosis.

For metazoa, there are obvious evolutionary benefits of possessing genetically encoded systems dedicated to the safe and timely removal of unwanted cells, but it is less clear how these systems might function and persist among unicellular organisms. However, in the mid 1990s, reports emerged that parasitic protozoa, including *Leishmania* and *Trypanosoma* spp., can undergo cell death accompanied by some features that are characteristic of mammalian apoptosis[10,11]. These reports inspired numerous subsequent studies centred on regulated cell death processes in parasitic protozoa. Recent research has generated a significant body of literature describing various protozoan death modalities, many using assays that were developed to detect apoptosis of higher-eukaryotic cells (reviewed in REFS 12,13). This has perhaps led many to naturally accept regulated protozoan death as a proven concept. However, despite the widespread detection of biochemical

and morphological features reminiscent of regulated mammalian cell death, more than 15 years of research has failed to convincingly establish the dedicated molecular pathways that orchestrate protozoan cell death. Even in light of plausible evolutionary concepts that explain why unicellular organisms might 'commit suicide' (REF. 14), robust experimental evidence demonstrating regulated cell death in parasitic protozoa is still lacking. Consequently, a better understanding of the biology of death in parasitic protozoa is required to distinguish clearly between necrosis, incidental cell death and regulated cell death.

In this Opinion article, we describe the instances when cell death occurs during the life cycles of selected parasitic protozoa, and critically analyse and evaluate the evidence for and against the presence of regulated cell death pathways in these organisms. We propose that the current evidence suggests that cell death in parasitic protozoa is not regulated and instead should be characterized as one of two types, incidental death or unregulated necrosis, until and unless additional strong support for regulated death is forthcoming.

Cell death during the life cycle

An appropriate starting point to assess the presence of regulated cell death pathways in parasitic protozoa is the identification of dead or dying cells during the natural life cycle. All the parasitic protozoa on which we focus here have a unidirectional, multiphase life cycle involving replicative and non-replicative developmental stages (FIG. 2). One feature of these life cycles is the wide variety of cellular differentiation events that is required to allow the parasites to adapt and establish in the different environments that they encounter[15]. It is this complexity which has led to the hypothesis that regulated cell death operates to limit proliferation, virulence and pathogenicity, thereby promoting transmission of the parasites[16–20].

Trypanosoma *spp.* The life cycle of *Trypanosoma brucei*, the causative agent of human and animal trypanosomiasis, includes replicating stages such as the long slender bloodstream trypomastigote in mammals and the procyclic trypomastigote in tsetse flies; these replicative forms set up and establish infection (FIG. 2a). The parasite also has non-dividing life cycle stages, notably the stumpy trypomastigote and metacyclic trypomastigote, which are pre-adapted for transmission to a new host[21]. These forms are quiescent and have a limited lifespan — for example, an estimated 48 hours for the stumpy trypomastigote[22,23] — and they undergo senescence and cell death if they are not transmitted to a new host (FIG. 2a). The generation of stumpy forms is stimulated through a quorum sensing-type mechanism involving the production of a parasite-derived soluble factor (stumpy induction factor; SIF) that accumulates and induces differentiation of the slender form[24]. This example of cell density-dependent signalling provides evidence for cell–cell communication in trypanosomes and a mechanism by which the parasite can avoid the depletion of host nutrients and optimize transmission. Experimental analyses coupled to mathematical modelling have convincingly shown that this tightly controlled developmental pathway is sufficient to achieve a long-lasting infection and efficient transmission[21] without the need for regulated cell death.

Leishmania *spp.* *Leishmania* spp. parasites cause an array of human diseases known as the leishmaniases, which can range from relatively mild cutaneous disease to lethal visceral infection. The motile, non-dividing metacyclic promastigote is inoculated into the skin of a mammalian host following the bite of a sandfly, but this form might undergo senescence and cell death if it is not transmitted to a new host (FIG. 2b). Transmission is enhanced by a parasite-derived and secreted virulence factor, filamentous proteophosphoglycan, as well as by sandfly saliva[25,26]. It has also been shown that the virulent inoculum of *Leishmania major* promastigotes contains a mixture of live and dead (or dying) parasites and that the dead parasites induce transforming growth factor-β (TGFβ)-mediated silencing of macrophages, thereby promoting survival of the viable *L. major* in the population[20,27]. Similarly, one study found that a population of *Leishmania amazonensis* metacyclic promastigotes isolated from the sandfly contained dead or dying parasites and that these parasites, in combination with viable parasites, enhanced pathogenesis[28]. The authors of this study proposed that some *L. amazonensis* parasites undergo an altruistic form of apoptosis to promote the survival of the population as a whole[27,28]. However, it is also possible that these parasites have a limited lifespan or that a nutrient shortage within the sandfly mouthparts causes some cells in the parasite population to die through starvation, so that their death is not necessarily altruistic, but nonetheless released nutrients could enable other cells to remain viable. Dead or dying parasites can also induce the secretion of TGFβ by neutrophils following parasite inoculation into the mammalian host. There is no evidence in *Leishmania* spp. for the existence of a non-dividing form in mammals that is pre-adapted for transmission, although amastigotes can grow slowly, and persistent, long-lasting but largely latent infections certainly occur. There has been no description to date of amastigotes undergoing cell death subtypes, although PS potentially acquired from the host and exposed on the surface of amastigotes leads to host phagocyte inactivation, a phenomenon known as apoptotic mimicry[29,30].

Plasmodium *spp.* *Plasmodium falciparum* causes malaria in humans and is transmitted by mosquitoes of the genus *Anopheles*. This parasite also has a number of life cycle stages that are non-dividing and pre-adapted for transmission (the sporozoite) or invasion (the merozoite), or are non-dividing because they are sexual stages (the gametocytes, the zygote and the ookinete) (FIG. 2c). There are few reports of cell death occurring during the replicative stages of the *P. falciparum* life cycle, although the appearance of abnormal parasites, known as crisis forms, during the erythrocytic cycle of *P. falciparum* indicates the possibility of fever-induced (that is, heat-induced) parasite cell death[31]. In the more readily studied rodent malaria parasite *Plasmodium berghei*, up to 50% of the diploid ookinetes die before mosquito gut invasion[32]. This has led to the hypothesis that this death is a mechanism to limit the intensity of infection in the mosquito and should be classified as apoptosis[32]. However, there is a paucity of hard evidence to support this being regulated cell death, and the available data do not exclude this death from being simply the natural senescence of a non-dividing life cycle form.

Stress-induced cell death

Numerous *in vitro* studies have involved subjecting parasitic protozoa to non-physiological stress conditions or drug treatment and then assessing the cell death modalities using a variety of morphological and biochemical assays for mammalian apoptosis (BOX 1), as detailed in several recent reviews[12,20,33,34].

In this context, *Leishmania* spp. have received the most attention, with many reported phenotypes accompanying cell death, including cell shrinkage, DNA fragmentation, activation of caspase-like peptidases, loss of $\Delta\Psi_m$, release of cytochrome *c*, PS exposure and translocation

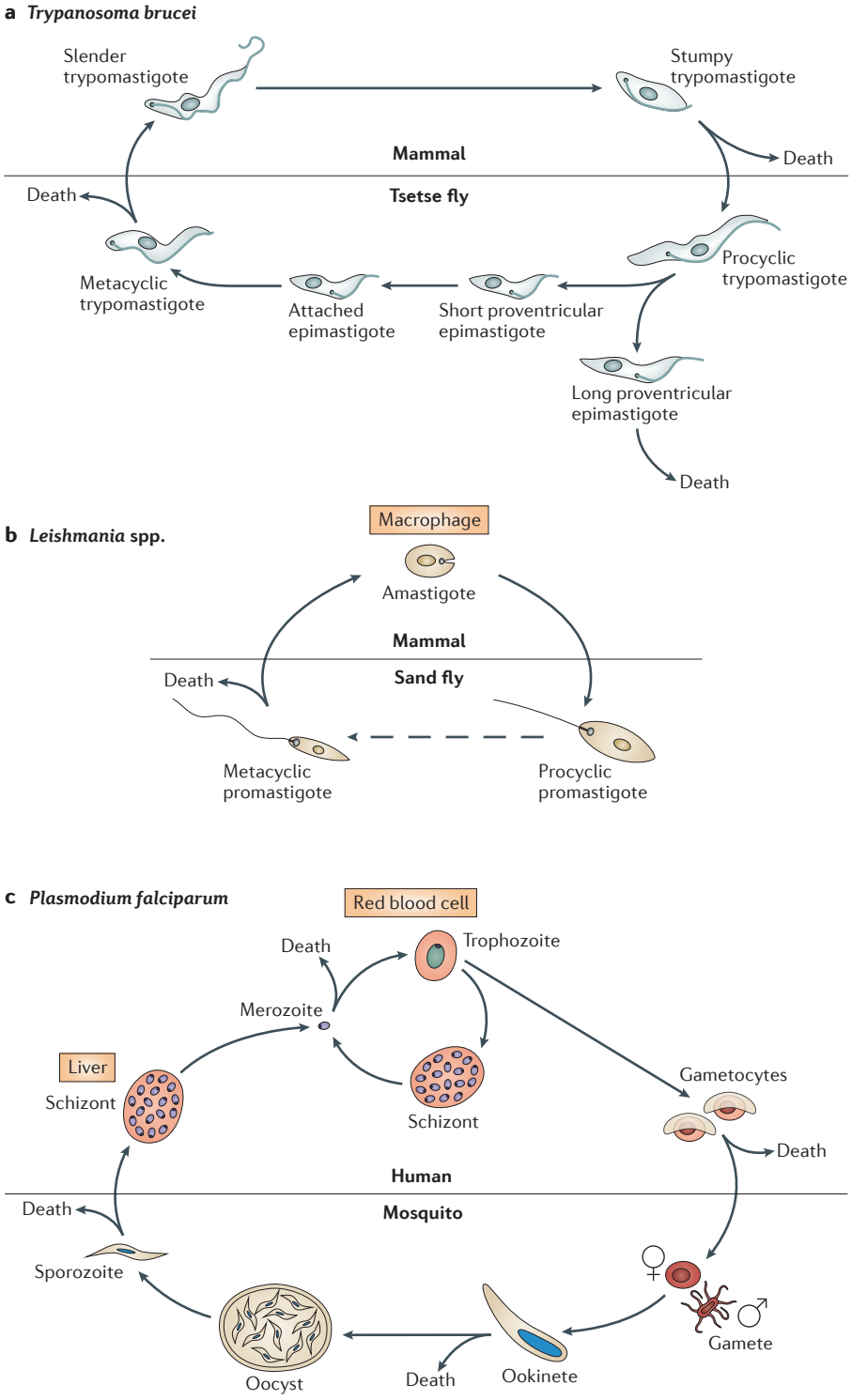


Figure 2 | **Life cycles and cell death of parasitic protozoa. a** | *Trypanosoma brucei*. The metacyclic trypomastigote is inoculated into the mammalian host by the bite of a tsetse fly and differentiates into a proliferative long slender trypomastigote to establish infection. The parasite then differentiates into a short stumpy form, which is quiescent (that is, cell cycle arrested) and primed for transmission. If taken up by a tsetse fly, the stumpy trypomastigote differentiates into a procyclic trypomastigote. If the stumpy form is not taken up by a fly, it undergoes senescence and cell death. The procyclic trypomastigote differentiates into the proventricular epimastigote, which undergoes asymmetric division to generate a short and a long form. The short form attaches to the salivary gland and transforms into the proliferative epimastigote, whereas the long form undergoes senescence and cell death. The proliferative epimastigote differentiates into the non-dividing metacyclic trypomastigote to complete the cycle. The metacyclic trypomastigote might die if not transmitted. **b** | *Leishmania* spp. A non-dividing metacyclic promastigote residing in the foregut or mouthparts of the sandfly is inoculated into the skin of a mammalian host, where it is taken up by a macrophage and differentiates into an amastigote. Metacyclic promastigotes that are not transmitted are thought to undergo senescence and cell death. The amastigote proliferates in the macrophage and can be taken up during a sandfly blood meal. In the sandfly gut, the procyclic promastigote develops into a metacyclic promastigote via several intermediate stages (dashed arrow). **c** | *Plasmodium falciparum*. The sporozoite form is injected by a mosquito into a human, where it travels to the liver, invades a hepatocyte and undergoes development into an exoerythrocytic schizont, which yields merozoites. These are released into the bloodstream and invade erythrocytes, inside which they develop into trophozoites and then schizonts, which generate further merozoites. These are released into the bloodstream to initiate another round of replication. The merozoite is a short-lived cell and undergoes cell death if it does not rapidly invade an erythrocyte. The parasite can also develop into sexual forms, the gametocytes, which subsequently differentiate into gametes if they are taken up by a mosquito during a blood meal. These gametes fertilize each other to form a zygote, which develops into an ookinete, escapes the midgut and then embeds itself into the exterior gut membrane of the mosquito. There, it develops into an oocyst, producing sporozoites to complete the cycle.

of endonuclease G[16,35]. These morphological and biochemical features have been described for a variety of *Leishmania* spp. under different stress stimuli, including exposure to reactive oxygen species (ROS), nitric oxide (NO), hydrogen peroxide (H_2O_2), increased temperature and leishmanicidal drugs. Similarly, stress-induced cell death in both trypanosomes and *Plasmodium* spp. has been linked to the appearance of multiple markers of mammalian apoptosis. However, it should be noted that substantial inconsistencies in the detection of such apoptosis-like phenotypes in *Plasmodium* spp.[36–38] and *T. brucei*[39] have created a degree of uncertainty regarding these observations[40].

The types of *T. brucei* death that are induced by prostaglandin D_2 (PGD_2) treatment[41] and spliced leader RNA silencing (SLS)[42] are of particular interest. It has been proposed that PGD_2 released by *T. brucei* stumpy trypomastigotes induces a selective apoptosis-like cell death of other stumpy forms in the blood of mammals,

Box 1 | **Evaluation of some biochemical and morphological features used to define cell death in parasitic protozoa**

Phosphatidylserine exposure
In mammalian cells, an early event in apoptosis is the loss of plasma membrane asymmetry, which leads to Ca^{2+}-dependent exposure of the phospholipid phosphatidylserine (PS) on the outer leaflet of the membrane. This can be detected with fluorescently conjugated annexin V or with PS-specific antibodies. The surface binding of annexin V has been used by many investigators as a marker to define apoptosis in parasitic protozoa; however, potential problems exist with using this assay. For example, recent data indicate that PS is absent (or at least below the level of detection) in *Leishmania* spp. promastigotes[57,69], raising doubts about the specificity of annexin V binding in parasitic protozoa. Moreover, annexin V is known to bind other *Leishmania* spp. phospholipids[69] that are not linked to apoptosis, and many parasitic protozoa have a dense surface comprising proteins and a glycocalyx that might prevent annexin V from gaining access to the plasma membrane. Thus, increased annexin V binding might not necessarily be indicative of cell death.

DNA fragmentation
Apoptosis in mammalian cells leads to DNA fragmentation, which can be detected as a DNA ladder on an agarose gel or by a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay. Numerous accounts of stress- or drug-induced DNA fragmentation exist for parasitic protozoa, but controversy remains about the link with regulated cell death. Protozoan parasites contain several homologues of mammalian nucleases with a role in apoptosis, such as mitochondrial endonuclease G, TATD and flap endonuclease 1 (FEN1), but some caution is advised when interpreting this finding, as many of these nucleases also have non-apoptotic functions. Furthermore, stress might induce the release of highly active nucleases from organelles, leading to incidental cell death in which DNA fragmentation occurs as a by-product.

Loss of mitochondrial membrane potential
Mitochondrial dysfunction is an established feature of apoptosis. The maintenance of a mitochondrial membrane potential ($\Delta\Psi_m$) across the inner membrane is required for correct mitochondrial function and ATP generation. In metazoa, apoptotic $\Delta\Psi_m$ dissipation occurs when pro-apoptosis members of the BCL-2 family (namely, BAX and BAK) cause mitochondrial outer-membrane permeabilization, which promotes the release of toxic intermembrane-space (IMS) proteins and destabilizes the mitochondria by exposing key mitochondrial proteins to the deleterious effects of peptidases such as caspases (for example, the IMS protein cytochrome *c* activates caspases). Accordingly, $\Delta\Psi_m$ reflects mitochondrial health, and $\Delta\Psi_m$-sensitive probes are widely used in apoptosis assays in higher eukaryotes. Numerous $\Delta\Psi_m$-sensitive probes have also been used in parasitic protozoa to show dissipation of $\Delta\Psi_m$ in response to a range of stresses (reviewed in REF. 12). However, little is known about $\Delta\Psi_m$ dissipation in protozoan parasites, and it remains to be established whether a dedicated molecular machinery regulates this process.

Caspase activity
In mammalian cell extracts, caspase activity can be detected specifically using fluorogenic peptides, such as Z-DEVD-AMC, and inhibited with substrate analogues, such as Z-DEVD-FMK[4]. Protozoa lack caspases, but activity towards small peptide substrates such as DEVD has been detected on several occasions, presumably reflecting the myriad other highly active peptidases that occur in parasitic protozoa. Likewise, inhibition of this activity with Z-DEVD-FMK can be detected, but this does not definitively indicate the presence of caspases. Parasitic protozoa contain cysteine peptidases called metacaspases, which belong to the same family as caspases (clan CD, family C14 in the MEROPS database) but have arginine-directed substrate specificity and do not cleave caspase substrates (see BOX 2).

thereby promoting survival of the host and, consequently, the parasite by curtailing overpopulation[41]. However, this has yet to be defined experimentally using *in vivo* models. Moreover, the sensitivity of *T. brucei* to PGD_2 *in vivo* remains unknown, and no molecular mechanism for the induction or regulation of cell death has been elucidated. Crucially, *T. brucei* has yet to be identified as the source of the elevated PGD_2 levels observed in patients with sleeping sickness[41], raising the possibility that this PGD_2 is in fact derived from host cells and is thus produced as a host defence mechanism rather than as part of a parasite-induced cell death pathway. SLS is a unique trypanosome stress response mechanism that disrupts gene expression by depleting a key RNA, the SL RNA, which is required for the maturation of all *T. brucei* mRNAs[43]. Persistent ER stress induces SLS and parasite cell death that has some features similar to those of mammalian apoptosis[42]. However, it is necessary to discriminate fully the individual impacts of ER stress and SLS on trypanosome cell death before classifying SLS as a bona fide inducer of regulated cell death, as it is possible that ER disruption alone is sufficient to cause cell death.

Although it is clear that parasites die under conditions of extreme stress, the extent to which this cell death is governed by regulated processes remains uncertain. We contend that the detection of features which are apparently characteristic of mammalian apoptosis during protozoan cell death does not unequivocally demonstrate the existence of this regulated death process in protozoa. Indeed, some phenotypes that are taken to indicate apoptosis in mammalian cells, such as exposure of PS, potentially have significant limitations when applied to protozoa (BOX 1). Overall, more complete and better evidence is required before we can conclude that regulated cell death occurs in these organisms.

Possible cell death modalities

There is good evidence that a range of cell death modalities operate in higher eukaryotes (FIG. 1). A key question to address is whether the situation is the same in parasitic protozoa; the clear molecular and biochemical differences, as well as the great evolutionary divergence between multicellular and unicellular organisms, is the basis for our view of 'probably not'.

Extrinsic apoptosis is induced by extracellular stresses that are sensed and transduced by specific transmembrane receptors. Ligands such as tumour necrosis factor (TNF) activate various death receptors, such as TNF receptor 1 (TNFR1), and cell death is dependent on executioner caspases. Parasitic protozoa lack the same death receptors and caspases, and functionally equivalent molecules have yet to be identified, so this type of death can be discounted at present. The occurrence of caspase-dependent intrinsic apoptosis also seems hard to reconcile with the absence of caspases in protozoa. However, these discrepancies do not entirely preclude functional regulated cell death mechanisms, but suggest that if they do occur, they must differ from the processes in higher eukaryotes.

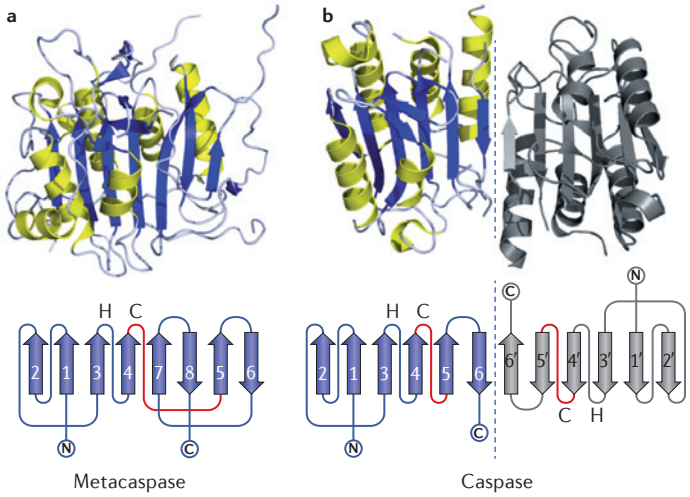
The apparent evolutionary diversification of the cell death molecular apparatus has spurred great interest in the caspase-family cysteine peptidases known as the metacaspases, which are found only in plants, fungi and protozoa[44] (organisms that lack caspases). Although plant metacaspases have been shown to have roles in cell death[45], distinct biochemical features of these proteins seem to have facilitated an expansion in the repertoire of metacaspase functions and allowed their participation in

Box 2 | **Are metacaspases caspases?**

The participation of the metacaspases in cell death events (reviewed in REF. 46) has prompted suggestions that metacaspases are synonymous to caspases. Although metacaspases have significant structural similarities with caspases, such as a conserved histidine-cysteine catalytic dyad set in a caspase-haemoglobinase fold, striking differences in the enzyme architecture confer distinct biochemical properties on the two groups, as evidenced by the recent X-ray crystallographic structure of *Trypanosoma brucei* metacaspase 2 (MCA2)[70].

Activation of effector caspases occurs by processing of an intersubunit linker, leading to dimerization with another activated caspase monomer[4]. By contrast, metacaspases are active monomers that do not require processing[71], and their homodimerization is prevented by their altered intersubunit properties and β-sheet organization (see the figure, which shows a structural comparison of *T. brucei* MCA2 and human caspase 7; Protein Data Bank accessions 4AF8 and 1SHL, respectively). Furthermore, metacaspase regulation requires a calcium-dependent conformational change to the extended amino-terminal domain, which obstructs the active site in the absence of calcium binding[70]. Additional differences in the active sites create distinct enzyme specificities: metacaspases cleave substrates only at basic arginine or lysine residues[71], whereas caspases strictly cleave at an aspartic acid residue[4]. For these reasons, we conclude that metacaspases are not caspases.

In the ribbon diagram and β′-sheet topology of the MCA2 monomer (see the figure, part **a**, top and bottom, respectively), loops are coloured light grey, β-strands are blue and surrounding α-helices are yellow. The histidine-cysteine catalytic dyad is labelled in the β-sheet topology diagram, and the catalytic loop, which does not undergo autoprocessing, is shown in red. For the caspase 7 dimer (see the figure, part **b**), one caspase monomer is shown coloured as for MCA2, and a second monomer is in dark grey; the intersubunit linker is in red. In both structures, the β-strands are numbered from the amino to the carboxyl terminus.



cellular processes outside of those defined for caspases[46]. Indeed, analyses of metacaspase gene deletion mutants for *T. brucei* (*MCA2*, *MCA3* and *MCA5* triple mutant)[47] and *P. berghei* (*MCA1* mutant)[36] could not identify a role for the encoded proteins in regulated cell death, although in both species the presence of additional metacaspase genes could provide functional redundancy. Overexpression of the single metacaspases of *Leishmania donovani* and *L. major* was shown to make these species more sensitive to H_2O_2-induced cell death, potentially owing to metacaspase-dependent mitochondrial impairment[48,49]; however, a null mutant for the single *Leishmania mexicana MCA* gene indicated that MCA is a negative regulator of amastigote proliferation, and there was no evidence for a role as a cell death regulator[50]. Thus, it seems that although metacaspases and caspases might have distant shared ancestry, significant differences have occurred during evolution that have given rise to two distinct enzyme families (BOX 2). Accordingly, the metacaspases of parasites cannot be viewed as caspase mimics or, consequently, as mediators of a similar regulated cell death.

Other peptidases do participate in parasite cell death. Peptidases from the cathepsin L-like or cathepsin B-like families are released from the lysosome in response to stress in *Leishmania* spp. and contribute to the death of the parasite[51,52]. Lysosomal membrane permeabilization has been implicated as a mechanism of regulated cell death in metazoa[53], but it is yet to be resolved whether lysosomal disruption in *Leishmania* spp. represents a regulated or incidental event in cell death[52]. Caspase-independent execution of intrinsic apoptosis via processes such as DNA fragmentation and ATP depletion can occur in higher eukaryotes. Although it has been demonstrated that various stresses can induce mitochondrial dysfunction, nuclease activation and DNA cleavage in parasitic protozoa[12], currently there is no firm evidence for cell death-specific signalling pathways. Thus, the observed phenotypes could be due to incidental cell death.

Autophagic cell death represents a potential protozoan regulated cell death modality. The cellular apparatus required for autophagy is widely conserved among parasitic protozoa[54], and robust molecular evidence confirms the existence of functional autophagic pathways in *Leishmania* spp., *Toxoplasma gondii* and trypanosomes[55–60]. Mechanistically, the pathways seem to be broadly similar to those of other eukaryotes; evidence from *Leishmania* spp. shows that autophagosome biogenesis occurs at the mitochondrial membrane and requires both autophagy-related protein 5 (ATG5)–ATG12 and ATG8–phosphatidylethanolamine in these species[57] (FIG. 3). Consistent with other organisms, parasitic protozoa undergo autophagy as a response to nutrient starvation[55–60]. Importantly, however, autophagy also directly influences parasite virulence by mediating cellular remodelling during life cycle differentiation in *Leishmania* spp. and *Trypanosoma cruzi* and by maintaining mitochondrial function in *L. major* and *T. gondii*[56–58,60].

The defining features of autophagic cell death have classically relied on morphological observations of increased autophagy during non-apoptotic cell death. Abiotic stress has been reported to cause autophagic cell death in parasitic protozoa[61–64]. However, autophagy has a central role in responding to stress, so whether autophagy activation has occurred in these cells to offer protection rather than mediate cell death is controversial[65]. To resolve this issue, it would be essential to show that this cell death occurs with increased autophagic flux and to combine this with definitive evidence implicating autophagy as an active cell death mechanism (for example, evidence that genetically and/or chemically inhibiting the autophagy pathway blocks this cell death)[1]. Such conclusive evidence has yet to be reported for parasitic protozoa, and indeed very few instances of bona fide autophagic cell death have been cited for any organism or cell, which could reflect the rarity of this type of death pathway[66]. Autophagy has been suggested as the mechanism that mediates cell death during starvation of procyclic-form *T. brucei*[59] and intracellular *T. gondii* tachyzoites[67], but more robust analysis is required to provide unambiguous evidence. The difficulties

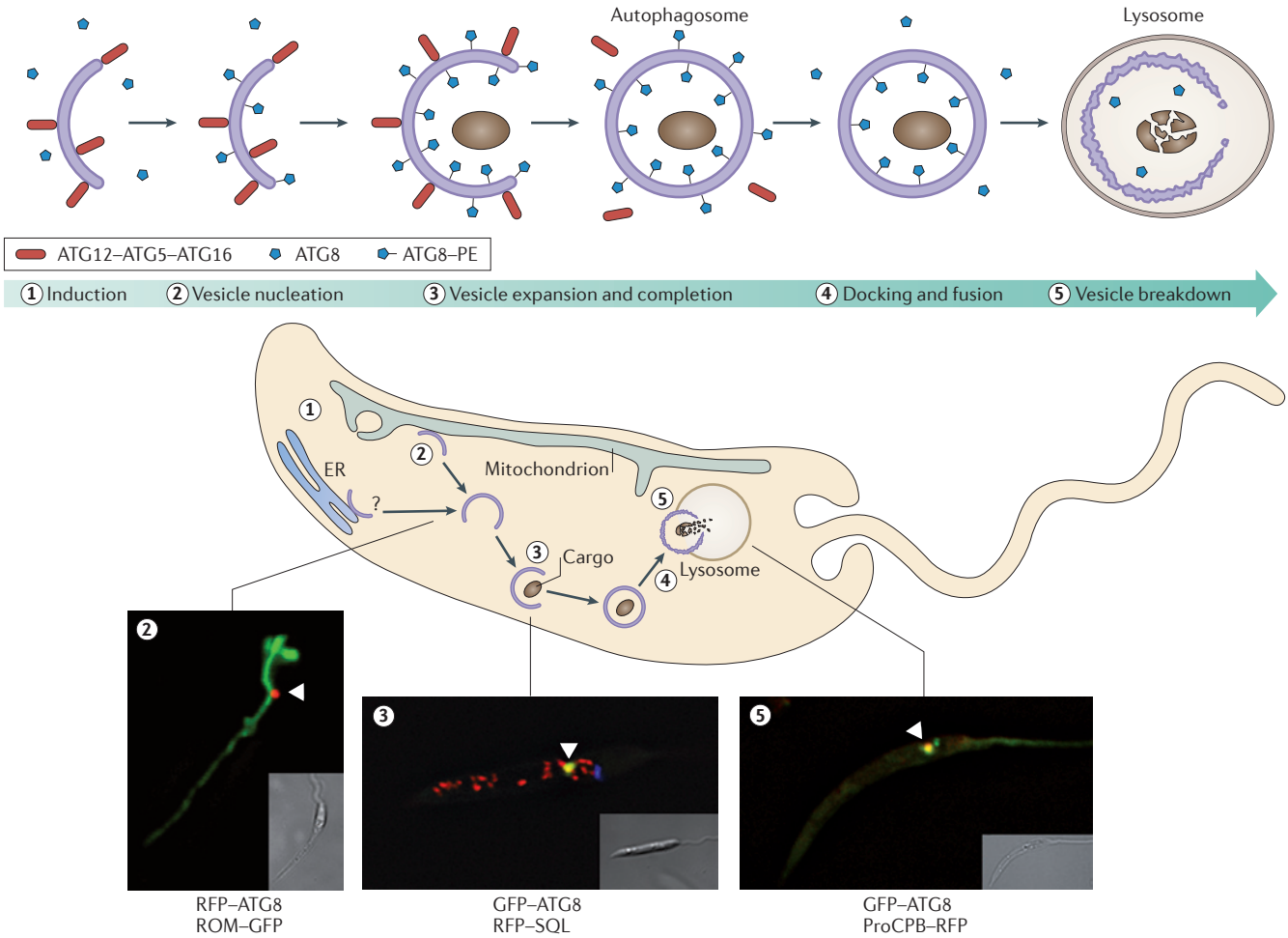


Figure 3 | **Autophagy in *Leishmania* spp.** The biogenesis and fate of autophagosomes in *Leishmania* spp., and images of different stages of autophagy within the parasite, obtained by fluorescence microscopy. After upstream signals induce autophagy (step 1), vesicle nucleation is initiated (in many cases, on the mitochondrial membrane) via the action of two ubiquitin-like conjugation systems involving autophagy-related protein 12 (ATG12) and ATG8 (step 2). This can be visualized by fluorescence microscopy with RFP-tagged ATG8 (arrow head) and the mitochondrial marker rhomboid (ROM) tagged with GFP[57] (bottom left panel). Following vesicle expansion, cargo is ensnared into autophagosomes (step 3), as can be visualized by the colocalization (arrow head) of GFP–ATG8-labelled autophagosomes and RFP tagged with the glycosomal import signal Ser-Gln-Leu (bottom middle panel). These autophagosomes are then trafficked to the lysosome. Cleavage of ATG8 from the surface of the autophagosome by ATG4 (not shown) allows the autophagosome to fuse with the lysosome and be degraded (steps 4 and 5), as indicated by the colocalization (arrow head) of GFP–ATG8 and the lysosomal marker procysteine peptidase B (ProCPB)–RFP (bottom right panel). Bottom panel images courtesy of B. Cull, University of Glasgow, UK.

in confidently identifying autophagic cell death are highlighted by the concerns regarding the effectiveness of 3-methyl adenine as a chemical autophagy inhibitor in *T. gondii*[58] and apparent contradictions in the absolute requirement for a functional autophagy pathway for the *T. brucei* cell death phenotype[59]. Nevertheless, given the current lack of evidence for biochemical pathways involved in other regulated cell death modalities in parasitic protozoa and the fact that the autophagy apparatus has been clearly shown to exist and operate in these organisms, it is tempting to consider that autophagic cell death might indeed be a form of regulated cell death that occurs in parasitic protozoa.

Implications and future directions

It is our opinion that until lethal signalling pathways and execution mechanisms are identified and shown to act in parasitic protozoa, it is difficult to view protozoan cell death as anything but incidental cell death or unregulated necrosis. This viewpoint does not imply that other mechanisms do not operate, and the theoretical reasoning that altruistic cell death occurs[14] makes a logical, if not compelling, case. We therefore suggest that to avoid confusion when describing cell death processes in parasitic protozoa, careful consideration should be given to the nomenclature used[1]. The current published data suggest to us that apoptosis, apoptosis-like cell death, programmed cell death and autophagic-like cell death are currently inappropriate terms for describing cell death processes in parasitic protozoa. In other fields, it has been valuable and necessary to formulate key criteria that form the benchmark for defining regulated cell death and autophagy pathways[1,5]. Accordingly, we suggest that to convincingly demonstrate regulated cell death in parasitic protozoa, it must be shown that the death process can be delayed or abolished by targeting key signalling or execution pathways. This could be achieved by genetic manipulation (for example, using RNAi or gene overexpression, or creating gene knockouts) or chemical perturbation (either inhibition or activation) of specific pathways. We believe

that such approaches should be actively adopted to discover whether cell death can be regulated in parasitic protozoa and, if it is, to elucidate the mechanisms that operate in this death.

This Opinion article is not intended to be overtly negative to the idea of regulated cell death in protozoan parasites. There are good reasons to study cell death mechanisms in these organisms, and the current data show that these death mechanisms are not identical to those operating in higher eukaryotes, perhaps suggesting that there are executioners and/or regulators that are specific for parasitic protozoa. This would not be surprising, given the large evolutionary divergence between protozoan parasites and mammals[68]. Perhaps shifting the focus away from established mammalian cell death subtypes and towards parasite-specific processes would be a beneficial, although undeniably challenging, line to pursue. To this end, system-wide analyses of parasitic protozoa during infections in the insect and mammalian hosts could provide useful information about natural cell death and also answer the question of whether senescence is regulated. Such insights could ultimately lead to the identification of key regulatory or executioner molecules that are central to regulated cell death. As well as helping to resolve the current confounding issues, such discoveries would potentially provide the basis of novel therapeutic strategies, an outcome that is most readily envisaged when there are distinct differences between the biology of the parasite and that of the mammalian host.

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Competing interests statement
The authors declare no competing financial interests.

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