Forum

Major cell death pathways at a glance

Linde Duprez a,b, Ellen Wirawan a,b, Tom Vanden Berghe a,b, Peter Vandenabeele a,b,*

a VIB, Department for Molecular Biomedical Research, Unit for Molecular Signaling and Cell Death, Technologiepark 927, B-9052 Ghent (Zwijnaarde), Belgium
b Ghent University, Department of Biomedical Molecular Biology, Unit for Molecular Signaling and Cell Death, Technologiepark 927, B-9052 Ghent (Zwijnaarde), Belgium

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Abstract

Cell death is a crucial process during development, homeostasis and immune regulation of multicellular organisms, and its dysregulation is associated with numerous pathologies. Cell death is often induced upon pathogen infection as part of the defense mechanism, and pathogens have evolved strategies to modulate host cell death. In this review, we will discuss the molecular mechanisms and physiological relevance of four major types of programmed cell death, namely apoptosis, necrosis, autophagic cell death and pyroptosis.

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1. Introduction

Different types of cell death are often defined by morphological criteria, and are classified as apoptotic, necrotic, autophagic or associated with mitotic catastrophe. Additionally, cell death is defined based on enzymological criteria, including the involvement of different classes of proteases (caspases, calpains, cathepsins and transglutaminases) and

Abbreviations: AICD, activation-induced cell death; AIM2, absent in melanoma 2; Ambra-1, activating molecule in Beclin-1-regulated autophagy; ANT, adenine nucleotide translocator; Apaf-1, apoptosis protease activating factor-1; ASC, apoptotic speck protein containing a CARD; Bcl-2, B-cell lymphoma-2; BH3, Bcl-2-homology 3; Bax interacting factor-1; CARD, caspase activation and recruitment domain; Cardinal, CARD inhibitor of NF-kB activating ligands; cFLIP, cellular FLICE-like inhibitory protein; cIAP, cellular inhibitor of apoptosis protein; CypD, cyclophilin D; Cyt c, cytochrome c; DAMP, danger/damage-associated molecular pattern; DED, death effector domain; DISC, death-inducing signaling complex; Diablo, direct IAP binding protein with low pI; DIF-1, differentiation-inducing factor-1; ER, endoplasmic reticulum; FADD, Fas-associated death domain; FIP200, focal adhesion kinase family interacting protein of 200 kD; HIN-200, hematopoietic interferon-inducible nuclear protein with a 200-amino-acid repeat; HIV-1, human immunodeficiency virus-1; HtrA2, high temperature requirement protein A2; IAP, inhibitor of apoptosis protein; ICE, interleukin-1 converting enzyme-β; IL-1β, interleukin-1β; IPaf, ICE-protease activating factor; I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein light chain 3; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; LRR, leucine-rich repeat; MAPK, mitogen activated protein kinase; MEF, mouse embryonic fibroblast; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; mTOR, mammalian target of rapamycin; NACHT, nucleotide binding and oligomerization domain; Nalp, NACHT/LRR/PYD-containing protein; Nec-1, necrostatin-1; NF-kB, nuclear factor kB; NLR, NOD-like receptor; nNOS, neuronal nitric oxide synthase; PAMP, pathogen-associated molecular pattern; PAR, poly(ADP-ribose); PARP-1, poly(ADP-ribose) polymerase-1; PE, phosphatidylethanolamine; PI3P, phosphatidylinositol-3-phosphate; PI3K, phosphatidylinositol-3-kinase; PI3K, phosphatidylinositol-3-kinase class 3; Pycard, PYD and CARD domain containing protein; PRR, pathogen recognition receptor; PS, phosphatidylserine; PYD, pyrin effector domain; RIP, receptor interacting protein; RLR, Rig-I-like receptor; RNAi, RNA interference; ROS, reactive oxygen species; Smac, second mitochondria-derived activator of caspase; TRADD, TNF receptor-associated death domain; TRAF2, TNF receptor-associated factor 2; TRAIL, TRAIL-related apoptosis-inducing ligand; TRAIL-R, TRAIL-receptor; ULK, UNC-51-like kinase; UVRAG, UV radiation resistance-associated gene; VPS34, vacuolar protein sorting 34; XIAP, X-linked inhibitor of apoptosis protein; zV AD-fmk, benzoxycarbonyl-Val-Ala-Asp-fluoro-methylketone.

* Corresponding author. VIB, Department for Molecular Biomedical Research, Unit for Molecular Signaling and Cell Death, Technologiepark 927, B-9052 Ghent (Zwijnaarde), Belgium. Tel.: +32 9 3313763; fax: +32 9 3313609.

E-mail address: peter.vandenabeele@dmbr.vib-ugent.be (P. Vandenabeele).

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nucleases, on functional aspects (programmed or accidental, physiological or pathological) or on immunological characteristics (immunogenic or non-immunogenic) [1]. The present paradigm is that caspase-dependent apoptosis is the predominant cell death pathway, but (1) that caspase-independent mechanisms can cooperate with (or substitute for) caspases in the execution of lethal signaling pathways, (2) that the major necrotic cell death pathway is mediated through the serine/threonine kinases receptor interacting protein 1 (RIP1) and 3 (RIP3), (3) that signaling involved in caspase-1-mediated cell death (termed pyroptosis) differs from classical caspase-dependent apoptosis, and (4) that “autophagic cell death” is a type of cell death occurring together with (but not necessarily by) autophagic vacuolization. In this review, we will discuss the four major forms of programmed cell death at the molecular, cellular and physiological levels.

2. Apoptotic cell death

In 1972, the term “apoptosis” was used for the first time to describe a form of cell death associated with specific morphological features. Since then, apoptosis has been extensively studied and underlying signaling events are now well characterized. Morphologically, apoptosis is associated with cell shrinkage, membrane blebbing, and chromatin condensation. It is a cell-intrinsic programmed suicide mechanism that results in the controlled breakdown of the cell into apoptotic bodies, which are subsequently recognized and engulfed by surrounding cells and phagocytes. Two main evolutionarily conserved protein families are involved in apoptosis, namely the Bcl-2 family of proteins, which control mitochondrial integrity [2], and the cysteiny1 aspartate-specific proteases or caspases, which mediate the execution phase of apoptosis [3].

2.1. Molecular mechanisms of apoptosis

In humans, twelve caspases belonging to the apoptotic and the inflammatory subfamilies of caspases have been described. Apoptotic caspases can be further subdivided into initiator (caspase-2, -8, -9, -10) and executioner (caspase-3, -6, -7) caspases. All caspases are expressed as inactive proenzymes consisting of an N-terminal prodomain of variable length, followed by a large subunit (p20) and a small C-terminal subunit (p10). The long prodomain of the initiator caspases contains protein–protein interaction motifs belonging to the death domain superfamily, namely death effector domains (DEDs) and caspase activation and recruitment domains (CARDs). Initiator caspases, present in the cell as inactive monomers, are recruited to platform molecules via these protein–protein interaction domains and are subsequently activated by oligomerization and proximity-induced autoproteolysis [4]. Short prodomain caspases exist as preformed dimers in the cell and their enzymatic activity requires proteolytic maturation by the action of upstream caspases [4].

In mammalian cells, caspases are activated by the intrinsic or the extrinsic apoptotic pathway (Fig. 1). The intrinsic pathway is activated by various stimuli, such as DNA damage and cytotoxic insults, and acts through the mitochondria, which are controlled by the Bcl-2 family of proteins [2]. In homeostatic conditions, the anti-apoptotic Bcl-2 family members maintain mitochondrial integrity by preventing the pro-apoptotic multidomain Bcl-2 family members Bax and Bak from causing mitochondrial damage. During cellular stress, Bcl-2-homology 3 (BH3)-only proteins are activated and antagonize the anti-apoptotic Bcl-2 family members. Consequently, the inhibition of Bax and/or Bak is relieved, leading to their oligomerization and formation of a channel through which cytochrome c (cyt c) is released into the cytosol. Then, cyt c associates with Apaf-1 and ATP, forming a platform for recruitment and activation of procaspase-9, also known as the apoptosome [5]. Active caspase-9 cleaves and activates the downstream executioner caspases-3, -6 and -7, which are crucial for the execution of apoptotic cell death. In addition, other pro-apoptotic proteins released from the mitochondria contribute to the cellular suicide mechanism. For example, Smac/Diablo antagonizes the action of inhibitor of apoptosis proteins (IAPs) such as XIAP, cIAP1, and cIAP2 [6]. Binding of Smac/Diablo to XIAP relieves its inhibitory interaction with caspase-9, -3 and -7. cIAP1 and cIAP2, unlike XIAP, do not directly inhibit caspases. Instead, they interact with tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2), which leads to their recruitment to TNFR1. There, they contribute to TNF-induced NF-kB activation by mediating the ubiquitination of RIP1 [6]. The NF-kB-mediated expression of anti-apoptotic genes might account for the anti-apoptotic function of cIAP1 and cIAP2.

The extrinsic pathway of apoptosis is induced upon stimulation of death receptors belonging to the TNFR family, such as TNFR, Fas and TRAIL-R. Signaling by these receptors can induce a variety of cellular responses, including proliferation, differentiation and cell death. Apoptosis is induced by the formation of a death-inducing signaling complex (DISC). In this complex, Fas-associated death domain (FADD) recruits the initiator caspases-8 and/or -10 via homotypic death domain interactions [7]. In contrast to signaling induced by Fas and TRAIL-R, TNFR1 aggregation leads to the sequential formation of two complexes [8]. Complex I consists of TNFR1, TNFR-associated death domain (TRADD), TRAF2, RIP1, cIAP1 and cIAP2 and is formed at the plasma membrane. These proteins are important mediators of NF-kB and MAPKs. Endocytosis of TNFR1 is followed by the formation of complex II, which is analogous to the receptor-proximal DISC induced by FasL and TRAIL and includes TRADD, FADD, and caspase-8 and/or -10. Activation of caspase-8 and -10 leads to activation of the downstream executioner caspases. In addition, caspase-8-mediated cleavage of the BH3-only protein Bid amplifies the death receptor-induced cell death program by activating the mitochondrial pathway of apoptosis. Recently, it was found that TNF induces one of two distinct caspase-8 activation pathways, depending on the cellular condition [9]. One pathway is RIP1 independent and is primarily inhibited by cFLIP, a protease-dead caspase-8 homologue that competes
for caspase-8 binding to FADD. The other pathway was discovered in experiments using treatment with TNF and Smac mimetics and is absolutely dependent on kinase active RIP1. Smac mimetics induce the autodegradation of cIAP1 and cIAP2, leading to release of RIP1 from the receptor complex to form a caspase-8 activating platform consisting of RIP1, FADD, and caspase-8 [9].

2.2. Physiological relevance of apoptosis

During development, apoptotic cell death has an important role in organ and tissue remodeling, e.g. in development of the lens, in shaping of the inner ear, in cardiac morphogenesis, in muscle development, and in removal of interdigital webs [10]. Furthermore, establishment of both the nervous and the immune systems are characterized by initial overproduction of cells and subsequent elimination of excess cells by apoptosis [11]. Apoptosis is crucial in maintaining homeostasis in the adult organism, as in post-lactational mammary gland regression, ovarian follicular atresia and post-ovulatory regression, and in terminating an immune response by eliminating of activated immune cells [11]. Because apoptotic cells are recognized and taken up by phagocytes before they lose plasma membrane integrity, it is generally believed that apoptotic cell death is immunologically silent and does not provoke inflammation [12].

Apoptosis of host cells during bacterial or viral infection functions as a defense mechanism by destroying the site of pathogen replication. Pathogens have therefore evolved several ways to prevent apoptosis, e.g. by protecting the mitochondria and preventing cyt c release, by activating cell survival pathways, or by preventing caspase activation [13,14]. Conversely, pathogens may also benefit from apoptosis, either by inducing apoptosis of infected cells, thereby favoring systemic infection, or by killing uninfected immune cells, thereby evading the host defense [13,14].

Dysregulation of apoptosis can result in the development of various pathologies. Insufficient apoptosis can lead to the
development of cancer and autoimmune diseases. Excessive or inappropriate apoptosis, on the other hand, contributes to the injury that accompanies several diseases, such as sepsis, stroke, myocardial infarction, ischemia, neurodegenerative diseases, and diabetes.

3. Necrotic cell death

For a long time, necrosis has been considered an accidental and uncontrolled form of cell death lacking underlying signaling events. This might be true for cell death resulting from severe physical damage, such as hyperthermia or detergent-induced cytolysis. However, accumulating evidence supports the existence of caspase-independent cell death pathways that can function even in a strictly regulated developmental context, such as interdigital cell death [15]. Necrotic cell death is characterized by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space.

3.1. Molecular mechanisms of necrosis

Over the past decade, it has become evident that in certain conditions, necrosis is the result of a strictly regulated interplay of signaling events, which are initiated by a diverse range of stimuli (Fig. 2). In most cell lines, death receptor ligands activate apoptosis rather than necrosis as the default cell death pathway. However, if caspase activation in this pathway is hampered, necrotic cell death might ensue instead, acting as a kind of back-up cell death pathway. zVAD-fmk is frequently used as a potent inhibitor of caspases, but off-target effects can also contribute to caspase-independent cell death. For example, zVAD-fmk binds and blocks the adenine nucleotide translocator (ANT), inhibits other proteases such as cathepsins, and generates the highly toxic fluoracetate due to metabolic conversion of the fluoromethylketone group [16,17].

FADD remains a crucial adaptor protein in Fas- and TRAIL-R-induced necrosis, but the importance of FADD in TNF-induced necrosis is controversial [18,19]. Recently, with the generation of the TRADD knockout mouse, it was demonstrated that TRADD is essential for TNF-induced necrosis in MEF cells [20]. RIP1 is a crucial initiator of death receptor-mediated necrosis [21] and the term necroptosis was introduced to designate programmed necrosis that depends on RIP1 [22]. The kinase activity of RIP1 is dispensable for the activation of NF-κB and MAPKs, but is required for necroptosis [19,22,23]. Necrostatin-1 (Nec-1) was identified as a small molecule inhibitor of necroptosis [22], and more recently, the RIP1 kinase activity was found to be the target of

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**Fig. 2. Schematic representation of necrotic signaling.** Stimulation of e.g. TNFR1 leads to the activation of RIP1, which induces a pro-survival pathway by activating transcription factors, such as NF-κB and AP-1. In addition, RIP1 interacts with RIP3 and both are crucial initiators of death receptor-induced necrotic signaling. Through RIP1 kinase activity, a wide range of necrotic mediators are activated, such as ROS, calcium, calpains, cathepsins, phospholipases, NO and ceramide. The same mediators can be activated by DNA damage or by triggering of TLR-3, TLR-4 and Nalp-3. See text for detailed sequence of events.
Nec-1 [24]. Furthermore, recent studies identified RIP3 as a crucial upstream activating kinase that regulates RIP1-dependent necroptosis [25–27]. TNF treatment induced the formation of a RIP1–RIP3 pro-necrotic complex and the kinase activity of both RIP1 and RIP3 was crucial for stable complex formation and subsequent induction of necrosis. During death receptor-induced apoptosis, RIP1 and RIP3 are cleaved by caspase-8, which suppresses their anti-apoptotic and/or pro-necrotic properties [28,29].

Besides death receptor-mediated necrosis, triggering of pathogen recognition receptors (PRRs) can also lead to necrotic cell death. Receptors of this family include the transmembrane toll-like receptors (TLRs), the cytosolic NOD-like receptors (NLRs) and the RIG-I-like receptors (RLRs). They all recognize pathogen-associated molecular patterns (PAMPs) found in bacteria or viruses, such as LPS, flagellin and double-stranded RNA (dsRNA), and stimulation of these receptors leads to the activation of innate immunity and/or cell death. In Jurkat cells and L929 cells, the recognition of synthetic dsRNA by TLR3 induces necrotic cell death, which was suggested to be RIP1-dependent [30]. TLR4 is expressed on macrophages and monocytes and is critical for the recognition of LPS from Gram-negative bacteria. Impeding caspase-8 activation switches TLR4-induced cell death from apoptosis to RIP1-dependent necrosis [31]. Pathogen-induced activation of NLRs results most commonly in caspase-1-dependent cell death or pyroptosis (see below). However, a recent report showed that the NLR member Nalp-3 mediates necrotic cell death of macrophages infected with Shigella flexneri [32]. RLR-induced activation of NF-κB and production of type I interferons are both dependent on FADD, RIP1 and TRADD [33,34]. Whether these proteins are also involved in RLR-induced cell death is unknown.

Extensive DNA damage causes hyperactivation of poly(ADP-ribose) polymerase-1 (PARP-1) and leads to necrotic cell death [35]. When DNA damage is moderate, PARP-1 participates in DNA repair processes. However, excessive PARP-1 activation causes depletion of NAD⁺ by catalyzing the hydrolysis of NAD⁺ into nicotinamide and poly(ADP-ribose) (PAR), leading to ATP depletion, irreversible cellular energy failure, and necrotic cell death. PARP-1-mediated cell death requires the activation of RIP1 and TRAF2 [36]. However, the mechanism by which these molecules sense the activation of PARP-1 has not been elucidated.

Many mediators are involved in the execution phase of necrotic cell death, including reactive oxygen species (ROS), calcium (Ca²⁺), calpains, cathepsins, phospholipases, and ceramide [37]. Oxidative stress leads to damage of cellular macromolecules, including DNA, proteins, and lipids. As discussed earlier, excessive DNA damage results in hyperactivation of PARP-1 and necrotic cell death. Modification of proteins by ROS leads to loss of the normal functions of proteins and enhances their susceptibility to proteolytic degradation. Other targets of ROS are the polyunsaturated fatty acid residues in the membrane phospholipids, which are extremely sensitive to oxidation. In mitochondria, lipid peroxidation affects vital mitochondrial functions. In addition, it destabilizes the plasma membrane and intracellular membranes of endoplasmic reticulum and lysosomes, leading to intracellular leakage of Ca²⁺ and lysosomal proteases, respectively. Among the different ROS, hydrogen peroxide (H₂O₂) plays a particularly important role because it diffuses freely across cellular membranes and can interact with iron in the Fenton reaction [37]. This reaction is favored in the lysosomes, because they are rich in free iron and do not contain H₂O₂-detoxifying enzymes. The resulting highly reactive hydroxyl radicals are among the most potent inducers of lipid peroxidation.

Ca²⁺ overload of mitochondria causes mitochondrial permeability transition (MPT) by the opening of large nonselective pores (the so called mitochondrial permeability transition pores, MPTPs) connecting the cytosol with the mitochondrial matrix [38]. MPT is accompanied by mitochondrial inner membrane depolarization, uncoupling of oxidative phosphorylation, matrix swelling, and outer mitochondrial membrane rupture [38]. If most mitochondria of the cell are disrupted, and glycolytic sources of ATP are inadequate, the cell becomes profoundly ATP-depleted. Cyclophilin D (CypD) might have an important role in MPT, as inhibition of CypD renders cells resistant to MPT, and CypD-deficient mice are more resistant to ischemic injury than wild type mice [39,40]. Besides affecting mitochondrial respiration, Ca²⁺ overload can activate phospholipases, proteases and neuronal nitric oxide synthase (nNOS), all of which contribute to the execution phase of necrotic cell death. For example, calpains are activated by elevated Ca²⁺ levels, which then cleave the Na⁺/Ca²⁺ antipporter in the plasma membrane, resulting in a sustained Ca²⁺ overload. Strong activation of calpains may also contribute to the release of cathepsins in the cytosol by causing lysosomal membrane permeabilization, as proposed in the “calpain–cathepsin” hypothesis by Yamashima and colleagues [41].

### 3.2. Physiological relevance of necrosis

Although apoptosis is indispensable for tissue remodeling during embryogenesis, necrosis can, at least in some conditions, substitute for apoptosis to eliminate unwanted cells. For example, removal of interdigital cells during the development of digits in the presence of the caspase inhibitor zVAD-fmk or in Apaf-1−/− mice occurs by a caspase-independent necrotic-like process [15]. Necrosis is also involved in physiologically relevant signaling processes, such as ovulation, the death of chondrocytes associated with the longitudinal growth of bones, and cellular turnover in the small and large intestines [42]. In addition, necrotic cell death participates in activation-induced cell death (AICD) of T lymphocytes, which is an important mechanism for reducing T cell numbers after an immune response [19]. However, it is important to point out that in most of the above-mentioned pathways, necrotic cell death is always observed together with apoptosis or in the presence of caspase inhibitors, suggesting that it functions as a back-up mechanism and is never the sole cell death pathway.
Necrotic cell death is often associated with pathological conditions. Necrosis has been observed during ischemia/reperfusion (I/R), which can lead to injury of organs, including heart, brain, liver, kidney, and intestine [43]. Necrotic cell death also contributes to excitotoxicity, which may be involved in stroke, traumatic brain injury, and neurodegenerative disorders [44]. More specifically, using Nec-1, it was shown that RIP1-dependent necrotic cell death or necroptosis contributes to a wide range of pathological cell death events, such as ischemic brain injury [22] and myocardial infarction [45]. Furthermore, RIP3−/− mice failed to initiate vaccinia virus-induced tissue necrosis and inflammation, resulting in much more viral replication and mortality [26]. Several other reports also illustrate the occurrence of necrotic cell death during infection by other pathogens, such as Shigella, HIV-1, West Nile virus, and Coxackievirus B [37]. In addition, patients carrying a disease-associated mutation in Nalp-3 show excessive necrotic-like cell death with features similar to the Shigella flexneri-induced Nalp-3-dependent necrosis [32].

In contrast to apoptosis, the recognition and uptake of necrotic cells by macrophages is slower, less efficient and occurs only after the loss of plasma membrane integrity [46]. As a result, necrotic cells initiate a proinflammatory response by the passive release of DAMPs (danger/damage-associated molecular patterns) [47]. In addition, necrotic cells actively secrete inflammatory cytokines due to the activation of NF-κB and MAPKs [48].

4. Autophagic cell death

Autophagy is an evolutionarily conserved catabolic pathway that allows eukaryotes to degrade and recycle cellular components. Proteins and organelles are sequestered in specialized double-membrane vesicles, designated autophagosomes, which are typical of autophagic cells. Basal levels of autophagy ensure the maintenance of intracellular homeostasis; however, many studies have revealed its diverse functions in important cellular processes, such as cellular stress, differentiation, development, longevity, and immune defense. Although a pro-survival role for autophagy is well-established, frequently debated is whether or not autophagy has a causative role in cell death. The presence of autophagic vacuoles in dying cells has led to the introduction of autophagic cell death, although autophagy often accompanies rather than causes cell death. It is plausible that massive autophagic activity could result in cellular demise. In addition, several interconnections exist between autophagy and apoptotic or necrotic cell death [49].

4.1. Molecular mechanisms of autophagy

Several types of autophagy have been described, including chaperone-mediated autophagy, microautophagy and macroautophagy. Macroautophagy (hereafter referred to as autophagy) typically occurs in severe stress conditions and is therefore the best characterized (Fig. 3). Macroautophagy functions by de novo formation of specialized vacuoles, the autophagosomes, in which the proteins are sequestered before being targeted to the lysosomes. So far, 30 autophagy-related (atg) genes have been identified in yeast, and several mammalian homologues have been isolated and functionally characterized [50].

The classical pathway of autophagic signaling acts through mTOR (mammalian target of rapamycin), a protein kinase that is important in controlling translation and cell-cycle progression and in negative regulation of autophagy. When mTOR is inhibited in response to starvation or treatment with rapamycin, the ULK-Atg13-FIP200 complex is activated, leading to the induction of autophagosome formation [51]. The synthesis of autophagic vacuoles requires vesicle nucleation, which is initiated by the assembly of another complex, the PI3KC3 (Vps34)-complex. Within this complex, Beclin-1 (Atg6) serves as a platform for binding PI3KC3 (Vps34), UVRAG, Bif-1 and Ambra-1, all of which positively regulate PI3KC3 activity [52]. Interestingly, Bcl-2, amongst other anti-apoptotic Bcl-2 family members, represses autophagy by binding of Beclin-1 and thereby abrogating autophagic signaling [53]. During starvation, Bcl-2 becomes phosphorylated, which releases Beclin-1 and stimulates autophagy [54]. Activation of the PI3KC3 (Vps34)-complex finally results in the generation of PI3P. Consequently, other Atg proteins that mediate vesicle membrane elongation are recruited. Two ubiquitin-like conjugation systems are implicated in this process. During a first conjugation step, an Atg12–Atg5–Atg16L multimediator complex that could be involved in vesicle curvature is formed [55]. During a second conjugation step, LC3 (Atg8) is lipidated by binding to phosphatidylethanolamine (PE). In contrast to the cytoplasmic localization of LC3, LC3–PE (mostly referred to as LC3-II) specifically binds to the autophagic membranes, and for that reason it is generally used as an autophagic marker. Finally, the autophagosome fuses with a lysosome, releasing its autophagic content into the lysosomal lumen for degradation by hydrolases.

The outcome of autophagic signaling mostly reflects its pro-survival function. In homeostasis, autophagy exerts its housekeeping role in cytoplasmic and protein turnover, while during stress, cells are protected from dying by elimination of harmful organelles and protein aggregates. However, several studies suggest a role for autophagy in cell death [56]. In cells in which apoptotic signaling is perturbed, a necrotic-like cell death is often induced as a back-up cell death mechanism, which can be associated with autophagy, as monitored by LC3-II accumulation. For example, treatment of apoptosis-deficient Bax/Bak double knockout MEF cells with DNA-damaging or ER-stress-inducing agents causes non-apoptotic cell death, which depends on the presence of Beclin-1 and Atg5 [57]. Another interesting study shows the involvement of FADD and caspase-8 in controlling autophagic signaling in proliferating T cells [58]. T cells lacking FADD or caspase-8 undergo extensive autophagy and succumb to RIP1-dependent necrotic cell death, but inhibition of autophagy rescued the T cells. Moreover, treatment with Nec-1 repressed LC3-II accumulation and prevented T cells from dying [58]. Similarly, in L929 cells, caspase inhibition with zVAD-fmk or caspase-8 RNAi also resulted in
a RIP1-dependent autophagic cell death [59]. Cell death was caused by severe ROS accumulation and cell damage. Surprisingly, elevated ROS after zVAD-fmk treatment was dependent on autophagy, which selectively degraded catalase, a major key enzyme in the anti-oxidant defense mechanism [60]. However, another group recently reported compelling data supporting a pro-survival instead of a cell killing role for autophagy in zVAD-fmk-induced cell death [61]. Rapamycin, a potent inducer of autophagy, blocked zVAD-fmk-induced cell death, whereas chloroquine, a lysosomal enzyme inhibitor, greatly sensitized L929 cells for this form of cell death. The authors suggested that zVAD-fmk, in addition to inhibiting caspases, also blocks cathepsins, resulting in decreased autophagosomal degradation and hence the appearance of typical autophagic hallmarks.

4.2. Physiological relevance of cell death associated with autophagy

Autophagic cell death has been described primarily in developmental processes that require extensive cell destruction
and elimination. During metamorphosis in *Drosophila*, death of salivary gland cells is associated with upregulation of *atg* genes and induction of autophagy, though this is accompanied with caspase activation and the appearance of typical apoptotic features, including DNA fragmentation [62]. However, studies of Atg8 mutant flies or flies overexpressing dominant negative Atg1 support the hypothesis that autophagy is needed for proper salivary gland degradation. Moreover, transgenic overexpression of Atg1 in salivary glands is sufficient to induce autophagic cell death, which is independent of caspase activation [63].

The protist, *Dictyostelium*, does not contain any caspase genes, implying that cell death in this organism is by default caspase-independent [64]. It has been shown that the autophagy-like cell death occurs in monolayer cultures of *Dictyostelium* cells subjected to starvation and differentiation-inducing factor DIF-1, mimicking the development of fruiting bodies. These fruiting bodies contain a mass of spores supported by a stalk composed of extensively vacuolated dead cells. However, *atg* gene disruption blocks autophagic vacuolization, but does not suppress cell death [65], suggesting that cell death occurs independently of autophagic signaling or that redundant cell death pathways exist. The non-vacuolar cell death in Atg1 mutants possesses classical characteristics of necrotic cell death [66].

Increasing evidence suggests a role for autophagy in cell death during ischemia/reperfusion (I/R). For example, Atg7-deficiency in neurons protects against neonatal hypoxic/ischemic brain injury [67]. Similarly, inhibition of autophagy blocks cardiac myocyte death after I/R and the size of myocardial infarction is significantly decreased in Beclin-1+/− mice [68]. Autophagy might also contribute to virus-induced cell death. For example, HIV-1 induces apoptotic cell death in uninfected bystander CD4+ T lymphocytes. However, cell death is associated with typical autophagic features and inhibition of autophagy significantly blocks T cell death [69].

Autophagy has also been implicated in another aspect of cell death, namely apoptotic cell clearance. Both Atg5−/− and Beclin-1−/− cells fail to express phosphatidylserine (PS) ("eat-me" signal) and secrete less lysophosphatidylcholine (LPC) ("come-and-get-me" signal) [70]. Interestingly, both PS and LPC exposure require ATP, suggesting that autophagy contributes to the production of engulfment signals by maintaining cellular ATP levels. Because effective engulfment also requires PS exposure on the phagocytes [71], it is possible that intact autophagy is also needed for the proper phagocytic function of the engulfment cells.

### 5. Pyroptosis

Pyroptosis is a more recently recognized form of regulated cell death with morphological and biochemical properties distinct from necrosis and apoptosis [72]. Pyroptosis has been described in monocytes, macrophages and dendritic cells infected with a range of microbial pathogens, such as *Salmonella, Francisella* and *Legionella*, and is uniquely dependent on caspase-1 [73]. In addition, non-infectious stimuli, such as DAMPs, can induce pyroptosis in non-macrophage cells.

#### 5.1. Molecular mechanisms of pyroptosis

Caspase-1, previously known as Interleukin-1 (IL-1β) Converting Enzyme (ICE), was the first mammalian caspase to be identified. As a member of the inflammatory caspses, it is not involved in apoptotic cell death [74]; vice versa, the apoptotic caspses usually do not contribute to pyroptosis [75]. Like all caspses, caspase-1 is present in the cytosol as an inactivezymogen. In analogy to activation of caspase-9 in the apoptosome, caspase-1 is activated in a complex called the inflammasome. This molecular platform includes NLR family members that recruit caspase-1 through adapter molecules, such as ASC/PyCARD and is formed through homotypic interactions between these inflammasome components (Fig. 4). Until now, four inflammasomes have been characterized and named after their NLR (Nalp-1, Nalp-3 and Ipaf) or HIN-200 protein (AIM2) [73,76]. Assembly of the inflammasome occurs when NLRs are triggered by intracellular bacterial, viral or host danger signals. For example, Nalp-1 recognizes cytosolic delivery of *Bacillus anthracis* lethal toxin, Ipaf recognizes cytosolic flagellin, and Nalp-3 responds to multiple DAMPs and PAMPs [73] (Fig. 4). Most NLRs consists of three distinct domains: an N-terminal CARD domain or pyrin effector domain (PYD), a central nucleotide binding and oligomerization domain (NACHT), and several C-terminal leucine-rich repeats (LRRs). In addition, Nalp-1 has a C-terminal extension that harbors a CARD domain. In contrast to human Nalp-1, the mouse orthologue Nalp-1b does not contain an N-terminal PYD domain. Upon stimulation, NLRs undergo oligomerization through homotypic NACHT domain interactions. Subsequently, the NLRs associate with the adaptor protein ASC through homotypic PYD interactions. In addition, Nalp-3 associates with the adaptor Cardinal in its inflammasome. These adaptor molecules then recruit caspase-1 through CARD—CARD interactions, resulting in its oligomerization and proximity-induced activation.

Recently, the AIM2 inflammasome was identified [76]. Through its HIN domain, AIM2 can directly bind to dsDNA, resulting in the activation of caspase-1 and maturation of pro-IL-1β. The source of the cytoplasmic dsDNA appears unimportant for AIM2 activation because viral, bacterial, mammalian and synthetic dsDNA could all activate caspase-1 [76]. Double stranded DNA-dependent cell death depends on AIM2, ASC and caspase-1 and shows features of pyroptosis [77].

Active caspase-1 is the central executor of pyroptotic cell death and acts mainly by inducing the formation of discretely sized ion-permeable pores in the plasma membrane, as analyzed by the use of membrane impermeable dyes and osmoprotective experiments [78]. The resulting osmotic pressure leads to water influx, cell swelling and ultimately cell lysis. Furthermore, caspase-1 activation initiates an inflammatory response by the cleavage of the proinflammatory cytokines pro-IL-1β and pro-IL-18, which are released by the cell upon their activation [79]. However, this inflammatory response is not required for the execution of cell death [80].
Although caspase-1 activation is inherently associated with an inflammatory response, it is still unclear whether it is inevitably linked to pyroptotic cell death. Which molecular mechanisms are implicated in the bifurcation between caspase-1-dependent inflammation and caspase-1-mediated pyroptosis is also an open question.

The digestome of caspase-1 comprises more than 40 caspase-1 substrates, among which are chaperones, cytoskeletal and translation machinery proteins, proteins involved in immunity, and a series of unexpected proteins along the glycolysis pathway [81]. Whether these caspase-1-dependent cleavage events contribute to pyroptosis is unknown. In
addition, by using a proteomics approach to search for novel caspase-1 targets, caspase-7 was identified as a caspase-1 substrate [75]. Also, it was reported that caspase-7 processing induced by *Salmonella* or *Legionella* infection required inflammasome signaling [75,82]. Caspase-7+/− macrophages infected with *Legionella* showed defective delivery of the organism to the lysosome, delayed cell death and substantial *Legionella* replication [82]. However, a role for caspase-7 in pyroptosis should not be generalized, as caspase-7+/− macrophages infected with *Salmonella* were not protected from cell death [75].

Cells dying by pyroptosis have biochemical and morphological features of both apoptotic and necrotic cells [73]. Pyroptotic cells lose their mitochondrial membrane potential and plasma membrane integrity and release their cytoplasmic contents into the extracellular milieu. As in apoptosis, pyroptotic cells undergo DNA fragmentation and nuclear condensation. However, this caspase-1-dependent nuclease-mediated cleavage of DNA does not exhibit the oligonucleosomal fragmentation pattern characteristic of apoptosis [83]. In addition, the DNA damage and concomitant PARP-1 activation associated with pyroptotic cell death are not required for cell lysis to occur [78].

5.2. Physiological relevance of pyroptosis

As a cellular suicide program, pyroptosis is part of the host defense system for fighting off pathogens. Death of the host cell destroys the pathogenic niche, thereby limiting microbial replication and exposing the pathogen to other antimicrobial mechanisms. On the other hand, host cell death can be beneficial for certain pathogens, as in the elimination of immune cells. Pathogens have therefore evolved strategies to efficiently inhibit or to induce pyroptotic host cell death. For example, poxviruses encode PYD-containing proteins that interact with ASC in the inflammasome, thereby inhibiting caspase-1 activation and promoting infection [84].

Because of its dependence on caspase-1 activity, pyroptosis is associated with the initiation of a proinflammatory response, which is further amplified by the release of the cytoplasmic content upon cell lysis. Since NLR-mediated activation of caspase-1 affects several cellular pathways, it is difficult to distinguish the precise role of caspase-1 in the cell death process itself. Caspase-1-deficient mice are more susceptible than wild type mice to infection, for example by *Salmonella*, and these mice are more susceptible than IL-1β and IL-18 double knockout mice [85]. On the other hand, caspase-1-deficiency confers protection against *Escherichia coli*-induced sepsis, again in contrast to wild type mice and IL-1β and IL-18 double knockout mice [80]. These observations suggest that the phenotype associated with caspase-1-deficiency is not only due to lack of these proinflammatory cytokines but that pyroptosis itself or other caspase-1-dependent events are involved in the control of infection.

Inappropriate or overwhelming activation of caspase-1 is associated with the pathogenesis of several diseases, such as myocardial infarction, cerebral ischemia, neurodegenerative diseases, inflammatory bowel disease and endotoxic shock [73]. These diseases are all characterized by inflammation and cell death. Caspase-1-deficiency, or its pharmacological inhibition, results in protection against inflammation and cell death associated with these diseases [86]. These studies suggest that the level of caspase-1 activation after recognition of danger signals plays a role in the host response [73]. Low levels of caspase-1 initiate cell survival responses, control intracellular bacterial growth and stimulate inflammatory cytokine production. In contrast, higher levels of caspase-1 may lead to the induction of pyroptosis, and inappropriate or overwhelming caspase-1 activation contributes to several inflammatory conditions.

6. Concluding remarks

It has become clear over the past decade that apoptosis is not the only cell death program available to mammalian organisms for removal of unwanted cells. Apoptotic cell death is mediated by caspases, which are responsible for its typical morphological features. Accidental necrosis results from physicochemical damage without involvement of underlying signaling events, but necrosis can also be the result of a programmed interplay of signaling events leading to plasma membrane rupture. Apoptosis and necrosis differ in their inflammatory outcome. Whereas apoptosis is immunologically silent, necrosis results in the release of the cytoplasmic contents with consequent inflammatory responses. Autophagic cell death can have characteristics of both apoptotic and necrotic cell death. Whether autophagy represents a distinct form of cell death or rather triggers or accompanies another form of cell death is debatable and needs further investigation. Pyroptosis is part of the host defense system to fight off pathogens and has morphological and biochemical properties distinct from necrosis and apoptosis. Because pyroptosis is associated with caspase-1-mediated maturation of pro-IL-1β and pro-IL-18 and ultimately results in release of the cytoplasmic content, it also elicits an immune response. As discussed throughout this review, all four forms of programmed cell death are involved in several pathological conditions. Better understanding of signaling events critical in these cell death pathways would not only give insight into disease pathogenesis, but could also lead to the development of new therapeutic strategies for treatment of cell death-related diseases.

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