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Review article

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A guide to cell death pathways

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Abstract

Regulated cell death mediated by dedicated molecular machines, known as programmed cell death, plays important roles in health and disease. Apoptosis, necroptosis and pyroptosis are three such programmed cell death modalities. The caspase family of cysteine proteases serve as key regulators of programmed cell death. During apoptosis, a cascade of caspase activation mediates signal transduction and cellular destruction, whereas pyroptosis occurs when activated caspases cleave gasdermins, which can then form pores in the plasma membrane. Necroptosis, a form of caspase-independent programmed necrosis mediated by RIPK3 and MLKL, is inhibited by caspase-8-mediated cleavage of RIPK1. Disruption of cellular homeostatic mechanisms that are essential for cell survival, such as normal ionic and redox balance and lysosomal flux, can also induce cell death without invoking programmed cell death mechanisms. Excitotoxicity, ferroptosis and lysosomal cell death are examples of such cell death modes. In this Review, we provide an overview of the major cell death mechanisms, highlighting the latest insights into their complex regulation and execution, and their relevance to human diseases.

Sections

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Introduction

Cell death is an essential component of organismal development and adult homeostasis. Elimination of superfluous cells in development is important to ensure normal morphogenesis and organogenesis. In adult life, elimination of auto-reactive immune cells, cancerous cells and damaged cells is essential to homeostasis. Either too much or too little cell death can lead to human disease — neurodegenerative diseases involve the death of neurons that should have been long-lived to maintain neurological functions, and one of the hallmarks of cancer is the failure to eliminate cells that carry cancerous mutations[1].

Different types of cell death can be classified on the basis of morphology. For example, apoptosis and necrosis have been described for their distinct morphological characteristics[2]. Apoptosis is characterized by cytoplasmic and nuclear condensation without compromising the integrity of the cytoplasmic membrane. In contrast, early loss of membrane integrity and the consequential expansion of the cytoplasmic compartment due to ionic imbalance are hallmarks of necrosis. The term 'programmed cell death' was proposed by Richard Lockshin and Carroll M. Williams in the 1960s to describe the degeneration of intersegmental muscles in Silkmoth during ecdysis (exoskeleton moulting), which implied genetic regulation of muscle degeneration[3,4], but insights into the molecular mechanisms of cell death came much later. Research in the last three decades has uncovered the dedicated molecular machinery that mediates apoptosis, necroptosis, and pyroptosis and identified the mammalian genes encoding its components. We propose to collectively refer to apoptosis, necroptosis and pyroptosis as 'programmed cell death'.

In addition to cell death resulting from the activation of programmed cell death pathways — as for apoptosis, necroptosis and pyroptosis — cells can die when key cell survival mechanisms that are required to maintain normal cellular homeostasis are inactivated or disrupted. Such examples may include the loss of cytoplasmic and/or intracellular membrane integrity, accumulation of misfolded proteins, excitotoxicity, oxidative stress and lipid peroxidation. Excitotoxicity results from the toxic accumulation of specific amino acids, such as glutamate, and occurs mainly in neurons[5]. Ferroptosis results from the inactivation of a cellular redox mechanism in defence against lipid peroxidation[6,7]. Pathological release of lysosomal acidic hydrolases, whose normal function is to break down macromolecules for cellular recycling, may lead to lysosomal cell death[8]. A cell may also be eaten alive by another cell in a process known as entosis[9].

As it is increasingly recognized that dysregulated cell death processes serve important roles in human diseases, substantial effort is being devoted to identifying the mechanisms of cell death involved in specific human pathological conditions — whether cell death results from the activation of programmed cell death mechanisms or the loss of cell survival mechanisms, or both. In this Review, we first provide an overview of the triggers, mechanisms and regulation of programmed cell death, including apoptosis, pyroptosis and necroptosis. We then describe the mechanisms underlying entosis, excitotoxicity, ferroptosis, lysosomal cell death and mitotic catastrophe, which are mediated by the disruption of different cellular homeostatic pro-survival mechanisms. We also discuss connections between these cell death modalities resulting from disruption of cell survival and programmed cell death. Lastly, we briefly discuss the latest insights into the involvement of cell death in human diseases.

Apoptosis

The machinery that mediates cell death was first characterized in *Caenorhabditis elegans*, and homologues of the *C. elegans* cell death effector proteins, which include caspases and BCL-2 proteins, were later identified in mammals (Box 1). Apoptosis of mammalian cells can be divided into intrinsic apoptosis and extrinsic apoptosis, which differ in how the apoptotic triggers are sensed and integrated by cells. Extrinsic apoptosis is mediated by the activation of death receptors localized on the plasma membrane, whereas intrinsic apoptosis is initiated without the involvement of death receptors (Fig. 1).

Intrinsic apoptosis

Intrinsic apoptosis can be activated following cellular alterations such as DNA damage, withdrawal of growth factors and mitochondrial damage (Fig. 1). Anoikis is also a form of intrinsic apoptosis that can be activated in certain cells by the loss of integrin-mediated cellular attachment to the extracellular matrix[10].

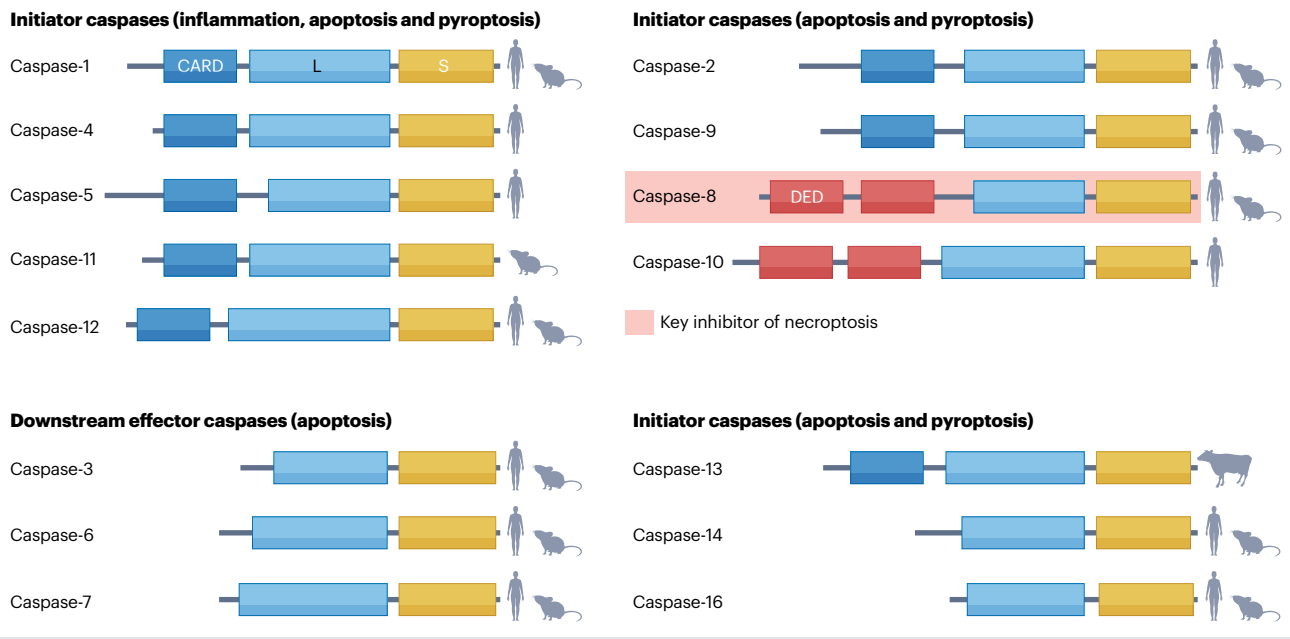
The BCL-2 family of proteins are the key upstream regulators of intrinsic apoptosis. BCL-2, a mammalian homologue of the *C. elegans* CED-9, was first isolated as an oncogene in human follicular lymphomas with the t(14;18) translocation and promotes cell survival rather than oncogenic cellular proliferation[11]. The mammalian BCL-2 family includes both anti-apoptotic as well as pro-apoptotic members, similar to that of CED-9 and EGL-1 in *C. elegans*[12,13]. The defining feature of the BCL-2 protein family, which includes both pro-apoptotic and anti-apoptotic family members, is the presence of one to four BCL-2 homology (BH) domains[12,14]. High levels of anti-apoptotic BCL-2 proteins suppress the activation of this cell death pathway. Apoptosis is crucial during animal development and adult life as genetic ablation of the anti-apoptotic proteins BCL-2, BCL-xL or MCL-1 can be lethal or lead to deleterious phenotypes in mutant mice[15–17]. The BH domains in the BCL-2 family mediate the interaction between pro-apoptotic and anti-apoptotic family members to inhibit or activate apoptosis[13]. In response to DNA damage, oxidative stress or nutrient deprivation, both transcriptional and post-translational activation of the pro-apoptotic BCL-2 family are critical in transducing the pro-apoptotic signal to drive mitochondrial outer membrane permeabilization (Fig. 1). In this process, pro-apoptotic BH3-only BCL-2 proteins, such as BIM, BID, BAD, NOXA and PUMA, utilize their BH3 domains to both actively inhibit anti-apoptotic BCL-2 proteins, such as BCL-2, BCL-xL, MCL-1 and BCL-W, and directly activate pro-apoptotic BAX and BAK to drive the formation of oligomeric pores in the mitochondrial outer membrane. The rupture of the outer mitochondrial membrane lead to the release of cytochrome c and second mitochondria-derived activator of caspase (SMAC, also known as DIABLO) to promote downstream caspase activation[14]. APAF1, the mammalian homologue of CED-4 and a member of the NOD family[18], forms a heptameric apoptosome[19,20] to promote the activation of caspase-9, which in turn mediates the cleavage of other downstream caspases, such as caspase-3 and caspase-7, that can cleave hundreds of protein substrates to promote cellular destruction[21] (Fig. 1). However, the view of a simple upstream and downstream cascade of caspases should be modified as the double knockout of caspase-3 and caspase-7 in mice can also delay mitochondrial damage and cell death[22]. Thus, apoptosis may be viewed as a circular cascade with the capability of self-amplification.

Activation of intrinsic apoptosis by DNA damage following ionizing irradiation or chemotherapeutic agents, such as etoposide and taxol, forms the basis of conventional cancer radiation therapies and chemotherapies. However, research over the past decade has uncovered redundancy in mammalian cell death mechanisms. Knockout of both *Bax* and *Bak1* in $Bax^{-/-}Bak1^{-/-}$ cells or $Bax^{-/-}Bak1^{-/-}$ double-knockout mice results in high resistance to apoptosis[23] and yet

Box 1

Mammalian caspase families

The first mammalian caspase, caspase-1, was identified as the IL-1β-converting enzyme involved in cleaving pre-IL-1β to generate mature IL-1β[218,219]. The molecular mechanism of programmed cell death in the nematode *Caenorhabditis elegans*, mediated by *Egl-1*, *Ced-9*, *Ced-3* and *Ced-4*, provides a prototypic example of apoptosis[220]. Mammalian caspases (see the figure) were found to be the sequence and functional homologues of *C. elegans Ced-3* in mediating apoptosis[221,222]. The genomes of mammals encode multiple caspases (the mouse genome encodes 11 caspases; the human genome encodes 13 caspases), which mediate cell death as well as inflammation[99]. Caspases are classified based on the prodomain length (short or long) and sequence features (CARD or DED) as well as the functions (initiator caspase or downstream caspase). Caspase-8 is an important inhibitor of necroptosis.



a small percentage of the mutant mice can still be born and survive for months[24]. This suggests that other cell death mechanisms may be activated and/or selected for in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells to eliminate unwanted cells when apoptosis fails during development. In addition, although DNA damage-induced cell death can activate apoptosis by promoting mitochondrial damage and downstream caspase activation, DNA damage-induced apoptosis may constitute only a part of the cell death response as severe DNA damage can lead to cell death by mitotic catastrophe (see below). Thus, when the programmed cell death mechanism is blocked, disruption of cellular homeostatic pro-survival mechanisms may contribute to cell elimination in mammals.

Extrinsic apoptosis

Extrinsic apoptosis is mediated by the activation of cytoplasmic membrane-localized death receptors that are characterized by the presence of an intracellular protein–protein interaction domain known as the Death domain (DD), such as Fas (also known as CD95), TNFR1, TRAIL (DR4) and TRAIL-R2 (DR5), by their cognate ligands[25,26] (Figs. 1 and 2). These pathways are relevant to human disease as genetic defects in Fas and its ligand FasL lead to a rare human autoimmune condition known as autoimmune lymphoproliferative syndrome, which can be phenocopied by Fas and FasL mutant mice[27–30]. FasL expressed in activated T cells induces apoptosis by promoting the recruitment of adaptor protein FADD and caspase-8 by interacting with the DD on Fas in target cells. Autoimmune lymphoproliferative syndrome-associated mutations in the Fas DD resulted in the loss of FADD recruitment and inhibition of caspase-8 activation. These findings underscore the importance of caspase-8 activation in Fas-mediated apoptosis[31].

Another example of death receptor-mediated extrinsic apoptosis that has important medical significance is TNF-mediated activation of TNFR1. Activation of TNFR1 by TNF leads to the rapid formation of a transient intracellular signalling complex, known as complex I, which plays a critical role in making the cellular life-or-death decision. The intracellular DD of activated TNFR1 recruits the DD-containing adaptor protein TRADD and RIPK1, a DD-containing kinase[32,33] (Fig. 2). TRADD, in turn, mediates the recruitment of TRAF2, TRAF5, cIAP1 and cIAP2, which catalyse K63-linked, K48-linked and K11-linked poly-ubiquitination on complex I[34–36]. The polyubiquitylated chains generated by cIAP1 and cIAP2 serve to recruit other complex I components such as the linear ubiquitin chain assembly complex (LUBAC). LUBAC comprises the

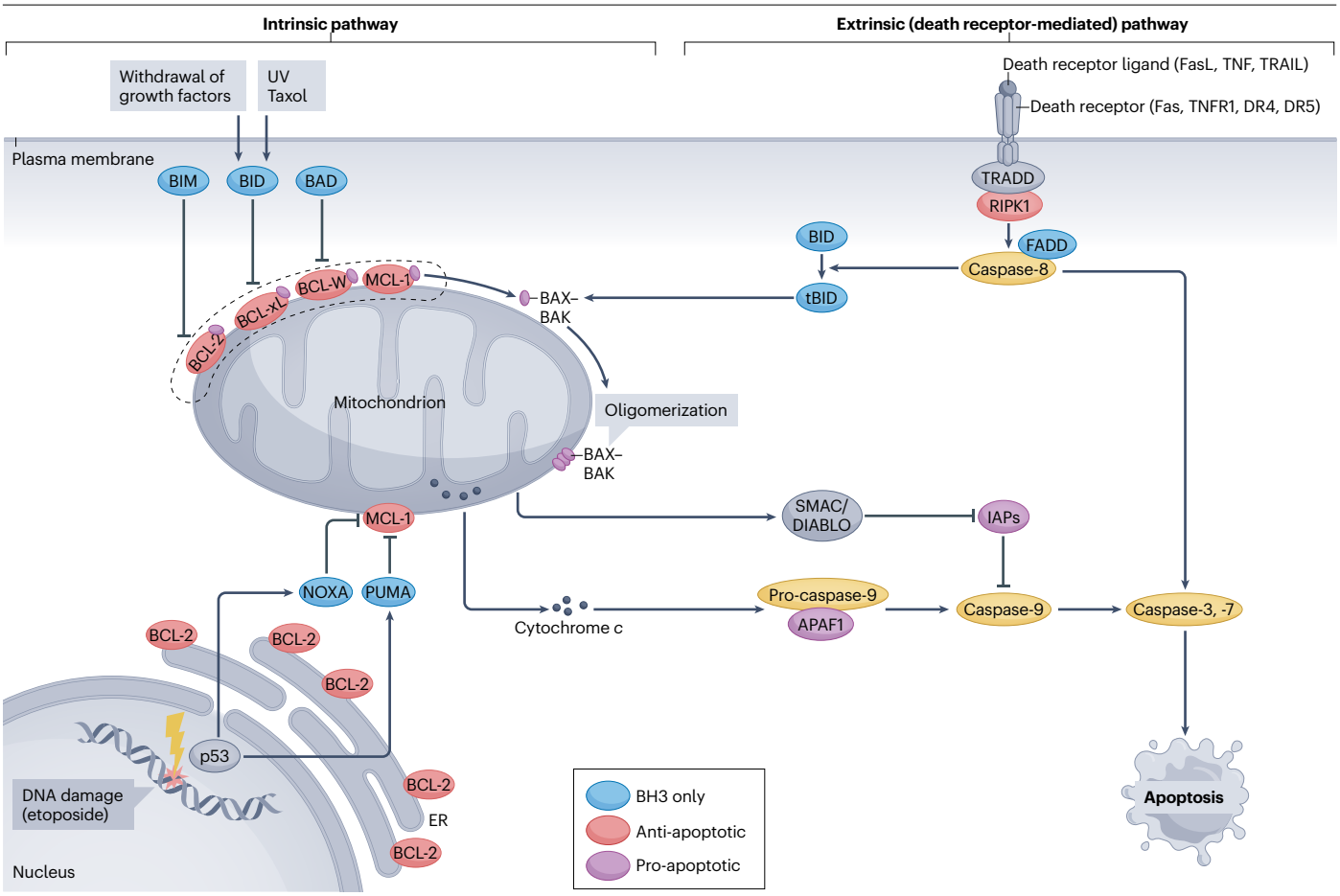


Fig. 1 | Intrinsic and extrinsic apoptosis. Extrinsic apoptosis (right) is mediated by the activation of plasma membrane-localized death receptors (such as TNFR1, Fas and TRAIL receptors DR4 and DR5) by their cognate ligands (such as TNF, FasL and TRAIL, respectively). Intrinsic apoptosis (left) can be activated by growth factor withdrawal, mitochondrial damage, DNA damage and chemotherapeutic drugs such as taxol. Activation of BH3-only members of the BCL-2 family, such as transcriptional induction of NOXA and PUMA by p53, post-translational modification of BAD and BIM, and cleavage of BID by caspase-8, induces mitochondrial damage by inactivating pro-survival BCL-2 family members, such as BCL-2, BCL-xL and MCL-1, and activating oligomerization of pro-death BCL-2 family members BAX and BAK. Mitochondrial damage leads to the release of cytochrome c and second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) to promote the activation of caspase-9 mediated by APAF1. Activated caspase-9 in turn cleaves downstream caspases, caspase-3 and caspase-7, to mediate the execution of intrinsic apoptosis. Activated caspase-3 and caspase-7 can also exert feedback activation of upstream caspases to allow amplification of caspase cascades. ER, endoplasmic reticulum; UV, ultraviolet.

catalytic components HOIP (RNF31), HOIL1 and SHARPIN that catalyse M1-linked ubiquitination on components of complex I such as RIPK1, NEMO, A20, TRADD and TNFR1[37–39]. The ubiquitin chains on complex I also serve to recruit key kinases, including the TAK1–TAB1–TAB2–TAB3 complex and NEMO–IKKα–IKKβ kinase complex, to activate NF-κB as well as to perform inhibitory phosphorylation on RIPK1[40–42]. In living cells, correctly assembled and ubiquitylated complex I is crucial for the activation of canonical NF-κB, a pro-survival and pro-inflammatory transcriptional pathway that controls the expression of *CFLAR* and *TNFAIP3*, which encode key regulators of apoptosis and inflammation[43–46]. A20, encoded by *TNFAIP3*, is a ubiquitin chain-editing enzyme that modifies the ubiquitination pattern of RIPK1 to control its kinase activity[44]. *CFLAR* encodes multiple c-FLIP isoforms, with similarity in amino acid sequence to caspase-8 but lacking enzymatic activity, that can directly bind to caspase-8 and modulate its activation[47].

Activation of TNFR1 by TNF may promote RIPK1-independent apoptosis or RIPK1-dependent apoptosis (RDA). Treatment with TNF and cycloheximide, which blocks the translation downstream of the NF-κB pathway, can induce RIPK1-independent apoptosis, while dysregulation of RIPK1 ubiquitination and phosphorylation can promote RDA. The loss of cIAP1, cIAP12, LUBAC or NEMO, inhibition of TAK1, TBK1 or IKKs, or mutating a Ub acceptor site in RIPK1 (K377R) results in the hyperactivation of RIPK1 kinase activity[48–53]. Activated RIPK1 mediates the formation of a cytoplasmic complex, known as complex IIa, that comprises RIPK1, FADD and caspase-8, to mediate the activation of caspase-8 to promote RDA[42,53–55] (Fig. 2). Thus, key mediators of NF-κB activation, including TAK1, IKKs, TBK1, NEMO, cIAP1, cIAP2 and LUBAC, also serve important roles in suppressing and modulating RDA. The activation of RIPK1 functions as a key checkpoint in the formation of complex IIa and inhibition of the kinase activity of RIPK1 is highly effective in blocking RDA[42,52,56].

Necroptosis

Necrosis was traditionally believed to be a passive form of cell death and therefore to be unregulated. The groundbreaking discoveries made since the mid-2000s identified RIPK1, RIPK3 and MLKL, encoded in the genomes of mammals and higher vertebrates, to mediate a necrotic programmed cell death pathway known as necroptosis[48,57–62]. Necroptosis represents the first discovered programmed cell death mechanism that mediates necrosis[57]. The activation of death receptors, including TNFR1, Fas, DR4 and DR5, by their cognate ligands in cells under apoptosis-deficient conditions can promote necroptosis. Necroptosis was first defined by the inhibition of caspase-independent necrosis upon treatment with necrostatin-1 (Nec-1)[57]. Nec-1 and its improved small-molecule analogues, such as Nec-1s, were later found to be inhibitors of RIPK1 kinase activity[48,63]. The activation of RIPK1 can mediate RDA under apoptosis-competent conditions and necroptosis under apoptosis-deficient conditions[34,42,64–68]. Thus, RIPK1 activation plays an important role in mediating multiple deleterious responses downstream of TNFR1 depending on genetic background, cell type and disease condition.

The caspase 8-mediated cleavage of RIPK1 in the intermediate domain (after D325 in mouse RIPK1 and D324 in human RIPK1) suppresses the activation of RIPK1 kinase activity by separating the N-terminal kinase domain from the intermediate domain and C-terminal DD, which mediates the dimerization of RIPK1 to promote its kinase activation[69,70]. Rare missense mutations in RIPK1 that alter the cleavage site by caspase 8 define a class of early-onset autoinflammatory diseases in humans characterized by periodic fevers[71,72].

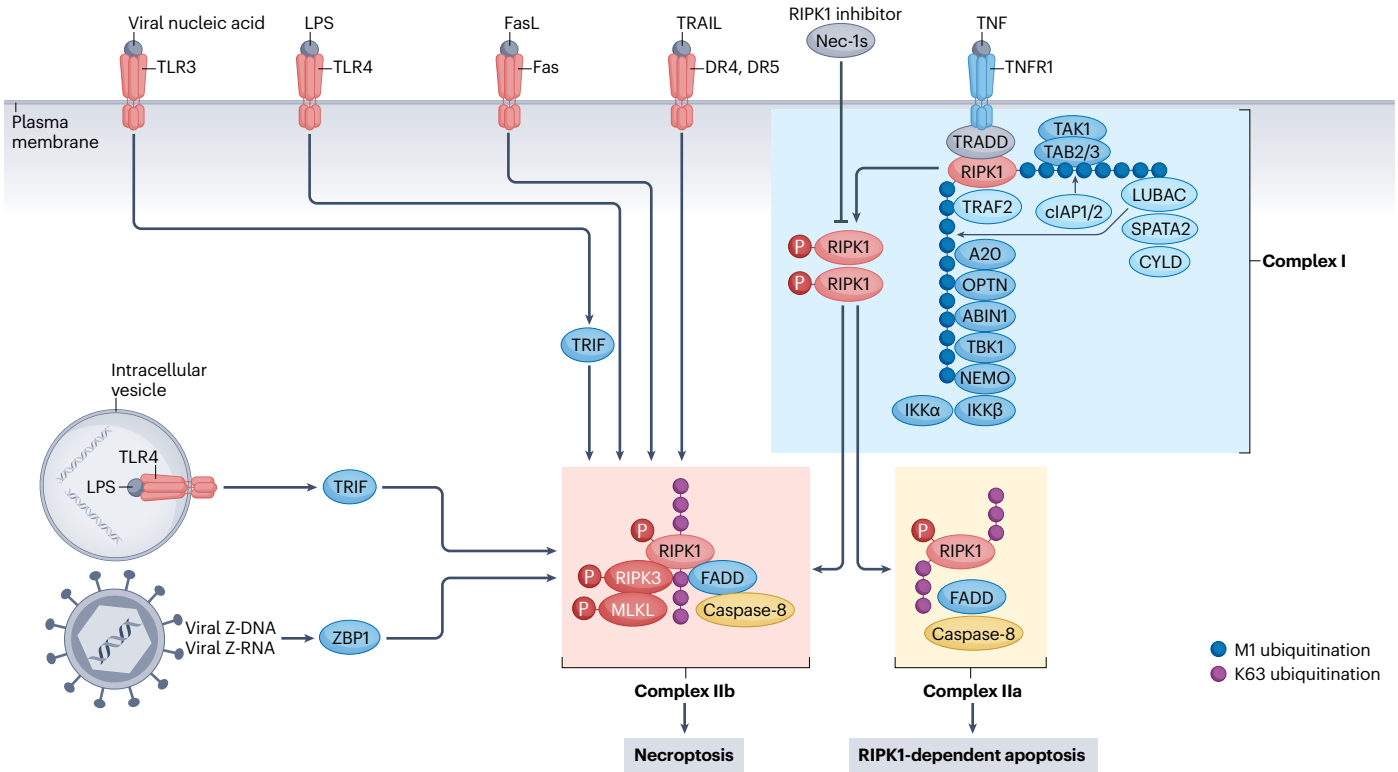


Fig. 2 | Necroptosis mediated by DD-containing receptors and pattern-recognition receptors. Upon activation by TNF, the cytoplasmic Death domain (DD) of trimerized TNFR1 mediates the formation of a transient intracellular complex, named complex I, which recruits adaptor protein TRADD, the DD-containing protein kinase RIPK1 and several E3 ubiquitin ligases, including TRAF2, cIAP1, cIAP2, the linear ubiquitin chain assembly complex (LUBAC), ubiquitin editing enzyme A20 and deubiquitylation enzyme cylindromatosis complex (CYLD and SPATA2) as well as multiple ubiquitin-binding proteins, including NEMO, ABIN1 and OPTN. In complex I, RIPK1 is rapidly polyubiquitylated by Lys63-linked and linear Met1-linked ubiquitin chains, which mediate the recruitment and activation of TAK1, TBK1 and IKK complexes. When RIPK1 kinase is not activated in living cells, the phosphorylation and subsequent ubiquitin–proteasome system-mediated degradation of IκB leads to the activation of NF-κB in the nucleus (not shown). Dysregulation of complex I promotes the activation of RIPK1 (as marked by pS166 RIPK1), which leads to the formation of two alternative cytosolic complexes, complex IIa or complex IIb, to mediate RIPK1-dependent apoptosis and necroptosis, respectively. Complex IIa includes the adaptor FADD protein, caspase-8 and RIPK1 to promote the activation of caspase-8, which in turn cleaves downstream caspases such as caspase-3, ultimately leading to apoptosis. When the activation of caspase-8 is inhibited, activated RIPK1 kinase binds to RIPK3 to form complex IIb. Activated RIPK3 in turn phosphorylates MLKL to mediate the execution of necroptosis by disrupting the integrity of the plasma membrane. The activation of Toll-like receptor (TLR3) and TLR4 by their cognate ligands, viral nucleic acids and bacterial lipopolysaccharides (LPS), respectively, in cells lacking caspase function can promote the binding of TIR domain-containing adaptor-inducing IFNβ (TRIF) to RIPK3 to mediate necroptosis. When activated by viral Z-DNA or Z-RNA, the binding of Z-DNA-binding protein 1 (ZBP1; also known as DAI) to RIPK3, mediated by their respective RHIMs (receptor-interacting protein homotypic interaction motifs), can also promote necroptosis in the absence of caspase function. The activation of DR4 or DR5 by TRAIL, Fas by FasL, and TLR4 by LPS has also been shown to promote necroptosis under certain conditions.

Activation of RIPK1 in caspase-deficient cells promotes the formation of complex IIb (necrosome), which includes RIPK3 and MLKL, to mediate necroptosis[59–61] (Fig. 2). During necroptosis, the interaction of activated RIPK1 with RIPK3 is mediated by their respective homotypic RIP homotypic interaction motifs (RHIM), which form a RHIM-mediated amyloid-like structure, to promote the activation of RIPK3[73,74]. Activated RIPK3, marked by the phosphorylation of Thr231 and Ser232 in murine RIPK3 and Ser227 in human RIPK3 (ref. 75), in turn mediates the phosphorylation of a pseudokinase MLKL at Ser358 in human MLKL or Ser345 in murine MLKL. This phosphorylation promotes MLKL oligomerization and the interaction of charged amino acids in its N-terminal four-helix bundle (4HB) domain with phosphatidylinositol phosphates in the plasma membrane. As a result, oligomerized MLKL inserts into the plasma membrane and forms pores that lead to necrosis[76–78]. Multiple lines of *Ripk1* kinase-dead knock-in mutant mice do not show developmental defects and have normal adult phenotypes but are highly resistant to TNF injection, which induces rapid lethality in wild-type mice[69,79–84]. *Ripk3*-null mice and *Mlkl*-null mice also show resistance to TNF injection[62]. These results indicate the importance of necroptosis in mediating TNF-mediated sepsis.

Similar to that of Fas and TNFR1, the activation of DD-receptors DR4 and DR5 by their cognate ligand TRAIL in cells lacking caspase activity can activate necroptosis[85,86]. Additionally, necroptosis can result from the activation of pattern-recognition receptors (PRRs), such as Toll-like receptor 3 (TLR3), TLR4 and Z-DNA-binding protein 1 (ZBP1), in cells lacking caspase activity[87–90] (Fig. 2). Necroptosis mediated by TLR3 and TLR4 is driven through TIR domain-containing adaptor-inducing IFNβ (TRIF, also known as TICAM1) by a RHIM-dependent engagement of RIPK3. Activation of ZBP1 by viral Z-RNA or Z-DNA with interferon stimulation can promote necroptosis as part of a host defence response, independent of RIPK1 and TNFR1[88,89]. ZBP1 is a sensor of cytoplasmic nucleic acids and can bind with viral Z-DNA and Z-RNA to promote the activation of necroptosis in response to viral infection by binding with RIPK3 via its two RHIM domains to form a necrosome[91]. Activation of these RHIM adaptor proteins can also lead to phosphorylation, oligomerization and membrane targeting of the necroptosis effector protein MLKL. The RHIM-dependent binding of ZBP1 to RIPK3 is inhibited by the scaffold function of RIPK1[50,92]. Thus, RIPK1 and ZBP1 may compete for their binding to RIPK3. Knockout of either *Zbp1* or core interferon signalling components prolongs the survival of *Ripk1*-null mice, suggesting the activation of a ZBP1-dependent interferon-mediated response upon the loss of RIPK1.

Metabolic stress and hypoxia can also promote the activation of RIPK1 and necroptosis independent of TNF or TNFR1[85,93]. Energy-stress activation of AMPK serves as a metabolic checkpoint[94]. Whilst activated AMPK mediates inhibitory phosphorylation of S416 in human RIPK1 and S415 in murine RIPK1, sustained glucose deprivation can promote the activation of RIPK1, RIPK3 and MLKL to induce cell death[85]. Moreover, under normoxic conditions, activation of RIPK1 is controlled by prolyl hydroxylation at multiple proline residues, such as Pro195, by EGLN proteins[93]. This leads to the recognition and suppression of RIPK1 activation by binding with phosphorylated von Hippel–Lindau disease tumour suppressor (pVHL) protein (an E3 ubiquitin ligase that is known to target the transcriptional factor hypoxia-inducible factor 1α, a key regulator of cellular response to oxygen concentration) for proteasomal degradation[93,95]. Prolonged hypoxia inactivates EGLN proteins and the loss of proline hydroxylation on RIPK1 as well as binding with pVHL1, which can promote the activation of RIPK1 without affecting its degradation. The interactions of RIPK1 with key mediators of metabolism and hypoxia, such as AMPK and pVHL, may contribute to the underlying mechanism for RIPK1 in mediating ischaemic damage in multiple organs, including brain, heart and kidney[57,96–98].

Pyroptosis

Caspases were found to mediate a form of programmed necrosis known as pyroptosis[99–102]. Thus, pyroptosis represents another type of programmed necrotic cell death regulated by dedicated genetic pathways in mammalian cells. The activation of the caspase 1 subfamily, including caspase-1 and caspase-11 in mouse and the corresponding caspase-1, caspase-4 and caspase-5 in human, promotes an inflammatory response in which mature IL-1β is generated by the cleavage of pro-IL-1β as well as other downstream caspases such as caspase-3[103–105] (Fig. 3). Activated caspase-1 and caspase-11 can mediate the cleavage of gasdermin D (GSDMD) to remove an autoinhibitory carboxy-terminal domain from GSDMD, enabling the N-terminal fragment to form pores on the plasma membrane and resulting in pyroptosis[101,106–108]. The pores formed by 16 GSDMD N-domain protomers have an inner diameter of 10–14 nm, which can mediate the release of cytokines without signal peptides such as mature IL-1β in living cells as well as necrosis[109,110]. Pyroptosis occurs predominantly in professional phagocytic cells, including macrophages, monocytes and dendritic cells, upon stimulation by pathogens, such as bacteria or viruses, or induced by pathogen-derived material such as lipopolysaccharide and viral DNA.

Release of pro-inflammatory factors, such as mature IL-1β, through gasdermin pores occurs via a charge-based mechanism independently of cell death[111,112]. The mechanism that mediates the release of cytokines without a signal peptide, such as mature IL-1β, has long been the subject of studies and debate. In human postmortem pathological samples, apparently live microglia and astrocytes that showed positive staining for cleaved GSDMD were found in close proximity to amyloid-β plaques, suggesting that the formation of pores by the GSDMD N-terminal domain triggers an inflammatory response[113]. Of note, both the inflammatory response and cell death are crucial for organismal survival in the presence of severe inflammation. For example, *Casp11*-null mice are highly resistant to lipopolysaccharide-induced lethality[103], which may be attributed to the inhibition of GSDMD pore-mediated release of pro-inflammatory cytokines such as mature IL-1β and IL-18, the inhibition of pyroptosis, or both. Future biomarker-based studies are needed to better understand the mechanism underlying reduced lethality.

Other members of the gasdermin family, GSDMA to GSDME and pejvakin, encoded in the human genome can also be cleaved by bacterial proteases or mammalian granzyme A. The bacterial SpeB-mediated cleavage of GSDMA in keratinocytes infected by group A *Streptococcus* (GAS) promotes pyroptosis[114]. Mutant mice with triple knockout of the three homologs of human GSDMA are sensitized to hypervirulent GAS strain invasion, suggesting a role of pyroptosis as a host defence mechanism. Granzyme A-mediated activation of GSDMB is part of the bacteriocidic activity of natural killer cells, which is targeted by IpaH7.8 in *Shigella*[115]. Interestingly, certain bacteria were found to encode gasdermin homologues with pore-forming activity used in defending against phage infection[116], suggesting that gasdermins may be an evolutionarily conserved ancient host defence mechanism.

Dead cell removal by efferocytosis

Elimination of dead cell bodies is important to prevent the release of intracellular content that may function as damage-associated molecular patterns to activate an inflammatory response and possibly lead to autoimmunity[117]. Efficient removal of apoptotic bodies by a

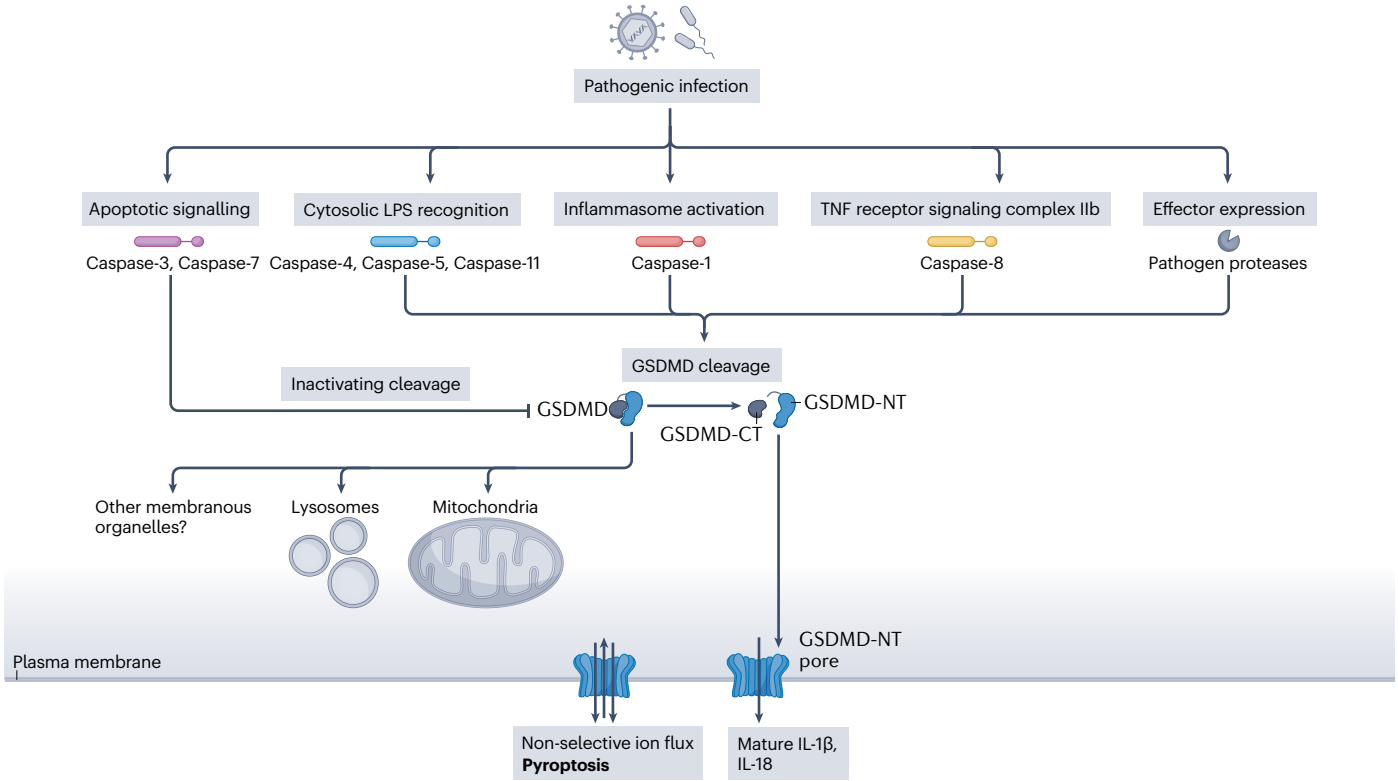


Fig. 3 | Pyroptosis induction and mature pro-inflammatory cytokine (IL-1β) release. Pathogenic infection by bacteria and viruses can promote the activation of caspase-1 and caspase-11 in mouse and caspase-1, caspase-4 and caspase-5 in human to mediate the cleavage of gasdermin D (GSDMD) to release the pore-forming N-terminal domain of GSDMD (GSDMD-NT) from its inhibitory C-terminal domain (GSDMD-CT). Activated caspase-8, caspase-3 and caspase-7 as well as pathogenic proteases can also mediate the cleavage of GSDMD. The pore formed by GSDMD-NT can mediate the secretion of mature IL-1β and IL-18 in living cells to promote inflammation and also promotes pyroptosis by cell lysis. LPS, lipopolysaccharides.

cleansing mechanism, known as efferocytosis, mediated by professional phagocytes such as macrophages and dendritic cells, is important for avoiding such auto-immunogenicity[118–120]. The caspase-mediated exposure of phosphatidylserine (PtdSer) on the surface of apoptotic cells serves as a recognition motif for efferocytosis. PtdSer is normally maintained in the inner leaflet of the plasma membrane in living cells by flippases, including ATP11A and ATP11C[120]. PtdSer becomes exposed on the surface of apoptotic cells because of caspase-3-dependent cleavage and inactivation of the flippases as well as simultaneous activation of the phospholipid scramblase XKR8, a homologue of CED-8 in *C. elegans*[121,122]. Efferocytosis by macrophages involves the recognition of apoptotic cells by various PtdSer receptors such as TIM4, TAM tyrosine-kinase receptors, MFGE8, GAS6, and Protein S. Similar to what is observed in *C. elegans*, small G proteins of the Rho and Rab family and their guanine nucleotide exchange factors in mammalian cells are involved in the execution of efferocytosis by regulating the formation of a phagocytic cup and maturation of phagosomes for the degradation of apoptotic bodies[118].

Consistent with the activation of caspases in pyroptosis, pyroptotic cells also display phosphatidylserine 'eat-me' signals on their outer plasma membrane and the dead cell bodies are rapidly removed by phagocytic cells[123,124]. These data suggest that both apoptotic and pyroptotic cells can be eliminated by phagocytes in vivo. Since necroptosis does not involve the activation of caspases, it is still unclear how necroptotic cell bodies are removed in vivo. Understanding the mechanism of efferocytosis for programmed necrosis, including necroptosis and pyroptosis, is important as the presence of a highly efficient efferocytosis mechanism might lead to the engulfment, and therefore the removal, of dying cells, before cell lysis and the release of intracellular DAMPs can occur.

Membrane rupture after cell death

Necrosis or failure in the efficient removal of apoptotic cell bodies may lead to the rupture of the plasma membrane in a process involving the membrane protein NINJ1[125]. In dying cells, NINJ1 proteins form filamentous assemblies with tightly packed fence-like arrays of transmembrane α-helices[126]. Application of anti-NINJ1 antibodies can limit tissue damage in animal models of liver injury, suggesting that targeting membrane damage may ameliorate pathology in diseases[127]. NINJ1 may be normally involved in the maintenance of functional synapses as *Ninj1*-null mice exhibit compulsive grooming-induced hair loss and self-made lesions as well as increased anxiety-like behaviours[128].

NINJ1 has been implicated in mediating cell–cell adhesion in the peripheral nervous system and central nervous system (CNS) after injury[129]. The levels of NINJ1 are increased in dorsal root ganglion neurons upon axonal injury and anterogradely transported to the site of injury[130]. NINJ1 may also be involved in inflammatory responses by promoting the activation of immune cells and transmigration of

Glossary

AMPK
AMPK is a highly conserved sensor of intracellular adenosine nucleotide levels and is activated when the AMP-to-ATP ratio is elevated under energy-stress conditions. AMPK activation promotes catabolic pathways to generate more ATP and inhibits anabolic pathways.

Amyloid-β plaques
An important pathological hallmark in the brain of patients with Alzheimer disease. Amyloid-β plaques contain fibrillar polymers of the amyloid-β cleavage products of APP protein as well as other components. Amyloid-β plaques can propagate and spread via prion-like self-assembly to drive neurodegeneration.

Apoptosome
A heptameric APAF1 protein complex shaped as a wheel-shaped structure with sevenfold symmetry that can drive the activation of caspase-9, which in turn cleaves and activates pro-caspase-3, in intrinsic apoptosis.

Autoimmune lymphoproliferative syndrome
A lymphoproliferative disease characterized by defects in the control of lymphocyte numbers that lead to enlargement of the lymph nodes (lymphadenopathy), the liver (hepatomegaly) and the spleen (splenomegaly).

Blood–brain barrier
A property of the blood vessels in the central nervous system that enables them to regulate the movement of molecules and cells between the blood and the brain, preventing many macromolecules from entering the brain through diffusion.

Centromere
A centromere is the point of attachment of the kinetochore, which is a structure that anchors the mitotic spindle during mitosis.

Damage-associated molecular patterns
Intracellular molecules with pro-inflammatory and immunogenic activity that are discharged to extracellular space as the result of damage to the cell membrane from necrotic cell death.

Efferocytosis
Process that mediates the removal of apoptotic cells by phagocytic cells and non-professional phagocytic cells.

Flippases
ABC transporter or P4-type ATPase families of transmembrane lipid transporter proteins to facilitate the movement of phospholipid molecules between the two leaflets of the cell plasma membrane.

Freidrich ataxia
A rare genetic disorder that leads to progressive movement disability.

Glutathione
Glutathione is a linear tripeptide of L-glutamine, L-cysteine and glycine with strong antioxidant activity.

Granzyme A
A serine protease present in cytotoxic T cells.

Integrin
Heterodimeric transmembrane receptors that mediate cell–cell and cell–extracellular matrix adhesion.

NF-κB
Nuclear factor-κ light chain enhancer of activated B cells is a family of highly conserved transcription factors that regulate many important cellular responses, including inflammation, proliferation, cellular growth and apoptosis.

NOD family
Nucleotide oligomerization domain (NOD) proteins NOD1 and NOD2, which can enable detection of intracellular bacteria and promote their clearance through initiation of the pro-inflammatory transcriptional programme and other host defence responses such as autophagy.

Normoxic condition
Having a normal oxygen concentration; typically 20–21% in the atmosphere or in tissue culture flasks.

Protomers
Structural units of an oligomeric protein.

Signal peptide
A peptide segment of 20–30 amino acids that acts as the N-terminal sorting signal that targets the linked protein to the secretory pathway in eukaryotes and prokaryotes.

Ub acceptor site
Ubiquitin is a 76-amino acid polypeptide that can be attached to proteins through the formation of an isopeptide bond between its carboxyl terminus and the Ub acceptor site, which can be the ε-amino group of lysin side chains on target proteins for K63, K48 and K11 ubiquitination or the N-terminal methionine as in M1-linked ubiquitination.

immune cells across the blood–brain barrier in models of multiple sclerosis and stroke[131,132]. Further studies are required to understand if the role of NINJ1 in regulating these conditions involves the disruption of cell membranes and cell death[133,134]. NINJ1-mediated plasma membrane rupture may be involved in pathologies involving a massive amount of cell death following acute neurological and tissue damage, which could 'overwhelm' dead cell clearance mechanism. Alternatively, genetic defects in efferocytosis may reduce the efficiency of dead cell removal leading to the accumulation of dead cells with ruptured cell membranes.

Interaction of programmed cell death pathways

While apoptosis, pyroptosis and necroptosis are executed by genetically designated cell death machineries, the evolutionary origins and functions of apoptosis, pyroptosis and necroptosis in organisms are dramatically different. The genes that regulate apoptosis are found in primitive organisms such as nematodes and flies, whereas the genes that regulate necroptosis, such as RIPK1, RIPK3 and MLKL, are only found in a subset of highly evolved vertebrates and mammals such as mouse and human[58,135]. The embryonic developmental defects seen in *Apaf1*-null mice, *Casp9*-null mice as well as *Casp3* and *Casp7* double-null mice demonstrate the functional importance of apoptosis in development[22]. In contrast, *Casp1*-null mice and *Casp11*-null mice are healthy and display resistance to inflammatory apoptosis and pyroptosis and do not release mature IL-1β and IL-18[103,107,136]. In addition, while *Ripk1*-null mice die perinatally[137], multiple mutant mice lines expressing catalytically inactive RIPK1 kinase are normal[69,79,80]. *Ripk3*-null mice and *Mlkl*-null mice are also normal[138]. Furthermore, blocking RIPK1 kinase and necroptosis can suppress early embryonic lethality of *Casp8*-null mice, *Fadd*-null mice, *Abin1*-null mice and *Tbk1*-null mice, suggesting that necroptosis may be involved in the removal of defective developing embryos[52,67,139,140]. Thus, apoptosis is involved in mediating development whilst necroptosis may have evolved to safeguard normal development by aborting defective embryos in higher vertebrates[58].

Distinct roles of apoptosis and necroptosis have been found in cancers. Amplification and increased expression of FADD are associated with the progression of many types of cancers, including breast

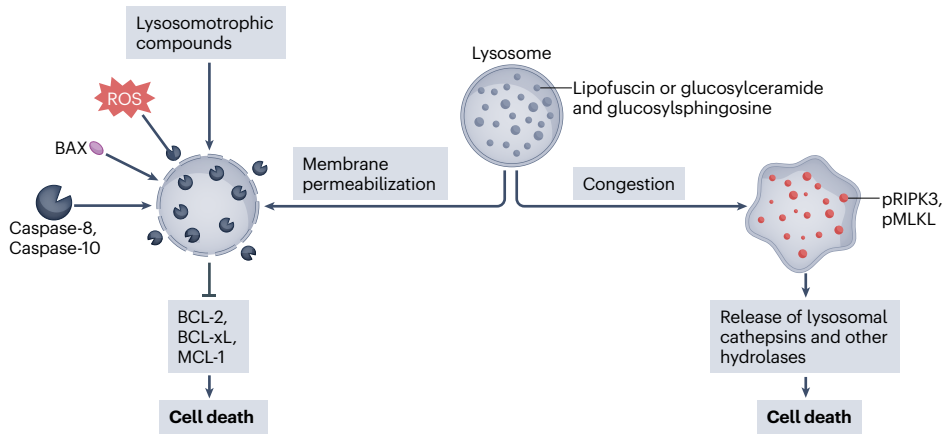


Fig. 4 | Cell death mediated by disruption of lysosomal function. Lysosomal membrane permeabilization (left), which may be induced by reactive oxygen species (ROS), lysosomotrophic compounds and activated BAX and caspase, leads to the release of cathepsins and other hydrolases from the lysosomal lumen to the cytosol. Lysosomal congestion (right), induced by the accumulation of glucosylceramide and glucosylsphingosine in Gaucher disease and of lipofuscin in Stargardt and dry age-related macular degeneration, can lead to the activation of RIPK3 and MLKL. pMLKL, phosphorylated MLKL; pRIPK3, phosphorylated RIPK3.

cancer, ovarian cancer and lung cancers[141]. Conversely, many cancers and cancer cell lines show decreased or loss of RIPK3 expression associated with necroptosis inhibition[142–144]. These findings indicate that apoptosis supports organismal development, whereas necroptosis functions as a checkpoint to block the progression of diseased states when caspases are dysregulated during embryonic development and in adult life. In particular, the prevalent loss of RIPK3 expression in cancer cells suggests the role of necroptosis as a checkpoint in cancer development.

Autophagy and lysosomal cell death

In the following sections, we discuss well-characterized examples of cell death due to the disruption of homeostatic pro-survival mechanisms that do not involve genetically programmed cell death mechanisms. Cells that die due to disruptions in cellular homeostasis may also exhibit certain hallmarks of apoptosis or necroptosis. However, inhibition of apoptosis or necroptosis per se may not be sufficient to rescue cell survival due to the loss of key cellular homeostatic pro-survival mechanisms under these conditions.

The term 'autophagic cell death' was first used to describe a form of cell death morphologically distinct from apoptosis in embryonic tissues[145]. Autophagic cell death has been described during metamorphosis of *Drosophila*[146]. However, autophagy is a normal physiological mechanism that delivers cytoplasmic contents to the lysosome for degradation and is important in promoting cell survival under stress[147,148]. Inhibition of autophagy can lead to necrosis[149]. Moreover, morphological evidence of autophagy activation is often associated with cell death processes such as necroptosis[57]. Blocking autophagy has minimal effect on cell death but increases the accumulation of cellular debris associated with necroptosis, which suggests that activation of autophagy in necroptosis promotes the degradation of cellular debris but not cell death. In the nervous system, autophagy is crucial for maintaining cellular homeostasis by promoting the degradation of aggregate-prone proteins and dysfunctional organelles such as damaged mitochondria. Defects in autophagy or lysosomal function are associated with ageing-related neurodegenerative diseases[150,151].

The lysosome is an important intracellular recycling centre filled with different hydrolases, such as lysosomal cathepsins, that can degrade cellular macromolecules to produce amino acids and other metabolic products for recycling and, thus, the lysosome is vital for cell survival. However, this organelle is also known as the 'suicide bag' as the rupture of lysosomal membranes has deadly consequences due to the leakage of lysosomal cathepsins that can lead to lysosomal cell death[8,152] (Fig. 4). Lysosomal cell death is executed by cathepsin-mediated cleavage and activation of key regulators of apoptosis, such as caspases and BCL-2 family members, as well as by the direct action of cathepsins on key pro-survival mechanisms[153]. Gaucher disease, caused by mutations in the *GBA* gene (encoding glucocerebrosidase (GBA)), is the most common lysosomal storage disease. Conditional loss of GBA in neurons in mice leads to increased expression of RIPK1 and RIPK3 and non-apoptotic cell death, which can be attenuated by *Ripk3* knockout[154]. In addition, lysosomal damage induced by spinal cord injury may mediate the recruitment of RIPK1 to the lysosome and promote the activation of RIPK1 and RIPK3[155]. Lysosomal permeabilization induced by the accumulation of lipofuscin, the cause for the degeneration of retinal pigment epithelial cells in Stargardt and dry age-related macular degeneration, can lead to atypical necroptosis by activating MLKL[156]. Furthermore, there is a large group of lysosomal storage diseases that are caused by altered function in this organelle and have severe manifestations in multiple organs, in particular the CNS[157,158]. A loss of lysosomal homeostatic cell survival mechanism may be a main driver of cell death in these patients.

Entosis

Entosis occurs when one cell engulfs and digests another living cell; thus, it may be considered a form of cellular cannibalism[9,159]. The engulfment process is triggered by cell detachment (of the cell being engulfed) from the extracellular matrix and involves the contractile activity generated by actin, myosin II, Rho-GTPase and Rho-associated protein kinase ROCK[160]. The internalized cell is eventually degraded by an autophagic lipidation-mediated lysosomal process, which shares similarities with the degradation of internalized pathogens such *Listeria monocytogenes*, GAS and *Shigella*[161–164]. Activation of AMPK under a glucose starvation condition can enhance the frequency of entosis, suggesting a role for metabolic stress in regulating entosis[165]. In addition, the activation of DR4 and DR5 by TRAIL can also stimulate entosis driven by the scaffold function of caspase-8 but not its protease cleavage activity. Entosis is found in human cancers, but the role of entosis in cancer needs further investigation[166].

Ferroptosis

Ferroptosis is mediated by iron-dependent lipid peroxidation and was originally defined as cell death that can be activated by small molecules such as erastin, an irreversible inhibitor of cystine/glutamate antiporter

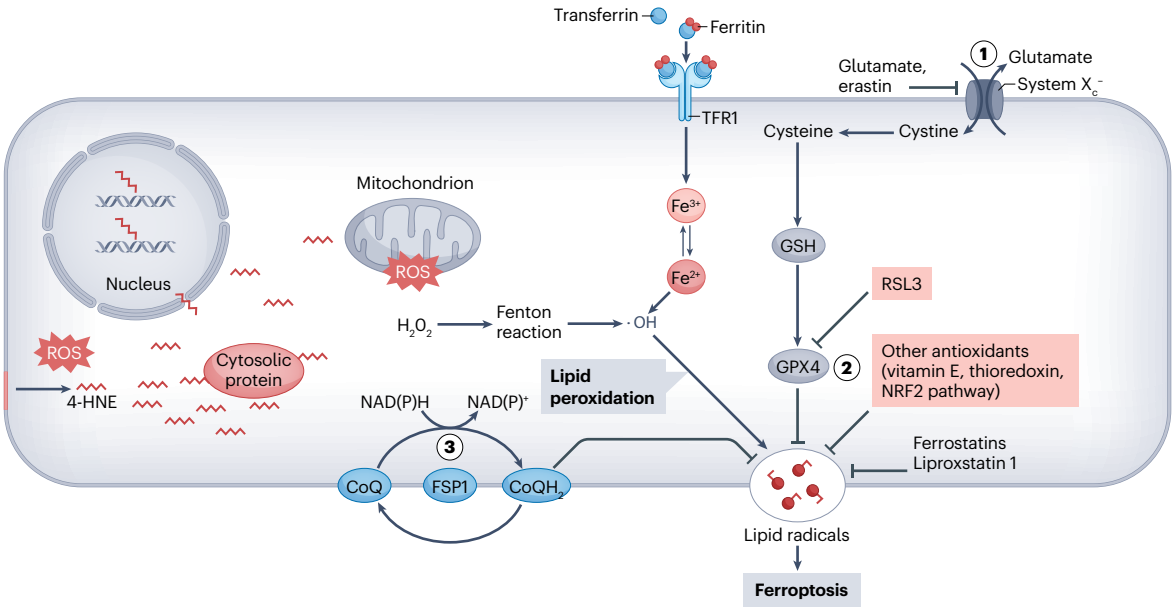


Fig. 5 | Lipid peroxidation and ferroptosis. Cellular mechanisms that protect against lipid peroxidation include (1) xCT (encoded by the gene *SLC7A11*), which is a heterodimeric amino acid antiporter specific for importing cystine, the oxidized form of cysteine, in 1:1 exchange for exporting glutamate. Reduction of cystine generates cysteine, the rate-limiting precursor in glutathione (GSH) synthesis that protects cells from oxidative stress. (2) GPX4, which protects against lipid peroxidation by converting lipid hydroperoxides into lipid alcohols. (3) Plasma membrane-localized FSP1 (previously known as AIFM2), which functions as an oxidoreductase that reduces coenzyme Q10 (CoQ) to generate a lipophilic radical-trapping antioxidant to block lipid peroxides. Lipid peroxidation of poly-unsaturated fatty acids generates reactive aldehydes, such as 4-hydroxynonenal (4-HNE), which can form relatively stable adducts with protein, lipid and DNA. Electrons may escape from cellular oxidation–reduction reactions and be captured by oxygen to form peroxides (H_2O_2). The Fenton reaction mediates oxidation by Fe^{2+} and H_2O_2, producing hydroxyl radicals to react with lipids to form lipid radicals, which initiate lipid peroxidation. Ferroptosis can be induced by the chemical inhibition of GPX4 by RSL3 or inhibition of xCT by erastin, which defeats cellular defence mechanisms against lipid peroxidation to induce cell death by massive iron-dependent lipid peroxidation. Ferrostatins and liproxstatin 1 inhibit lipid peroxidation by functioning as radical-trapping antioxidants. ROS, reactive oxygen species; TFR1, transferrin receptor 1.

xCT, or RSL3, a GPX4 inhibitor[6,167] (Fig. 5). Ferroptosis differs morphologically from apoptosis or necrosis and does not involve the cellular machinery that mediates apoptosis or necroptosis[6]. Maintenance of a cellular homeostatic redox balance is critical for cell and animal survival[168] (Fig. 5). Antiporter xCT, encoded by the gene *SLC7A11*, and GPX4 are two key regulators of cellular redox mechanisms that protect cells against lipid peroxidation. xCT is a heterodimeric amino acid antiporter specific for exporting glutamate in exchange for importing cystine[169]. Reduction of cystine generates cysteine, the rate-limiting precursor in the synthesis of glutathione that protects cells from oxidative stress. GPX4, a selenoprotein with glutathione-dependent peroxidase activity, is the major cellular defence mechanism against lipid peroxidation. Inactivation of GPX4 leads to the production of excessive amounts of reactive oxygen species (ROS), inducing lipid peroxidation, disruption of the plasma membrane and mitochondrial membrane and, consequently, activation of cell death. Inducible inactivation of GPX4 in mice leads to lipid oxidation-induced acute renal failure and animal death[170]. Lipid peroxidation of poly-unsaturated fatty acids, one of the consequences of increased cellular ROS, generates reactive aldehydes, such as 4-hydroxynonenal (4-HNE), which can form adducts with proteins, lipids and DNA in a cell type-dependent and concentration-dependent manner[171–173].

Lipid peroxidation can occur as a normal consequence of cell metabolism when oxidants attack lipids, especially poly-unsaturated fatty acids, which can be modulated by cellular redox mechanisms[174]. Increased lipid peroxidation is observed in living cells during inflammation and in diseased conditions. Ageing may lead to increased production of oxidized products, exceeding the detoxification capacity of a biological system[171]. Increased levels of 4-HNE-modified proteins detected by a HNEJ-1 monoclonal antibody were found in different organs of ageing mice when compared to young mice, suggesting that ageing is associated with increased lipid peroxidation[175]. Oxidative stress and lipid peroxidation have long been associated with a variety of ageing-related human diseases, including diabetes and neurodegeneration[176,177]. However, because we still do not have a specific biomarker for ferroptosis, caution should be exercised to differentiate lipid peroxidation (without cell death) and ferroptosis (with cell death).

The levels of lipid peroxidation are modulated by cellular redox mechanisms as a reversible process(Fig. 5). Accumulation of HNE-modified proteins can be reduced by increasing NRF2-mediated antioxidant defence gene expression[178]. Notably, Freidrich ataxia, caused by a mutation in the gene encoding frataxin leads to alterations in iron handling within mitochondria and impairment of mitochondrial respiration, which can be treated with NRF2 activators[179]. The production of cellular radical-trapping antioxidants, including vitamin E and vitamin K, can also provide defence against lipid peroxidation and ferroptosis. In addition to GPX4, lipid peroxidation is

regulated by coenzyme Q10 oxidoreductase FSP1, which catalyses the regeneration of the reduced form of ubiquinone (ubiquinol) with free radical-trapping antioxidant activity[180,181]. FSP1 can function to reduce vitamin K to hydroquinone, which has radical-trapping antioxidant activity[182]. In addition, induction of the phospholipid-modifying enzymes MBOAT1 and MBOAT2 by sex hormone receptors, such as oestrogen receptor and androgen receptor, can also modulate lipid peroxidation[183].

Excess accumulation of lipid peroxidation may promote inflammation independent of cell death. Iron is a key regulator of cellular homeostasis and survival as it is involved in mitochondrial respiration. The loss of transferrin receptor 1, which is involved in iron uptake in the brain, leads to neuronal iron deficiency and progressive loss of dopaminergic neurons[184]. Mutations in genes for ferritin light chain (FTL), which has an important function in iron storage, lead to rare Parkinson-like diseases known as neuroferritinopathies due to brain iron overload[185]. An inflammatory response to iron accumulation in microglial cells can be coupled to ferroptosis[186]. Thus, inactivating key cellular redox pro-survival mechanisms in defence against lipid peroxidation may include activation of inflammation as a consequence.

Excitotoxicity

Disruption of intracellular ionic balance after overactivation of specific ion channels can lead to a type of necrosis known as excitotoxicity (Fig. 6). Genetic studies on mechanosensitivity in *C. elegans* provide an elegant prototypical example of cell death induced by the loss of a cellular homeostatic pro-survival mechanism mediated by overactivation of ion channels[187,188]. Missense mutations in the genes *mec-4* and *deg-1*, which encode proteins similar to subunits of the vertebrate amiloride-sensitive epithelial Na^+ channel, lead to ionic imbalance, swelling and necrotic degeneration of touch-receptor neurons in *C. elegans*. The cell death mediated by mutant MEC-4 and DEG-1 is independent of CED-3 but partially mediated by aspartyl and calpain proteases, which are activated by increased intracellular Ca^{2+} (refs. 189,190).

Glutamate is a key excitatory neurotransmitter in the mammalian nervous system that drives Ca^{2+} influx into neurons[191] (Fig. 6). In a pathological state, altered Ca^{2+} signalling can result in excessive levels of this second messenger and lead to excitotoxicity from ionic imbalance. The cells are unable to survive with a cellular Ca^{2+} overload. The downstream cell death effectors of excitotoxicity may include both apoptotic and necroptotic processes, and potentially depend on the magnitude of this homeostatic disruption[192,193]. The clearance or recycling of glutamate by astrocytes may also determine the availability of glutamate as a neurotransmitter for proper signalling and prevention of neuronal hyperexcitation[194]. Excessive or persistent activation of glutamate-gated ion channels, such as the NMDA receptor, has been implicated in mediating necrotic cell death in the CNS after acute neuronal injury such as cerebral ischaemia, status epilepticus, traumatic CNS injury and hypoglycaemia[176]. The cytotoxicity of glutamate may be mediated by the ability of increased Ca^{2+} influx to promote the activation of neuronal nitric oxide synthase, which is also known to modulate many physiological functions such as synaptic plasticity, learning, memory and neurogenesis[195–197]. Excitotoxic events in neurons can also alter the oxidative state, which has been shown to drive s-nitrosylation[198].

Mitotic catastrophe

Mitotic catastrophe is a type of cell death induced by inappropriate mitotic entry due to chemical or physical stresses[199]. Biochemically, mitotic catastrophe is characterized by premature condensation of under-replicated or damaged chromosomes with double-stranded DNA breaks at the centromere[200]. Cancer cells with genetic defects in G2 checkpoint genes, such as *ATM*, *ATR*, *CHEK1*, *CHEK2* and polo-like kinase genes (*PLK1*, *PLK2*, *PLK3*), can be induced to undergo mitotic catastrophe by pharmacological agents targeting microtubules, such as taxanes, vinca alkaloids and colchicine, and DNA damaging agents. Cancer cells undergoing mitotic catastrophe may exhibit hallmarks of caspase activation. However, inhibition of caspase activity cannot

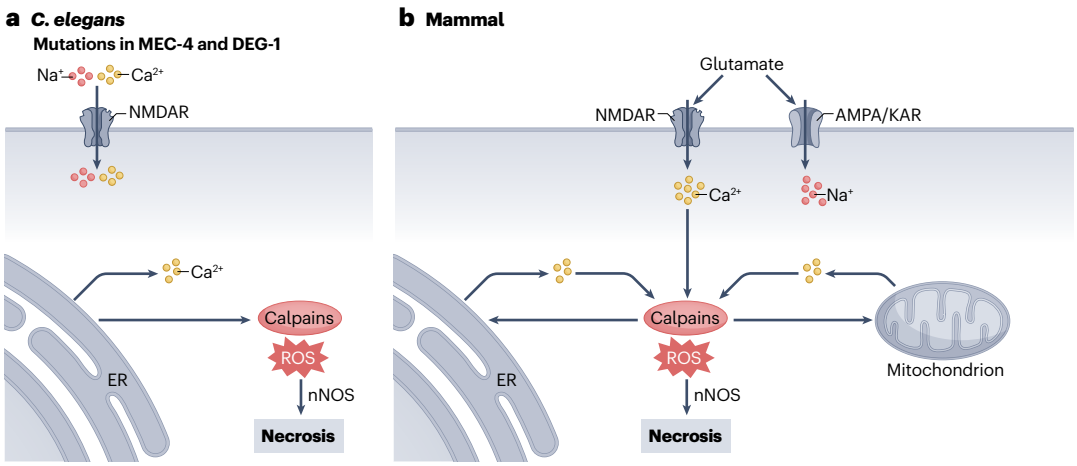


Fig. 6 | Necrosis and excitotoxicity induced by disruption of homeostatic ionic balance. Disruption of intracellular ionic balance after overactivation of MEC-4 and DEG-1, which are homologues of the vertebrate amiloride-sensitive epithelial Na^+ channel, leads to necrotic degeneration of touch-receptor neurons in *Caenorhabditis elegans*. The cell death is independent of CED-3 but partially mediated by aspartyl and calpain proteases, which are activated by increased intracellular Ca^{2+}. Elevated levels of glutamate under pathological conditions in the mammalian nervous system can also lead to cellular Ca^{2+} overload and loss of cell survival due to ionic imbalance. The downstream cell death effectors of excitotoxicity may include both apoptotic and necroptotic processes, and potentially depend on the magnitude of homeostatic disruption. ER, endoplasmic reticulum; nNOS, neuronal nitric oxide synthase; ROS, reactive oxygen species.

Box 2

Cell death mechanisms in drug discovery

The groundbreaking discoveries that increased our understanding of programmed cell death mechanisms have been translated into the successful development of venetoclax (BCL-2-specific inhibitor) and navitoclax (inhibitor of BCL-2, BCL-xL and BCL-W) for the treatment of leukaemias and lymphomas[11,202,203,223]. Developing strategies to selectively disrupt cell survival and homeostasis mechanisms has always been the goal of anticancer drug development, from traditional chemotherapeutic drugs to paclitaxel, which can induce mitotic catastrophe[199]. In this regard, small-molecule activators of ferroptosis may be considered as a potential new line of anticancer drug candidates acting by inhibiting the pro-survival mechanisms in defence against oxidative stress in therapy-resistant cancer cells. For example, icFSP1, a small-molecule inhibitor of FSP1, can trigger the relocalization of FSP1 from the membrane by promoting FSP1 condensates to synergize with ferroptosis-inducing agents to potentiate ferroptosis[224]. Conversely, inhibiting several steps along the ferroptosis pathway could block deleterious oxidative damage and lipid peroxidation that occur in ageing and disease. This is of particular interest in the neurodegenerative space as several studies have shown increased iron mishandling and oxidation in the brain[225]. First-generation molecules, like ferrostatin 1 and liproxstatin 1, for ferroptosis inhibition have mainly been radical-trapping antioxidants that work in a relatively non-specific manner; however, the therapeutic relevance of each component in this pathway requires further exploration. A recent study suggests that acute kidney injury in mouse models with increased lipid peroxidation may induce the activation of RIPK1 and thus simultaneous inhibition of RIPK1 and ferroptosis may be considered[226].

Pyroptosis regulates cellular response to pathogens; however, in the context of autoimmunity or neurodegeneration, the inflammasome components can be hijacked to mediate an aberrant hyperinflammatory response[227,228]. Since it is difficult to develop small inhibitors of caspases that are stable in vivo, alternative targets must be considered. In the context of neurodegeneration, the inflammasomes, for example, NLRP3, are thought to be activated by aggregated protein and drive a progressive neuroinflammatory response[229]. There are several ongoing clinical trials targeting NLRP3 as well as some of its interacting proteins like ASC[230,231]. This approach may have the potential to block both inflammation and pyroptosis.

The biology and structure of RIPK1 kinase make it suitable for developing small-molecule inhibitors for the treatment of human diseases[204]. Structurally, RIPK1 kinase has a hydrophobic pocket associated with its activation segment amenable for developing small-molecule inhibitors, which can stabilize its inactive conformation and allow blood–brain barrier passage[232,233]. Necroptosis has been implicated in mediating the pathogenesis of multiple human diseases characterized by cell death with inflammation such as ischaemic brain, heart, kidney or eye injuries, multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS)[49,66,204]. Biomarkers for the activation of RIPK1, RIPK3 and MLKL, including the phosphorylated forms (pRIPK1, pRIPK3 and pMLKL), have been demonstrated in postmortem pathological samples from patients with ALS, multiple sclerosis and Alzheimer disease[49,66,205,206]. Since RIPK1 is recruited exclusively to TNFR1 via Death domain-mediated interaction, the activation of RIPK1 kinase specifically mediates TNFR1 signalling. Thus, inhibition of RIPK1 kinase presents an opportunity to selectively inhibit the deleterious responses mediated by TNFR1 without affecting TNFR2. The RIPK1 inhibitor SAR443820 (previously known as DNL788) has been advanced into phase IIb human clinical trials for the treatment of ALS and MS.

The loss of RIPK3 expression, and therefore disabling necroptosis, has been found to be common in many cancers and cancer cell lines[142,234,235], which suggests that necroptosis may act as a cancer checkpoint during the development of cancers. In addition, targeting necroptosis may also modulate anticancer immunity. Necroptosis has been implicated in promoting cancer metastasis by mediating macrophage-induced suppression of T cell immunity in pancreatic ductal adenocarcinoma[236]. Inhibiting RIPK1 kinase and necroptosis may protect against oncogenic progression with the development of a highly immunogenic myeloid and T cell infiltrate.

block defective mitotic chromosome segregation and can thus lead to defective cell division and aneuploidy[201].

Cell death in human diseases

In this section, we briefly discuss the involvement of cell death and disruption of cellular homeostasis in human diseases, whereas Box 2 discusses the impact of our increased understanding of programmed cell death mechanisms on drug discovery.

Anti-apoptotic BCl-2 family as oncogenes

BCL-2 is transcriptionally activated in human follicular lymphomas as a consequence of t(14;18) chromosomal translocation, and overexpression of anti-apoptotic BCL-2 family members is also found in many types of cancer cells[13]. The pro-oncogenic activity of the anti-apoptotic BCL-2 family functions by prolonging the survival of cancer cells under stressful conditions, rather than promoting cell proliferation[11]. The critical role of this family in regulating the balance between death and survival is underscored by the successful development of venetoclax (BCL-2-specific inhibitor) and navitoclax (inhibitor of BCL-2, BCL-xL and BCL-W) for the treatment of leukaemias and lymphomas[202,203].

Necroptosis in neurodegenerative diseases

Necroptosis has been implicated in mediating the pathogenesis of multiple human diseases characterized by cell death with inflammation, such as ischaemic brain injury, glaucoma, multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS)[49,66,204]. Biomarkers for the activation of RIPK1, RIPK3 and MLKL, including the phosphorylated forms (pRIPK1, pRIPK3 and pMLKL) have been identified in postmortem pathological samples from patients with ALS, MS and Alzheimer disease[49,66,205,206]. Since RIPK1 is recruited exclusively to TNFR1 via

DD-mediated interaction, the activation of RIPK1 specifically mediates TNFR1 signalling. Thus, inhibition of RIPK1 presents an opportunity to selectively inhibit the deleterious responses mediated by TNFR1 without affecting TNFR2.

Inflammation mediated by RIPK1 and caspases

The two main pro-inflammatory pathways include RIPK1-mediated pro-inflammatory cytokine production downstream of TNFR1 and inflammasome-mediated caspase-1 activation. While the upstream activators of these two pathways may show both stimulus and cell specificity, the production and release of downstream cytokines often include a common set of pro-inflammatory factors, such as TNF, IL-1β, IL-6 and IFNγ, although the difference in the levels, timing and tissue specificity of such release may be crucial to determine the consequences on the organism. Inactivation of caspase-8-mediated cleavage of RIPK1 leads to autoinflammatory disease in humans[71,72]. Activated RIPK1 has been detected in the nucleus under necroptotic and inflammatory conditions such as in ALS[207]. Nuclear RIPK1 interacts with a striking array of transcriptional activators and coactivators, such as p65 (also known as RELA), SP1 and JUNB, as well as almost all components of the BAF chromatin remodelling complex, to promote a transcriptional induction of pro-inflammatory factors by regulating chromatin dynamics[208].

Activation of caspase-1, caspase-11 and the related caspases is mediated by multi-molecular complexes, known as inflammasomes, including different PRRs such as NLRP3, NLRP1, NAIPs, NLRC4, AIM2, Pyrin and adaptor proteins like ASC. The downstream signalling of these complexes is determined in both a PRR-dependent and stimulus-dependent manner[209]. The NLRP3 inflammasome has recently been shown to be activated in the context of neurodegenerative diseases, leading to both inflammation and degeneration[210]. The cleavage of GSDMD regulates the release of mature IL-1β and IL-18 to promote inflammation as a key pro-inflammatory mechanism in human inflammatory conditions[111].

Disruption of homeostasis in diseases

Disruption of protein homeostasis, the proper balance between protein synthesis and degradation, leads to the accumulation of misfolded and aggregated proteins in neurodegenerative diseases[211]. The restoration of cellular homeostasis is an important mechanism to consider, particularly for neurodegenerative diseases[151]. Targeting TRADD, an important adaptor in the TNFR1 signalling pathway, presents an opportunity to inhibit RIPK1-dependent apoptosis and to activate autophagy to restore cellular homeostasis by promoting the degradation of misfolded proteins[212].

Disruption of redox homeostasis leads to the accumulation of ROS, which is common in patients with chronic inflammatory diseases[213]. In addition, the accumulation of lipid peroxidation, as indicated by the elevated levels of 4-HNE, can lead to cellular damage and ferroptosis. Owing to its consumption of a disproportionally high amount of oxygen (~20%) in relation to only accounting for ~2% of body weight, the brain is highly sensitive to oxidative damage. Lipid peroxidation is an early pathological event in the brain of patients with amnestic mild cognitive impairment and in early-stage Alzheimer disease[214]. Oxidative stress and lipid peroxidation may be involved in promoting the formation of amyloid-β plaques in animal models of Alzheimer disease[215].

Conclusions and perspectives

Genetic studies of programmed cell death in the nematode *C. elegans* provided the first insights into the molecular mechanisms (mediated by EGL-1, CED-9, CED-3 and CED-4) that control developmental apoptotic cell death[216]. This evolutionarily conserved cell death mechanism has been significantly expanded in mammalian cells, presumably through gene duplications, to include genes that encode multiple homologues of CED-3 (caspase family) and CED-9 (BCL-2 family) that mediate not only developmental cell death but also disease-associated cell death and inflammation. Activation of necroptosis, mediated by RIPK1, RIPK3, MLKL and ZBP1, may have been added late in evolution because of additional demands to eliminate cells in complex multi-cellular organisms and during defence against pathogens. In addition, the cleavage of GSDMDs by caspases, including caspase-11, caspase-1, caspase-3 and caspase-8, can promote pyroptosis, a form of necrosis, that can also mediate the release of cytokines without a signal peptide, such as mature IL-1β, to drive inflammation under pathological conditions.

The consequences of activating specific cell death and inflammation pathways in vivo may differ depending on cell type and on the expression levels of different mediators of these pathways. For example, activation of RIPK1 has been associated with increased production of pro-inflammatory cytokines rather than cell death in microglia, whereas activation of RIPK1 in oligodendrocytes may result in cell death and dysmyelination due to the loss of mature oligodendrocytes and lead to compensatory regeneration of new oligodendrocytes[49,66,205]. Furthermore, the activation of caspase-1 and caspase-11 can promote the activation of downstream caspases (caspase-3 and caspase-7) as their specificities are similar to other long-prodomain caspases such as caspase-9[104,217]. The observation that caspase-1 and caspase-11 as well as caspase-8 and caspase-3 can cleave both apoptotic substrates and pyroptotic substrates is consistent with their cleavage specificities. However, whether the activation of caspases leads to apoptosis or necrosis may depend upon the expression levels of key caspase substrates, among the hundreds that are cleaved, in the dying cells.

The involvement of diverse cellular homeostatic mechanisms in mediating cell survival explains the complexity and increasing number of different cell death modalities being discovered. Since inactivation of cellular homeostatic pro-survival mechanisms may provide a wide variety of options to selectively induce the death of cancer cells, targeting selective cancer-preferred pro-survival mechanisms, by chemical or genetic manipulation, may provide exciting new options for the treatment of cancers in the future. Moreover, while many strategies for therapeutic intervention have focused on blocking aberrant activation of pro-death pathways, the possibility of enhancing specific pro-survival mechanisms for the treatment of neurodegenerative diseases should be further explored. Identifying which cell survival signals are disrupted and developing specific therapeutic strategies to restore them may enable cells, for example, neurons or pancreatic β-cells, to survive in the context of a deleterious milieu under specific disease conditions. Thus, understanding the signalling pathways that connect cellular homeostasis and cell death may enable the development of new therapeutic avenues that inhibit cell death and restore cell homeostasis and survival.

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