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Emerging connectivity of programmed cell death pathways and its implications in health and disease

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Competing interests

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Abstract

Removal of superfluous, infected or potentially neoplastic cells is driven by programmed cell death (PCD) pathways, which thus have important roles in homeostasis, host defence against pathogens, cancer and a range of other pathologies. Several types of PCD pathways have been described, including apoptosis, necroptosis and pyroptosis; they employ distinct molecular and cellular processes and differ in outcomes, such as their capacity to trigger inflammatory responses. Recent genetic and biochemical studies have revealed remarkable flexibility in the use of these PCD pathways and indicate a considerable degree of plasticity in their molecular regulation that, for example, allows inflammatory caspases despite primarily inducing pyroptosis, to induce apoptosis and, conversely, apoptotic stimuli to trigger pyroptosis. Intriguingly, this flexibility is most pronounced in cellular responses to infection, whilst apoptosis is the dominant cell death process through which organisms prevent the development of cancer. In this Review, we summarise the mechanisms of the different types of PCD and describe physiological and pathological processes engaging the crosstalk between these pathways, focusing on infections and cancer. We discuss the intriguing notion that the different types of PCD could be seen as a single, coordinated cell death system, in which individual pathways are highly inter-connected and can flexibly compensate for another.

H1 Introduction

The development and homeostasis of multi-cellular organisms not only depends on regulated cell proliferation, but also hinges on the disposal of cells that are no longer needed or pose a potential danger for the organism. This includes removal of damaged cells at risk of neoplastic transformation or those hijacked by microbes for pathogen replication. Programmed cell death (PCD) represents the primary means through which the organism coordinates the elimination of such cells[1-3]. It can be induced by developmental programs and stress-induced signals stimulating membrane-bound and cytosolic proteins that trigger cell death via intricate cascades of transcriptional changes and post-translational protein modifications. Work over the past three decades has defined several distinct types of PCD[4]. Apoptosis represents the coordinated disintegration and typically 'immunologically silent' removal of dying cells, while pyroptosis and necroptosis refer to comparatively 'violent' types of cell death that are characterised by the bursting of the dying cell and the resulting release of potent inducers of inflammation and, in the case of infected cells, also pathogens and its varying components, such as antigens (Fig. 1).

A simple model of the biological relevance of distinct forms of PCD stipulates that apoptosis ensures normal development and tissue homeostasis in mature organisms, while pyroptosis and necroptosis are reported to protect the host from pathogens and other external threats[4]. This view is supported by the analysis of mice with genetic disruptions of the different PCD pathways. Mice with defects in apoptosis, such as those lacking pro-apoptotic BCL-2 (B cell leukaemia/lymphoma-2) family **[G]** members BAX (BCL-2 associated X), BAK (BCL-2 antagonist/killer 1) and BOK (BCL-2 related ovarian killer), typically die during late stages of development or soon after birth with rare survivors into adulthood showing marked accumulations of lymphoid cells and pathologies arising from a lack of normal removal of

certain epithelial tissues (e.g. webbed paws, imperforate vagina)[5,6]. Conversely, mice deficient in pyroptosis or necroptosis are born healthy but are defective in their responses to certain pathogens or other external insults[7-9]. Having said so, there is now mounting evidence that the different PCD pathways are interconnected at multiple levels and should perhaps be thought of in less discrete terms. Here, we discuss the changing views about the roles of the different types of PCD in health and disease. We summarise the latest insights into molecular and functional connections between the different PCD pathways and interrogate the idea that all these pathways form integral components of a single extended system that responds with different kinetics and modalities to a complex set of challenges, particularly those imposed by infectious pathogens.

H1 Mechanisms of key PCD pathways

PCD pathways can be loosely grouped into those causing lytic *vs* non-lytic forms of cell death (Box 1). Lytic types of cell death, such as pyroptosis and necroptosis, result from the formation of pores or other breaches in the plasma membrane[4]. The breakdown of cellular integrity during lytic cell death is caused by water influx, loss of membrane potential and cellular swelling that ultimately results in the rupture of the cell. Consequently, intracellular content as well as material from intra-cellular pathogens, if present, such as RNA, DNA, proteins and lipids, is released into the extracellular space, where they act as danger-associated molecular patterns **[G]** (DAMPs) and pathogen-associated molecular patterns **[G]** (PAMPs). These danger signals stimulate pattern recognition receptors **[G]** and other receptor systems expressed by neighbouring macrophages and other bystander cells, triggering the release of pro-inflammatory cytokines[10] (Fig. 1). Non-lytic forms of PCD, such as apoptosis, are characterised by the coordinated disintegration of dying cells into smaller fragments, the so-called apoptotic bodies, which ensure sequestration of intracellular content, including DAMPs (and PAMPs, if

pathogen infected). Macrophages recognise apoptotic cells primarily through their TAM receptors **[G]** and the resulting removal of these dying cells is thought to prevent inflammation (Fig. 1).

H2 Cell death by apoptosis.

Cellular demolition during apoptosis is mediated by caspases[11]. These aspartate specific cysteinyl proteases are produced as inactive zymogens and are highly conserved throughout evolution[11]. A variety of upstream cell death signalling processes[12] cause the consecutive activation of 'initiator' and then 'effector' caspases that induce the various processes involved in cell demolition. This includes DNA fragmentation by DNAses[13], chromatin condensation and actomyosin-driven membrane blebbing generating apoptotic bodies[14].

Two distinct, albeit converging pathways can lead to apoptosis. Growth factor or nutrient withdrawal, developmental cues associated with tissue reorganisation or loss of cell attachment, as well as steroid hormones or treatment with diverse cytotoxic agents can trigger the 'intrinsic' (also called mitochondrial or BCL-2-regulated) pathway of apoptosis (Fig. 2a). This apoptotic pathway involves transcriptional and/or post-transcriptional increases in the expression of pro-apoptotic BH3-only proteins **[G]** that bind with high affinity to members of the pro-survival BCL-2 protein family[15,16], which in healthy cells, keep the effectors of apoptosis, BAX and BAK, in inactive states. When all pro-survival BCL-2 proteins within a cell are functionally neutralised by BH3-only proteins, BAK and BAX are unleashed to oligomerise and assemble into structures that cause a breach of the outer mitochondrial membrane, thereby inducing mitochondrial outer membrane permeabilisation **[G]** (MOMP)[17,18]. MOMP causes the release of several mitochondrial proteins, and some of these drive critical downstream processes in apoptosis. For example, cytochrome *c* binds to apoptotic peptidase activating factor 1 (APAF-1) upon translocation into the cytosol, promoting formation of the apoptosome. Monomeric,

121 enzymatically inactive pro-forms of the 'initiator' caspase 9 are recruited into the apoptosome,
122 resulting in caspase 9 activation thereby promoting the downstream proteolytic activation of
123 the 'effector caspases' 3 and 7 (ref. 19). Activation of this caspase cascade can be attenuated
124 by XIAP (X-linked inhibitor of apoptosis), one of the inhibitor of apoptosis proteins **[G]** (IAPs).
125 XIAP can prime certain caspases for proteasomal degradation[20]. MOMP also causes release of
126 SMAC (Second Mitochondrial Activator of Caspases, also known as DIABLO) and HTR2
127 (HTRA serine peptidase 2), which both can block XIAP and thereby prevent it from inhibiting
128 caspases[21].

129 Apoptosis can also be initiated through the death receptor (also known as 'extrinsic') pathway
130 upon ligation of so-called death receptors **[G]** on the cell surface (Fig. 2b). Stimulation of these
131 members of the tumour necrosis factor receptor (TNFR) family containing intra-cellular 'death
132 domains', such as FAS and TNFR1 [22], results in the recruitment of the adaptor protein FADD
133 (FAS associated via death domain), and for some receptors this also involves TRADD
134 (TNFRSF1A associated via death domain) through the action of death domains that are present
135 in both the receptors and their adaptors. Homotypic interactions between death effector
136 domains (present in FADD and pro-caspase 8) then promote recruitment of pro-caspase 8 into
137 the death inducing signalling complex (DISC), leading to activation of this initiator caspase[12,23] .
138 The activity of caspase 8 can be modulated by cFLIP (cellular CASP8 and FADD-like
139 apoptosis regulator) **[G]** [24]. Caspase 8 can proteolytically activate the executioner caspases to
140 unleash the downstream cell demolition processes of apoptosis. Caspase 8 can also convert the
141 BH3-only protein BID into its pro-apoptotic form, tBID, which triggers BAX/BAK-mediated
142 MOMP and apoptosis through the intrinsic pathway (see above)[25].
143
144 **H2 Cell death via pyroptosis.**

Caspase 1 and caspase 11 (caspase 4 and caspase 5 are the human homologues of mouse caspase 11) have important roles in a lytic form of cell death, termed pyroptosis, which is widely considered to be involved in defending the organism against pathogens[26,27]. Caspase 1 is synthesised as an inactive zymogen that undergoes autocatalytic activation within large disc-shaped platforms, called inflammasomes **[G]** (Fig. 3). These are formed upon triggering of members of the NLR (nucleotide-binding domain and leucine-rich repeat containing) family **[G]**[28,29], such as NLRP3 (NLR family pyrin domain containing 3), which engage pro-caspase 1, a process that is often facilitated by activating adaptors, such as ASC (apoptosis-associated speck-like protein containing) in the case of NLRP3 (refs.[11,29]). Many different cellular disturbances can cause NLRP3 inflammasome activation, including alterations in intracellular ion concentrations or failure to degrade inert structures, such as crystals, that can rupture lysosomal membranes[30]. It is still unclear how NLRP3 is activated, although a role for NEK-7 (NIMA related kinase 7) as an NLRP3 activator is emerging[31]. Binding of pathogen-derived DNA to AIM2 (Absent in melanoma 2) also promotes assembly of inflammasomes[32]. Inflammasomes can also incorporate NLRC4 (NLR family CARD domain containing 4), which is activated downstream of NAIPs (NLR family apoptosis inhibitory proteins) that recognise bacterial components, such as the rod-structure of the type III secretion (T3SS) apparatus **[G]** and flagellin **[G]**[33]. Although inflammasomes can differ substantially in the upstream sensors and therefore the activating ligands, they all result in caspase 1 activation[33]. Active caspase 1 cleaves several targets amongst which the gasdermin family **[G]** member, gasdermin D (GSDMD) is essential for pyroptosis[34]. GSDMD is cleaved by caspase 1 into an N-terminal and a C-terminal fragment (Fig. 3), with the latter exerting auto-inhibitory activity in unprocessed GSDMD. Liberated N-terminal GSDMD fragments are recruited to the inner leaflet of the cell membrane where they form transmembrane pores that create a breach between the cytosol and the extracellular space[8,35] (Fig. 3). This results in potassium efflux and

water influx, thereby destabilising the plasma membrane potential and causing rupture of the cell[34]. In addition to activating GSDMD to form pores, caspase 1 also promotes the proteolytic conversion of the precursors of the pro-inflammatory cytokines, pro-IL-1β and pro-IL-18, into their bioactive forms that drive wide-ranging inflammatory responses[36]. These cytokines lack the leader sequences required for secretion[37], and cell rupture allows these cytokines to be released into the extracellular space, providing further drivers of inflammation in addition to the release of DAMPs[38]. Recent findings suggest that IL-1β and IL-18 can also be released through GSDMD pores, even when only few such pores have formed and membrane repair mechanisms dependent on ESCRT **[G]** (endosomal sorting complexes required for transport) machinery are still able to keep cells alive[39]. However, more research is needed to better define how frequently such events occur and how relevant such transient pores are in the context of cellular responses to varying stressors at both the cellular and the organism level. Doing so will require overcoming challenges related to experimentally disentangling the relative roles of GSDMD in mediating pyroptosis *vs* the release of bio-active IL-1β and IL-18. While mice lacking IL-1β and/or IL-18 help decipher the specific contribution of the lytic cell death machinery downstream of inflammasomes (without a confounding influence of these cytokines), it is currently not possible to separate the role of these cytokines from lytic cell death, as they depend on GDSMD for release (either through lytic death or transient pores).

Human caspases 4 and 5, and their murine homologue caspase 11, are closely related in amino acid sequence to caspase 1. In the mouse, the genes for caspases 1 and 11 are located in close proximity[40] (which caused the first caspase 1 deficient animals to also lack caspase 11). There are, however, some notable differences between caspase 1 and caspase 11. Although caspase 11 can cleave GSDMD with similar efficiency as caspase 1, it is unable to convert pro-IL-1β and pro-IL-18 into their bio-active forms[11]. Caspase 11 also differs from caspase 1 in that it is

not constitutively expressed but depends on transcriptional induction through interferon alpha receptor and/or NFκB signalling[41,42]. Of note, caspase 11 and its human orthologues caspases 4 and 5 can bind to and be directly activated by lipopolysaccharide **[G]** (LPS)[43] as opposed to caspase 1, which is activated downstream of pattern recognition receptors. This suggests a particularly important role of caspase 11 in mice, and caspases 4 and 5 in humans, in host defence against gram-negative bacteria **[G]**. In fact, caspase 11-deficient mice are resistant to LPS-induced septic shock[44,45], indicating that the pro-inflammatory responses triggered by LPS alone through engagement of its cell-surface Toll-like receptor **[G]**, TLR4, (ref. 46) are not sufficient to cause lethal septic shock but that this pathology requires additional intra-cellular activation of caspase 11 by LPS[45]. These differences have led to the description of the machinery for caspase 11 activation as non-canonical inflammasomes. Disentangling the relative roles of caspase 1 and caspase 11 *in vivo* remains challenging, as the pore-inducing capacity of caspase 11 through activation of GSDMD promotes secondary NLRP3 inflammasome activation, most likely driven by the resulting potassium efflux[47,48]. This essentially 're-connects' caspase 11 to the full suite of pro-inflammatory signals, including caspase 1-mediated production of bio-active IL-1β and IL-18. It is perhaps useful to interpret these findings with the view that caspase 1 and caspase 11 operate in a complementary manner as products of gene duplication events[11] that provide the host with two overlapping processes for activating pyroptosis to dispose of cells invaded by gram-positive pathogens.

H2 Cell death via necroptosis.

Necroptosis is a pathway for genetically programmed lytic cell death that is thought to have a role in the killing of pathogen-infected cells and/or damaged cells during certain degenerative or inflammatory disorders[49]. Necroptosis can be induced by multiple innate immune signalling pathways, including those initiated by the stimulation of RIG-I-like Receptors **[G]**, TLRs and

death receptors[49]. These signalling pathways all lead to the phosphorylation and activation of necroptotic kinase RIPK3 (receptor-interacting serine-threonine kinase 3) (Fig. 4), which in the case of death receptor-induced necroptosis also requires RIPK1 activity[50]. RIPK3 activates the pseudokinase MLKL (mixed lineage kinase domain like) through phosphorylation, thereby causing substantial conformational changes that allow trafficking of MLKL to the plasma membrane, where it induces membrane permeabilisation[51]. Although the resulting loss of membrane integrity and cytosolic osmolarity accounts for the lytic nature of the cell death associated with necroptosis, more work is necessary to determine whether MLKL does form well-defined pores, as is the case for GSDMD, or causes a breach of the plasma membrane in some other way. Like for pyroptosis, during the early stages of MLKL activation, cell membrane repair processes involving ESCRT appear to be able to halt or possibly even prevent cell killing[52].

H1 Functions of PCD pathways in disease

The programmed suicide of cells in mature, fully developed organisms likely serves the purpose to combat infections by removing replicative niches for intracellular pathogens and to prevent cancer through the elimination of nascent neoplastic cells. Conversely, aberrant cell death has been postulated to contribute to pathologies associated with acute injuries and chronic degenerative diseases. PCD pathways also have roles in activating adaptive immune responses that are vital in the fight of the host to combat pathogens and cancers. For example, intracellular pathogens released from dying cells can be engulfed by nearby macrophages and neutrophils, whose subsequent activation results in the secretion of cytokines and chemokines that support the immune response (f.ex. via recruitment of cells involved in adaptive immunity). DAMPs, PAMPs and antigens released from dying cells are also sensed and engulfed by dendritic cells, and this primes these potent antigen presenting cells **[G]** to stimulate T

lymphocytes enabling them to find and destroy additional infected cells as well as aiding in the differentiation of B cells that produce pathogen-specific antibodies (Fig. 5).

H2 The role of PCD in host defence against infection.

Many of the sensors that initiate inflammasome activation and pyroptosis respond to components from intracellular pathogens, such as bacteria and viruses[53], implying that these processes may be indispensable for protecting the host from such infections. Early *in vitro* studies supported this view, showing that loss of caspase 1 abrogated the killing of macrophages infected with a variety of bacterial pathogens, including *Salmonella enterica*[54,55], *Pseudomonas aeruginosa*[56] or *Franciscella tularensis*[57], or viruses, like murine cytomegalovirus[58]. NLRP3 inflammasomes are also activated during influenza virus infections. This may involve direct roles of viral antigens, such as PB1-F2 **[G]**, acting as NLRP3 agonists[59] or could be an indirect consequence of the viral M2 protein **[G]** causing calcium flux from endosomes and the trans-Golgi network into the cytosol[60]. Despite these initial findings suggesting critical roles for pyroptosis in host defence against pathogens, there are also studies reporting comparatively minor defects in responses to bacterial infections caused by the absence of caspase 1, and even the combined lack of caspase 1 and caspase 11 (refs. 61,62). This surprising resistance of mice unable to rely on pyroptosis for host defence appears to be most prominent in infectious disease models involving relatively low doses of pathogen inoculation[63]. This is remarkable as infections of humans are thought to often commence with rather low pathogen doses. This implies that pyroptosis may have its most significant role under conditions of overwhelming pathogen encounters. Newer investigations showed that macrophages deficient for both caspase 1 and 11 could actually still die upon infection with intracellular pathogens but did so with protracted kinetics when compared to wild-type macrophages[64]. It thus appears likely that pyroptosis has an important role in limiting the

spread of large numbers of pathogens by rapidly killing infected cells, yet it is unlikely to be the only cell death pathway that the host relies on to remove infected cells.

The relative reliance on pyroptosis during host defence is likely influenced by microbial strategies to evade recognition and prevent inflammasome activation. For example, *Salmonella enterica serovar* Typhimurium rapidly downregulate flagellin expression once the bacteria have infected a cell, and *Salmonella* strains engineered to sustain flagellin expression are more efficiently removed from the host than wild-type bacteria[65]. Similarly, exposure of mice to *Legionella pneumophila* only results in productive infection when either the host lacks NLRC4 or the bacteria do not produce flagellin[66,67]. Removing a key trigger of NLRC4 inflammasomes and pyroptosis therefore affords the bacteria a replicative advantage[68]. The capacity to evade pyroptosis is likely a consequence of the continuing battle between these pathogens and the host. This notion is supported by the observation that microbes that do not typically infect mammalian hosts and have therefore not evolved under the pressure of mammalian immune systems, such as *Chromobacterium violaceum*, are non-pathogenic in wild-type mice even when administered at high doses, but cause lethal disease in mice deficient in pyroptosis or lacking IL-18 (refs. [63,69]) .

The role of certain other PCD pathways also seem to be linked to microbial evasion strategies. Some bacterial pathogens, such as enteropathogenic *Escherichia coli* (EPEC), utilise T3SS systems to inject effector proteins into host cells to promote their survival in the epithelial layers of the gut[70]. One of these effectors, NLEB1, has been shown to inhibit FADD to thereby prevent death receptor-induced apoptosis of host cells[71]. Accordingly, mice deficient in FAS-induced apoptosis showed delayed clearing of the EPEC-like mouse pathogen *Citrobacter rodentium*[71]. A critical role for death receptor induced apoptosis in host defence against

bacterial pathogens can also be inferred from rapid pathogen dissemination in caspase 8 deficient mice after infection with *Yersinia pestis, Y. pseudotuberculosis* or influenza virus[72,73]. However, as outlined above, caspase 8 is required to prevent necroptosis and therefore all these experiments were performed in mice lacking not only caspase 8 but also core components of the necroptosis machinery (i.e. RIPK3 or MLKL). This makes it difficult to determine whether the aforementioned failures in host defence resulted from the lack of either death receptor induced apoptosis or necroptosis, or possibly both. Findings that RIPK3-deficient mice are impaired in controlling Herpes simplex virus[74] or Vaccinia virus[75] have been used to argue for roles of necroptosis in these infections. In addition, a recent study showed that influenza virus and other orthomyxoviruses are associated with the generation of Z-RNAs **[G]**, which are bound by the ZBP1 protein that initiates RIPK-3-dependent activation of MLKL and necroptosis. Accordingly, mice lacking ZBP1 were more susceptible to infection[76]. However, the interpretation of these data is made difficult by RIPK3 also being involved in the activation of caspase 8-driven apoptosis, as well as the role of this kinase in the production of certain cytokines and chemokines[77,78]. Moreover, there are conflicting reports showing increased, unaffected or even decreased susceptibility to influenza virus infection in mice lacking key components of the necroptotic pathway[79-81]. Together with unperturbed control of *Y. pestis* or *Y. pseudotuberculosis* infection in mice deficient for necroptosis[73] and classes of mammals, such as marsupials and carnivorous placentals, lacking key components of the necroptosis machinery[82], these considerations question whether necroptosis exerts essential functions in host defence against pathogens.

Thus, although the literature suggests critical roles for PCD in controlling infections, it appears that no single pathway is universally responsible. Rather, the specifics and modalities associated with a particular infection, such as the type of pathogen (i.e. viral, bacterial, fungal

or parasitic), the infectious dose and host genetic makeup, as well as the actions of varying microbial immune evasion strategies[68], determine what type of PCD pathway(s) the host employs to achieve control and clearance of the invader.

H2 The role of PCD in cancer.

The role of apoptosis in cancer has been recognised for over 30 years. Indeed, the first regulator of programmed cell death, BCL-2, was discovered in human follicular lymphoma, where the t[14;18] chromosomal translocation causes BCL-2 overexpression[83,84]. Somatically acquired copy number amplifications of loci encoding the related pro-survival proteins MCL-1 or BCL-XL are found in ~10% or ~3% of human cancers, respectively[85]. Over-expression of pro-survival BCL-2 (ref. [86]), or absence of pro-apoptotic BIM[87] or PUMA[88], were shown to accelerate *c-Myc* oncogene-driven lymphoma development, altogether providing solid evidence that evasion of apoptosis can promote tumorigenesis. Notably, on their own, defects in apoptosis are not potent drivers of tumorigenesis, with only ~5% of mice over-expressing BCL-2 in lymphocytes developing lymphoma over an 18 month period[89]. Therefore, inhibition of apoptosis appears to aid tumour development by keeping alive cells that would normally die, thus facilitating their acquisition of oncogenic lesions (e.g. c-MYC over-expression) that then cooperate in neoplastic transformation.

Many, possibly all, anti-cancer agents kill malignant (and also normal) cells at least in part by inducing the intrinsic pathway to apoptosis[90], whereas direct activation of the death receptor induced apoptotic pathway does not appear to be required for cell killing downstream of these anit-neoplastic therapeutics[91]. However, the up-regulation of FAS and TRAIL receptors — often mediated by the tumour suppressor p53 — may sensitise malignant cells to the ligands of these death receptors. These ligands are expressed on activated cytotoxic lymphocytes **[G]**

and the ensuing death receptor-induced killing of malignant cells may contribute to the overall response of a tumour to therapy in vivo[92]. Studies using gene knock-out mice and cell lines have shown that different anti-cancer agents induce apoptosis in a manner dependent on distinct BH3-only proteins. PUMA and to a lesser extent NOXA are required for cell killing induced by agents that cause DNA damage (e.g. radiation, etoposide)[93,94], and they are both transcriptionally regulated by the tumour suppressor p53, which is inactivated in ~50% of human cancers. The induction of apoptosis by glucocorticoids (e.g. dexamethasone) depends on BIM and PUMA[95], and BIM is also critical for the killing of tumour cells by inhibitors of oncogenic kinases (e.g. inhibitors of BCR–ABL, mutant B-RAF or mutant EGFR)[96]. The demonstration of the critical role of BH3-only proteins in the killing of malignant cells by anti-cancer agents stimulated the development of small molecule mimetics of these initiators of apoptosis, so-called BH3 mimetic drugs **[G]**, for cancer therapy[97] (for recent reviews see[98,99]). Experiments using inducible gene deletion and later BH3 mimetic drugs revealed that the survival of certain tumour cells is safeguarded mainly by a single pro-survival BCL-2 family member, such as BCL-2 in chronic lymphocytic leukaemia[100] or MCL-1 in c-MYC driven lymphoma[101,102]. However, efficient killing of most malignant cells necessitates inhibition of two or more pro-survival BCL-2 family members. This can be achieved by using two BH3 mimetic drugs that target distinct pro-survival proteins or by combined treatment with a BH3 mimetic drug that inhibits a select pro-survival BCL-2 family member (e.g. venetoclax to inhibit BCL-2) plus another anti-cancer agent that causes an increase in pro-apoptotic BH3-only proteins (e.g. BIM, PUMA) that will inhibit the non-targeted pro-survival BCL-2 proteins present in the tumour cells[103].

Contrasting with the contribution of defects in the regulation of apoptosis in malignancy, so far cancer genome analyses have not identified recurrent mutations in genes encoding effectors

of necroptosis or pyroptosis. There have been reports suggesting a role for necroptosis in pancreatic cancer, whereby necroptosis would promote tumorigenesis (in contrast to the tumour suppressive role of apoptosis discussed above). Blockade of the necrosome **[G]** a protein complex composed of RIPK1, RIPK3 and MLKL, enhanced the *in vitro* proliferation of cell lines derived from pancreatic cancers, yet loss or inhibition of RIPK3 or RIPK1 diminished the growth of pancreatic cancers *in vivo* in mice. The discrepancy between these *in vitro* vs *in vivo* observations was attributed to the finding that loss of RIPK1 or RIPK3 function in cells in the tumour micro-environment created a more immunogenic milieu, by abrogating immunosuppressive signalling associated with necroptosis of tumour-associated macrophages[104,105]. However, these findings have since been challenged[106].

The roles of pyroptosis and inflammasomes, more generally, in the development of cancer and cancer therapy are currently also not clear. There is little evidence to suggest that induction of pyroptosis in cells undergoing neoplastic transformation functions as a major mechanism of tumour suppression, but the release of IL-1β and IL-18 could lead to the infiltration and activation of immune cells into tumour sites, which could support anti-tumour immune responses[107]. However, opposing evidence suggests that pyroptosis, through promoting release of IL-1β and IL-18, could support tumorigenesis. In this context, inflammasome-driven secretion of IL-1β and IL-18 could induce in neighbouring non-malignant cells the expression of angiogenic proteins and growth factors, such as vascular endothelial growth factor (VEGF), IL-6, IL-8, TNF (tumour necrosis factor) and TGF-β (transforming growth factor β), that fuel tumour growth[107]. Hence, it is not clear under which circumstances IL-1β- and IL-18-induced inflammation and GSDMD-mediated pyroptosis would promote or hinder tumour development. Sustained inflammation has clearly been implicated in the development of several cancers, including those of the liver, stomach and colon[108]. Yet, to our knowledge, essential mediators

of pyroptotic cell death, such as GSDMD, or components of the various inflammasomes have not emerged from whole genome CRISPR- or shRNA-based screens as tumour suppressors or resistance factors against anti-cancer agents. However, recent studies showed that GSDME, a relative of GSDMD that is also thought to be able to perforate the plasma membrane, can act as a tumour suppressor in certain solid cancers[109,110]. Many human cancer-derived cell lines have inactivating mutations in GSDME and low expression of GSDME was associated with decreased survival of patients with certain cancers[109,110]. Additionally, it was reported that GSDME can be activated by effector caspases in tumour cells treated with chemotherapeutic drugs[111]. This was proposed to change the death of the tumour cells from non-lytic apoptosis to a lytic pyroptosis-like death. Such GSDME-mediated tumour cell lysis may underlie the tumour lysis syndrome **[G]** with the associated massive cytokine release (cytokine release syndrome) that cancer patients experience when their phagocytic system is overwhelmed by very large numbers of cancer cells undergoing drug induced apoptosis in a short amount of time[13]. Consistent with this, it has recently been demonstrated that GSDME-mediated pyroptosis of cancer cells induced by therapeutic CAR (chimeric antigen receptor) T cells **[G]** could cause cytokine release syndrome. DAMPs released by dying cancer cells were reported to cause activation of caspase 1 with consequent GSDMD processing and cytokine release in macrophages. This non-desired effect of pyroptosis on macrophages could be one of the factors limiting the effectiveness of CAR-T cell therapy in cancer[112]. These findings suggest that therapies that induce PCD directly in large numbers of cells (e.g. BH3 mimetic drugs or CAR-T cells) could be exploited more effectively and safely by combining them with strategies that limit the detrimental 'cytokine storm'.

H2 PCD in diseases associated with traumatic or degenerative cell loss.

Cell death has long been recognised as a feature of several acute as well as chronic degenerative pathologies[1]. Many degenerative disorders are characterised by aggregates of misfolded proteins that are thought to result in pathological cell death, for example the loss of motor neurons in amyotrophic lateral sclerosis (ALS) or reductions in cortical and hippocampal neurons during Alzheimer disease[16]. Several cell death processes have been implicated in the pathological loss of cells in these diseases. For example, insoluble complexes incorporating active RIPK1, RIPK3 and MLKL were found in the brains of Alzheimer disease patients but not in healthy controls[113]. Pharmacological blockade or genetic ablation of MLKL in a mouse model of Alzheimer disease was reported to result in partial improvements of functional defects[114] and the absence of RIPK1 activity was shown to delay the onset of symptoms in a mouse model of ALS[115]. Interestingly, amyloid plaques **[G]** isolated from brains of Alzheimer disease patients have abnormally high levels of active caspase 1 and mouse models suggest a role for NLRP3 inflammasomes and pyroptosis in this disease, possibly triggered by these pathological protein aggregates[116]. Inflammasome activation has also been linked to fronto-temporal dementia[117], ALS and Parkinson disease[116].

Apoptotic cell death has long been hypothesised to have a critical role in neurodegeneration, with reports showing that Aβ fibrils associated with Alzheimer disease can activate caspase 3 in cultured neurons and that this is accompanied by increased expression of pro-apoptotic BAX and decreased expression of pro-survival BCL-2 proteins[118,119]. Similar changes in the expression patterns of regulators of apoptosis have also been documented in the hippocampi of patients with Alzheimer disease[120]. While all these lines of evidence suggest that necroptosis, apoptosis and pyroptosis are active in the diseased tissues, it remains unclear whether this constitutes a cause or consequence of the underlying pathologies. The fact that the genetic loss of BIM, caspase 1 and/or caspase 11 or necrosome components (MLKL, RPIK3 or RIPK1) did

not afford marked protection in several mouse models of neurodegenerative diseases makes it unlikely that aberrant activation of a single PCD pathway is the critical driver of these complex pathologies. This highlights the need for more research in this area.

Contribution of PCD is also considered in traumatic tissue damage. Disruption of oxygen supply, loss of nutrient provision and failure to remove cellular waste products promote cell death and tissue injury during ischaemic events caused by blood vessel blockage or luminal occlusion via thrombosis or embolism. While this causes un-regulated necrotic cell death of the immediately affected ischaemic tissue, the slower, secondary death of more distant cells may be mediated by apoptosis triggered as a result of the loss of support from cells that had been killed in the initial traumatic event[121]. This view is supported by demonstration of smaller infarct areas in mice lacking pro-apoptotic BAX, BAK or PUMA or the death receptor FAS[122]. Necroptosis has also been implicated in the aforementioned pathologies, but genetic loss of mediators of necroptosis (i.e. RIPK3) did not offer marked benefit in mouse models of ischaemic injury of the brain[123]. Loss of RIPK3 or the kinase function of RIPK1 did, however, afford significant protection in murine models of ischaemic injury of the kidney and heart[123]. Of note, loss of MLKL afforded less protection[123], suggesting roles for RIPK1 and RIPK3 in these pathologies that extend beyond their function as necroptosis inducers. This may involve the functions of these kinases in the induction of apoptosis (via caspase 8) or the production of cytokines and chemokines[78]. There are also indications of a potential role for ferroptosis (Box 1), another PCD pathway, in certain ischaemic diseases that are associated with abnormal cell killing. For example, an inhibitor of ferroptosis was shown to protect renal tubular cells from death in culture[124].

Inflammasome activation has also been implicated in inflammatory diseases involving tissue damage that are associated with unwanted cell death. For example, in murine models of periodic fever syndrome **[G]**, loss of activators of inflammasomes or loss of GSDMD were reported to markedly reduce pathology associated with the severe inflammation, such as pharyngitis, stomatitis and adenitis[125]. Given the role of GSDMD in the cellular release of IL-1β and IL-18, it is, however, not clear whether this protection was due to the prevention of pyroptotic cell death, the reduction in the release of these pro-inflammatory cytokines, or both.

Clearly, we still know too little about the role of the various PCD pathways in these diverse disorders. Nevertheless, it is tempting to speculate that treatment with inhibitors of multiple PCD mechanisms in combination with agents that target the insult that caused the pathology in the first place could have therapeutic value, although the exact implementation and safety of such hypothetical combination treatments needs to be established.

H1 Connectivity between PCD pathways

The findings discussed above highlight important roles for the different types of cell death in both the maintenance of homeostasis and a broad variety of pathological conditions. However, they also make it abundantly clear that the biological relevance of the different cell death pathways is complex and that the individual PCD pathways do not seem to have unique and isolated roles, but rather operate in synergy to eliminate cells. Having said so, it is intriguing that host responses to infection make a broad use of the different types of PCD, yet embryonic development, tissue homeostasis and cancer development are most strongly associated with apoptosis, indicating the existence of perhaps some form of specificity in the engagement of different PCD pathways. In the following section, we will take a closer look at the interplay

between different types of PCD and compare and contrast the relevance of connections between PCD pathways in infection and cancer.

H2 Switching between apoptosis, necroptosis and pyroptosis.

The inhibition of caspase 8 or its adaptor FADD would be expected to cause an increase in the number of cells in tissues owing to the ensuing blockade of death receptor induced apoptosis. However, this is not the case suggesting that other types of cell death can be activated in cells unable to undergo death receptor induced apoptosis. The discovery of aberrant necroptosis induction in cells lacking caspase 8 (ref. 126) or FADD127 provided the first glimpse of the possibility that different types of PCD are functionally intertwined78,128,129. A likely explanation for this capacity of cells to substitute apoptosis with necroptosis may be found in strategies of several pathogens that have evolved varying means to prevent caspase 8 activation and consequently apoptosis. For example, the latent *K13* gene of the Human Herpesvirus (HHV)-8 encodes a viral equivalent of cFLIP, called vFLIP. vFLIP binds to and prevents activation of pro-caspase 8 and thus blocks death receptor induced apoptosis in HHV-8-infected cells130. Other examples include: cowpox viruses, which encode the serpin **[G]** CrmA capable of blocking caspase 1 and caspase 8 (ref. 131); HSV preventing caspase 8 activation via ICP6 (ref. 132); and cytomegalovirus (CMV) promoting production of viral mitochondria-localised inhibitor of apoptosis, a protein with structural similarities to anti-apoptotic BCL-XL that blocks MOMP by sequestering BAX133. Necroptosis may therefore have evolved to provide a back-up process to kill cells infected with pathogens that evade apoptosis. This option is of course limited to cells equipped with the molecular machinery for necroptosis, which at least in some types of neurons (and all cells of certain species; see above) does not appear to be the case134.

517 Functional backup of PCD pathways is not unique to necroptosis. Macrophages infected with
518 *Yersinia pseudotuberculosis* typically die by undergoing apoptosis. However, macrophages
519 lacking the apoptotic executioner caspases still die in response to *Y. pseudotuberculosis*
520 infection but this appears to occur via pyroptosis instead[135]. This functional link between
521 apoptosis and pyroptosis also seems to have a role during embryonic development. Most
522 functions of caspase 8 have been attributed to its enzymatic activity, but recent reports suggest
523 that it may also function as a scaffolding protein. These scaffold functions have been revealed
524 in mice that expressed a version of caspase 8 with a selective defect in its proteolytic activity.
525 Notably, these mice display signs of uncontrolled pyroptosis in the intestine[136-139], indicating
526 that the enzymatic activity of caspase 8 not only is critical for apoptosis and keeping
527 necroptosis in check, but may also prevent pyroptosis. It will be interesting to investigate
528 whether pyroptosis is triggered when the enzymatic activity of caspase 8 is inhibited because
529 expression of enzymatically inactive caspase 8 induces the formation of aggregates containing
530 caspase 8 and ASC that have the capacity to activate caspase 1 in a manner similar to some
531 other protein aggregates (e.g. certain crystals or those implicated in neurodegenerative
532 disorders)[136]. Regardless, these findings suggest that caspase 8-mediated cell death is
533 functionally backed-up by several processes. Interestingly, cell death back-up mechanisms are
534 not only relevant when apoptosis is blocked but also appear to come into play when the
535 magnitude of apoptotic events is induced to such a level that it exceeds the capacity of the
536 phagocytotic system to safely remove the dying cells. In this case, cells undergo a lytic type of
537 cell death, initially referred to as ‘secondary necrosis’, which is akin to the unregulated death
538 that is induced by excess heat or freezing. At the molecular level, this secondary lytic cell death
539 likely involves channel-forming molecules, such as pannexin-1 (ref. [140]) that act downstream
540 of apoptotic caspases. This is followed by a decrease in intracellular potassium concentrations
541 in cells at late stages of apoptosis thereby driving NLRP3 inflammasome activation and

pyroptosis[141]. This raises the prospect of a caspase 3-activated pathway towards pyroptosis, which springs into action when the processes for the removal of cells undergoing apoptosis are hampered or overwhelmed. Although, so far, this switch from apoptosis to secondary lytic cell death has mostly been examined under *in vitro* conditions[141], these mechanisms are likely involved in the tumour lysis syndrome with excessive release of cytokines that can occur in lymphoma patients treated with apoptosis-inducing BH3 mimetic drugs[142]. Perhaps this detrimental tumour lysis syndrome could be attenuated using inhibitors of caspases. It must, however, be noted that upon caspase inhibition cells will generally still undergo cell death[143], likely owing to the mitochondrial dysfunction and energetic collapse of a cell following extensive MOMP. Importantly, this caspase-independent cell death is pro-inflammatory, and therefore may engender the potential to promote anti-cancer immune responses[144].

The ability of cells to alternate between different cell death pathways is perhaps best documented in the cellular response to TNF. Under physiological conditions, TNFR1 stimulation mostly triggers signalling pathways that promote cell survival, proliferation and differentiation[145] (Fig. 6). Stimulation of TNFR1 by its ligand, TNF, induces the formation of a so-called type I signalling complex, comprising TRADD, RIPK1, TRAF2 (TNF receptor associated factor 2) and/or TRAF5 and the E3 ligases cIAP1 (cellular inhibitor of apoptosis protein 1) and cIAP2. cIAP1 and cIAP2, together with the linear ubiquitination complex (LUBAC), ubiquitylate TNFR1 signalling complex components (which is counteracted by deubiquitylating enzymes, such as CYLD and A20, to attenuate signalling)[146]. The ubiquitylation of components of the type I TNFR1 signalling complex promotes the recruitment, phosphorylation and retention of key regulators that control activation of the canonical and non-canonical NFκB pathways **[G]**, including mitogen-activated protein kinase 7 (TAK1), IKK-α–IKK-β (inhibitor of nuclear factor kappa-B kinase complex) and NEMO

(NF-kappa-B essential modulator)[147], as well as the recruitment of JNK kinase that activates AP1 (adaptor protein complex 1) transcription factor[148]. NFκB and AP1 transcription factors can activate genes encoding pro-survival factors, including the pro-survival BCL-2 family members BCL-XL, BCL-2 and A1 (ref. [149]) as well as cFLIP (Fig. 6), thereby blocking both the intrinsic and the death receptor-induced apoptotic pathways to promote the survival of TNF-stimulated cells[150]. These mechanisms can have a role in pathogen defence. For example, *Yersinia Spp.* facilitate intracellular infection through T3SS-mediated injection of a variety of effector molecules. One of these, YopJ, can directly block the activation of TAK1 (refs. [151,152]), thereby preventing infected cells from responding to TNF through NFκB pathway activation, which beyond regulating cell survival is also important for stimulating immune responses that would target the pathogen. However, in this case, pathogen-induced blockade of NFκB does not act in the pathogen's favour, because upon TNFR1 stimulation this blockade triggers death receptor-mediated apoptosis, which prevents efficient pathogen replication[145]. This dramatic change in cellular outcome — switching from survival mode to apoptosis — in response to TNF is caused by the formation of an alternative, so-called type II complex of TNFR1 signalling, comprising TRADD, FADD and pro-caspase 8, the formation of which is otherwise inhibited by TAK1 and the IKK complex[153]. The absence or blockade of these NFκB signalling components alleviates this inhibition and allows the type II TNFR1 complex to recruit and activate pro-caspase 8 and thus links TNFR1 to the triggering of apoptosis. Bearing in mind that certain pathogens, such as *Pseudomonas Spp.*, *Vibrio Spp.* or enteroviruses, target TAK1 (refs. [154-156]), it is tempting to speculate that the switch in cellular response to TNFR1 signalling from promoting cell survival and proliferation to apoptosis reflects a host strategy aimed at offsetting microbial attempts at evading NFκB and/or AP-1 driven responses. This cell death may even be seen as a guardian of intact NFκB and AP-1 function that ensures the removal of cells in which these vital responses are corrupted by pathogens or other noxious stimuli.

The above considerations raise the important question of whether similar flexibility encompassing the possibility to activate back-up cell death pathways also occurs in the context of cancer and other conditions, where cell death has an important role. As discussed above, evasion of apoptosis can promote tumorigenesis, but there is only little evidence that other pathways of PCD can also suppress tumorigenesis or exert a compensatory role in tumour suppression when apoptosis is blocked. For example, c-MYC over-expression in addition to causing aberrant cell proliferation also increases the predisposition of cells to die[157]. This cell death is entirely blocked by over-expression of anti-apoptotic BCL-2 family members[86,158,159] or loss of pro-apoptotic BIM[87] or PUMA[88]. This indicates that cells with deregulated c-MYC expression do not compensate for the lack of apoptosis by undergoing necroptosis, pyroptosis or some other form of cell death, as a compensatory tumour suppressive process. Moreover, malignant and non-transformed cells lacking the essential effectors of apoptosis, BAX and BAK, show impressive long-term survival when treated with anti-cancer agents, and retain the capacity to proliferate again after the cytotoxic insult has been removed[6,160]. This supports the notion that in cancer therapy there is no major functional back-up by other processes of PCD that would help eliminate tumour cells. Having said so, it has been reported that under certain circumstances autophagic cell death (Box 1) can contribute to tumour suppression, for example in cells undergoing replicative crisis, where the release of telomeric DNA into the cytosol induces autophagy via intracellular DNA sensor cGAS **[G]** [161]. However, there is also evidence that autophagy can enhance tumour growth under conditions of stress, for example by providing nutrients and energy[162]. Autophagy has also been associated with reduced expression of class I major histocompatibility complexes (MHC), thereby protecting malignant cells from attack by cytotoxic lymphocytes[163]. Whether the appearance of non-apoptotic types of cell death in tumour tissues reflects secondary inflammatory responses, perhaps arising from

necrosis of malignant cells caused by hypoxia, or rather indicates a more intricate regulatory relationship between apoptosis and other cell death pathways in cancer remains to be investigated.

The situation is equally complex with regards to switching between PCD pathways in the context of degenerative disorders. Experimental data indicate that neurodegenerative diseases are associated with apoptosis, necroptosis and pyroptosis, but the slow progression of these diseases makes it difficult to discern whether the potential involvement of various PCD pathways is the consequence of switching between the pathways or simply represents secondary effects, whereby the triggering of one type of PCD ultimately causes tissue damage that can result in other types of PCD. The observation that abnormal elevation of IL-1β in the brains of Alzheimer disease mouse models are dependent on RIPK1 may suggest the latter[164]. It is also interesting to consider that the developing brain is characterised by enhanced susceptibility to apoptosis compared to the adult brain and this may in fact be an important feature for building and establishing new neuronal connections. Upon maturation of the organism, most neurons will transition into a post-mitotic stage and this is thought to reverse the initial susceptibility to apoptosis towards a state in which they are refractory to cytotoxic insults[16]. It is tempting to speculate that the proposed contributions of pyroptosis and necroptosis to neurodegenerative disorders might be related to decreased susceptibility of neurons to apoptosis, which would be reminiscent of infected cells that undergo necroptosis when the pathogen expresses inhibitors of apoptosis.

H2 Molecular plasticity in the induction of the known PCD pathways.

Recent findings suggest that the different PCD pathways are also inter-connected at the molecular level in a manner that goes well beyond their consecutive activation as part of a fail-

642 safe system (Fig. 7). As discussed above, NLRC4 activation driven by bacterial components,
643 such as flagellin, stimulates inflammasome formation and the resulting caspase 1 activation
644 promotes pyroptosis via GSDMD cleavage[35]. Of note, cells lacking caspase 1 or GSDMD still
645 die in response to flagellin-induced NLRC4 activation, but this death not only sets in with
646 delayed kinetics but also takes on morphological characteristics of apoptosis[165]. Such dying
647 cells contain active caspase 8, likely complexed with ASC and NLRC4 (refs. [67,166]). This
648 indicates that cells with sustained inflammasome activation that are unable to undergo
649 pyroptosis (e.g. lacking GSDMD) can die by apoptosis instead (Fig. 7). Interestingly, the likely
650 physical interaction between caspase 8 and inflammasome components is not exclusive to
651 NLRC4, but has also been demonstrated upon stimulation of NLRP1b by lethal anthrax toxin[167].
652 Inflammasomes thus appear capable of incorporating and activating not only caspase 1 but also
653 caspase 8. There are reports that inflammasome activation may also induce apoptosis through
654 additional molecular switches, for example via caspase 8 mediated processing of the BH3-only
655 protein BID into pro-apoptotic tBID with subsequent triggering of the intrinsic apoptotic
656 pathway through activation of BAX/BAK and induction of MOMP[168] (Fig. 7). A recent study
657 indicated that caspase 1 may also directly proteolytically activate pro-caspase 3 in macrophages
658 infected with *Salmonella enterica serovar* Typhimurium that lack GSDMD[64]. Interestingly,
659 macrophages can still undergo PCD in response to *Salmonella* infection even if they lack all
660 known executioner molecules and under such circumstances, they appear to rely on caspase 1
661 and caspase 8 acting not only as initiators but also as executioners of PCD[169]. These findings
662 challenge our previous understanding that apoptosis and pyroptosis are functionally distinct
663 cellular responses that serve very different purposes. The existence of inflammasomes that can
664 drive apoptosis rather than pyroptosis is also conceptually intriguing as it links recognition of
665 PAMPs, such as flagellin or bacterial toxins, to apoptosis with potentially concomitant
666 production of anti-inflammatory cytokines by macrophages engulfing apoptotic cells (Fig. 1).

667 It remains to be determined whether the resulting changes in the type of the cell death response
668 from pro-inflammatory to anti-inflammatory serve to apply immune regulation in the context
669 of potentially overwhelming anti-microbial responses. It is also possible that inflammasome-
670 driven forms of apoptosis might be particularly relevant to cell types that do not express
671 GSDMD, such as neurons or mast cells **[G]**[64]. Perhaps this represents a form of protection for
672 cells that directly connect to many other cells in the body, as is the case for neurons. These
673 alternative mechanisms of PCD induction, favouring apoptosis over lytic cell death pathways,
674 could then be seen as means of preventing damage to neighbouring cells caused by cell lysis,
675 concomitant release of DAMPs as well as other mediators of inflammation, such as histamine
676 and TNF, as would be the case for mast cells.
677
678 Recent reports indicate that a reverse connection may also be at play, whereby caspase 8 drives
679 pyroptotic responses. This appears to be particularly relevant under conditions in which death
680 receptor induced NFκB signalling is disrupted (see also previous sub-section). For example,
681 stimulation of *Yersinia Spp.*-infected macrophages with LPS and a TAK1 inhibitor was shown
682 to result in caspase 8 mediated GSDMD cleavage and pyroptosis[170]. Similarly, *Yersinia Spp.*
683 infection of macrophages defective in the apoptotic executioner caspases caused proteolytic
684 activation of GSDMD in a manner dependent on YopJ-mediated inhibition of TAK1 (ref. [135]).
685 Interestingly, a recent study suggested that the association between caspase 8 and pyroptosis
686 can be inhibited through NFκB-dependent induction of the long form of the apoptosis inhibitor
687 cFLIP[171]. The precise molecular mechanism that connects caspase 8 to pyroptosis is not clear.
688 Caspase 8 appears capable of cleaving GSDMD directly but does so with about 30-times lower
689 efficacy compared to caspase 1 (ref. [140]). The pyroptotic activity of caspase 8 has also been
690 linked to its incorporation into ripoptosomes **[G]**, which resemble the type II complex of
691 TNFR1 signalling comprising RIPK1, FADD and caspase 8 (Fig. 7)[172]. However, whether and

how ripoptosomes engage with inflammasome components remains to be determined. Caspase 8 has also been implicated in the pyroptotic expulsion of epithelial cells during intestinal *Salmonella* Typhimurium infection, where it was shown to be activated downstream of NLRC4. Such NLRC4–caspase 8 inflammasomes were shown to be sufficient to drive pyroptosis in the absence of caspase 1 (ref. [173]). This efficient cell removal process might explain why most human *Salmonella Spp.* infections are rather mild and limited to gastroenteritis and do not advance to typhoid fever[174].

The above findings support two important conclusions. First, they indicate that the biochemical and cellular consequences of one type of cell death can have profound influence on the activity of another type of cell death. Second, they show that the known molecular pathways underpinning our current understanding of PCD are characterised by surprising levels of plasticity, where upstream triggers of apoptosis can cause inflammatory cell death and, conversely, inflammatory caspases, such as caspase 1, can drive 'immunologically silent' apoptosis.

H1 Conclusions and perspectives

While much of the research so far has focused on the molecular mechanisms underpinning individual types of cell death, it is now evident that the different PCD pathways do not operate in isolation. In fact, a more likely interpretation of the current knowledge around the varying possibilities in triggering and rewiring the PCD signalling cascades suggests that apoptosis, pyroptosis and necroptosis constitute a single, coordinated cell death system, in which one pathway can flexibly compensate for another. This back-up system is also characterised by remarkable plasticity enabling classical apoptotic triggers to induce pyroptosis, while inflammatory caspases can induce apoptosis, which further blurs the lines between the

supposedly ‘immunologically silent’ character of apoptotic death and the highly inflammatory consequences of pyroptosis or necroptosis.

It is also interesting to consider the kinetics with which the fail-safe systems of using different PCD pathways for elimination of pathogens spring into action. The apoptotic death ensuing in cells unable to undergo pyroptosis takes place with a considerable delay compared to the rapid onset of pyroptosis. Perhaps pyroptosis is much faster because its execution depends on the enzymatic activation and cleavage of pre-formed caspase 1 and subsequent proteolytic activation of pre-formed GSDMD. Apoptosis, by contrast, involves more steps to ensure the coordinated and ‘clean’ removal of a cell, which may be more time-consuming. Although apoptosis might not be evident in infected cells, its machinery might nonetheless be activated ‘in the background’ in cells undergoing pyroptosis or necroptosis, and hence can run its course when these lytic forms of PCD are not available or blocked. We hypothesise that the different cell death processes have evolved to provide multicellular organisms with a failsafe system to offset varying and ever evolving attempts by pathogens to evade host cell suicide. An answer to the question of why cells have so many ways to die could therefore be that this versatility is needed to allow the existence of multicellular organisms in a world dominated by single-cell organisms.

In contrast to infection, for tumour suppression, apoptosis appears clearly much more important than all other PCD pathways. Also, all currently used drugs, in particular the BH3 mimetics, induce apoptosis in malignant cells[98,175]. This rather strict dependence on apoptosis for cancer cell death is noteworthy, as in principle any type of PCD could lead to tumour shrinkage. Clearly more work is required to better understand the context in which PCD pathways are

coordinated as a failsafe system, what the underlying drivers are and why the interplay between PCD pathways appears to differ between cell types and forms of cell stress.

Regardless of the underlying biology and fascinating conceptual questions about cause and consequence in the context of cell death, therapeutic manipulations of cell death pathway have enormous promise. This includes the use of BH3 mimetic drugs in haematological malignancies, which is already in place[98], but likely also pertains to other means of cell killing and their future therapeutic use in the treatment of cancers more generally (i.e. not only leukaemias and lymphomas). For example, agonists of death receptors[22] would be expected to synergise with BH3 mimetic drugs, activating both apoptotic pathways, although the safety of such combination therapies requires careful assessment. Other therapeutic approaches might involve GSDME, which when introduced in its active state into cancer cells can promote anti-tumour immunity *in vivo* through the induction of pyroptosis[109]. The killing of tumour cells also underpins the success of immune checkpoint **[G]** inhibitors, whose capacity to reinvigorate exhausted T cells has caused remissions and possibly even cures in certain types of cancer, such as melanoma[176-178]. There may also be scope for cell death-inducing drugs in infections, as was shown in the context of pulmonary *Legionella Spp.* infections[179]. By contrast, blocking PCD pathways could be explored as a therapeutic approach in degenerative diseases[180]. We are hopeful that future investigations of the complicated molecular interactions between the different PCD pathways underpinning their functional complementation may lead to the identification of central signalling hubs that could be exploited as drug targets for various human diseases.

Glossary

BCL-2 (B cell leukaemia/lymphoma-2) family: A family of proteins that has been named after its original member, BCL-2, which regulate the intrinsic apoptotic pathway.

Damage-associated molecular patterns (DAMPs, also known as alarmins): Intracellular molecules, such as HMGB1 or S100A8, whose release from cells undergoing lytic cell death triggers distinct receptors in innate immune cells and causes inflammation.

Pathogen-associated molecular patterns (PAMPs): Evolutionary conserved molecular components of pathogens, such as LPS expressed by gram-negative bacteria, causing inflammatory responses by innate immune cells.

Pattern recognition receptors: Membrane-associated or cytosolic receptors capable of recognising and responding to PAMPs through the induction pro-inflammatory responses.

TAM receptors: Family of receptor tyrosine kinases (TYRO3, AXL, MERTK) that promote apoptotic cell clearance by binding to phosphatidylserine exposed on apoptotic cells using GAS6 and PROTEIN S as bridging ligands.

BH3-only proteins: Pro-apoptotic members of the BCL-2 protein family, which share only one out of the four BCL-2 homology (BH) domains, namely the BH3 domain, with the remainder of the family. BH3-only proteins are induced transcriptionally and/or activated post-transcriptionally in response to developmental cues or cytotoxic stimuli that initiate the intrinsic apoptotic cell death pathway.

Mitochondrial outer membrane permeabilisation (MOMP): Perforation of the outer mitochondrial membrane, causing leakage of content from the mitochondrial intermembrane space, including the apoptosis inducers cytochrome *c* and SMAC. MOMP can result in the translocation of mitochondrial DNA into the cytosol, leading to the production of type I interferons and thereby drive inflammatory responses.

Inhibitor of apoptosis proteins (IAPs): Family of proteins with structural homology (i.e. Baculovirus IAP repeat). Some of the IAP proteins have E3 ubiquitin ligase function, allowing them to ubiquitylate their target proteins. XIAP inhibits apoptosis by binding to and promoting degradation of caspases 3 and 7, whereas cIAP1 and cIAP2 promote pro-survival signalling from TNFR1 by enhancing NF**κ**B activation.

Death receptors: Subsets of the Tumour Necrosis Factor Receptor superfamily that contain an intra-cellular death domain, which upon ligation can induce killing of the cells on which they are expressed through FADD adaptor protein mediated activation of caspase 8.

cFLIP (CASP8 and FADD like apoptosis regulator): Protein with structural similarity to caspase 8 but lacking enzymatic activity. There are two forms of FLIP: FLIP short and FLIP long. FLIP short inhibits apoptosis by preventing activation of caspase 8. FLIP long can form heterodimers with caspase 8 and this heterodimer inhibits necroptosis by cleaving RIPK1. High levels of FLIP long can also inhibit caspase 8 activation and apoptosis.

Inflammasomes: A multimeric protein complex, activated by various events, including ion flux, reactive oxygen species or mitochondrial dysfunction. It is comprised of sensors, such as

NLR molecules, often depends on the adaptor protein ASC and pro-caspase 1, which together causes the autocatalytic activation of caspase 1 with consequent proteolytic processing of pro-IL-1β and pro-IL-18 into their bio-active forms to cause inflammation and proteolytic activation of GSDMD to drive pyroptotic cell death.

NLR (nucleotide-binding domain and leucine-rich repeat containing) family: Evolutionary conserved diverse family of proteins further classified into NLRA, NLRB, NLRP and NLRC in accordance with their N-terminal domains and the presence or absence of CARD domains. Certain (but probably not all) NLRs function in innate immune sensing of pathogens and infection-associated cellular changes. They contribute to the protection of the infected host by instructing anti-microbial defence, including inflammatory responses.

Type III secretion (T3SS) apparatus: Complex molecular machines used by bacteria to inject effector proteins into eukaryotic host cells.

Flagellin: Subunit protein of the flagellum, which endows bacteria with motility.

Gasdermin family: Conserved family of proteins in vertebrates, named after the restriction of gasdermin A to gut and skin epithelial cells, although it is now clear that these proteins are much more widely expressed. At least some of the gasdermins can form pores in membranes after proteolytic cleavage (e.g. processing of gasdermin D by caspase 1 or 11).

ESCRT (Endosomal sorting complexes required for transport): Multiprotein machinery that enables membrane bending/budding away from the cytoplasm.

Lipopolysaccharide (LPS): Large molecules comprising of a lipid and a complex polysaccharide found in the outer membrane of gram-negative bacteria.

Gram-negative bacteria: Diverse group of bacteria defined by their inability to retain crystal violet (or Gram) stains due to the architecture of their cell envelope being composed of an inner cytoplasmic and outer bacterial cell membrane separated by a thin peptidoglycan cell wall. Typical examples include *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis* and *Yersinia pestis*.

Toll-like receptor: Family of transmembrane receptors that recognise PAMPs and DAMPs and upon stimulation can induce diverse pro-inflammatory responses.

RIG-I-like receptors: Cytosolic pattern recognition receptors that respond to double-stranded RNA.

Antigen presenting cells: While all nucleated cells can present antigens, the group of professional antigen presenting cells comprising macrophages, dendritic cells and B cells are capable of priming naïve T cells by processing and presenting antigen derived peptides in the context of class I or class II major histocompatibility complex (MHC) molecules and by delivery of co-stimulatory signals.

PB1-F2: A protein encoded by Influenza A virus that contributes to its pathogenicity.

M2 protein: Protein encoded by Influenza A virus that is part of the viral envelope; it is capable of forming a tunnel between host cell compartments.

Z-RNAs: Left-handed form of double stranded RNA, which is bound by proteins, such as ADAR, ZBP1, or their viral homologues.

Cytotoxic lymphocytes: $CD8^+$ T cells and natural killer (NK) cells can kill infected or malignant cells via diverse mechanisms, including delivery of perforin plus granzymes, using FAS ligand to activate the death receptor FAS on the target cells and IFN-γ.

BH3 mimetic drugs: Small molecule inhibitors of pro-survival BCL-2 proteins. They mimic the action of the pro-apoptotic BH3-only proteins, the natural cellular inhibitors of the pro-survival BCL-2 proteins that are critical for the initiation of the intrinsic apoptosis signalling pathway.

Necrosome: Protein complex consisting of RIPK1, RIPK3 and FADD. This complex is formed in response to TNFR1 stimulation when both the activation of NFκB and caspase 8 activity are blocked. This signalling complex causes activation of the pseudokinase MLKL, the critical effector of necroptosis.

Tumour lysis syndrome: Caused by the failure to safely remove large numbers of dying tumour cells during anti-cancer therapy. This can cause renal failure, cardiac abnormalities, seizures and sudden death.

CAR (chimaeric antigen receptor) T cells: T lymphocytes engineered to express artificial antigen receptors capable of directly recognising proteins on cancer cells and killing these malignant cells.

Amyloid plaques: Beta-amyloid protein aggregates implicated in the destruction of nerve connections causing degenerative disorders, such as Alzheimer disease.

Periodic fever syndrome: Group of rare genetic auto-inflammatory diseases in which patients develop periodic fevers with a range of inflammatory pathologies, including stomatitis, aphtitis and adenitis.

Serpin: Superfamily of proteins sharing structural homology, where some (e.g. CrmA from cowpox virus) but not all members have serine protease inhibitory activity.

Canonical and non-canonical NF-κB pathways: Induced by stimulation of a variety surface receptors (e.g. TLRs, members of the TNFR superfamily and antigen receptors), the two distinct NF-kB signalling pathways involve different members of the NF-κB/REL protein family. The classical/canonical NF-κB pathway operates via heterodimers of NF-κB1 (its cleavage product p50) with RELA or c-REL, whereas the non-canonical pathway is mediated mainly by heterodimers of NF-κB2 (its cleavage product p52) with RELB.

cGAS: Intracellular DNA sensor that induces an interferon response.

Mast cells: Tissue-resident cells involved in immune defence against parasitic infections and allergic responses.

Ripoptosomes: Signalling platforms comprising RIPK1, RIPK3, FADD and caspase 8 that can induce apoptosis or necroptosis depending on the state of the cell.

Immune checkpoint signalling: Signalling pathways that attenuate the activity of immune cells, mainly T lymphocytes, thereby regulating immunological immune responses and preventing destruction of self-tissues (contributing to self-tolerance). Inhibition of immune checkpoint regulators, such as PD-1 or CTLA-4, can enhance $CD8_+$ cytotoxic T cell mediated killing of cancer cells.

Complement system: Evolutionary ancient system of protein cascades capable of lysing bacteria by perforating their outer membrane, opsonising pathogens and activating cells of the immune system.

Box 1: Other pathways of programmed cell death

Autophagic cell death

A unique programmed cell death (PCD) pathway that does not require caspases and is not characterised by typical morphological features of apoptosis, necroptosis or pyroptosis. *In vivo*, this cell death is important for the involution of salivary glands during *Drosophila melanogaster* development and has been implicated in the death of cultured mammalian cells deprived of nutrients that are incapable of undergoing apoptosis (e.g. due to the absence of the essential effectors of apoptosis, BAX and BAK). A characteristic feature of autophagic cell death is the accumulation of large vacuoles within the cytosol of a self-digesting dying cell[181].

Oxeiptosis

A caspase-independent, non-inflammatory apoptosis-like cell death pathway, that can be induced by reactive oxygen species (ROS) production in response to viral infection. It was reported to require the ROS sensor KEAP1, the phosphatase PGAM5 and the pro-apoptotic factor AIFM1 (ref. [182]).

Ferroptosis

An iron-dependent, non-apoptotic oxidative cell death, which is morphologically, biochemically and molecularly distinct from apoptosis, necroptosis, pyroptosis and autophagy. It is similar to glutamate-induced cell killing, in which blockade of cellular cysteine uptake interferes with anti-oxidant cellular defences and eventually causes cell death[183]. Ferroptosis is initiated by failure in or blockade of glutathione-dependent antioxidant defences of a cell. This leads to unchecked lipid peroxidation and ultimately the killing of the cell. Lipophilic anti-oxidants and iron chelators can prevent ferroptotic cell death.

Secondary necrosis

This is the secondary event that is not programmed but occurs in cells undergoing apoptosis when they are not engulfed and digested by phagocytes at an early stage, for instance owing to the overload of the phagocytic capacity in response to extensive apoptosis, as can occur following highly effective anti-cancer treatments that cause apoptosis of large numbers of malignant cells in a short time frame. The morphology of a cell undergoing secondary necrosis is reminiscent of un-programmed necrotic death, such as that caused by burning or freezing of cells.

NETosis

One of mechanisms through which activated neutrophils kill invading pathogens. In this process, the neutrophils release granule proteins and chromatin that form extracellular fibres called neutrophil extracellular traps (NETs) to entrap and kill pathogens. Because of the release of vital cellular material, NETosis is often associated with neutrophil cell death, and it is morphologically distinct from apoptosis or necrosis[184].

Cytotoxic T lymphocyte (CTL)- and natural killer (NK) cell-induced cell killing

Virus infected cells or malignant cells can be killed by activated $CD8^+$ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells in a manner that has features of both lytic and non-lytic PCD[185]. Perforins secreted by CTLs and NK cells induce pores in their plasma membrane of target cells (similar to the complement **[G]** system) and allows the entry of granzymes into their cytosol[186]. The resulting loss of cellular integrity causes the cell to die. Granzymes have been reported to kill target cells by multiple mechanisms, including induction of apoptosis through proteolytic activation of the BH3-only protein BID[187] or proteolytic activation of

974 effector caspases[188], as well as by induction of a pyroptosis-like cell death through proteolytic
975 activation of gasdermin E[110]. Activated CTLs and NK cells can also trigger death receptor-
976 induced apoptosis using membrane-bound FAS ligand or TNF. IFN-γ also plays an important
977 role in mediating the cytotoxic effects of T cells and NK cells. *In vitro* studies suggest that
978 IFN-γ can activate the intrinsic apoptotic pathway through the BH3-only protein BIM and it
979 may also enhance death receptor-induced apoptosis by increasing the expression of TRAIL and
980 TNFR1 (refs. [189,190]).
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Figure Legends

Figure 1: Different forms of programmed cell death lead to different bystander responses. Cells can engage various pathways of programmed cell death (PCD); these pathways are characterised by distinct machineries and differ in their activation and outcomes. **a.** Apoptosis can be triggered by the extrinsic (also called death receptor induced) or the intrinsic (also called mitochondrial or BCL-2-regulated) pathway; although death receptors can also engage the intrinsic pathway of apoptosis. The death receptor pathway is activated at the plasma membrane by ligands (e.g. FAS ligand) binding to their cognate death receptors, such as FAS, leading to activation of caspase 8. The intrinsic pathway can be induced by a vast number of different stress stimuli, including DNA damage and growth factor withdrawal, and also by developmental cues. This pathway to cell death is associated with mitochondrial outer membrane permeabilisation (MOMP), with consequent release of cytochrome *c* leading to apoptosome formation and the activation of the initiator caspase 9. Both apoptotic pathways converge upon proteolytic activation of the effector caspases 3 and 7 by the initiator caspases. The effector caspases cleave hundreds of cellular proteins and thereby, amongst other processes, cause inter-nucleosomal DNA fragmentation and the exposure of phosphatidylserine (PtdSer) on the outer cell membrane of a dying cell. They also cause actin reorganisation leading to membrane blebbing, which supports the formation of apoptotic bodies. Collectively, these caspase-induced processes propagate the dismantling of the cell. **b.** Surface-exposed PtdSer is recognised by TAM receptors on neighbouring macrophages allowing clearance of apoptotic cells before any intracellular content is exposed to the extracellular environment. PtdSer recognition also triggers an anti-inflammatory response in phagocytes mediated by the secretion of IL-10, TGF-β (transforming growth factor-β) and prostaglandins, and the inhibition of the production of pro-inflammatory cytokines, including TNF and IL-1β. **c.** Apoptosis of very large numbers of cells in a short amount of time can

exceed the removal capacity of phagocytes. This can lead to so-called secondary necrosis, a lytic cell death with exposure of intracellular content from the dying cells to phagocytes, thereby fuelling inflammation. **d-f.** Necroptosis and pyroptosis are the main pro-inflammatory lytic forms of PCD. They are associated with cell lysis, which leads to the release of damage-associated molecular patterns (DAMPs) as well as pathogen-associated molecular patterns (PAMPs) in the case of cells infected with a pathogen. DAMPs and PAMPs are recognised by neighbouring phagocytes, leading to the production of pro-inflammatory cytokines. Necroptosis is induced via TNFR1 or Toll-like receptor (TLR) stimulation (and certain other signals), causing the activation of the receptor-interacting serine-threonine kinases RIPK1 and RIPK3. This causes conformational changes and activation of the pseudo-kinase mixed lineage kinase domain like (MLKL), which then translocates to the cell membrane, where it induces membrane rupture (possibly by pore formation) (**d**). Pyroptosis is a key PCD pathway triggered in response to pathogen infection, whereby pathogen products promote the formation of inflammasomes that act as platforms for caspase 1 activation. In addition, LPS was reported to directly stimulate the activation of caspase 11 (caspase 4 and caspase 5 in humans). Both caspase 1 and caspase 11 cleave gasdermin D (GSDMD), the N-terminal fragments of which assemble into pores in the cell membrane resulting in loss of membrane integrity and cell lysis. Caspase 1 also proteolytically processes the pro-forms of the pro-inflammatory cytokines IL-1β and IL-18 into their bio-active forms, which are released via GSDMD-induced membrane pores (**f**). IFN, interferon; IRF, interferon regulatory transcription factor; MAPK, mitogen-activated protein kinase.

Figure 2: Molecular mechanisms of apoptosis pathway activation. The intrinsic apoptotic pathway (**a**) is activated by various stresses (e.g. ER stress, growth factor deprivation, DNA damage) and by developmental cues. This induces, transcriptionally and/or post-

transcriptionally, pro-apoptotic members of the BCL-2 family called BH3-only proteins, such as BIM, PUMA, BAD or NOXA. The BH3-only proteins bind and neutralise pro-survival BCL-2 proteins, such as BCL-2, BCL-XL, MCL-1 and A1 (BFL-1 in humans), thereby liberating the critical effectors of apoptosis, BAX and BAK (members of a second pro-apoptotic sub-group of the BCL-2 family), which then assemble into large complexes that cause breaches in the mitochondrial outer membrane (MOMP: mitochondrial outer membrane permeabilisation). This causes release of apoptogenic factors, such as cytochrome *c* and SMAC (also known as DIABLO). Certain BH3-only proteins, such as BIM and PUMA, have been reported to also directly bind and activate BAX and BAK to induce MOMP. Cytochrome *c* via binding to apoptotic peptidase activating factor 1 (APAF1) causes formation of the apoptosome. The initiator caspase 9 is activated in this complex, and caspase 9 then proteolytically activates the effector caspases 3 and 7. SMAC blocks the activity of the caspase inhibitor XIAP. Death receptor induced apoptosis (**b**) is triggered by activation of death receptors, such as FAS by FAS ligand that is present on neighbouring cells. This leads to the recruitment of pro-caspase 8 to the intracellular region of the death receptor via the adaptor protein FADD (FAS associated via death domain), resulting in the formation of the so-called death inducing signalling complex (DISC) that catalyses the activation of caspase 8. Active caspase 8 induces cell killing either by direct proteolytic activation of the effector caspases 3 and 7 or indirectly through proteolytic activation of the BH3-only protein BID into tBID, thereby engaging the intrinsic apoptotic pathway. Effector caspases activated by either pathway cleave hundreds of intra-cellular proteins to induce the typical apoptotic morphology and prevent leakage of intra-cellular damage-associated molecular patterns (DAMPs) that would lead to inflammatory responses. Effector caspases directly or indirectly activate ROCK-1 kinase, which induces plasma membrane blebbing through actin contraction and caspase-activated DNase (CAD) (by cleaving its inhibitor ICAD), which leads to inter-nucleosomal DNA cleavage and chromatin

condensation. Effector caspases also proteolytically inactivate lipid flippases, such as ATP11, and proteolytically activate the lipid scramblase, XKR8, and collectively this causes exposure of phosphatidylserine (PtdSer) on the outer leaflet of the cell membrane. This acts as an 'eat me' signal for phagocytic cells and promotes engulfment of cells undergoing apoptosis.

Figure 3: Molecular features of inflammasome activation and pyroptosis. Cells encountering varying combinations of damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), inert structures or disturbances in intracellular potassium (K^+) concentration can undergo inflammasome activation and consequent pyroptotic cell death. In the cytosol, the aforementioned disturbances cause the stimulation of NLRs (nucleotide oligomerisation domain-like receptors), such as NLRC4 or NLRP3. This triggers formation of inflammasomes, large protein aggregates acting as sites of caspase 1 activation. At the cell membrane, DAMPs and PAMPs can trigger TLRs (Toll-like receptors) such as TLR4, which then activate IRAK1 (interleukin-1 receptor-associated kinase 1) and the mitogen-activated protein kinase TAK1 via the adaptor protein MYD88. This triggers the IKK (inhibitor of NFκB kinase) complex to phosphorylate the NF-κB pathway inhibitor IκB, leading to its ubiquitin-dependent proteasomal degradation. This liberates c-REL–p50 or RELA–p50 NF-κB complexes to enter the nucleus and activate NF-κ- dependent target genes, such as those encoding pro-IL-1β, pro-IL-18 and pro-caspase 11. Type I interferons (e.g. IFN-α/β) can also induce expression of pro-caspase 11 through the activation of type I IFN receptors on the cell membrane and the resulting activation of JAK–STAT pathway signalling. Activated caspases 1 and 11 can both cleave gasdermin D (GSDMD). The N-terminal fragments of GSDMD cause plasma membrane lysis via pore formation. Whereas caspase 1 is activated in inflammasomes, caspase 11 has been shown to be activated directly by intracellular lipopolysaccharide (LPS). Caspase 1 (but not caspase 11) also causes

proteolytic conversion of pro-IL-1β and pro-IL-18 into their bioactive forms that are secreted via GSDMD pores or are released as the cell undergoes lysis.

Figure 4: Induction of necroptosis. When inhibitors of apoptosis cIAP1 and cIAP2 are inhibited and caspase 8 activity is blocked (see Figure 6), binding of tumour necrosis factor (TNF) to tumour necrosis factor receptor 1 (TNFR1) leads to the recruitment and phosphorylation of receptor-interacting serine-threonine kinase 1 (RIPK1) via the adaptor protein TRADD. RIPK1 in turn phosphorylates and thereby activates RIPK3. RIPK3 can also be activated by Toll-like receptor (TLR) signalling through a process involving the adaptor TRIF. Active RIPK3 phosphorylates mixed lineage kinase domain like (MLKL) pseudo-kinase. This causes conformational changes in MLKL allowing it to translocate to and breach the integrity of the plasma membrane (possibly by forming pores). The resulting influx of water and sodium (Na^+), and potassium efflux leads to the swelling of the cell, disruption of membrane potential and eventually lysis of the cell. Heterodimers of caspase 8 with the cell-death regulator cFLIP long can cleave RIPK1 and thereby inhibit TNFR1 mediated induction of necroptosis.

Figure 5: The role of cell death in host responses to infection. Intracellular bacteria, such as *Salmonella Spp.* or *Legionella Spp.*, enter the host and infect target cells, including epithelial cells lining the gastrointestinal or respiratory tract. Following replication, some bacteria escape into the sub-epithelial tissue, where they can enter macrophages to establish additional replicative niches. This involves the injection of bacterial products into the cytosol and the repurposing of subcellular compartments as bacteria containing vesicles. The resulting contamination of the cytosol with bacterial components is detected through cellular sensors that promote the formation of inflammasomes (see Figure 3), which promote caspase 1

activation. Caspase 1 causes pyroptotic cell death by proteolytic activation of gasdermin D (GSDMD). The N terminal fragments of GSDMD form pores in the plasma membrane and thereby cause cell lysis. The resulting pyroptotic death purges the bacteria from their intracellular niches, exposing them alongside intracellular host molecules acting as damage-associated molecular patterns (DAMPs) to cells of the immune system. Neighbouring macrophages are activated by the DAMPs and pathogen-associated molecular patterns (PAMPs). This, together with the concomitant release of bioactive IL-1β and IL-18 from pyroptotic cells augments macrophage capacity to phagocytose and destroy bacteria. Bacteria and their components are also taken up by dendritic cells that migrate to the local draining lymph nodes, where they display to T cells antigenic fragments from pathogen encoded proteins in the context of class I and class II major histocompatibility complex (MHC) molecules. Together with the provision of appropriate membrane-bound costimulatory signals and cytokines that are triggered by the exposure to DAMPs and PAMPs, dendritic cells then induce proliferation and activation of effector functions of bacteria-specific T cells in the lymph nodes. Here, activated $CD4_+$ T cells aid in the activation of B cells and their differentiation into plasma cells that produce antibodies that help clear the infection through opsonisation and neutralisation as well as by facilitating complement-mediated lysis and phagocytosis **[Au:OK?]** yes ok. Upon migration to the site of infection, activated $CD4_+$ T cells locally support bacterial killing by secreting cytokines, amongst which interferon γ (IFN-γ) enhances anti-bacterial activity of macrophages. Activated $CD8_+$ T cells kill infected cells displaying bacterial antigen derived peptides on class I MHC molecules through release of perforin plus granzyme, FAS ligand induced activation of FAS death receptor mediated apoptosis and through the provision of IFN-γ. In addition to dendritic cells, also macrophages can serve as antigen-presenting cells activating T cells at the site of infection.

Figure 6. The molecular mechanisms of TNFR1 signalling. Stimulation of tumour necrosis factor receptor 1 (TNFR1) by TNF in unstressed (healthy) cells results primarily in the activation of NFκB and AP1 transcription factors (the latter not shown), leading to cell survival, proliferation and production of pro-inflammatory cytokines. Stimulation of TNFR1 by TNF engages an intra-cellular signalling complex involving the recruitment of the adaptors TRADD and TRAF2 and/or TRAF3 and the E3 ubiquitin ligases cIAP1 and cIAP2 that ubiquitylate receptor-interacting serine-threonine kinase 1 (RIPK1). Additionally, the linear ubiquitination complex (LUBAC) is recruited into this signalling complex where it causes linear ubiquitylation of several signalling components, including the NFκB activator complex IKKα–IKKβ–NEMO. This is required for optimal signalling and activation of the canonical (RELA or c-REL with p50 or p52) and non-canonical (RELB with p52) NFκB pathways that induce cell survival by induction of the caspase 8 inhibitor cFLIP (long or short form) and the pro-survival BCL-2 family members, BCL-XL, BCL-2 and A1.

Figure 7: Overlap and back-up of apoptosis and pyroptosis to induce cell death. Diverse initiator and effector molecules involved in apoptosis or pyroptosis are interchangeably used to guarantee cell killing. For example, the apoptotic caspase 8 is able to induce pyroptosis by mediating gasdermin D (GSDMD) cleavage. This has been shown to occur in cells that are unable to activate NFκB signalling downstream of tumour necrosis factor receptor 1 (TNFR1) stimulation (see Figure 6) and was associated with the formation of ripoptosomes, whereby pro-caspase 8 assembles with RIPK1 (receptor-interacting serine-threonine kinase) and FADD (FAS associated via death domain) (1). Caspase 8-mediated GSDMD cleavage and pyroptotic cell death was also shown to occur downstream of caspase 8-containing inflammasomes (2), indicating that in addition to the pyroptosis-inducing caspase 1, also caspase 8 can be activated within inflammasomes. In cells unable to undergo pyroptosis (e.g. ones lacking GSDMD or

caspase 1 and 11) this inflammasome-mediated caspase 8 activation can induce apoptosis (3). As an additional link between pyroptosis and apoptosis, caspase 1 can activate apoptosis by proteolytic activation of caspase 3 (4) or by conversion of BID to tBID (5). Of note, in cells lacking all known executioner molecules, the intiator caspases 1, 8 and 9 can also serve as executioners to directly induce apoptosis (not shown). APAF1, apoptotic peptidase activating factor 1; NLR, nucleotide-binding domain and leucine-rich repeat containing.

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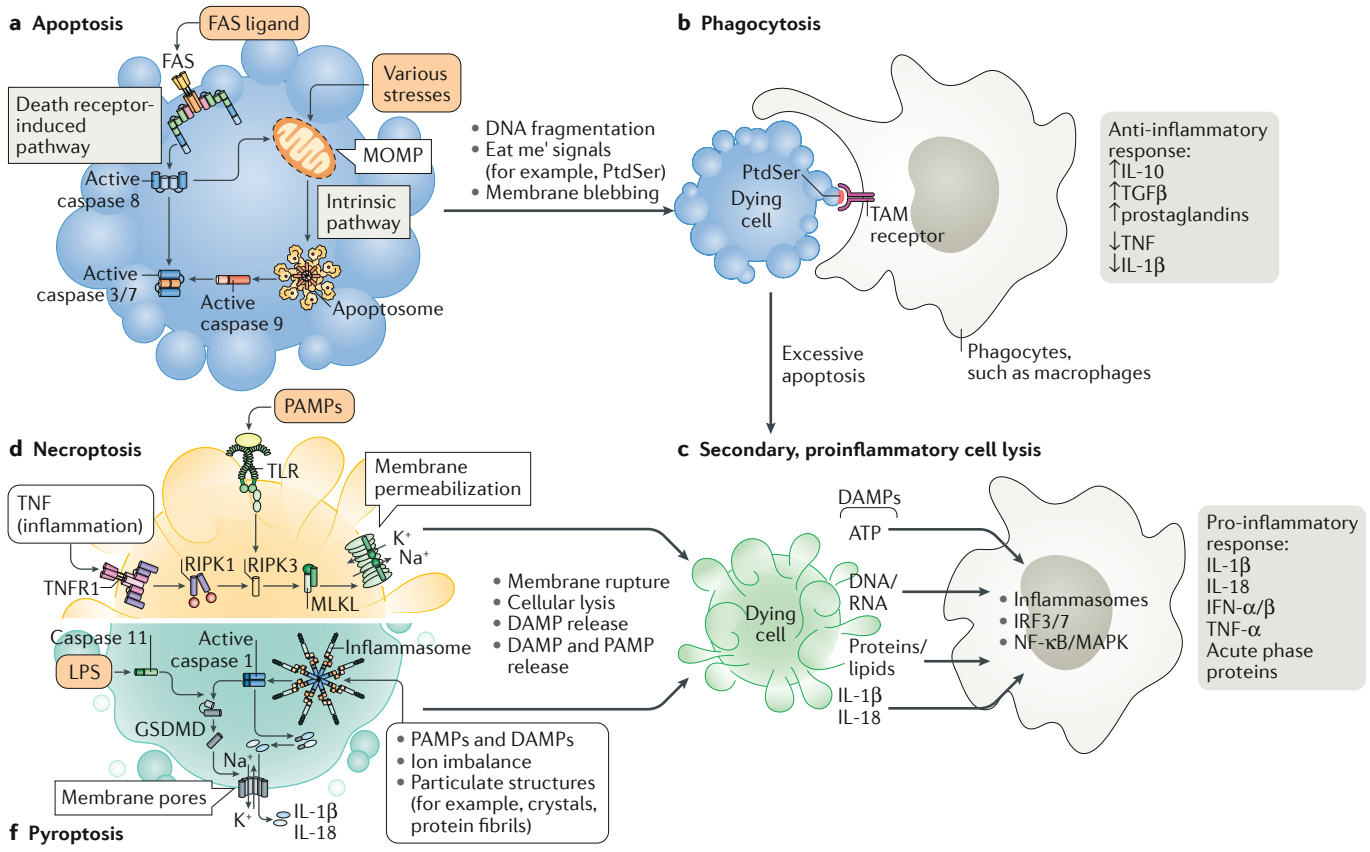
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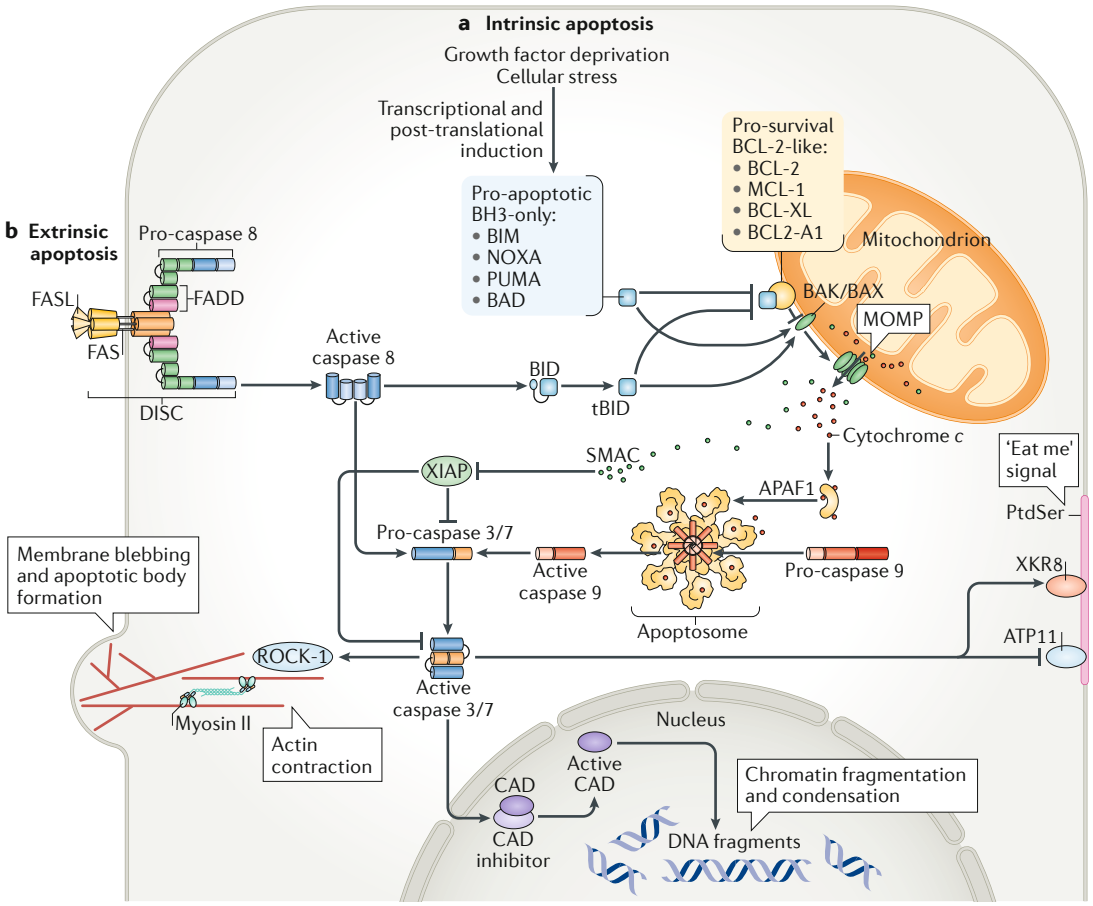
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Fig 1



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Fig 2



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Fig 3

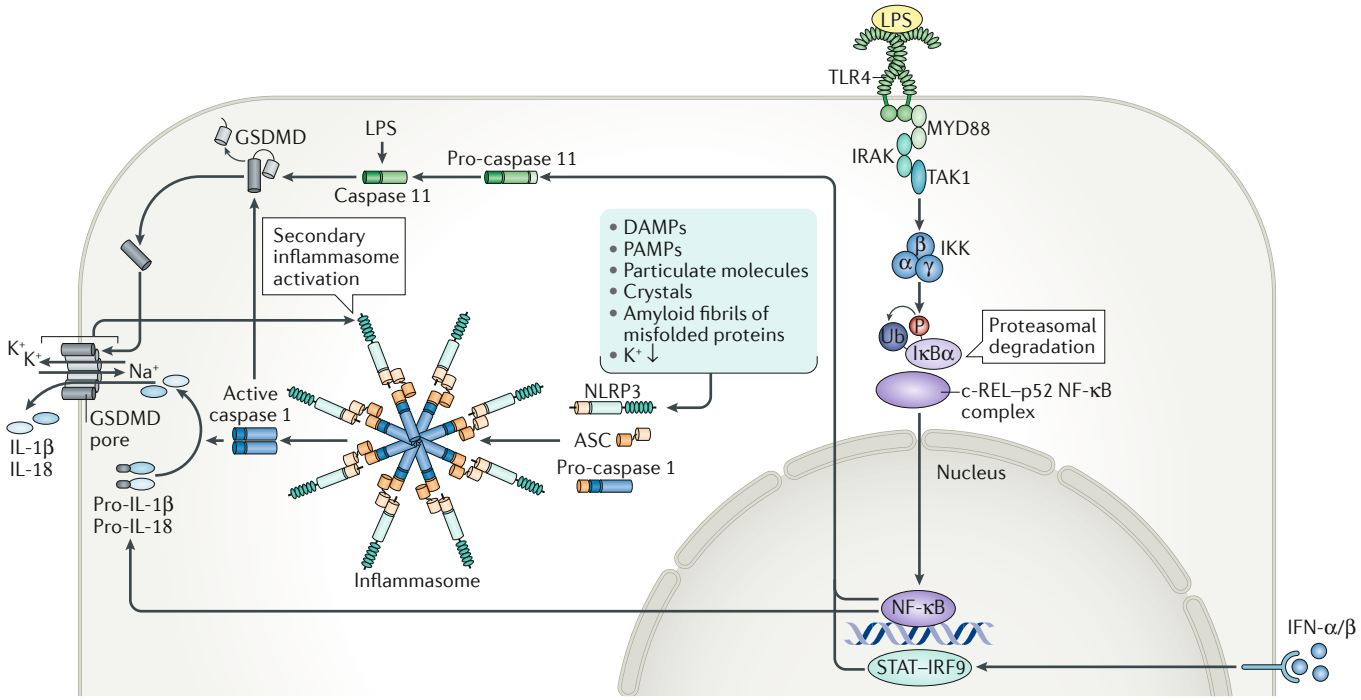
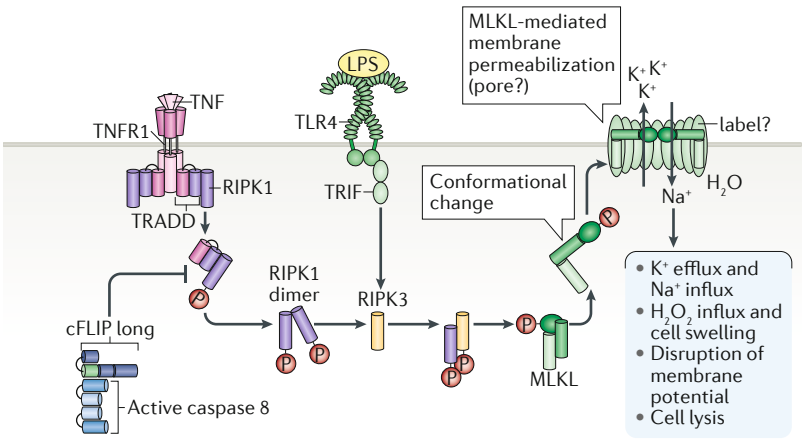


Fig 4



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Fig 5

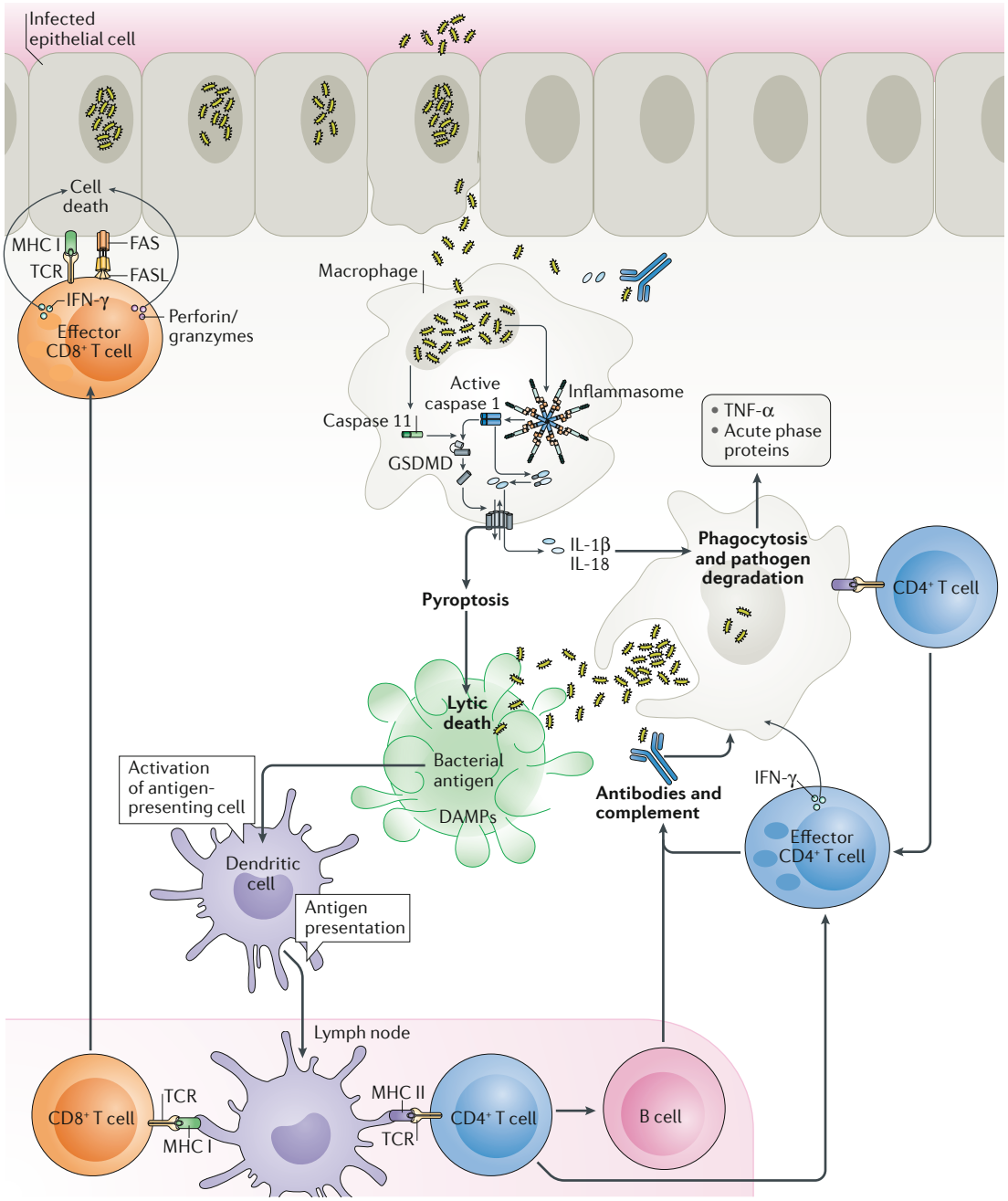


Fig 6

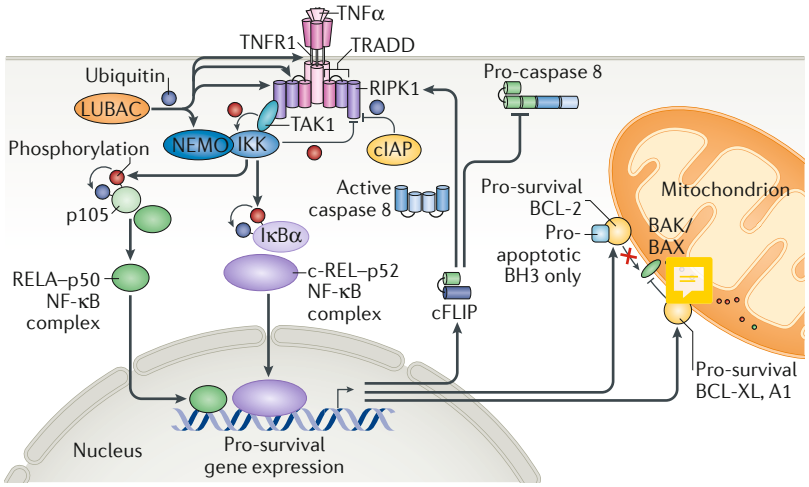
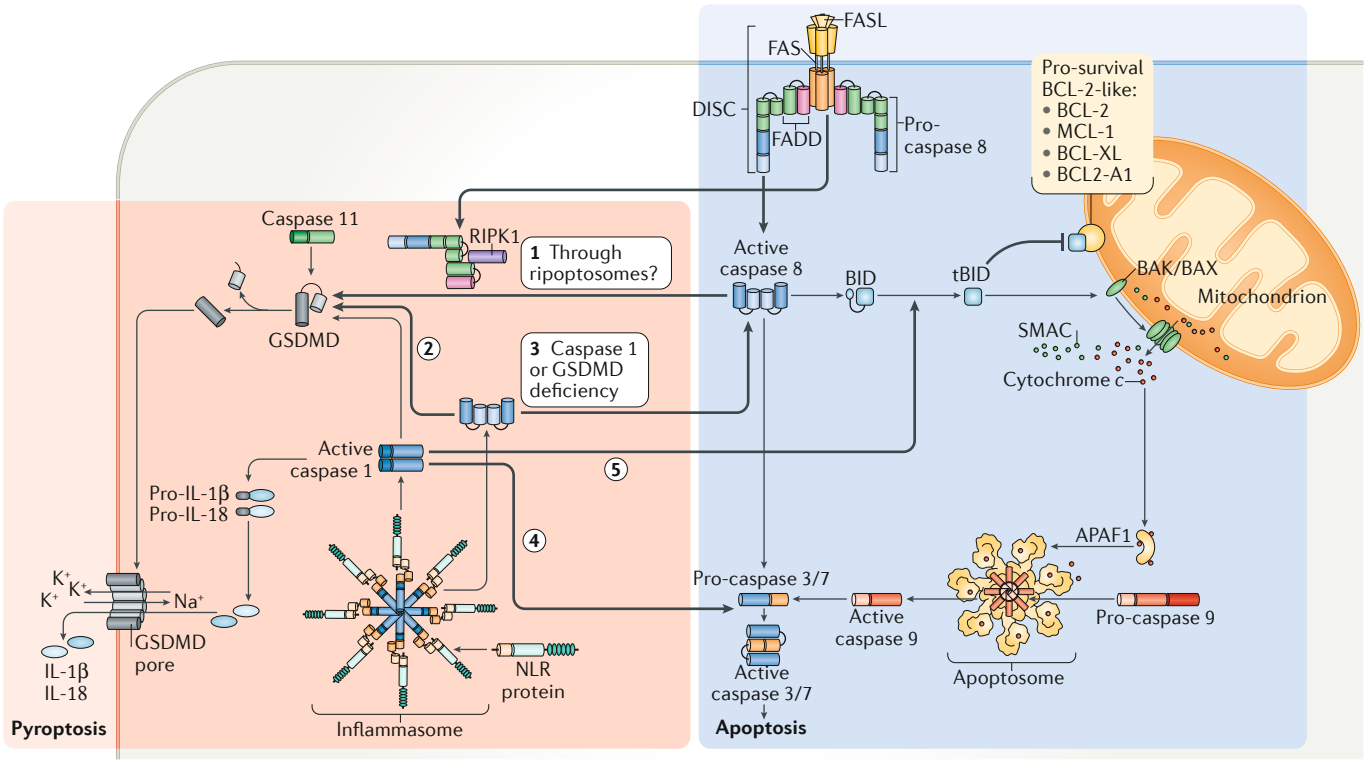

TNFα
TNFR1
TRADD
Ubiquitin
LUBAC
RIPK1
TAK1
NEMO
IKK
cIAP
Phosphorylation
p105
IκBα
RELA–p50 NF-κB complex
c-REL–p52 NF-κB complex
Active caspase 8
Pro-caspase 8
cFLIP
Pro-survival BCL-2
Pro-apoptotic BH3 only
BAK/ BAX
Mitochondrion
Pro-survival BCL-XL, A1
Nucleus
Pro-survival gene expression

Fig 7


FASL
FAS
DISC
FADD
Pro-caspase 8
Pro-survival BCL-2-like:
• BCL-2
• MCL-1
• BCL-XL
• BCL2-A1
Caspase 11
RIPK1
1 Through ripoptosomes?
Active caspase 8
BID
tBID
BAK/BAX
Mitochondrion
GSDMD
2
3 Caspase 1 or GSDMD deficiency
SMAC
Cytochrome c
Active caspase 1
5
Pro-IL-1β
Pro-IL-18
4
APAF1
K+ K+ K+
Na+
GSDMD pore
IL-1β
IL-18
NLR protein
Pro-caspase 3/7
Active caspase 9
Pro-caspase 9
Active caspase 3/7
Apoptosome
Pyroptosis
Inflammasome
Apoptosis