New insights into the regulation of apoptosis, necroptosis and pyroptosis by RIPK1 and caspase-8

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Summary

Necroptosis and pyroptosis are inflammatory forms of regulated necrotic cell death as opposed to apoptosis that is generally considered immunologically silent. Recent studies revealed unexpected links in the pathways regulating and executing cell death in response to activation of signalling cascades inducing apoptosis, necroptosis and pyroptosis. Emerging evidence suggests that RIPK1 and caspase-8 control the cross-talk between apoptosis, necroptosis and pyroptosis and determine the type of cell death induced in response to activation of cell death signalling.

Introduction

Cell death is an essential biological process for multicellular organisms including fungi, plants and animals [1], with important functions for development, tissue homeostasis and host defence. This article focuses on recent developments on cell death regulation and function in mice and humans with particular emphasis on the role of cell death in tissue homeostasis, inflammation and disease. Apoptosis was the first pathway of regulated cell death identified, initially described based on its distinct morphological features including cytoplasmic and nuclear condensation, membrane blebbing and the formation of apoptotic bodies. The discovery that apoptosis was induced by specific cysteinyl aspartate proteases termed caspases provided the proof that this type of cell death is controlled by distinct molecular machineries and should be considered a form of cellular suicide. Two pathways of apoptosis have been described based on the upstream initiator caspases that induce them [2]. Extrinsic apoptosis is induced by caspases 8 and 10, which are activated mostly in response to death receptor stimulation, while intrinsic apoptosis is induced by caspase-9, which is activated by cytochrome c released to the cytosol in response to mitochondrial outer membrane permeabilisation (MOMP). Initiator caspases activate the effector caspases 3, 6 and 7, which proteolytically process multiple cellular substrates to induce the controlled demise of the cell [2].

Until recently, apoptosis was considered the only molecularly controlled type of cell death, as opposed to necrosis that was classically described as non-regulated accidental cell death. However, genetic, biochemical and functional evidence as well

as the discovery of specific chemical inhibitors of necrotic cell death have redefined necrosis as a molecularly controlled form of cell death [3,4]. Several types of regulated necrotic cell death have been recently identified, which share common morphological features, including increase of the cellular volume, swelling of organelles and disruption of the plasma membrane, but are activated in response to different triggers and are executed by distinct biochemical pathways [4]. Necroptosis is a form of regulated necrotic cell death induced by receptor interacting protein kinase 3 (RIPK3) and its substrate mixed lineage kinase-like (MLKL) [3]. RIPK3 is activated by upstream pathways via RIP homotypic interaction motif (RHIM)-dependent protein-protein interactions with the three additional proteins in the mammalian genome that contain conserved RHIMs, namely RIPK1, TRIF and ZBP1/DAI [3,5]. RIPK1 links RIPK3 to death receptor (DR) signalling, TRIF mediates RIPK3 activation downstream of TLR3 and TLR4, whilst ZBP1/DAI mediates RIPK3 activation primarily in response to certain viruses [3,5]. Necroptosis execution requires the RIPK3-dependent phosphorylation of MLKL, which subsequently damages the plasma membrane via poorly understood mechanisms inducing the death of the cell [3,5].

Pyroptosis is a type of regulated necrotic cell death induced by inflammatory caspases, namely caspase-1 as well as caspase-11 (in mouse cells) or caspase-4 and caspase-5 (in human cells) [2]. Both caspase-1 and caspase-11 (or caspases-4, -5) induce pyroptosis by proteolytically processing Gasdermin D (GSDMD) yielding an N-terminal fragment that opens pores on the plasma membrane leading to cell death [6,7]. In addition to cleaving GSDMD, caspase-1 processes the precursor cytokines pro-IL-1β and pro-IL-18 to produce the mature bioactive forms that are released through the GSDMD pores and the lysed cells triggering inflammation [2]. Pyroptotic cell death is triggered by the assembly and activation of inflammasomes, macromolecular protein complexes that serve as platforms for the activation of caspase-1. The NOD-like receptors (NLRs) Nlrp3, Nlrp1b and Nlrc4, the cytosolic DNA sensor AIM2 (absent in melanoma 2) as well as Pyrin, engage the so-called canonical inflammasomes by recruiting and activating caspase-1 either directly or through the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) [2].. The non-canonical inflammasome is triggered by intracellular lipopolysaccharide (LPS), which activates caspase-11 (caspase-4, -5 in human cells) [2].

These developments have sparked a large number of studies addressing the mechanisms regulating the different types of regulated cell death and their role in physiology and disease, which have been covered in a number of comprehensive reviews during the last years [3,5,8-10]. The aim of the present short article is not to comprehensively cover the large literature on cell death, but rather to discuss most recent studies that shed light on new mechanisms regulating and connecting necroptosis and pyroptosis, focusing particularly on the role of caspase-8 and RIPK1 as essential players in a complex network of cell death signalling.

RIPK1 regulation by ubiquitination and phosphorylation

RIPK1 functions as a signalling hub within apoptotic and necroptotic cell death pathways and exhibits kinase-dependent and -independent functions that are important for the regulation of cell survival, cell death and inflammation. Studies in

genetic mouse models showed that RIPK1 acts as a scaffold independently of its kinase activity to prevent apoptosis and necroptosis in different tissues and that this function is important for preventing inflammation and maintaining tissue homeostasis [11-19]. Children with RIPK1 deficiency were found to suffer from severe immunodeficiency and autoinflammatory pathologies [20-22], suggesting that RIPK1 scaffold functions are essential for tissue homeostasis also in humans. On the other hand, genetic and pharmacological inhibition of RIPK1 kinase activity revealed an important role of RIPK1 kinase-dependent apoptosis and necroptosis in driving inflammatory and degenerative pathologies in multiple tissues [23-30]. These studies highlighted the important but opposing roles of RIPK1 in preventing or promoting cell death and suggested that the balance between its kinase-independent and -dependent functions must be tightly regulated to ensure tissue homeostasis and prevent cell death and disease.

Several studies revealed that ubiquitination of RIPK1 with lysine 63 (K63), K11, K48 as well as M1 (linear) ubiquitin chains regulates its function by promoting its pro-survival role within the TNFR1 signalling complex and preventing the formation of death-inducing protein complexes. In particular, the role of K63 and linear ubiquitin chains has been thoroughly investigated biochemically and genetically in numerous studies that could not be covered in this short article but have been discussed extensively in recent reviews [31-35]. Two recent studies revealed that mutation of lysine 376 of RIPK1, which was shown to be ubiquitinated by K63-linked chains [36], in murine RIPK1 caused embryonic lethality that was fully prevented by combined inhibition of caspase-8-mediated apoptosis and RIPK3-MLKL-dependent necroptosis [37,38], providing functional evidence that K376 (K377 in humans) is a critical residue for ubiquitin-dependent regulation of RIPK1.

In addition to ubiquitination, multiple phosphorylation events are implicated in regulating RIPK1 function. Three independent studies reported that phosphorylation of RIPK1 by MK2 on multiple serine residues in the intermediate domain including serine 321 in mouse (320 in human) restrains its kinase activity and cell death-inducing potential [39-41]. TAK1 was also suggested to mediate RIPK1 phosphorylation [42], but it remains unclear if this function of TAK1 is indirect by inducing MK2-dependent RIPK1 phosphorylation. Furthermore, RIPK1 phosphorylation by IκB kinases (IKKs) on multiple residues including serine 25 was reported to prevent RIPK1-mediated cell death [43]. Mutation of serine 25 of RIPK1 to glutamic acid to mimic phosphorylation indeed protected cells and mice from TNFR1-mediated cell death and inflammation by inhibiting RIPK1 kinase activity [44]. These reports revealed the important role of IKK-mediated phosphorylation in preventing RIPK1-mediated cell death and inflammation, consistent with studies showing that mice with tissue specific knockout of IKK subunits developed inflammatory pathologies due to RIPK1-mediated cell death [26,29]. Two independent recent studies showed that TBK1 and IKKε also phosphorylate RIPK1 to suppress its cytotoxic potential [45,46], a function important during embryonic development as TBK1-deficient mice die between embryonic days 13.5 and 14.5 due to RIPK1 kinase dependent cell death [45]. Together, these studies revealed that RIPK1 ubiquitination and phosphorylation prevent the activation of RIPK1 kinase activity, which induces cell death by both apoptosis and necroptosis. Autophosphorylation is considered to be the critical function of RIPK1 kinase activity, with multiple residues identified as autophosphorylation sites in human (serines 14/15,

20, 161 and 166) and mouse (serines 14/15, 161 and 166 as well as threonine 169) RIPK1 [30,44,47,48]. Mechanistically, autophosphorylation is currently thought to impose a conformational change in RIPK1 facilitating the formation of the signalling complexes inducing apoptosis and necroptosis, however the specific function of individual autophosphorylation events remains to be experimentally validated in relevant *in vivo* models.

Caspase-8 cleaves RIPK1 to prevent cell death and inflammation

Caspase-8 inhibits necroptosis but the underlying mechanisms remain poorly understood. Caspase-8 was shown to cleave several proteins involved in the regulation of necroptosis, including RIPK1 [49], RIPK3 [50], CYLD [51] and cFLIP [52], however, the role of these cleavage events in the regulation of cell death and inflammation have remained unknown until recently. Four independent studies have now identified RIPK1 as a critical substrate that is cleaved by caspase-8 to prevent excessive cell death and inflammation [53-56] **(Figure 1)**. In three of these studies, the authors generated knock-in mice expressing RIPK1 with mutation of aspartic acid at position 325 to alanine (D325A), which prevents its cleavage by caspase-8, and found that these mice died between embryonic days E10.5-E12.5 [53,55,56]. The embryonic lethality of *Ripk1*[D325A/D325A] mice could not be rescued by RIPK3 or MLKL deficiency but could be fully prevented by combined inhibition of FADD-caspase-8-mediated apoptosis and RIPK3-MLKL-mediated necroptosis [53,55,56], showing that caspase-8-dependent cleavage of RIPK1 prevents both apoptosis and necroptosis. Interestingly, mice expressing RIPK1 with combined mutations inhibiting both its kinase activity (D138N) and caspase-8-mediated cleavage (D325A) developed to term but exhibited severe systemic inflammatory pathology resulting in early postnatal death [55,56], suggesting that inhibition of RIPK1 cleavage triggers cell death and inflammation by both kinase-dependent and -independent functions. Consistent with the genetic studies, mouse embryonic fibroblasts expressing RIPK1 with mutation of D325 underwent both apoptosis and necroptosis in response to TNF stimulation [54-56]. Notably, patients with heterozygous missense mutations of the caspase-8 cleavage site of human RIPK1 (D324) were reported to suffer from a syndrome characterised by periodic fever and lymphadenopathy [54,56], revealing that RIPK1 cleavage by caspase-8 has a critical physiological role in preventing inflammation also in humans. Together, these studies identified RIPK1 cleavage by caspase-8 as a critical event that limits RIPK1-dependent cell death and inflammation in both mice and humans. Mechanistically, RIPK1 cleavage seems to limit apoptosis and necroptosis by dismantling the respective cell death inducing signalling complexes (**Figure 1**).

Caspase-8 inhibits inflammasome activation

In addition to its functions in inducing apoptosis and inhibiting necroptosis, recent studies revealed a novel role of caspase-8 in inhibiting inflammasome activation [57,58] **(Figure 1)**. Knock-in mice expressing caspase-8 with mutation of its catalytic cysteine (C362) died at around embryonic day 10.5 similarly to mice lacking caspase-8 or FADD [55,57,58]. However, while MLKL deficiency could rescue embryonic lethality and support survival of caspase-8 or FADD knockout mice to adulthood[59],

MLKL knockout could only prolong the survival of mice expressing catalytically inactive caspase-8 until birth [55,57,58]. This finding suggested that necroptosis-independent mechanisms caused perinatal death of mice expressing catalytically deficient caspase-8. Formation of ASC specks was detected in tissues from these animals [57,58], suggesting that inflammasome activation could contribute to the pathology. Indeed, mice expressing catalytically inactive caspase-8 could survive to adulthood when crossed into combined *Mlkl*$^{-/-}$ *Asc*$^{-/-}$ or *Mlkl*$^{-/-}$ *Caspase-1*$^{-/-}$ genetic backgrounds [57,58], showing that ASC-Caspase-1-dependent mechanisms cause perinatal lethality in these mice. Furthermore, mice expressing catalytically inactive caspase-8 specifically in intestinal epithelial cells (IECs) were viable and showed mild intestinal inflammation, however when these mice were crossed into an *Mlkl*$^{-/-}$ genetic background they died perinatally and showed formation of ASC specks and expression of IL-1β in their intestines [57,58], suggesting that inhibition of necroptosis was detrimental by causing increased activation of the inflammasome. A rational interpretation of these results is that IECs expressing catalytically inactive caspase-8, which would normally undergo necroptosis, persist in the absence of MLKL and by mechanisms that depend on the presence of catalytically inactive caspase-8 but remain poorly understood ASC is activated inducing caspase-1-dependent inflammatory pathology. Interestingly, gasdermin D (GSDMD) deficiency could not rescue the *Casp8*$^{C362A/C362A}$ *Mlkl*$^{-/-}$ mice [57], suggesting that ASC and caspase-1 mediate the pathology by pyroptosis-independent mechanisms. Moreover, RIPK3 deficiency could rescue mice expressing catalytically inactive caspase-8 to adulthood [57,58], suggesting that necroptosis-independent functions of RIPK3 contribute to the activation of the ASC-caspase-1 inflammasome. Taken together, these studies revealed another layer of cell death regulation controlled by caspase-8, which acts in a catalytic activity-dependent manner to restrain the activation of the ASC-caspase-1 inflammasome. Catalytically inactive caspase-8 acts as a scaffold to induce ASC-caspase-1 activation, though the underlying molecular mechanisms remain elusive. Interestingly, necroptosis seems to act as a mechanism inhibiting inflammation in the intestinal epithelium by limiting ASC-caspase-1 activation by catalytically inactive caspase-8. Caspase-8-deficient patients were found to suffer from immune deregulation, lymphocytic infiltrations in multiple organs and very early onset inflammatory bowel disease [60-62], showing that caspase-8 has critical functions for the maintenance of immune homeostasis, in particular in the intestine, also in humans.

Cross-talk between apoptosis, necroptosis and pyroptosis

Despite the progress made in delineating the molecular machineries controlling and executing apoptosis, necroptosis and pyroptosis, the cross-talk between these cell death pathways has not been appreciated until lately. Recent findings provided evidence that these cell death pathways are interconnected at multiple levels and can be activated simultaneously or consecutively within the same cell **(Figure 1)**. Caspase-3, the executioner caspase in apoptosis, was shown to cleave and activate GSDME resulting in pyroptosis-like necrotic cell death in cells expressing sufficient amounts of GSDME [63,64], a phenomenon suggested to contribute to tissue damage in response to chemotherapeutic agents [63]. Caspase-8 was also recently suggested to cleave and activate GSDMD inducing pyroptosis in response to activation of the extrinsic

apoptosis signalling pathway [65-67]. An increased complexity of the regulation of GSDMD activation was revealed by experiments showing that caspase-3 cleaves GSDMD within its N-terminal domain thus limiting GSDMD-induced pyroptosis [65,68]. These studies revealed that apoptotic caspases such as caspase-8 and caspase-3 can also trigger pyroptosis-like necrotic death in cells expressing GSDME and GSDMD, suggesting that the type of cell death induced is not determined by the caspases activated but by the availability of different caspase substrates that execute apoptotic or necrotic forms of cellular demise.

A link between RIPK3-MLKL-induced necroptosis and NLRP3 inflammasome activation was suggested by studies showing that caspase-8 deficiency or treatment with caspase inhibitors caused NLRP3 activation that depended on RIPK3 and/or MLKL expression [69,70]. Although different mechanisms were proposed to explain this role of RIPK3 and MLKL, a recent study convincingly demonstrated that MLKL-mediated permeabilisation of the plasma membrane triggers NLRP3 activation by inducing potassium efflux [71]. MLKL-dependent activation of the NLRP3 inflammasome was shown to be physiologically important in mice with myeloid cell specific knockout of A20, where MLKL knockout could prevent NLRP3-Caspase-1-dependent arthritis development [72]. Moreover, a number of studies reported that exposure of myeloid cells to agents promoting extrinsic or intrinsic apoptosis caused activation of NLRP3 resulting in inflammatory cell death [73-78], suggesting that apoptosis is also linked to inflammasome activation. Activation of the extrinsic and intrinsic apoptotic pathways was suggested to induce NLPR3 activation by activating pannexin-1 channel formation on the plasma membrane resulting in potassium efflux [65].

Concluding remarks

Together, the recent studies discussed here revealed new mechanisms regulating cell death, highlighting the complexity and plasticity of the pathways controlling if and how a cell dies. A key take home message is that cell death pathways do not operate in isolation as linear signalling cascades as often depicted in schematic models. On the contrary, the pathways regulating the different modalities of cell death are interconnected at multiple levels. The final outcome of a cell death inducing stimulus will depend on many parameters related to the expression and activation state of many factors that will determine if and how the cell dies. In certain cases, more than one cell death pathways may be induced in parallel influencing the immunogenic properties of the dying cells. This plasticity in simultaneously engaging diverse cell death pathways provides the cell with the capacity to fine-tune the cross-talk between dying and bystander cells in the tissue microenvironment and eliciting optimal responses ensuring effective tissue regeneration and host defence with minimal tissue damage. Elucidating the underlying mechanisms will be important in order to understand the role of cell death pathways in physiology and disease and exploit this knowledge for developing novel therapeutic approaches.

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Declaration of competing interests

M.P. received consulting and speaker fees from Genentech, GSK, Boehringer Ingelheim and Sanofi.
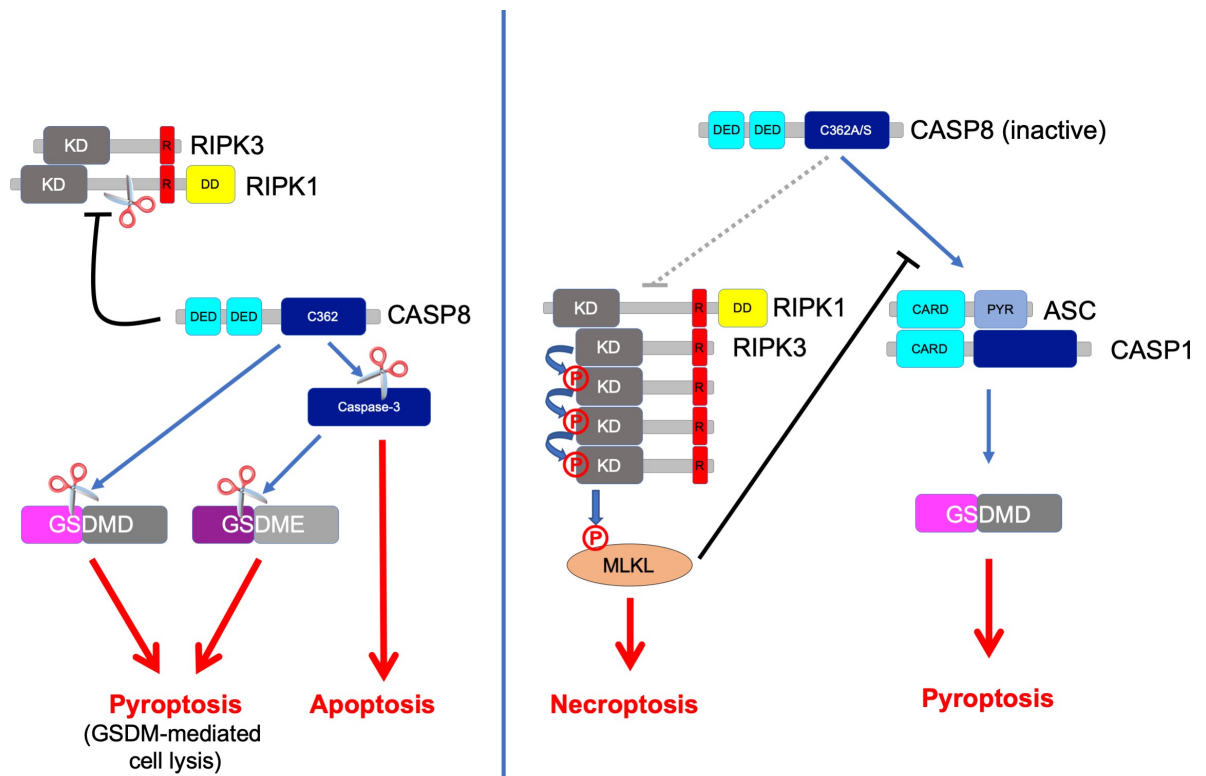
Figure 1. Caspase-8 regulates apoptosis, necroptosis and pyroptosis.
Schematic depiction of the functions of caspase-8 in regulating apoptosis, necroptosis and pyroptosis. Left panel: caspase-8 acts as the initiator caspase in the extrinsic apoptosis pathway inducing activation of the effector caspases 3, 6 and 7 causing apoptotic cell death. GSDME cleavage by caspase-3 as well as GSDMD cleavage by caspase-8 can trigger lytic cell death resembling pyroptosis. Caspase-8 also inhibits necroptosis by cleaving RIPK1 and perhaps other proteins regulating necroptosis such as RIPK3. Right panel: loss of caspase-8 activity allows activation of necroptosis. Expression of catalytically inactive caspase-8 causes the activation of the ASC/caspase-1 inflammasome resulting in GSDMD-mediated pyroptosis. KD, kinase domain; DD, death domain; DED, death effector domain; R, RHIM; CARD, caspase recruitment domain; PYR, pyrin domain.