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Review

Molecular mechanisms of regulated necrosis

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ABSTRACT

It is now clear that apoptosis does not constitute the sole genetically encoded form of cell death. Rather, cells can spontaneously undertake or exogenously be driven into a cell death subroutine that manifests with necrotic features, yet can be inhibited by pharmacological and genetic interventions. As regulated necrosis (RN) plays a major role in both physiological scenarios (*e.g.*, embryonic development) and pathological settings (*e.g.*, ischemic disorders), consistent efforts have been made throughout the last decade toward the characterization of the molecular mechanisms that underlie this cell death modality. Contrarily to initial beliefs, RN does not invariably result from the activation of a receptor interacting protein kinase 3 (RIPK3)-dependent signaling pathway, but may be ignited by distinct molecular networks. Nowadays, various types of RN have been characterized, including (but not limited to) necroptosis, mitochondrial permeability transition (MPT)-dependent RN and parthanatos. Of note, the inhibition of only one of these modules generally exerts limited cytoprotective effects *in vivo*, underscoring the degree of interconnectivity that characterizes RN. Here, we review the signaling pathways, pathophysiological relevance and therapeutic implications of the major molecular cascades that underlie RN.

Contents

Abbreviations: AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; CsA, cyclosporin A; CYPD, cyclophilin D; $\Delta\psi_m$, mitochondrial transmembrane potential; Fer-1, ferrostatin-1; MAMP, microbe-associated molecular pattern; MLKL, mixed lineage kinase domain-like; MPT, mitochondrial permeability transition; Nec-1, necrostastin-1; PAR, poly(ADP-ribose); PARP1, PAR polymerase 1; PPIF, peptidylprolyl isomerase F; PRR, pattern recognition receptor; PTPC, permeability transition pore complex; RIPK, receptor-interacting protein kinase; RN, regulated necrosis; ROS, reactive oxygen species; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; TNFR1, TNFα receptor 1.

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1. Introduction

The Nomenclature Committee for Cell Death has recently proposed to use the adjective "programmed" to identify instances of cell death that occur in a completely physiological setting such as (post)embryonic development or the preservation of tissue homeostasis. Conversely, the term "regulated" should be employed to refer to cases of cell death that can be inhibited by specific pharmacological or genetic interventions, implying that they rely on a defined (though sometimes known to partial extents) molecular machinery. Thus, each instance of programmed cell death is by definition regulated, but not *vice versa*. Finally, the expression "accidental cell death" has been put forward to indicate cell death instances that cannot be controlled, as they generally originate from very harsh microenvironmental perturbations (Fig. 1) [1–3].

Until recently, apoptosis was considered as the only form of regulated cell death, possibly because: (1) the stereotyped morphological appearance of this cell death modality has been recognized as early as in the 1960s, mostly owing to the pioneer work of Sir Richard Lockshin [4]; and (2) the biochemical processes that regulate and execute apoptosis (including the massive activation of a class of cysteine proteases known as caspases) have emerged quite rapidly, at least in part following the milestone discoveries made by Robert Horvitz in *Caenorhabditis elegans* [5–7]. Conversely, necrosis was viewed as a merely accidental subroutine of cell death, mostly resulting from very harsh stimuli including steep changes in temperature, osmotic pressure or pH [8]. As necrosis was conceived as a (pharmacologically) incontrollable process, for a long time it generated limited interest within the scientific community. Accordingly, necrosis was mainly defined in a negative fashion, as a cell death subroutine not manifesting with apoptotic features or with an extensive vacuolization of the cytoplasm (which was considered as a sign of autophagic cell death) [9]. This begun to change only with the late 1980s, when tumor necrosis factor α (TNFα) was shown to kill cancer cells while promoting either an apoptotic or a necrotic phenotype, in a cell type-dependent fashion [10]. The possibility that – similar to apoptosis – necrosis might also occur in a regulated fashion continued to gather momentum throughout the 1990s [11–13], and was definitively confirmed in 2005, when the team of Junying Yuan discovered a groups of molecules that inhibit several instances of necrotic cell death, namely, necrostatins [14,15]. In 2008, the same authors identified receptor-interacting protein kinase 1 (RIPK1), a kinase that so far had been involved in NF-κB and apoptotic signaling, as the cellular target of necrostatin 1 [16,17]. This ignited an intense experimental effort that led to the precise characterization of the signal transduction cascade whereby TNFα can promote necrosis, at least under some circumstances [18–22].

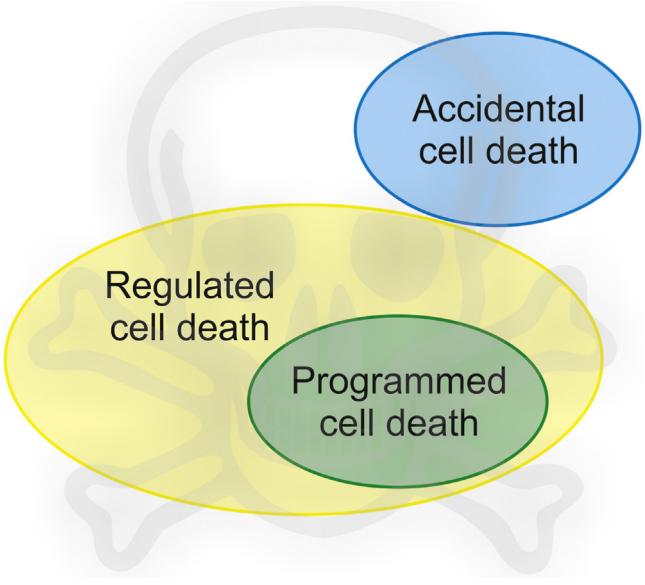

Fig. 1. Cell death nomenclature. At odds with "accidental" instances of cell death, which by definition cannot be controlled, "regulated" cell death relies upon a genetically encoded molecular machinery that can be pharmacologically modulated. Regulated cell death, be it apoptotic or necrotic, can be triggered by exogenous stimuli or occur as part of a genetically encoded physiological program, for instance (post)embryonic development or the maintenance of tissue homeostasis. Such as physiological type of regulated cell death is generally indicated as "programmed".

Since then, our knowledge on the molecular mechanisms that control and execute regulated necrosis (RN) has significantly improved [23–25]. Alongside, it has become clear that RN plays a significant role in both physiological scenarios (*e.g.*, embryonic development) and pathological settings (*e.g.*, ischemic conditions), suggesting that the pharmacological modulation of RN might provide consistent therapeutic benefits to patients affected by a large panel of disorders [23–25]. Furthermore, it rapidly turned out that RN does not occur only in caspase-incompetent cells upon the activation of the RIPK1 homolog RIPK3. Rather, there are multiple molecular circuitries that can drive RN including (but not limited to) necroptosis, mitochondrial permeability transition (MPT)-dependent RN, and parthanatos. Interestingly, the inhibition of only one of these modules generally provides limited cytoprotective effects *in vivo* [26], underscoring the elevated degree of interconnectivity of the RN signaling network. Here, we discuss the signal transduction cascades, pathophysiological relevance and therapeutic implications of the major molecular circuitries underlying RN.

2. Mechanisms of regulated necrosis

2.1. Necroptosis

The term "necroptosis" has originally been introduced in 2005 to indicate a necrostatin 1 (Nec-1)-inhibitable, and hence RIPK1-dependent, regulated form of non-apoptotic cell death triggered by TNFα receptor 1 (TNFR1) in the presence of genetic or pharmacological caspase inhibition [14]. For the next few following years, this term has been widely (but improperly) employed as a strict synonym of RN [27]. Nowadays, following the milestone discovery that RIPK1 transduces pro-necrotic signals by engaging in physical and functional interactions with its homolog RIPK3 [18–20] and the identification of multiple RIPK3-dependent but RIPK1-independent instances of RN [28–31], necroptosis is rather defined as a RIPK3-dependent molecular cascade promoting RN [32].

During necroptosis, RIPK3 gets activated in the context of a supramolecular complex that may or may not involve RIPK1, hence acquiring the ability to phosphorylate mixed lineage kinase domain-like (MLKL), a pseudokinase that binds ATP but is not catalytically active [21,33] The multiprotein complex promoting the activation of RIPK3 is commonly referred to as the necrosome [34]. Of note, when RIPK1 is not involved in the necrosome other factors that share with RIPK1 and RIPK3 a RIP homotypic interaction motif (RHIM) are [35,36]. Only two murine and human proteins other than RIPK1 and RIPK3 are known to contain a RHIM, namely, Z-DNA binding protein 1 (ZBP1, also known as DAI) and Toll-like receptor (TLR) adaptor molecule 1 (TICAM1, best known as TRIF) [35,36]. As both these proteins have already been involved in instances of necroptosis [35,36], RHIMs appear to be critical for the activation of the necrosome.

RIPK3 only engages in transient interactions with MLKL, resulting in the exposure of a positively charged N-terminal stretch [33]. This said, the precise molecular mechanisms whereby MLKL

plays an essential non-enzymatic role in necroptosis [37] remain relatively obscure. Indeed, while phosphoglycerate mutase family member 5 (PGAM5) was initially thought to execute RIPK3- and MLKL-dependent necroptosis by promoting dynamin 1-like (DNM1L)-mediated mitochondrial fragmentation [22], accumulating evidence indicates that both PGAM5 and mitochondria are dispensable for the pro-necrotic effects of MLKL [33,38]. Very recently, MLKL has been suggested to respond to RIPK3-mediated phosphorylation by forming homo-oligomers (most likely homotrimers) that localize to the plasma membrane [39,40]. Such a relocalization appears to be a strict requirement for necroptosis, possibly because it triggers the influx of Ca^{2+} or Na^{+} ions into the cytoplasm and hence disrupts osmotic homeostasis [39,40]. As an alternative, MLKL oligomers might be involved in the activation of an oxidative burst by plasma membrane redox-active enzymes such as NADPH oxidase 1 (NOX1), a process that has previously been involved in the execution of necroptosis [41]. Experimental evidence in support of this latter hypothesis, however, is missing.

The list of physiological and pathological stimuli that trigger necroptosis is constantly growing, nowadays encompassing not only various death receptor ligands but also so-called "microbe-associated molecular patterns" (MAMPs), *i.e.*, microbial components that generally alert the immune system of an ongoing infection [28]. Death receptors including TNFR1 and FAS (also known as CD95) generally promote the activation of RIPK3 *via* RIPK1, a process that is constitutively inhibited by a supramolecular complex including caspase-8, the long isoform of CASP8 and FADD-like apoptosis regulator (CFLAR), best known as $FLIP_L$, and Fas (TNFRSF6)-associated *via* death domain (FADD) [11,42–47]. Interestingly, the embryonic lethality of *Casp8*$^{-/-}$ mice is prevented on a *Ripk3*$^{-/-}$ background [48], perhaps suggesting that at least part of the apoptotic machinery may have evolved to safeguard multicellular organisms from necroptosis [49]. As it stands, necroptosis appears to be dispensable for the embryonic and post-embryonic development of higher eukaryotes, as demonstrated by the absence of obvious physiological defects in *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice [37], but plays a major role in multiple pathological conditions (see below).

At odds with death receptor ligands, MAMPs usually trigger RIPK1-independent variants of necroptosis [30,31,36]. The pro-necrotic activity of MAMPs (as well as their immunogenic potential) relies on their recognition by specific pattern-recognition receptors (PRRs) [50]. In particular, double-stranded RNA molecules (which are a virus-specific product) and lipopolysaccharide (LPS, the major component of the outer membrane of Gram-negative bacteria) have been shown to elicit an instance of necroptosis that requires the RHIM-containing TLR adaptor molecule TICAM1 upon binding to TLR3 and TLR4, respectively [31,36,51]. Along similar lines, cytosolic DNA (a sign of ongoing viral infection) has been demonstrated to promote the recruitment of the necrosome in a RIPK1-independent fashion upon recognition by ZBP1 [30]. Interestingly, in the course of infection several PRRs including (but not limited to) various TLRs respond to MAMPs by stimulating the secretion of type I interferons (IFNα, β and ω), which promote the establishment of an antiviral state among neighboring cells [52]. Type I interferons are actually capable of triggering RIPK1-dependent necroptosis by a mechanisms that relies on eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2, best known as PKR) and is under the control of FADD and caspases [53,54]. These observations suggest that necroptosis participates in the first-line defense of multicellular organisms against invading pathogens, in particular those that express caspase inhibitors [55]. As a matter of fact, necroptosis has been shown to be determinant for the control of vaccinia virus infection in mice [18], and the genome of some viruses (*e.g.*, cytomegalovirus) codes for RIPK3 inhibitors [56,57].

Importantly, the pathophysiological relevance of necroptosis is not limited to infectious diseases. The pharmacological inhibition of RIPK1 with Nec-1 and robust genetic evidence based on *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice have indeed demonstrated that necroptosis contribute to the etiology of a wide panel of conditions characterized by excessive cell loss, including (but perhaps not limited to): ischemia-reperfusion injury [14,58–62], chronic neurodegenerative disorders [63], acute neurotoxicity [64], retinal detachment [65,66], sepsis [67–69], and pancreatitis [19,20,37]. In addition, (1) a loss-of-function mutation in *MLKL* (resulting in a L291P substitution) has been identified in human adenocarcinoma samples [33]; and (2) low expression levels of MLKL have been correlated with poor disease outcome in a cohort of ovarian cancer patients [70]. These latter observations suggest that necroptosis may exert anti-neoplastic functions, although it cannot be formally excluded that these effect originate from hitherto uncharacterized necroptosis-independent functions of MLKL.

2.2. MPT-dependent regulated necrosis

The expression "mitochondrial permeability transition" has been employed throughout the last two decades to indicate an abrupt increase in the permeability of the inner mitochondrial membrane to small solutes [71,72]. In this setting, osmotic forces drive a massive entry of water into the mitochondrial matrix, leading to (1) an immediate cessation of the bioenergetic and biosynthetic functions of mitochondria that depend on the transmembrane potential ($\Delta\psi_m$), and (2) the release of multiple mitochondrial proteins, including various activators of the intrinsic pathway of apoptosis, into the cytoplasm [2,71,72]. Indeed, in several experimental and pathophysiological scenarios, the MPT represents one of the major gateways to mitochondrial apoptosis, the other being a process that is mediated and regulated by proteins of the Bcl-2 family, namely, mitochondrial outer membrane permeabilization (MOMP) [73,74]. However, the MPT is also capable of triggering a peculiar subroutine of regulated necrosis that critically relies on peptidylprolyl isomerase F (PPIF, best known as cyclophilin D, CYPD) [75–77].

The importance of CYPD for MPT has been suspected for a long time, mostly due to the consistent cytoprotective effects mediated *in vitro* and *in vivo* by the pharmacological CYPD inhibitor cyclosporin A (CsA) [71,72]. However, it's only in 2005 that CYPD has been conclusively identified as the central component of an otherwise poorly characterized MPT-inducing supramolecular entity assembled at the junctions between the inner and outer mitochondrial membranes, the so-called "permeability transition pore complex" (PTPC) [75–77]. Great efforts were made before and after this discovery to identify (additional) PTPC constituents that would be truly critical for its lethal functions, with no success. Indeed, robust genetic evidence from double/triple knockout mice ruled out a critical contribution of several proteins that had been involved in the execution or regulation of MPT *in vitro*, including the main isoforms of adenine nucleotide translocase (ANT, an antiporter embedded in the inner mitochondrial membrane) [78] and voltage-dependent anion channel (VDAC, a protein of the outer mitochondrial membrane) [79,80]. Nowadays, CYPD remains the only confirmed functional constituent of the PTPC, although accumulating findings suggest that other proteins, notably the c subunit of the mitochondrial F_0 ATPase [81,82], may also play a prominent role in MPT. However, genetic evidence in support of this hypothesis is still lacking.

In response to several stimuli, including the overproduction of reactive oxygen species (ROS) and an abrupt increase in the cytosolic concentration of Ca^{2+} ions, the PTPC assumes a high-conductance state, hence mediating MPT [71,72]. Often (but not

always, see above) this results in the activation of RN. In line with this notion, both the administration of CsA and the *Ppif*$^{-/-}$ genotype have been shown to limit necrotic cell death, *in vitro* as well as *in vivo*, in a variety of pathophysiological settings including ischemia-reperfusion injuries of the heart [75,83,84], brain [77,85] and kidneys [26,86]. Interestingly, the pro-necrotic activity of CYPD appears to be regulated by a mitochondrial pool of the oncosuppressor protein p53 [85]. However, the precise molecular mechanisms that execute MPT-dependent RN remain obscure. Along similar lines, it remains to be formally demonstrated whether CYPD mediates RN as part of the PTPC or independently from the assembly of such a supramolecular complex. As mentioned above, so far no PTPC components other than CYPD have been shown to be indispensable for MPT *in vivo*. This may indicate (1) that CYPD can assemble the PTPC in a relatively unspecific manner, based on virtually any (or at least several distinct) protein(s) that is (are) locally available; (2) that the knockout models employed so far failed to delete the genes encoding for all isoforms of a specific protein (as is indeed the case for ANT) [87]; or (3) that CYPD exerts PTPC-independent lethal functions, at least in some settings.

2.3. Parthanatos

The term "Parthanatos" has been coined by the Ted and Valina Dawson a few years ago to indicate a non-apoptotic cell death subroutine that critically relies on the (hyper)activation of poly(ADP-ribose) (PAR) polymerase 1 (PARP1) [88,89]. PARP1 belongs to a large family of enzymes that catalyze the addition of PAR moieties (an NAD^+-dependent reaction also known as ADP ribosylation) to multiple substrates, hence regulating a wide panel of cellular processes [90,91]. In particular, PARP1 plays a critical role in the repair of single- or double-strand DNA breaks *via* the base-excision repair (BER) and non-homologous end joining (NHEJ) pathways [90,92]. In this setting, PARP1 is recruited to DNA at the site of damage (*via* a zinc finger DNA-binding domain) and thus becomes able to catalyze the ADP ribosylation of various proteins, including histones. This generates an abundance of negative charges that allow for the recruitment of multiple factors involved in DNA repair, including (but not limited to) X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) and DNA ligase III [92]. In the presence of persistent DNA damage, however, PARP1 becomes hyperactivated, a situation that has been associated with multiple outcomes, including the execution of RN [93–95].

One of the first consequences of PARP1 hyperactivation is the depletion of NAD^+, which has a dramatic bioenergetic impact [96]. Indeed, this not only inhibits mitochondrial ATP synthesis as it limits the availability of substrates for the generation of the $\Delta\psi_m$ (which is an absolute requirement for the activity of the F_1F_0 ATPase), but also inhibits the glycolytic flux at the level of glyceraldehyde phosphate dehydrogenase [96]. Such a profound depletion in the pool of ATP and reducing equivalents (owing to a mass effect triggered by overconsumption of NAD in ADP ribosylation reactions) has been thought to constitute a sufficient cause for necrotic cell death, mostly based on the assumption that apoptosis could not be activated in these conditions as it impinges on various ATP-dependent processes [97]. Although it is true that a minimal level of ATP is required for the execution of apoptotic cell death [97], which may also explain why PARP1 is inactivated by caspase-3 [98], PARP1-dependent necrosis relies on additional molecular mechanisms [94,95,99]. In particular, it has been demonstrated that PARP1 hyperactivation results in the release of apoptosis-inducing factor, mitochondrion-associated, 1 (AIFM1, best known as AIF) from the mitochondrial intermembrane space [94,95,99] and its translocation to the nucleus, where it operates as an endonuclease to promote large-scale chromatin degradation [100].

Interestingly, AIF has first attracted attention owing to its ability to exit mitochondria (in a calpain-dependent fashion) in response to specific lethal stimuli (*e.g.*, the DNA damaging agent *N*-methyl-*N*′-nitro-*N*′-nitrosoguanidine, MNNG), resulting in the activation of a cyclophilin A-dependent but caspase-independent variant of cell death manifesting with apoptotic features [101–103]. Conversely, in the course of parthanatos (which is associated with a necrotic morphotype), AIF appears to be released from mitochondria by a mechanism that does not depend on calpains [104] but requires the binding of AIF to PAR moieties [94].

Accumulating evidence suggest that AIF (which functions as a NADH oxidase) is way more relevant for its ability to stabilize and/or promote the enzymatic activity of the mitochondrial respiratory chain than for its role as a caspase-independent cell death executioner, at least in a majority of physiological settings [105–107] Indeed, mice lacking *Aifm1* at the whole body level die *in utero* by embryonic day E9 [106], and the targeted deletion of *Aifm1* in the cardiac and skeletal muscle or in the liver results in significant metabolic alterations (for the most part reflecting defects in mitochondrial respiration) rather than in cytoprotection against lethal insults [108,109]. This said, PARP1 and AIF have convincingly been shown to contribute to the (most-often non-apoptotic) demise of neurons exposed to a variety of cytotoxic stimuli *in vivo*. Such neurotoxic triggers include (but presumably are not limited to) MNNG [104], *N*-methyl-D-aspartate (NMDA) [104,110], glutamate [111], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [112], irradiation [113], trauma [114,115], retinal detachment [116], and perinatal [117–119] as well as adult cerebral ischemia [102,120–122]. Of note, most of these findings have been obtained with chemical inhibitors of PARP1, with *Parp1*$^{-/-}$ mice or with so-called Harlequin mice, which express low levels of AIF owing to a hypomorphic, X-linked recessive mutation in *Aifm1* [123]. Why PARP1 and AIF appear to play a relevant role in cell death predominantly in the central nervous system, but much less so in other tissues/organs, remains an open conundrum. Nonetheless, targeting the molecular machinery of parthanatos stands out as a promising approach for the development of novel neuroprotective interventions [124,125].

2.4. Other forms of regulated necrosis

During the last decade, additional cell death subroutines have been shown to manifest with a necrotic morphotype and to be responsive to specific pharmacological or genetic interventions, hence representing (at least theoretically) instances of RN [2]. However, it often remains to be formally demonstrated whether these cell death modalities, including (but not limited to) ferroptosis and pyroptosis, are truly distinct from necroptosis, MPT-mediated RN and parthanatos [126].

By means of large chemical screens, Brent Stockwell and colleagues identified at least 3 compounds (*i.e.*, erastin, RSL3 and RSL5) that trigger an iron-dependent form of non-apoptotic demise in cancer cells bearing oncogenic *RAS* mutations [127,128]. These authors named such a cell death subroutine "ferroptosis" and proposed it to be "morphologically, biochemically, and genetically distinct from apoptosis, necrosis, and autophagy" [129]. Indeed, erastin-elicited ferroptosis appears to be insensitive to the pan-caspase inhibitor Z-VAD.fmk as well as to Nec-1 and CsA [130], but can be inhibited by a cell-permeant 3,4-diaminoethylbenzoate-based antioxidant called ferrostatin 1 (Fer-1) [129]. Fer-1 effectively prevents erastin-induced cell death as well as glutamate-induced neurotoxicity (in rat organotypic hippocampal slice cultures) by inhibiting the accumulation of reactive oxygen species (ROS) and consequent lipid peroxidation [129]. Of note, ferroptosis and glutamate-induced

excitotoxicity [131,132] share several biochemical features, including the involvement of ROS and lipid peroxidation as well as the sensitivity to antioxidants other than Fer-1 [133]. As a matter of fact, erastin has been shown to initiate ferroptosis by inhibiting the Na^+-independent cystine/glutamate antiporter known as system x_C^- [129], which is known to participate in the excitotoxic responses of neurons to glutamate (which generally proceeds *via* MPT) [134]. In addition, the lethal effects of erastin and RSL5 (but not RSL3) reportedly depend on VDAC2 and/or VDAC3 [127,130], which are known to participate in the PTPC, at least under some circumstances [135,136]. Finally, by promoting the depletion of intracellular glutathione, erastin robustly inhibits the enzymatic activity of various glutathione peroxidases, including glutathione peroxidase 4 (GPX4) [137], thus promoting the establishment of oxidative stress (one of the major inducers of MPT) [137,138]. GPX4 is involved in the reduction of lipid peroxides and has recently been attributed a critical role in ferroptosis as triggered by a variety of compounds, including (but not limited to) erastin, RSL5 and RSL3 (whose cytotoxic activity does not rely on the inhibition of system x_C^- and does not require VDAC2 or VDAC3) [137]. GPX4 turned out not only to constitute the actual target of the ferroptosis-inducing activity of RLS3, but also to be required for the survival of cancer cells expressing oncogenic RAS mutants [137]. Notably, the selective cytotoxic activity of GPX4-targeting small-interfering RNAs (siRNAs) was inhibited by the administration of cell-permeant iron chelators as well as by antioxidants, both of which also efficiently inhibit the opening of the PTPC [71,137]. Taken together, these observations suggest that ferroptosis may constitute a peculiar type of MPT-driven RN that occurs in the context of oncogenic *RAS* mutations, rather than an independent signaling pathway leading to necrotic cell death. Robust genetic evidence confirming or disconfirming that ferroptosis is independent of CYPD is urgently awaited. Two distinct inducers of ferroptosis have recently been shown to exert robust antineoplastic activity against tumors that express oncogenic RAS variants developing in immunocompromised mice [137]. It will now be interesting to evaluate the cytoprotective potential of Fer-1 *in vivo*, in rodent models of ischemia-reperfusion injury, neurodegeneration and sepsis.

The term "pyroptosis" has initially been introduced by Molly Brennan and Brad Cookson to describe the caspase-1-dependent demise of macrophages infected by *Salmonella enterica* serovar *Typhimurium* [139]. However, it quickly become clear (1) that several bacterial and non-bacterial stimuli can trigger a pyroptosis-like cell death subroutine; (2) that cell types other than macrophages can die *via* a mechanism that critically relies on caspase-1 (but not caspase-3, the central executioner of apoptosis); and (3) that pyroptotic cells can exhibit a spectrum of morphotypes that range from purely necrotic to purely apoptotic [126,140,141]. Besides allowing for the maturation and secretion of the pyrogenic cytokines interleukin (IL)-1β and IL-18, the activation of caspase-1 (which can occur in the context of either of two supramolecular complexes known as inflammasome and pyroptosome) [142,143] has been proposed (1) to cause the formation of pores in the plasma membrane that would rapidly lead to osmotic cell lysis [144], and (2) to drive the activation of caspase-7, possibly accounting for the apoptotic manifestations of pyroptosis [145]. Of note, an inactivating passenger mutation affecting *Casp11* has been identified in 2011 in the most commonly employed strain of *Casp1*$^{-/-}$ mice, calling for the reinterpretation of a large bundle of data generated with this model [146,147]. In this setting, recent evidence tends to endow caspase-11, rather than caspase-1, with a critical function in pyroptosis [147,148]. Irrespective of these issues, pyroptosis is currently viewed as a peculiar type of necroptosis (or apoptosis, depending on the morphological phenotype exhibited by dying cells, possibly correlating with the degree of caspase-7 activation) rather than as a cell death subroutine of its kind [126]. In support of this notion, *Salmonella enterica* serovar *Typhimurium* has been recently shown to trigger the RIPK3-dependent demise of infected macrophages upon the production of type I IFNs [54]. Moreover, both RIPK1 and RIPK3 reportedly promote the activation of the inflammasome [149–151]. In this scenario, necroptosis stands out as a prominent defense mechanism against viral challenges, not only as it executes the death of infected cells to avoid viral replication, but also as it allows for the delivery of multiple danger signals (including type I IFNs and pyrogenic cytokines) to neighboring cells and the entire organism.

3. Interconnectivity of regulated necrosis

The signaling pathways leading to RN exhibit a consistent degree of crosstalk with the molecular cascades that control and execute apoptosis [2,23]. This functional interplay – which often occurs in the context of cell-wide responses to stress [152] – mainly reflects: (1) the existence of shared signal transducers, which can activate either apoptotic or necrotic cell death (*e.g.*, TNFR1, AIF, p53), depending on the specific circumstances [19,85,94]; and (2) the existence of negative feedback circuitries whereby one cell death subroutine (most often apoptosis) actively inhibits the other (most frequently necrosis) (Fig. 2) [153]. At least in part, such feedback circuitries are responsible for the delayed discovery of RN, which in a vast number of experimental settings is actively blocked by caspase-8/$FLIP_L$/FADD [42–47]. In addition, the active inhibition of RN by apoptosis coupled to the highly immunogenic nature of the former (but not of the latter, at least in a majority of settings) [154] suggests that the apoptotic machinery might have evolved as a refined system to prevent the potentially dangerous consequence of (at least some pathways of) RN [49].

RN not only is closely interconnected with apoptosis (and autophagy) [152,153], but also exhibits an elevated degree of intrinsic interconnectivity. Accumulating evidence suggests indeed that inhibiting a single signaling pathway leading to RN does not provide optimal cytoprotective effects. We have recently demonstrated this point using a murine model of renal ischemia-reperfusion injury [26]. In this setting, both *Ppif*$^{-/-}$ and *Ripk3*$^{-/-}$ mice were significantly protected from a relatively mild ischemic injury as compared with wild-type animals, the latter more so than the former. However, only *Ppif*$^{-/-}$*Ripk3*$^{-/-}$ survived a severe ischemic episode, while both *Ppif*$^{-/-}$ and *Ripk3*$^{-/-}$ animals failed to do so [26]. Similar results were obtained by treating mice with Nec-1 and the CYPD inhibitor sanglifehrin A, either as standalone interventions or in combination [26]. On a slightly different note, Temkin and colleagues reported as early as in 2006 that the co-administration of TNFα and Z-VAD.fmk kills monocytic cells *via* a RIPK1-dependent pathway that (1) results in the inhibition of ANT, and (2) can be rescued by CYPD overexpression [155]. It is difficult to reconcile these latter findings with the critical role of CYPD in MPT. Nonetheless, taken together, these observations demonstrate that necroptosis and MPT-dependent RN do not constitute completely independent signal transduction cascades, but exhibit some degree of overlap. Interestingly, this is also the case for the two major signal transduction cascades leading to apoptosis (the so-called extrinsic and intrinsic pathways), which are linked by the caspase-8 mediated cleavage of the BH3 only protein BID, at least in some cell types including hepatocytes [156]. Thus, regulated cell death stands out as a critical process for multicellular organisms, as demonstrated by the highly interconnected machinery that ensures its regulation or execution, be it apoptotic of necrotic. This has profound therapeutic implications, as it implies that complete cytoprotection, if possible at all, can be achieved only *via* a combinatorial strategy that simultaneously inhibits multiple signal transduction cascades leading to regulated cell death.

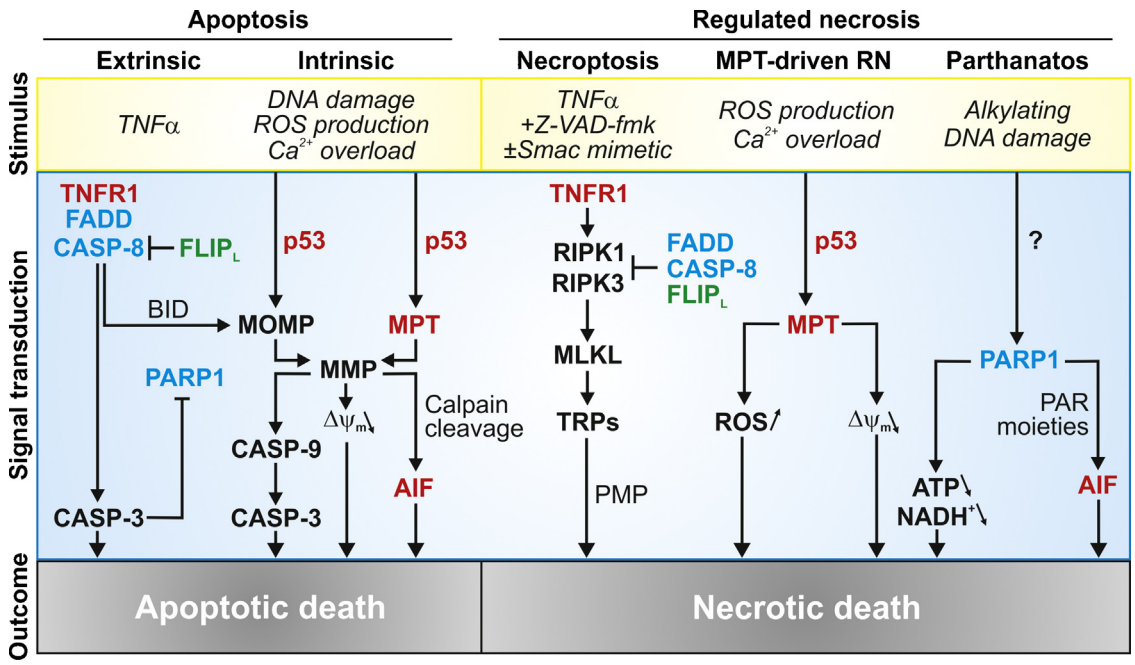

Fig. 2. Interconnectivity of regulated cell death modalities. Regulated necrosis (RN) can follow the activation of at least three signal transduction cascades, namely, necroptosis, mitochondrial permeability transition (MPT)-dependent RN and parthanatos. These signaling pathways not only exhibit a relevant degree of mutual crosstalk, but also functionally (and sometimes physically) interact with the molecular machinery that regulate and execute apoptotic cell death. Thus, depending on contextual parameters, some proteins and processes can actively promote either apoptosis or RN (in red). This is the case, for instance, of tumor necrosis factor α (TNFα) receptor 1 (TNFR1), apoptosis-inducing factor (AIF) and the oncosuppressor protein p53. Along similar lines, some inhibitory components of the apoptotic cell death machinery, such as the long isoform of CASP8 and FADD-like apoptosis regulator (CFLAR), best known as $FLIP_L$, also suppress forms of RN (in green). Finally, proteins like Fas (TNFRSF6)-associated *via* death domain (FADD), caspase (CASP)-8 and poly(ADP-ribose) (PAR) polymerase 1 (PARP1) stimulate one subroutine of cell death while inhibiting another (in blue). $\Delta\psi_m$, mitochondrial transmembrane potential; MLKL, mixed lineage kinase domain-like; MMP, mitochondrial membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization; PMP, plasma membrane permeabilization; RIPK, receptor-interacting protein kinase; ROS, reactive oxygen species; TRP, transient receptor potential cation channel.

4. Concluding remarks

As discussed above, necrosis is no longer considered as a purely accidental, and hence completely uncontrollable, cell death subroutine. Rather, it is now clear that several distinct, yet intimately interconnected, signal transduction cascades are in place to trigger necrotic cell death, at least under some circumstances. Moreover, accumulating evidence indicates that several regulators and executors and RN functionally (and sometimes physically) interact not only with the apoptotic cell death machinery, but also with the molecular systems that underpin adaptive stress responses such as autophagy. The development of pharmacological agents that inhibit or activate RN for therapeutic applications is highly warranted, as the former could be employed in the treatment of ischemic or neurodegenerative conditions, while the latter may be harnessed to promote the demise of cancer cells (which are frequently resistant to apoptosis). This said, accumulating data indicate that molecules inhibiting just one of the signaling pathways that lead to RN (including necroptosis, MPT-dependent RN and parthanatos) may exerted limited cytoprotective effects. We suspect that combinatorial strategies that simultaneously target most (if not all) the signal transducers that are critical for RN, and perhaps even key pro-apoptotic molecules, are required to achieve clinically relevant levels of cytoprotection.

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