

## Research Highlight

## RIP Kinases Initiate Programmed Necrosis

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**Some lethal stimuli can induce either apoptosis or necrosis, depending on the cell type and/or experimental setting. Until recently, the molecular bases of this phenomenon were largely unknown. Now, two members of the receptor-interacting serine-threonine kinase (RIP) family, RIP1 and RIP3, have been demonstrated to control the switch between apoptotic and necrotic cell death. Some mechanistic details, however, remain controversial.**

Apoptosis and necrosis constitute two distinct cell death modalities that are associated with specific morphological and biochemical features. Prototypic apoptosis involves massive activation of caspases and manifests with chromatin condensation and reduction in cell volume, while the barrier function of plasma membranes is (at least initially) retained. In contrast, necrosis leads to early plasma membrane permeabilization, cytoplasmic swelling and no signs of nuclear shrinkage. The co-existence of apoptotic and necrotic traits as well as caspase-independent instances of apoptosis have also been reported (Kroemer et al., 2009). For decades, necrosis has been considered as a merely fortuitous and unregulated event, whereas programmed cell death (PCD, as it occurs in many developmental and pathophysiological settings) was entirely ascribed to apoptosis. However, recent pharmacological and genetic evidence suggests that necrosis can occur in a tightly regulated fashion and mediate PCD (Golstein and Kroemer, 2007).

Mitochondrial dysfunction has been linked to both apoptosis and necrosis (Kroemer et al., 2007). The enzymatic complexes of the respiratory chain, which function at the interface between the mitochondrial matrix and the mitochondrial intermembrane space (IMS), the mitochondrial inner membrane (IM), generate the majority of intracellular ATP, but also represent a source of harmful reactive oxygen

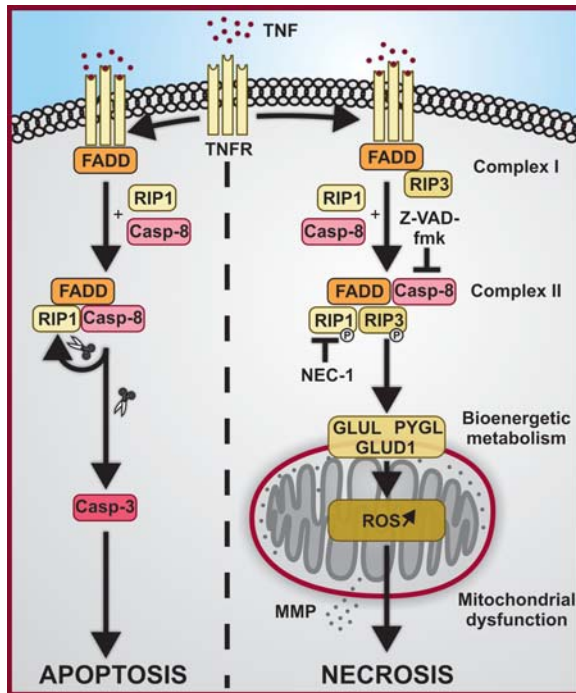
species (ROS). Moreover, mitochondria constitute the battleground at which pro-death and pro-survival signals oppose each other. When the lethal signals predominate, mitochondrial membrane permeabilization (MMP) occurs and brings about a series of catastrophic consequences, including (but not limited to) dissipation of the mitochondrial transmembrane potential ( $\Delta\psi_m$ ), overgeneration of ROS, arrest of ATP synthesis and cytosolic spillage of toxic IMS proteins. This results in the activation of multiple caspase-dependent and -independent executors of cell death (Kroemer et al., 2007; Galluzzi et al., 2009).

MMP can be ignited at the mitochondrial outer membrane (OM) by the pore-forming activity of pro-apoptotic members of the Bcl-2 protein family, in particular Bax and/or Bak. Alternatively, MMP can result from the opening of the permeability transition pore complex (PTPC), a supramolecular entity that is assembled at the junctions between the IM and the OM. Whereas during Bax/Bak-mediated mitochondrial outer membrane permeabilization (MOMP) the  $\Delta\psi_m$  is initially maintained, the so-called mitochondrial permeability transition (MPT) leads to immediate  $\Delta\psi_m$  dissipation and hence to the instantaneous cessation of mitochondrial ATP synthesis (Kroemer et al., 2007). On the basis of this (and other) observation(s), it has been proposed that MOMP and MPT would preferentially lead

to apoptosis and necrosis, respectively (Galluzzi and Kroemer, 2008).

Several cell death inducers are known to promote either apoptosis or necrosis, depending on the specific experimental setting (Figure 1). Thus, some cell types (e.g. NIH 3T3 murine fibroblasts) respond to ligation of the tumor necrosis factor (TNF) receptor (TNFR) by activating the extrinsic pathway of apoptosis that involves the sequential activation of caspase-8 and caspase-3 (Hitomi et al., 2008; Zhang et al., 2009). Conversely, L929 murine fibrosarcoma cells react to TNF by triggering necroptosis, a form of programmed necrosis that requires the receptor-interacting serine-threonine kinase 1 (RIP1) (Hitomi et al., 2008). Until recently, the precise molecular mechanisms of this phenomenon were poorly understood. Since several apoptotic processes require ATP, early models suggested that the bioenergetic status of the cell would influence the decision between apoptosis (which requires high ATP levels) and necrosis (which occurs in conditions of ATP shortage). However, more recent studies suggest that a novel type of 'molecular switches' determine whether cells succumb to apoptosis or necrosis.

In recent issues of *Science* and *Cell*, three research groups independently demonstrated that, upon death receptor ligation, the two kinases RIP1 and RIP3 interact and mediate programmed necrosis (Cho et al.,



**Figure 1** RIP1 and RIP3 drive programmed necrosis via mitochondrial dysfunction. In some cell types, ligation of death receptors (e.g. tumor necrosis factor receptor, TNFR) results in the assembly of a supramolecular platform composed of caspase-8 (Casp-8), the adaptor protein FADD and the receptor-interacting serine-threonine kinase 1 (RIP1). Active caspase-8 cleaves RIP1, thereby inactivating it. Moreover, caspase-8 ignites the caspase cascade, which executes the extrinsic pathways of apoptosis. In cells that express the RIP1 homologue RIP3 and cannot properly activate caspases (for instance, due to presence of the pancaspase inhibitor Z-VAD-fmk, or upon infection by viral strains that encode serpins), TNFR ligation leads to the assembly of a multiprotein complex that involves caspase-8, FADD, RIP1 and RIP3. In this setting, RIP3 becomes able to interact with several bioenergetic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1), thereby enhancing their catalytic activity. Enhanced glycogenolysis and glutaminolysis provide additional respiratory substrates and ultimately result in the overgeneration of reactive oxygen species (ROS). Excess ROS, in turn, can trigger mitochondrial membrane permeabilization (MMP), thereby mediating TNF-induced programmed necrosis.

2009; He et al., 2009; Zhang et al., 2009). Both RIP1 and RIP3 were scored among the top 10 hits in two distinct small interfering RNA (siRNA) screens aimed at identifying modulators of the necrotic response of immortalized human cells to TNF (alone or associated with the pancaspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, Z-VAD-fmk) (Cho et al., 2009; He et al., 2009). In addition, RIP3 was found to be differentially expressed in two NIH 3T3 cell lines, called A and N cells, which respond to TNF by undergoing apoptosis or caspase-independent necrosis, respectively (Zhang et al., 2009). Along the same lines, human colon cancer HT-29 cells and mouse embryonic fibroblasts (MEFs) die from apoptosis upon treatment with TNF and appropriate sensitizing

agents (i.e. a Smac mimetic or cycloheximide), yet they undergo necrosis when Z-VAD-fmk is co-administered (He et al., 2009). Thanks to these and other experimental models, it has been firmly established that both RIP1 and RIP3 must be catalytically active and reciprocally interacting (via the RIP homotypic interaction motif, RHIM) for the initiation of programmed necrosis (but not of apoptosis) *in vitro* and *in vivo*. The pro-necrotic role of RIP1 and RIP3 has been substantiated by several complementary experimental approaches: (i) depletion of RIP1 and RIP3 by RNA interference (RNAi) in RIP-proficient cells; (ii) ectopic overexpression of wild-type (WT), kinase dead (KD) or RHIM-mutated RIP1 and RIP3 in RIP-deficient and RIP-proficient cells; (iii) pharmacological inhibition of

RIP-1 with Necrostatin-1; (iv) co-immunoprecipitation of RIP1 and RIP3 interacting partners, within and outside the TNFR signaling complexes I and II, before and after induction of necrosis; (v) *in vitro* kinase assays with [<sup>32</sup>P] γ-ATP on purified WT and/or KD RIP1 and/or RIP3; (vi) induction of acute necrotizing pancreatitis in WT and *rip3*<sup>-/-</sup> mice; and (vii) infection with vaccinia virus of WT and *rip3*<sup>-/-</sup> mice (Cho et al., 2009; He et al., 2009; Zhang et al., 2009).

Although these data unequivocally point to the implication of RIP1 and RIP3 in the early steps of programmed necrosis (Figure 1), some controversial issues remain to be clarified. While He et al. (2009) suggests that during necrosis RIP3 undergoes autophosphorylation at Ser199, Cho and colleagues propose that another yet unidentified kinase activated by RIP1 would account for this phenomenon, based on the fact that WT RIP1 failed to phosphorylate KD RIP3 *in vitro*, yet Necrostatin-1 prevented RIP3 phosphorylation in MEFs undergoing necrosis (Cho et al., 2009). However, since WT (but not KD) RIP3 exhibited high levels of phosphorylation *in vitro* (Cho et al., 2009), these results might indicate that in necrotic cells RIP3 can indeed undergo autophosphorylation following interaction with RIP-1, in turn depending on RIP1-mediated phosphorylation of a substrate other than RIP3, maybe RIP1 itself. Intriguingly, whereas stable expression of KD RIP3 inhibited the necrotic response of HT-29 cells in a dominant negative fashion (He et al., 2009), both KD RIP1 and RIP3 failed to do so in other RIP-proficient cells (Cho et al., 2009). Whether this may reflect a cell type-specific effect remains to be clarified. Similarly, it is still unclear whether RIP3 represents a downstream effector of a RIP1-initiated signaling cascade (He et al., 2009; Zhang et al., 2009), or whether RIP3 might act upstream of RIP1, as suggested by the fact that RIP3 is constitutively associated with the adaptor protein Fas-associated via death domain (FADD) while RIP1 is recruited to complex II only in pro-necrotic conditions (Cho et al., 2009). Notably, in 14 human and murine cell lines, responsiveness to necrosis correlated with RIP3 protein levels, whereas RIP1 was expressed to similar extents in all cell types (He et al., 2009).

In NIH 3T3 N cells, TNF-activated RIP3 interacts with glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1), thereby increasing their enzymatic activity and promoting a considerable metabolic burst (Zhang et al., 2009). This in turn may propagate the overgeneration of ROS elicited by TNF, thereby favoring mitochondrial dysfunction and cell death (Figure 1). By using specific inhibitors of the respiratory chain, Zhang et al. (2009) demonstrated that ROS generated at and upstream of the ubiquinone site have a key role for TNF cytotoxicity in NIH 3T3 N cells. While Cho et al. (2009) also implicated ROS in the necrotic response of MEFs, ROS quenching failed to prevent TNF-induced necrosis in HT-29 cells (He et al., 2009), suggesting that programmed necrosis might require ROS in some (but not all) cell types.

During programmed necrosis, RIP3 was found to redistribute from a diffuse cytosolic staining to filamentary and punctuated structures, yet failed to exhibit an obvious colocalization with intracellular organelles including mitochondria (He et al., 2009). Although this argues against a direct effect of the RIP1/RIP3 complex on a specific organelle, it does not invalidate the hypothesis that RIP3 indirectly triggers MMP via ROS. PTPC components including the adenine nucleotide translocase and cyclophilin D (CypD) are known to operate at the crossroad between bioenergetic metabolism and

cell death regulation (Kroemer et al., 2007). CypD-deficient MEFs were partially resistant to necrosis induction by TNF + Z-VAD-fmk + a Smac mimetic, though not to the same level as *rip3*<sup>-/-</sup> cells (He et al., 2009), suggesting that CypD is not the sole mediator of RIP3-driven MMP and cell death.

The necrotic pathway ignited by the interaction between RIP1 and RIP3 is relevant *in vivo*, in at least two distinct pathophysiological settings. Thus, as compared to WT animals, *rip3*<sup>-/-</sup> mice are protected against cerulein-induced acute pancreatitis (He et al., 2009; Zhang et al., 2009) and fail to control viral infection, presumably because necrosis-dependent inflammation is severely compromised (Cho et al., 2009).

In summary, further investigation is required to elucidate the mechanistic details of the pro-necrotic signaling cascade triggered by distinct members of the RIP protein family. Irrespective of the unresolved issues, RIPs (including RIP2) (Galluzzi and Kroemer, 2009) emerge as promising therapeutic targets for the modulation of necrotic cell death and innate immune responses.

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