

Review

Many stimuli pull the necrotic trigger, an overview

N Vanlangenakker^{1,2}, T Vanden Berghe^{1,2} and P Vandenabeele^{*1,2}

The lab of Jürg Tschopp was the first to report on the crucial role of receptor-interacting protein kinase 1 (RIPK1) in caspase-independent cell death. Because of this pioneer finding, regulated necrosis and in particular RIPK1/RIPK3 kinase-mediated necrosis, referred to as necroptosis, has become an intensively studied form of regulated cell death. Although necrosis was identified initially as a backup cell death program when apoptosis is blocked, it is now recognized as a cellular defense mechanism against viral infections and as being critically involved in ischemia-reperfusion damage. The observation that RIPK3 ablation rescues embryonic lethality in mice deficient in caspase-8 or Fas-associated-protein-via-a-death-domain demonstrates the crucial role of this apoptotic platform in the negative control of necroptosis during development. Here, we review and discuss commonalities and differences of the increasing list of inducers of regulated necrosis ranging from cytokines, pathogen-associated molecular patterns, to several forms of physicochemical cellular stress. Since the discovery of the crucial role of RIPK1 and RIPK3 in necroptosis, these kinases have become potential therapeutic targets. The availability of new pharmacological inhibitors and transgenic models will allow us to further document the important role of this form of cell death in degenerative, inflammatory and infectious diseases.

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Facts

- The kinase activities of RIPK1 and RIPK3 are crucial for necroptosis.
- The FADD/caspase-8 apoptotic platform negatively regulates RIPK1/3-mediated necroptosis.
- RIPK1 and RIPK3 kinase activities contribute to pathogenesis in IR injury, pancreatitis, photoreceptor cell loss and intestinal epithelial cell loss.
- RIPK1 and RIPK3 kinase activities contribute to an appropriate immune response during viral and microbial infections.
- Some forms of regulated necrosis act independently of RIPK1 or RIPK3 kinase activity.

Open Questions

- What is the point of convergence of the molecular mechanisms initiating regulated necrosis elicited by different stimuli?

- Are common executioner mechanisms operating in regulated necrosis elicited by different stimuli?
- Are there common or differential biomarkers for necrosis triggered by different stimuli?
- Which are the molecular nodes and regulatory mechanisms that determine the cellular cell death outcome initiated by different stimuli?
- How are RIPK1 and RIPK3 kinase activities connected with the execution mechanisms of necroptosis?

The term ‘necrosis’ originates from the Greek word ‘nekros’, which is translated as ‘dead body’. Necrosis is morphologically characterized by rounding of the cell, a gain in cell volume (also known as oncosis), organelle swelling, lack of internucleosomal DNA fragmentation, and plasma membrane rupture.¹ As a consequence of plasma membrane permeabilization and cell lysis, the intracellular content is spilled and the damage-associated molecular patterns (DAMPs) may modulate inflammation. Necrosis, as a form of

¹Department for Molecular Biomedical Research, VIB, Zwijnaarde-Ghent, Belgium and ²Department of Biomedical Molecular Biology, Ghent University, Zwijnaarde-Ghent, Belgium

*Corresponding author: P Vandenabeele, Department for Molecular Biomedical Research, VIB-Ghent University, Technologiepark 927, Zwijnaarde-Ghent, 9052 Belgium. Tel: +32 09 33 13760; Fax: +32 09 33 13609; E-mail: Peter.Vandenabeele@dibr.vib-UGent.be

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Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; ASK1, apoptosis signal-regulating kinase 1; cIAP, cellular inhibitor of apoptosis protein; CID, caspase-independent cell death; cFLIP, cellular FLICE-like inhibitory protein; CypA, cyclophilin A; CypD, cyclophilin D; DAI, DNA-dependent activator of interferon regulatory factor; DAMP, damage-associated molecular patterns; DR, death receptor; EDAR, Ectodermal dysplasia receptor; FADD, Fas-associated protein via a death domain; FasL, Fas ligand; H&E, hematoxylin and eosin; H₂O₂, hydrogen peroxide; HIV-1, human immunodeficiency virus type-1; HMGB1, high-mobility group box 1 protein; HSV-1, herpes simplex virus type-1; IR, ischemia-reperfusion; LPS, lipopolysaccharide; LTβR, lymphotoxin-β receptor; MCMV, murine cytomegalovirus; MDA5, melanoma differentiated-associated gene 5; MEF, mouse embryonic fibroblasts; MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; Nec-1, necrostatin-1; NLR, NOD-like receptor; NLRP3, NOD-like receptor family pyrin domain-containing protein 3; NMDA, *N*-methyl-D-aspartate; Nod1, nucleotide-binding oligomerization domain-containing 1; PAMP, pathogen-associated molecular patterns; PARP1, poly(ADP-ribose) polymerase 1; PDT, photodynamic therapy; PRR, pattern recognition receptor; RHIM, RIP homotypic interaction motif; RIG-I, retinoic acid-inducible gene-1; RIPK, receptor-interacting protein kinase; RLR, retinoic acid-inducible gene-1-like receptors; IITLR, Toll-like receptor; TNFR, tumor necrosis factor receptor; TRADD, TNFR-associated death domain protein; TRAIL-R, TNF-related apoptosis-inducing ligand receptor; TUNEL, Tdt-mediated dUTP nick end labeling; TWEAKR, TNF-like weak inducer of apoptosis receptor; VV, vaccinia virus; WNV, West Nile virus

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caspase-independent cell death (CID), has for a long time been regarded as an accidental, uncontrolled mode of cell death. However, accumulating evidence shows that some forms of necrosis actively involve defined signaling pathways that contribute to the cellular demise, as is the case for apoptosis. The connotation of 'caspase-independent' is not completely correct, because in case of TNF (tumor necrosis factor)-induced necroptosis, caspase-8 apparently negatively regulates necrosis and its inhibition in fact strongly sensitizes cell death.² The term 'pyroptosis' has been introduced by Cookson and colleagues^{3,4} to describe necrotic-like cell death that depends on caspase-1 activation, which has an essential role in the proteolytic activation of pro-IL1 β , which once released, acts as a pyrogen. Because of its dependency on caspase-1 activity, this type of cell death is confined to caspase-1-expressing cells such as monocytes, dendritic cells, epithelial cells and keratinocytes.⁵⁻⁷ Whether other inflammatory caspases such as caspase-11 in mouse, and caspase-4 and -5 in human, are functionally redundant in their capacity to mediate pyroptosis is unclear. How caspase-1 is precisely implicated in the cell death process through the activation of the IL1 β release mechanism via pore formation,⁸ proteolysis of cell death-associated substrates,⁹ or a combination of both is unclear. Because of the morphological similarities between pyroptosis and necrosis, such as cytoplasmic swelling and plasma membrane rupture and consequently release of the intracellular content,^{8,10} it is tempting to speculate that common executioner mechanisms such as those leading to osmotic swelling may be partially involved.

Different forms of necrotic cell death can be distinguished based on their initiating mechanisms. Much of the knowledge is based on the study of TNF-induced necroptosis.^{11,12} Necrosis dependent on the kinase activities of receptor-interacting protein kinase 1 (RIPK1)¹³⁻¹⁵ and RIPK3¹⁶⁻¹⁸ has been defined as necroptosis.^{14,19} The necrotic process can be subdivided into several subroutines: preconditioning, initiation, propagation, execution and exposure or release of DAMPs. Preconditioning toward TNF-induced necroptosis includes increased glycolysis and glutaminolysis,^{18,20,21} which increase the metabolic flux toward the Krebs cycle. In the propagation and execution phase of TNF-mediated necroptosis, the mitochondrial complex I-mediated production of reactive oxygen species has been shown to be crucial, as well as lipid peroxidation and lysosomal leakage²² (Figure 2e). Because these necrotic executioner mechanisms are not within the scope of this review, the reader is referred to earlier reviews for detailed descriptions.^{12,23}

We will also discuss the initiation process as similar mechanisms may also be implicated in necrosis elicited by other stimuli. TNF-induced necroptosis is highly modulated by proteolysis, ubiquitylation and deubiquitylation events, and kinases (Figure 1). An important regulator of necroptosis is cylindromatosis, which has been shown in cells²⁴ and *in vivo* in intestinal epithelial cells.²⁵ This deubiquitylase counteracts the activity of ubiquitylating enzymes such as cellular inhibitor of apoptosis protein 1 (cIAP1), which is involved in survival signaling.²⁶⁻²⁸ Also the linear ubiquitin chain assembly complex, involved in the linear ubiquitylation of NF- κ B essential modifier, is crucial in survival signaling^{29,30} and its

counteraction promotes cell death.³⁰⁻³³ In addition, transforming growth factor- β -activated kinase 1 negatively regulates the formation of a cell death-inducing complex.³⁴ Recently, an important negative regulatory mechanism of necroptosis has been repeatedly reported by the finding that the embryonic lethality in mice lacking Fas-associated protein via a death domain (FADD) or caspase-8 is due to massive necrosis and can be rescued by RIPK1 or RIPK3 deletion, respectively.³⁵⁻³⁷ Moreover, caspase-8 forms with its enzymatically inert homolog cellular FLICE-like inhibitory protein long (cFLIP_L) an active complex that prevents RIPK3-dependent necroptosis.³⁶ These data demonstrate that FADD and caspase-8, but also cFLIP_L, counteract RIPK1- and RIPK3-dependent necroptosis during development.³⁵⁻³⁷ More than 13 years ago, the concept of an anti-necrotic role of caspase-8 was already suggested by Vercammen *et al.*,² who reported on the observation that CrmA-transfected L929 cells were more sensitive to TNF-mediated necroptosis. In addition, the loss of RIPK3 rescues caspase-8-deficient T-cells from their defective proliferation, which is caused by necroptosis and results in lymphoproliferative disease,^{35,38} indicating also a role for necroptosis during lymphoid homeostasis. The critical role for caspase-8 and FADD in suppressing RIPK3-mediated necroptosis during intestinal homeostasis has been recently confirmed.^{25,39} Indeed, conditional deletion of FADD²⁵ or caspase-8³⁹ in intestinal epithelial cells leads to spontaneous necrotic cell death of Paneth cells and goblet cells, and an enhanced susceptibility to colitis, which was rescued by genetic deletion of RIPK3²⁵ or treatment with the RIPK1 kinase inhibitor necrostatin-1 (Nec-1).^{14,15,39} Importantly, enhanced levels of RIPK3 in human Paneth cells and increased necroptosis in the ileum of patients with Crohn's disease were identified, strongly suggesting a role for necroptosis in the pathology of this disease.³⁹ Also ablation of caspase-8 in keratinocytes leads to enhanced necroptosis⁴⁰ and inflammation.⁴¹ Similar to the observations in these epithelial cell pathologies, necrotic cell death has also been observed upon acute liver injury in liver specific caspase-8-deficient mice.⁴² Furthermore, RIPK3-dependent necroptosis in particular has also been observed during pancreatitis¹⁷ and photoreceptor cell loss⁴³ and it serves as a defense strategy against viral infections.^{16,44} Pharmacological inhibition by administration of Nec-1, an allosteric inhibitor of RIPK1 kinase,^{14,15} showed that RIPK1 kinase activity contributes to brain^{14,45} and myocardial⁴⁶ ischemia-reperfusion (IR) injury. Together, these studies demonstrate the (patho)physiological importance of targeting RIPK1 and RIPK3 kinase activity. However, the observation that Nec-1 inhibits a pathology does not directly imply a role for necroptosis in that pathology. It is clear that under conditions of IAP inhibition also RIPK1-mediated apoptosis can occur.⁴⁷⁻⁵⁰ It is therefore conceivable that the *in vivo* efficiency of Nec-1 is related to interfering both with necrotic as well as apoptotic processes. The rescue of a lethal phenotype in RIP3 knockout is often used as an argument for the implication of necroptosis. However, strictly spoken, as no clear biochemical markers of necroptosis are available, this should still be considered with caution (see below).

A growing list of triggers such as cytokines, pathogen-associated molecular patterns (PAMPs), alkylating DNA

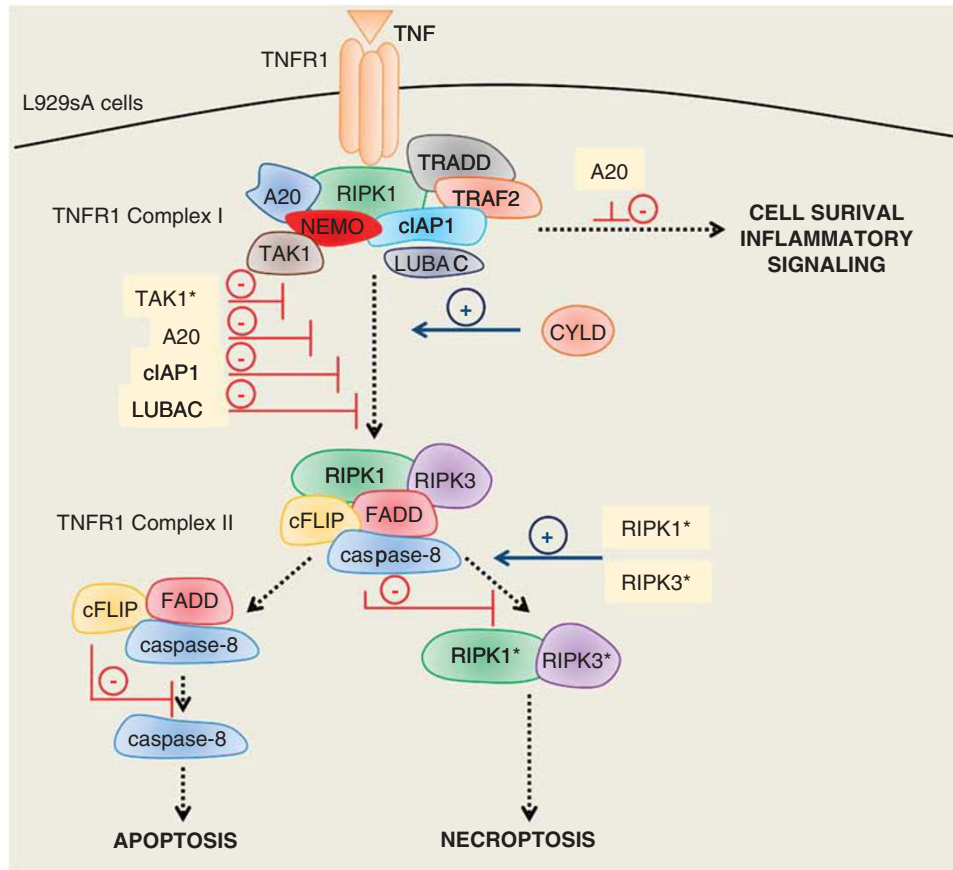


Figure 1 Breaks and gears on TNF-induced necroptosis. Upon TNF stimulation, TNFR1 complex I, important for cell survival and inflammatory signaling, is formed at the plasma membrane. Within this TNFR1 complex I, A20, an ubiquitin-editing enzyme, cIAP1, an ubiquitylating enzyme, LUBAC, a linear ubiquitylating enzyme complex, and TAK1* negatively regulate TNF-induced necroptosis in L929sA cells. The transition from TNFR1 complex I to the cytosolic death-inducing TNFR1 complex II requires the activity of cylindromatosis (CYLD), a deubiquitylating enzyme. The composition of TNFR1 complex II determines the cell death outcome: apoptosis or necroptosis. Within TNFR1 complex II, the apoptotic machinery FADD, c-FLIP and caspase-8 suppresses the induction of necroptosis, which requires the kinase activity of RIPK1* and RIPK3*. *Refers to the implication of the kinase activity in the function indicated

damage, excitotoxins, irradiation or oxidative stress can initiate necrotic cell death (Table 1), showing an emanating paradigm of an intricate interrelation between necrosis and inflammation.^{12,51} However, it should be noted that cell death initiated by these triggers is not limited to necrosis because depending on the cellular context, other cell death modalities such as apoptosis and pyroptosis can also occur. In this review we describe the triggers that are known to induce necrotic cell death in certain conditions, which does not exclude that they may also elicit other types of cell death. We will discuss the similarities and differences in necrosis initiated by these stimuli (Figure 2 and Table 1).

How to Determine Necrosis?

To date, there are no specific positive discriminative biochemical biomarkers for the *in situ* detection of necrosis *in vitro* and *in vivo*. The release of intracellular proteins such as high-mobility group box 1 protein (HMGB1)⁵² and cyclophilin A (CypA)⁵³ has been proposed as a candidate necrotic biomarker. However, HMGB1 and CypA can also be passively released from cells dying by secondary necrosis following apoptosis^{53,54} or actively secreted from activated

immune cells or cells dying from pyroptosis.^{55,56} Seemingly, what is really distinctive is not the release itself but the immunostimulatory activity of HMGB1. During apoptosis, HMGB1 undergoes oxidation, which neutralizes its immunostimulatory activity,⁵⁴ whereas in contrast, necrotic cell debris from HMGB1-deficient cells showed an impaired induction of proinflammatory cytokines.⁵⁷ Beside HMGB1 release, the ratio between caspase-cleaved cytokeratin-18 released from apoptotic cells and intact cytokeratin-18 released from cells dying from other causes, including necrosis, has also been proposed as a marker to determine qualitatively and quantitatively the extent of both types of cell death,⁵⁸ but should again be taken with caution.⁵⁹

Because of the absence of positive discriminative markers, people use combined immunohistochemical methods and electron microscopy to show the presence of necrotic dying cells. Typically, hematoxylin and eosin (H&E) stained tissues are analyzed for the presence of intact extracellular nuclei remaining from necrotic dying cells (apoptotic nuclei are condensed and fragmented) and infiltrating immune cells.^{16,17,25,37,39} Often, these H&E stainings are supplemented with electron microscopic pictures to illustrate the morphological characteristics of necrotically dying cells.^{39,60}

Table 1 Overview of different classes of necrotic stimuli and the regulatory mechanisms implicated

Trigger	Regulatory mechanism	Reference
<i>Ligand/cytokine-induced necrosis</i>		
TNF	RIPK1-RIPK3-dependent TNFR2 stimulation promotes TNFR1 signaling Negatively regulated by FADD, caspase-8 and cFLIP _L	13,16–18,36,37,68
FasL	CypD-dependent Requires caspase inhibition Requires FADD	13,14,17,34,35,64,65
TRAIL	RIPK1-RIPK3-dependent Requires caspase inhibition Requires FADD	13, 7,67
EDAR	RIPK1-RIPK3-dependent	70
LT β	?	72,73
TWEAK	ASK1-dependent Requires caspase inhibition Promotes TNFR1 signaling	78–80
<i>Pathogen-induced necrosis</i>		
HIV-1	RIPK1-independent	83,84,87
HSV-1	Inhibited by Nec-1 treatment	85
WNV	WNV-E protein inhibits RIPK1 ubiquitylation	86,88
VV	Sensitizes TNF-induced necroptosis	16,68,89
MCMV	RIPK1-RIPK3-dependent M45 protein protects from TNF-induced necroptosis M45-deficient MCMV strain induces RIPK3-dependent necroptosis	44,90
<i>Neisseria gonorrhoeae</i>	ASC/NLRP3-dependent HMGB1 release	94
<i>Porphyromonas gingivalis</i>	ASC/NLRP3-dependent HMGB1 release	95
<i>Klebsiella pneumoniae</i>	ASC/NLRP3-dependent HMGB1 release	96
<i>Shigella flexneri</i>	Myeloid cells: ASC/NLRP3-dependent; HMGB1 release Non-myeloid cells: ASC/NLRP3-independent; negatively regulated by Nod1 and RIPK2; CypD –dependent	93,102
<i>Mycobacterium tuberculosis</i>	NLRP3-dependent	98–101
<i>Toxoplasma Gondii</i>	?	103
<i>Bordetella bronchiseptica</i>	?	104,105
<i>PAMP-mediated necrosis</i>		
Poly(I:C)	RIPK1-dependent	24,34,111
LPS	RIPK1-RIPK3-dependent	18,113
Unmethylated CpG	?	114
<i>Physico-chemical stress induced necrosis</i>		
H ₂ O ₂	Role of RIPK1 is controversial RIPK3-independent PARP1-dependent Dependent on intralysosomal iron	16,22,37,140,144–147
Ischemia-reperfusion	CypD-dependent PARP1-dependent Inhibited by Nec-1 treatment	14,45,46,141–143,147,148
Calcium overload	CypD-dependent	147,148
Glutamate/NMDA	CypD-dependent PARP1-dependent Inhibited by Nec-1 treatment	140,146,151–153
MNNG	CypD-dependent PARP1-dependent	140,157
Photodynamic therapy	?	160–163
Ionizing irradiation	Increased RIPK1 levels	165,166
Etoposide	IAP and cFLIP depletion	50
IAP antagonists	Induces ripoptosome assembly IAP depletion Induces ripoptosome assembly	49

Abbreviations: EDAR, ectodermal dysplasia receptor; H₂O₂, hydrogen peroxide; HSV-1, herpes simplex virus type-1; LPS, lipopolysaccharide; LT β , lymphotoxin- β ; MCMV, murine cytomegalovirus; MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; NMDA, *N*-methyl-D-aspartate; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-like weak inducer of apoptosis; VV, vaccinia virus; WNV, West Nile virus.

Different triggers can initiate necrotic cell death regulated by distinct mechanisms. Note that these triggers can also induce other types of cell death such as apoptosis or pyroptosis depending on the cellular context and conditions

In addition, Tdt-mediated dUTP nick end labeling (TUNEL) and anti-active caspase-3 staining are often used to determine the type of cell death.^{25,39,42} Typically, cells that stain

positive for TUNEL but negative for active caspase-3 are considered as necrotic cells. To investigate whether cells are dying by necroptosis *in vivo*, RIPK1 and RIPK3 expression

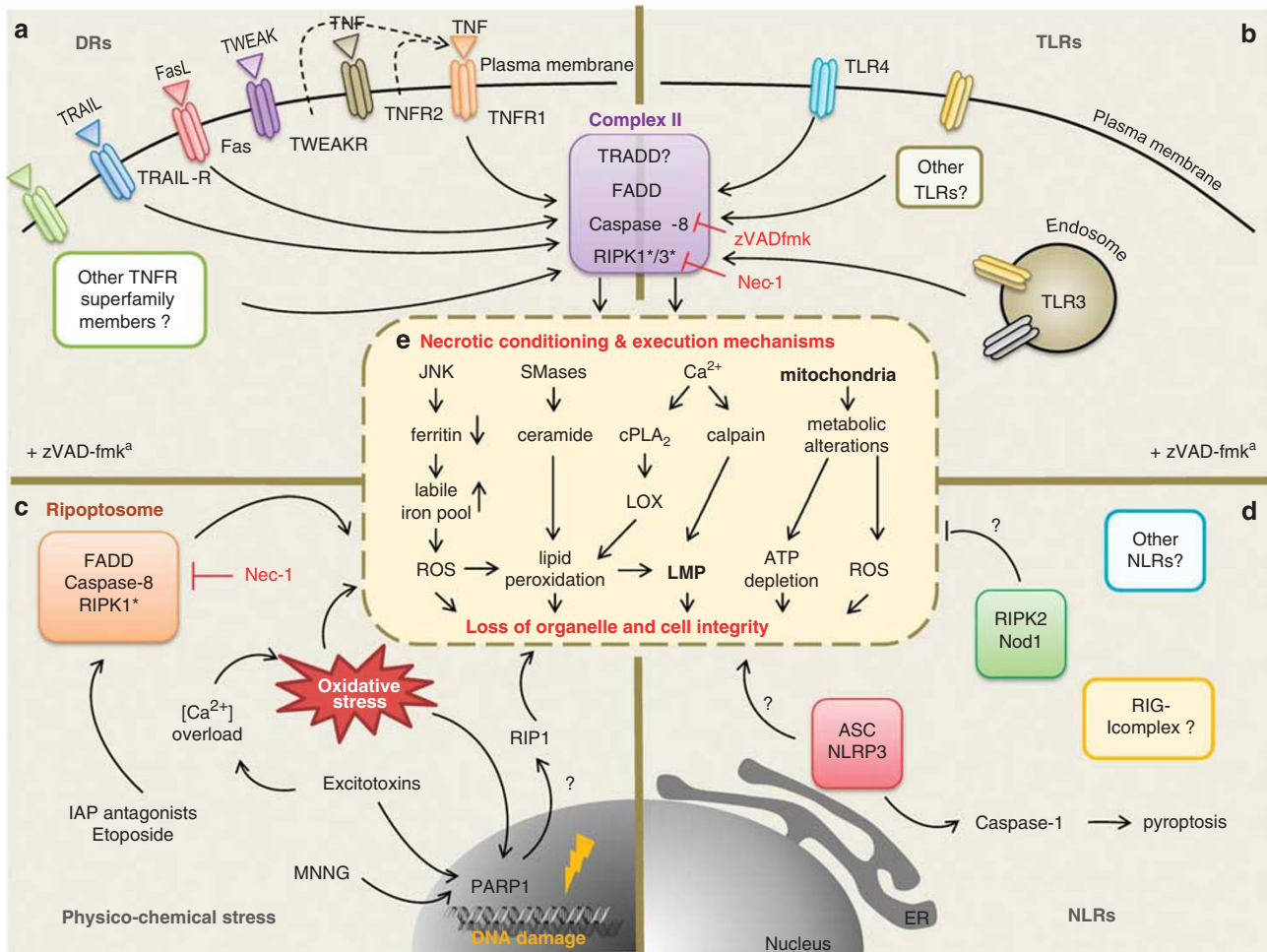


Figure 2 Overview of different necrotic triggers and regulatory mechanisms. Necrosis can be elicited by a wide range of stimuli. (a) Necroptosis induced by DR (TNFR1, TRAIL-R or Fas) stimulation depends on the kinase activity of RIPK1* and RIPK3*. RIPK1 and RIPK3 are present with FADD, caspase-8, and possibly TRADD in TNFR1 complex II, which can induce apoptosis or necroptosis. The latter depends on the functional assembly of a RIPK1*/RIPK3* necrosome complex, which is inhibited by Nec-1. (b) TLR3 and TLR4 triggering induce necroptosis through RIPK1* and RIPK3*-mediated signaling (see text). (c) Physico-chemical stress-mediated necrotic cell death. Oxidative stress-, excitotoxin- or MNNG-induced necrosis require PARP1 activation. IAP depletion by etoposide or IAP antagonist treatment induces the spontaneous RIPK1-mediated assembly of the ripoptosome. (d) NLR stimulation can induce necrosis depending on the cellular context. Microbial infection of cells with *S. flexneri*, *K. pneumoniae* and *N. gonorrhoeae* triggers NLRP3/ASC-dependent necrosis in myeloid cells. In non-myeloid cells, *S. flexneri*-induced necrosis does not require NLRP3 or ASC and is negatively regulated by Nod1 and RIPK2. Whether the executioner mechanism of NLR-mediated necrosis is similar to necroptosis requires further research. (e) Upon initiation of necrosis, several factors become involved in the conditioning and execution of necrotic cell death. Important mediators are: the activities of cytosolic phospholipase A₂ (cPLA₂), lipoxygenase (LOX) and sphingomyelinase (SMase), which contribute to an increased reactive oxygen species (ROS) production and lipid peroxidation that damages cellular membranes, calcium-mediated calpain activation that results in lysosomal membrane permeabilization (LMP), activation of JUN N-terminal kinase (JNK) that triggers the degradation of ferritin thereby increasing the labile iron pool and consequently ROS generation and LMP, and alteration of the mitochondrial energy metabolism, which causes an enhanced ROS production and ATP depletion. zVAD-fmk^a: in certain cellular conditions, the induction of necrosis requires caspase inhibition (see text for more details)

levels are measured in tissues via western blot analysis or immunohistochemistry,^{17,37,39} sometimes combined with a colocalisation study of RIPK1 and RIPK3.⁴² Another indication for necroptosis *in vivo* is the detection of RIPK1 and RIPK3 protein¹⁶ or complex activity⁴² after the isolation of protein complexes from tissue extracts. Moreover, necroptosis is suggested when the amount of necrotic lesions in tissues suspected upon treatment with Nec-1³⁹ or genetic deletion of RIPK3.^{16,17,25} To determine different types of cell death *in vitro*, we refer the reader to detailed reports.^{60–62} In summary, necrotic cell death *in vitro* or *in vivo* cannot be

determined using a single method and preferably should be identified by a combination of different methods.

Ligand/Cytokine-induced Necrosis

The TNF receptor (TNFR) superfamily consists of different members that can be roughly divided in two groups, dependent on the presence or absence of a cytosolic death domain. Necroptosis triggered by death receptor (DR) TNFR1 relies on the activity of two serine-threonine kinases, RIPK1¹³ and RIPK3.^{16–18,36,44} In certain cell types, TNF-induced

necroptosis can occur in the absence of caspase inhibitors,⁶³ whereas necroptosis upon stimulation of the DRs Fas^{13,64,65} and TNF-related apoptosis-inducing ligand receptor 1 and -2 (TRAIL-R1/2 or DR4/5)^{13,66} requires the inhibition of caspases or the absence of the caspase-8-activating adaptor, FADD.¹³ Similar to TNF-induced necroptosis, Fas ligand (FasL)^{13,14,17,34,35} or TRAIL^{13,17,67} initiate necroptosis (Figure 2a). Unlike the requirement for FADD in necroptosis triggered by FasL or TRAIL stimulation,¹³ necroptotic cell death initiated by TNF is negatively regulated by the presence of FADD,^{13,68} probably by favoring an apoptotic pathway and suppressing the necroptotic pathway.³⁷ In contrast, FADD-deficient mouse embryonic fibroblasts (MEF) are resistant to TNF-induced necroptosis in the presence of cycloheximide and caspase inhibitors,⁶⁹ suggesting that mechanistic differences may exist between different cell types. CID has also been observed upon overexpression of the DR ectodermal dysplasia receptor (EDAR),⁷⁰ but EDAR signaling does not involve FADD or TNFR-associated death domain protein (TRADD) recruitment.^{70,71} It remains to be defined if this form of dying has necrotic features or is dependent on the kinase activity of RIPK1 or RIPK3.

Necrosis can be induced by triggering the lymphotoxin- β receptor (LT β R) in the absence of caspase inhibitors and requires the kinase activity of apoptosis signal-regulating kinase 1 (ASK1).^{72,73} Because RIPK1 has been suggested to act upstream of ASK1,⁷⁴ it is conceivable that LT β -induced CID involves RIPK1. Stimulation of the death domain-lacking receptors TNFR2 or TNF-like weak inducer of apoptosis receptor TWEAKR activates the non-canonical NF- κ B pathway, thereby inducing endogenous TNF production, which favors TNFR1-induced apoptosis.^{75–78} Recently, it has been reported that the autocrine TNF signaling during TWEAK stimulation triggers apoptosis by promoting the assembly of a RIPK1–FADD–caspase-8 complex.⁷⁹ In caspase inhibitory conditions, it has been observed that triggering of TNFR2⁸⁰ or TWEAKR induces necrotic cell death.⁸⁰ As TNFR2 and TWEAKR lack a death domain, endogenously produced TNF may stimulate TNFR1-mediated necroptosis, as has been demonstrated recently for TWEAKR-mediated apoptosis.⁷⁹ Finally, triggering of TNFR superfamily member CD40 induces cell cytotoxicity by upregulating the death ligands FasL, TRAIL and TNF.^{76,81} Recently, RIPK1 was shown to be required for CD40 ligand-induced apoptosis.⁸² Whether necrotic cell death can occur upon CD40 triggering is currently not known to the best of our knowledge.

Pathogen-induced Necrosis

Beside cytokines, necrosis can also be induced by multiple viruses such as human immunodeficiency virus type-1 (HIV-1),^{83,84} herpes simplex virus type-1 (HSV-1),⁸⁵ West Nile virus (WNV),⁸⁶ vaccinia virus (VV)¹⁶ and murine cytomegalovirus (MCMV)⁴⁴ (Figure 3). Although RIPK1 deficiency does not protect HIV-1-infected T cells from necrosis,⁸⁷ cell viability upon HSV-1 infection is increased when the infection is preceded by a treatment with the RIPK1 kinase inhibitor Nec-1.⁸⁵ Although it is unclear if WNV-induced necrosis is RIPK1-dependent, the WNV envelope protein has been reported to inhibit the antiviral response by interfering

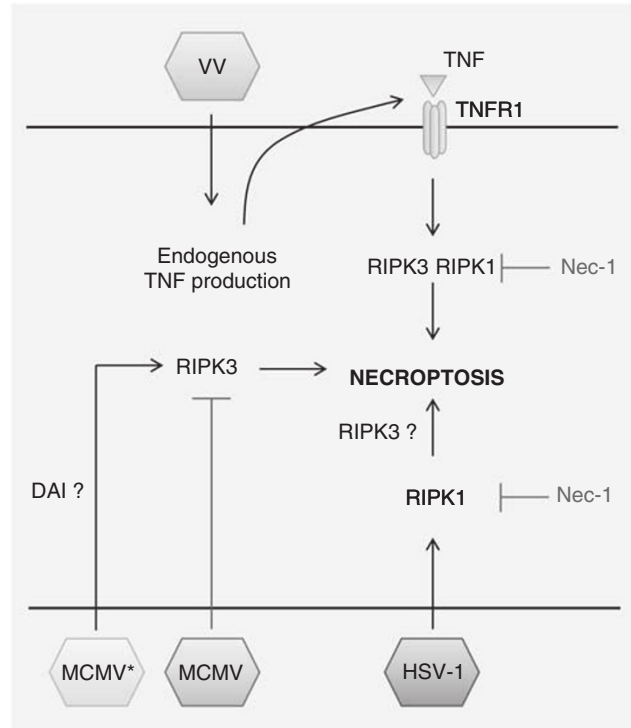


Figure 3 Virus-induced necroptosis: VV infection enhances TNF-induced necroptosis, probably by endogenous TNF production. In contrast, infection with MCMV rescues cells from TNF-induced necroptosis through M45-mediated inhibition of RIPK1–RIPK3 interaction. Infection with a RHIM-mutated M45 or M45-deficient MCMV strain (MCMV*) induces RIPK1-independent, RIPK3-dependent necroptosis. HSV-1 infection induces necroptosis, which can be blocked by Nec-1 treatment

with dsRNA-induced RIPK1 polyubiquitylation and NF- κ B activation.⁸⁸ VV infection sensitizes TNF-resistant cells to TNF-induced cell death^{16,68,89} and this sensitization requires the presence of RIPK1⁶⁸ and RIPK3.¹⁶ Moreover, as in TNF-induced necroptosis, VV infection induces the formation of a pro-necrotic RIPK1–RIPK3 complex, probably due to the endogenous production of TNF¹⁶ (Figure 3). As a consequence, RIPK3-deficient mice do not suffer from VV infection-induced necrosis and liver inflammation, but are unable to control viral replication,¹⁶ suggesting that RIPK1- and RIPK3-dependent necroptosis is important for the inflammatory response against virus infections. In contrast to VV infection, MCMV-infected cells are resistant to TNF-induced necroptotic cell death^{44,90} and this resistance is mediated by the RIP homotypic interaction motif (RHIM) of MCMV's M45 protein, which allows M45 to interact with RIPK1 and RIPK3^{90,91} (Figure 3). Consequently, MCMV strains lacking the M45 protein or containing a RHIM-mutated M45 protein induce necrosis that relies on RIPK3 but not on RIPK1 or endogenous TNF production.^{44,90} As a result, viral replication of RHIM-mutated M45 MCMV strains is restored in RIPK3-deficient mice,⁴⁴ again suggesting that RIPK3-dependent necroptosis is essential for antiviral host defense.

Depending on the cellular context, microbial pathogens can trigger apoptosis, necrosis or caspase-1-dependent cell

death, also called pyroptosis.^{3,4,19} Here, we will focus on necrotic cell death triggered by microbial infections; reviews discussing pathogen-induced apoptosis and pyroptosis can be found elsewhere.⁹² Infection of macrophages with *Shigella flexneri*,⁹³ *Neisseria gonorrhoeae*,⁹⁴ *Porphyromonas gingivalis*⁹⁵ or *Klebsiella pneumoniae*⁹⁶ or infection of the human monocytic cell line NOMO-1 with *Staphylococcus aureus*⁹⁷ induces regulated necrosis that is dependent on apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and NOD-like receptor (NLR) family pyrin domain-containing protein 3 (NLRP3; Figure 2d), requires cathepsin B, and is associated with HMGB1 release. However, see our critical remarks above regarding the specificity of this process. Apparently, this particular type of bacterial infection related to cell death does not rely on the catalytic activity of caspase-1.⁹³ Therefore this form of cell death has been named 'pyronecrosis'.⁹³ However, the Nomenclature Committee on Cell Death 2012 advises researchers not to use the term pyronecrosis because it still lacks a truly functional definition.¹⁹ Interestingly, mice deficient in NLRP3 or ASC exhibit reduced lung necrosis, an attenuated inflammation and strongly reduced HMGB1 serum levels as compared to wild-type mice, but have an increased mortality upon pulmonary infection with *K. pneumoniae*.⁹⁶ This suggests that ASC/NLRP3-dependent necrosis is crucial for inducing an appropriate innate immune response against microbial infection. Recently, it has been demonstrated in human monocytic THP-1 cells that ASC-mediated necrosis is not affected by blocking RIPK1 kinase activity using Nec-1,⁹⁷ suggesting distinct regulatory mechanisms for ASC-dependent necrosis. Remarkably, in the same study it was shown that knockdown of caspase-1, but not the inhibition of the catalytic activity of caspase-1, suppresses *S. aureus*-induced ASC-mediated necrosis in NOMO-1 cells,⁹⁷ suggesting that caspase-1 might fulfill a platform function in ASC-mediated necrosis. Necrotic cell death is also observed upon infection of mouse⁹⁸ or human macrophages^{99–101} with a virulent *Mycobacterium tuberculosis* strain. Wong and Jacobs¹⁰⁰ have shown that *M. tuberculosis*-induced necrosis in THP-1 cells decreases upon targeting NLRP3 using pharmacological inhibition or RNA interference but not upon inhibition of caspase-1 activity, indicating that *M. tuberculosis* induces necrosis and not pyroptosis in THP-1 cells. Although it has been suggested that ASC/NLRP3-mediated necrosis depends on cathepsin B activity,^{93,94,96,97} it seems that NLRP3-mediated necrosis can also occur in conditions of cathepsin B inhibition.^{100,101} In contrast to macrophages, *S. flexneri*-induced necrosis in non-myeloid cells is distinct because this type of necrosis is independent of ASC, NLRP3 or cathepsin B, and is negatively regulated by the NLR nucleotide-binding oligomerization domain-containing protein 1 (Nod1) and RIPK2,¹⁰² suggesting that the regulatory mechanism differs depending on the cell type (Figure 2d). Other pathogens that trigger a necrotic response are the parasite *Toxoplasma Gondii*,¹⁰³ the bacterium *Bordetella bronchiseptica*^{104,105} and the bacterial toxin nigericin from *Streptomyces hygroscopicus*.¹⁰⁶ Whether necrotic cell death induced by these different pathogens is controlled by ASC, NLRP3 or a different mechanism requires further investigation.

PAMP- and DAMP-mediated Necrosis

The cell recognizes pathogens upon binding of the so-called PAMPs to PRRs. In addition, there is increasing evidence showing that these PRRs also sense endogenous danger signals, known as DAMPs that are released by necrotic cells.¹⁰⁷ The PRR group consists of Toll-like receptors (TLRs), NLRs, retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and C-type lectin receptors. Depending on the cellular context, PRR triggering can induce different types of cell death. To date, TLR-mediated necrotic cell death has been described in cells triggered by TLR3, -4 and -9. Recognition of dsRNA or poly(I:C) (synthetic dsRNA analog) by TLR3 and lipopolysaccharide (LPS) by TLR4 triggers the recruitment of an adaptor called Toll-interleukin-1 receptor domain-containing adaptor inducing interferon- β ,^{108,109} which interacts with both RIPK1 and RIPK3 via its RHIM domain,¹¹⁰ suggesting the possible involvement of RIPK1 and RIPK3 in TLR3- and TLR4-induced necrosis. Indeed, poly(I:C)-induced necroptotic cell death in the presence of interferon- β is inhibited in RIPK1-deficient cells¹¹¹ or when RIPK1 kinase activity is blocked.^{24,34} Recently, it has been reported that poly(I:C) stimulation in a steatohepatitis disease model induces necrosis that is correlated with an increase in RIPK3 expression, indicating a possible role for RIPK3 in poly(I:C)-induced regulated necrosis *in vivo*.¹¹² Triggering TLR4 by LPS prevents necrotic cell death of macrophages when either RIPK1 or RIPK3 is absent by RNA interference-mediated knockdown.^{18,113} Together, these data suggest that TLR3 and TLR4 stimulation may induce RIPK1- and RIPK3-dependent necroptosis (Figure 2b).

CID has been observed in progenitor B-cells upon triggering of TLR9 with unmethylated CpG.¹¹⁴ Whether TLR9-induced necrosis, like TLR3- and TLR4-induced necroptosis, involves RIPK1 or RIPK3 remains to be investigated. Viral RNA is not only sensed by TLR3 but also by RLR members RIG-I¹¹⁵ and melanoma differentiated-associated gene 5 (MDA5).¹¹⁶ The antiviral interferon response is induced by a mitochondria-associated RIG-I sensing and signaling complex involving RIPK1, FADD and TRADD^{117,118} is negatively regulated by caspase-8-mediated cleavage of RIPK1.¹¹⁹ To date, necrosis has not been reported in this RIG-I/MDA5 pathway, but this may depend on the cellular context and the presence of RIPK3. In addition to TLR9, exogenous DNA is also detected by the cytosolic sensor DNA-dependent activator of interferon regulatory factor (DAI).¹²⁰ Interestingly, DAI-induced NF- κ B activation is dependent on the RHIM-mediated interaction with RIPK1 and RIPK3 and is inhibited by MCMV's M45 protein.^{121,122} Whether MCMV inhibits RIPK3-dependent necroptosis and the antiviral immune response by acting at the level of DAI is an interesting speculation and subject for future research. Beside the NLRs Nod1 and NLRP3, which are important for *S. flexneri*-induced regulated necrosis in non-myeloid cells and macrophages, respectively,^{93,102} no other NLRs have been linked to necrotic cell death.

Endogenous molecules such as uric acid,¹²³ HMGB1,⁵² RNA,¹²⁴ DNA¹²⁵ and ATP¹²⁶ are released from necrotic cells and are recognized by PRRs. For instance, TLR2 and TLR4 recognize HMGB1,¹²⁷ TLR3 senses RNA,^{124,128} TLR9 is

activated by endogenous genomic¹²⁵ or mitochondrial DNA,¹²⁹ absent in melanoma 2 also detects cytoplasmic DNA,^{130–132} and NLRP3 detects ATP,^{126,133} uric acid^{123,134} and endogenous DNA.¹³⁵ Whereas PAMP detection by PRRs is able to trigger necrosis, recognition of DAMPs by the same PRRs results in a sterile inflammatory response^{51,123,125,126} or pyroptosis.^{136,137}

Physico-Chemical Stress-induced Necrosis

Physico-chemical stressors such as IR, oxidative stress, calcium overload, chemicals, DNA damage and irradiation can trigger necrotic cell death (Figure 2c). The insufficient blood flow to tissues results in a limited oxygen supply or hypoxia. Reoxygenation upon reperfusion has been shown to induce necrotic cell death mediated by oxidative stress.^{138,139} Oxidative stress-induced necrosis caused by exposing cells to hydrogen peroxide (H₂O₂)¹⁴⁰ and necrosis upon hypoxia-reoxygenation¹⁴¹ are dependent on poly(ADP-ribose) polymerase 1 (PARP1). Interestingly, Nec-1 treatment protects against IR-injury *in vivo*.^{14,45,46,142,143} In contrast to the requirement for RIPK1 in TNF-induced necroptosis,¹³ the role of RIPK1 in H₂O₂-induced necrosis is controversial. FADD-deficient MEF cells are apparently hypersensitive to H₂O₂-induced necrosis whereas MEF cells lacking RIPK1 show resistance.¹⁴⁴ In addition, the sensitivity of FADD-deleted MEF cells to H₂O₂ is reversed by RIPK1 deficiency or Nec-1 treatment,³⁷ suggesting a similar mechanism of FADD/caspase-8-mediated control of necrosis sensitivity as observed *in vivo*.^{35–37} However, we and others observed that RIPK1^{16,22,145,146} and RIPK3¹⁶ are dispensable during necrosis triggered by H₂O₂. A possible RIPK1/RIPK3-independent mechanism involves the stability of lysosomes, which are immediately permeabilized upon exposure to H₂O₂ by a mechanism involving free iron.²² Intralysosomal iron chelation, but not cathepsin B inhibition, rescues cells from H₂O₂-induced necrosis.²² Beside lysosomes, mitochondria are also implicated in H₂O₂-induced necrosis. Cells lacking cyclophilin D (CypD), a component of the mitochondrial permeability transition pore, are resistant to necrosis triggered by H₂O₂.^{147,148} *In vivo*, CypD deficiency strongly reduces oxidative stress-mediated necrosis upon IR.^{147,148} In addition to H₂O₂, necrotic cell death initiated by TNF in the presence of caspase inhibitors,¹⁷ calcium overload^{147,148} and *S. flexneri* infection¹⁰² is inhibited by CypD loss, suggesting a common mechanism. Notably, Nec-1 treatment fails to protect CypD-deficient animals from IR-injury,¹⁴⁹ indicating that Nec-1 may act at the level of the mitochondria. In addition to oxidative stress, nitrosative stress (e.g. peroxynitrite) has recently been reported to trigger necrosis and HMGB1 release.¹⁵⁰

Stimulation with glutamate- or *N*-methyl-D-aspartate (NMDA) increases intracellular calcium levels, thereby triggering necrotic cell death, known as excitotoxicity. Similar to oxidative stress-induced necrosis, this form of necrosis also relies on PARP1 and CypD.^{140,151,152} In addition, studies have shown that NMDA- and glutamate-induced necrosis are inhibited by Nec-1 treatment,^{146,153} indicating a role for RIPK1 kinase activity in excitotoxicity.

Exposing cells to the chemical *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) induces DNA damage and results in

necrosis.¹⁵⁴ Like TNF-(although controversial), glutamate- and H₂O₂-induced necrosis,^{140,155,156} MNNG-induced necrosis is dependent on PARP1 activation leading to polyADP-ribosylation and NADH depletion.^{140,157} Whereas RIPK1 kinase activity is essential for TNF-induced necroptosis,¹³ its role in MNNG-induced necrosis is less clear. In contrast to RIPK1-deficient MEFs that are resistant against MNNG-induced necrosis,¹⁵⁸ hippocampal HT-22 cells treated with the RIPK1 kinase inhibitor Nec-1 are not.¹⁵¹ Besides MNNG as a DNA damaging agent, genotoxic stress induced by etoposide treatment has recently been shown to trigger necroptosis as well as apoptosis depending on the cellular content.⁵⁰ Etoposide causes the depletion of cIAPs, which results in the spontaneous assembly of the 'ripiptosome', a cytosolic multiprotein death-inducing complex containing RIPK1-, FADD-, caspase-8-containing complex, independently of DR activation.⁵⁰ Similarly, the ripiptosome is spontaneously formed upon treatment with IAP antagonists, which deplete cIAP levels,⁴⁹ suggesting that IAP levels control the formation of the RIPK1/FADD/caspase-8-containing death-inducing complex. Although the 'spontaneous' formation of the ripiptosome has been demonstrated to occur independently of autocrine TNF,^{49,50} in other cell types a similar complex formation upon genotoxic stress and resulting in IAP depletion, has been shown to operate through an autocrine loop of TNF.⁴⁸ The concept that different forms of cellular stress may propagate the formation of the ripiptosome complex is a very attractive one, indicating that beside the apoptosome also other cytosolic death complexes may sense cellular stress and translate it to apoptosis or necroptosis.¹⁵⁹ Importantly, the assembly of the ripiptosome and ripiptosome-mediated cell death depends on the kinase activity of RIPK1.⁵⁰ Although ripiptosome-induced necroptosis is RIPK3-dependent, RIPK3 could not be detected in the ripiptosome,^{49,50} so whether the ripiptosome initiates necroptosis directly or indirectly requires further research.

Finally, necrotic cell death can also be induced by irradiation. For instance, photodynamic therapy (PDT), which is the treatment of cells with a photosensitizer followed by irradiation, triggers necrosis.^{160,161} Indeed, it has been shown that treatment with the photosensitizer hypericin in combination with UV irradiation induces necrotic cell death in colon adenocarcinoma HT-29 cells¹⁶² and melanosome-containing cells.¹⁶³ Recently, it was demonstrated that the presence or absence of RIPK3 determines the cell death modality by PDT.¹⁶⁴ Moreover, ionizing irradiation (X-ray) combined with hyperthermia has recently been shown to induce necrosis associated with HMGB1 release.¹⁶⁵ Interestingly, necrotic cell death induced in colon carcinoma cells upon hyperthermia and radiotherapy has been associated with increased RIPK1 expression levels.¹⁶⁶

Concluding Remarks and Future Perspectives

Today, increasing evidence demonstrates that regulated necrosis is not anymore an isolated observation of a particular cell line or in certain conditions, but is also present *in vivo* during the development, homeostasis, immune response and pathology. The knowledge on the signal transduction and regulation of necrosis is one of the hot issues in cell death research. Because of the absence of clear and distinctive

markers, it remains difficult to study necrotic cell death *in vivo* and to understand its contribution to development, homeostasis and pathogenesis. The most distinctive biochemical marker is the dependency on RIPK3 kinase activity, which makes it possible to examine necrotic cell death by the use of RIPK3 knockout mice.¹⁶⁷ The absence of any spontaneous phenotypic change suggests that RIPK3 apparently is not involved in embryonic development and homeostasis.¹⁶⁷ However, genetic deletion of RIPK3 rescues caspase-8-deficient mice from embryonic lethality,^{35,36} demonstrating that RIPK3-dependent necroptosis is suppressed by apoptotic regulatory mechanisms, a remarkable example of how cellular processes tightly control each other and that there may be a good physiological reason why the apoptotic pathway blocks the necrotic pathway.

Several studies have demonstrated a role for RIPK3-dependent necroptosis in T-cell homeostasis.^{35,38} Furthermore, RIPK3-dependent necroptotic cell death is crucial to control viral replication^{16,44} whereas ASC/NLRP3-dependent necrosis is important to elicit an antibacterial immune response.⁹⁶ Necrotic cell death has also been shown in glutamate-induced excitotoxicity,^{146,153} which is linked to neurological disorders such as Parkinson's disease, Huntington's disease and Alzheimer's dementia. IR-injury^{147,148} and glutamate-induced neurotoxicity rely on the mitochondrial component CypD,¹⁵² making it an attractive pharmacological target for clinical practice.

The *in vivo* results with Nec-1, which acts by blocking RIPK1 kinase activity,^{14,15} but has also other targets,^{48,168} suggests that RIPK1 targeting could be a promising strategy for future therapy development against stroke, heart failure and neurological disorders because Nec-1 treatment has been shown to reduce IR-injury^{14,45,46,142,143} and to ameliorate the symptoms of Huntington's disease *in vivo*.¹⁶⁹ Interestingly, the protective effect of Nec-1 on IR-injury is abrogated when CypD is absent,¹⁴⁹ suggesting that Nec-1 or RIPK1 kinase activity might act at the level of or upstream of CypD. As discussed above, certain conditions, such as ripoptosome formation in the absence of IAPs^{49,50} also revealed a contribution of RIPK1 kinase activity to apoptosis, suggesting that the *in vivo* efficacy of Nec-1 may rely on its ability to target both types of cell death. Studying IR-injury and neurotoxicity in RIPK3-deficient or conditional RIPK1 knockout mice will be required to identify the precise role of RIPK1 targeting and necroptosis. Moreover, also RIPK3 targeting could be desirable in view of the existence of RIPK1-independent but RIPK3-dependent necrotic cell death processes. Indeed, although RIPK1 kinase activity has been shown to be essential for the initiation of necroptosis,^{15–17} RIPK1-independent RIPK3-dependent necroptosis can occur upon overexpression of RIPK3 in RIPK1-deficient MEF cells,¹⁸ upon infection with MCMV⁴⁴ or during TNF-induced necroptosis in RIPK1/caspase-8 double knockdown L929 cells.³³ This implies that in certain cellular conditions, the need for the kinase activity of RIPK1 to activate RIPK3 and initiate necroptosis could be bypassed. In this respect, efforts to develop specific RIPK3 kinase inhibitors may be very successful.

To conclude, necrosis can be induced by a plethora of triggers and seemingly, depending on the necrotic stimulus, different programs may be initiated eventually leading to a

necrotic cell death phenotype (Figure 2 and Table 1). Although the regulation of the initiation of necrosis by these stimuli differs, it might still be possible that a common execution mechanism of necrosis exists. Intriguingly, the same stimulus can elicit apoptosis or necrosis, depending on the cellular context. This suggests that during evolution the induction of necrotic cell death has been advantageous for the organism. In this respect, necrosis has been shown to be crucial to fight against viral and bacterial infections, and maybe also against cancer. Undoubtedly, elucidating the underlying molecular mechanisms regulating necrosis initiated by these different stimuli will improve therapy development and hopefully lead to the identification of specific necrotic biomarkers. The research activities of Jürg Tschopp have inspired many of us in necrotic cell death research. He has been the first to propose the RIPK1 kinase activity as an important initiator¹³ and to identify components of complex I and II in TNF signaling.¹⁷⁰ His very instructive talks and his structured way of conceptualizing signaling pathways and molecular complexes in functional modules (e.g. inflammasome) that can regulate multiple cellular outcomes had a large impact and boosted the research in the cell death and inflammation field.

Conflict of Interest

The authors declare no conflict of interest.

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