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MULTI-AUTHOR REVIEW

Tumor necrosis factor-mediated cell death: to break or to burst, that's the question

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Abstract In this review, we discuss the signal-transduction pathways of three major cellular responses induced by tumor necrosis factor (TNF): cell survival through NF-κB activation, apoptosis, and necrosis. Recruitment and activation of caspases plays a crucial role in the initiation and execution of TNF-induced apoptosis. However, experimental inhibition of caspases reveals an alternative cell death pathway, namely necrosis, also called necroptosis, suggesting that caspases actively suppress the latter outcome. TNF-induced necrotic cell death crucially depends on the kinase activity of receptor interacting protein serine-threonine kinase 1 (RIP1) and RIP3. It was recently demonstrated that ubiquitination of RIP1 determines whether it will function as a pro-survival or pro-cell death molecule. Deeper insight into the mechanisms that control the molecular switches between cell survival and cell death will help us to understand why TNF can exert so many different biological functions in the etiology and pathogenesis of human diseases.

Keywords Tumor necrosis factor · Apoptosis · Necrosis · Receptor interacting protein kinase 1

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Introduction

Cell death is a fundamental cellular response that plays a crucial role in shaping our body during development and in regulating tissue homeostasis by eliminating unwanted cells. Three major morphologies of cell death have been described: apoptosis (type I), cell death associated with autophagy (type II), and necrosis (type III) [1]. Apoptosis involves a sequence of specific morphological changes in the dying cell: condensation of the cytoplasm and nuclear chromatin, followed by breakage of cells into membrane-bound apoptotic bodies containing a variety of cytoplasmic organelles and nuclear fragments, which are then engulfed by neighboring cells and macrophages [2, 3]. In mammalian cells, the apoptotic response is mediated either by an intrinsic or an extrinsic pathway, depending on the origin of the death stimulus, and it is almost always caspase-dependent. The importance of caspases and other proteases to cell death is discussed by Schrader et al. in this issue. Necrosis is characterized by swelling of the endoplasmic reticulum, mitochondria, and cytoplasm, with subsequent rupture of the plasma membrane and lysis of the cells [3]. Necrosis has long been considered an accidental and uncontrolled form of cell death. However, accumulating evidence shows that necrotic cell death is sometimes as well controlled and programmed as caspase-dependent apoptosis. The aim of this article is to provide a general overview of the current knowledge on signaling events that

result in apoptosis or necrosis. We will focus mainly on TNF-induced apoptosis and necrosis, and on the interplay between apoptotic, necrotic, and inflammatory signaling pathways. Another form of cell death, autophagy, is foremost a survival mechanism that is activated in cells deprived of nutrients or obligate growth factors. If cellular stress persists, cell death either continues by autophagy alone or becomes associated with features of apoptotic or necrotic cell death, depending on the stimulus and cell type. This kind of cell death is discussed extensively by Fimia and Piacentini in a separate review in this issue [4]. For the role of autophagy and other forms of cell death in the control of infections, we refer to Bortoluci and Medzhitov in this issue [5].

TNF-receptor-mediated apoptosis

Members of the TNF-receptor (TNF-R) superfamily are characterized by extracellular cysteine-rich domains that bind their respective ligands, and by intracellular interaction motifs, such as the death domain (DD) and the TRAF (TNF-receptor-associated factor)-binding domain [6]. In general, these receptors can initiate signaling cascades leading to transcription factor activation and/or cell death. In 1990, two different TNF receptors were cloned (for review see [7]): TNF-R1, which is expressed on most cell types, and TNF-R2, which is primarily expressed on haematopoietic cells. In contrast to TNF-R1, TNF-R2 lacks a cytoplasmic death domain. The biological role of TNFR2 is not fully understood, although recent evidence suggests that it can modulate the actions of TNF-R1 on immune and endothelial cells. The TNF-R superfamily comprises the so-called death receptors (DRs), namely TNF-R1, Fas, TRAIL-R1 and -R2, TRAMP, DR6, EDAR, and p75NTR, all of which contain a cytoplasmic death domain. We focus in this review on TNF-receptor-induced apoptosis as a model of DR-induced cell death signaling and discuss the different signaling phases and their control. For extensive reviews on other DR-induced cell-death pathways we refer the reader to recent reviews [8, 9].

Engagement of TNF-R1 leads to activation of NF-κB (nuclear factor kappa B) and/or cell death. NF-κB activation induced by TNF-R1 is thought to depend on the receptor interacting protein serine-threonine kinase 1 (RIP1) [10] (see Fig. 1). However, the absolute requirement for RIP1 in TNF-induced NF-κB activation was recently challenged by the observation that TNF-induced NF-κB is only partially inhibited in RIP1-deficient cells [11]. In most cell lines, RIP1 is essential for TNF-R1-induced apoptosis [10, 12, 13]. Involvement of RIP1 in both signaling pathways is related to its structural features that allow binding of proteins for activation of both pathways. On the one hand, RIP1 is linked to the apoptotic cell death program by virtue of its N-terminal death domain. The RIP1-DD links DD-containing DRs, such as TNF-R1, Fas, TRAIL-R1, and TRAIL-R2 with adaptor proteins that initiate the apoptotic machinery, such as TRADD (TNF-receptor associated via DD) and FADD (Fas associated via DD) [14]. On the other hand, the RIP1 intermediate domain (ID) allows direct association with proteins that are crucial for activation of NF-κB, such as TRAF2, IKKγ/NEMO and TAK1 (TGF-β activated kinase 1) [14]. This RIP1-ID contains a RIP1 homotypic interaction motif (RHIM) that allows interaction with RIP3, a protein suggested to modulate RIP1 activity towards TNF-R1-induced NF-κB activation [15]. However, this could not be confirmed in RIP3$^{-/-}$ cells [16]. The RIP1 kinase activity used to be considered essential only for signaling to necrosis [17], but it was recently shown to be also essential for formation of an alternative caspase-8 activation complex that is not sensitive to inhibition by cFLIP (cellular FLICE-like inhibitory proteins) [13].

TNF-R1-bound TRADD recruits FADD through DD interaction (see Fig. 1). In turn, FADD recruits via its dead effector domain (DED) procaspase-8 or -10, which are activated by proximity. This activation is sufficient to initiate a signaling cascade that induces apoptosis [18, 19]. It was found that induction of apoptosis and activation of NF-κB are initiated from different receptor-bound and intracellular signaling complexes dynamically formed after TNF stimulation [18]. The first complex (complex I) is formed on the cell membrane where TNF-R1 binds adaptor proteins, such as TRAF2, RIP1, and TRADD to activate NF-κB. Subsequently, several adaptor proteins are reshuffled to form a second cytosolic complex (complex II) that may or may not contain TNF-R1. This second complex then attracts FADD and caspase-8 to initiate apoptosis. In some cases, FADD/caspase-8 association depends on high molecular weight complexes containing unubiquitinated RIP1 as scaffold [13]. This caspase-8 activating platform leading to induction of cell death is called the death-inducing signaling complex (DISC). When complex I formation is successful, NF-κB-regulated anti-apoptotic gene products efficiently block initiation of apoptosis from complex II [18, 19]. According to this model, apoptosis is induced after NF-κB activation but there is evidence that a very early attempt to signal for apoptosis precedes activation of NF-κB. The intracellular part of TNF-R1 binds a factor associated with neutral sphingomyelinase (FAN) activation [20]. FAN mediates neutral sphingomyelinase (nSMase)-dependent production of ceramide from the cell membrane; ceramide induces lysosomal membrane permeabilization and apoptosis [21]. nSMase activity is indeed observed before TNF-R1 internalization and NF-κB activation, but it is repressed upon TNF-R1 internalization.

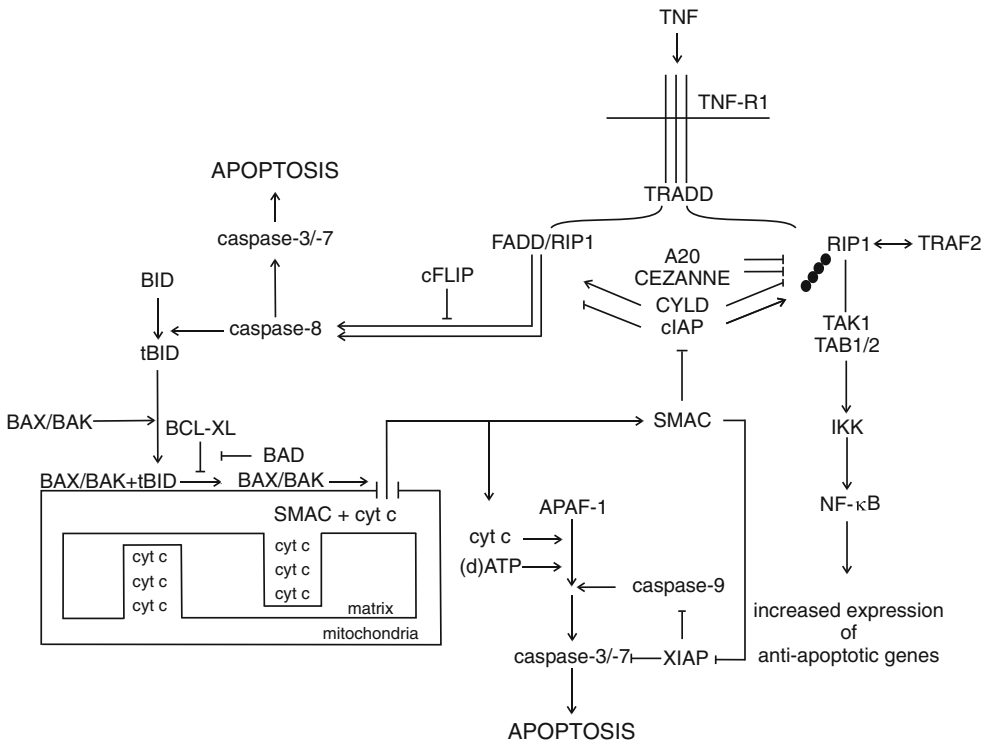

Fig. 1 NF-κB activation and apoptosis induced by TNF-R1 activation. Engagement of TNF-R1 activates a pathway that induces NF-κB activation through ubiquitinated RIP1. The NF-κB gene products counteract apoptosis at different levels and induce inflammatory responses. TRADD/FADD/RIP1 associations lead to activation of caspase-8 and apoptosis through a pathway that either is or is not cFLIP-sensitive. The cFLIP-insensitive pathway requires the kinase activity of RIP1 to form FADD/RIP1/caspase-8 complexes, and RIP1 must not be ubiquitinated. Caspase-8 mediated tBID/BAX translocation to the outer membrane of mitochondria induces MOMP with release of cyt C and SMAC. Cytochrome *c*, together with (d)ATP allows rearrangements of APAF-1, leading to recruitment and activation of caspase-9 and apoptosis. Release of SMAC creates a permissive condition for caspase activation. Some SMAC mimetics inhibit XIAP activity. See text for details

This early FAN-mediated signal, however, is enough to initiate apoptosis in some cells [19]. Recently, it was demonstrated that caspase-8 is rapidly recruited and activated on a FADD/RIP1 complex to induce apoptosis quickly. However, this interaction and the early activation of apoptosis is blunted when RIP1 becomes ubiquitinated and IKKγ (inhibitor of κB kinase γ) binds to RIP1 to induce the activation of NF-κB [22]. This explains why cells expressing a form of RIP1 that cannot be ubiquitinated are extremely sensitive to TNF-induced apoptosis [12]. In several cell lines, TNF-R1 engagement produces sufficient amounts of active caspase-8 to cleave and activate executioner caspase-3 and -7 in order to carry out the apoptotic cell death program. However, when caspase-8 activation is insufficient, the cell death signal has to be amplified. Such amplification is provided by the mitochondria.

Mitochondrial control of TNF-R1-mediated apoptosis

Almost all stress stimuli, including DR engagement [23], that use mitochondria to execute their apoptotic program rely on proteins of the BCL-2 family (see Fig. 1). Details about the mitochondrial control of cell death can be appreciated in a separate review in this issue by Pradelli et al. [24]. All BCL-2 proteins contain between one and four BCL-2 homology domains (BH). The pro-apoptotic BCL-2 family members BAK (BCL-2 antagonist/killer) and BAX (BCL-2-associated X protein) are crucial in regulating a wide range of apoptotic stimuli [25] and become activated by BCL-2 family members that have only the BH3 domain, e.g., BID (BH3 interacting domain death agonist) [26]. Cytosolic BAX has to be activated before it can translocate and oligomerize into the outer mitochondrial membrane. This activation is a complex process involving early conformational changes in the cytosol and is induced by phosphorylation, deubiquitination, and increases or decreases in intracellular pH [27, 28]. Interactions between the BH1 of BAX and the BH3 of BID are needed for exposure of the mitochondrial addressing sequence of BAX [28]. The BID-BH3 domain is shielded, and it is exposed only after processing of BID into a truncated form (tBID) by proteases such as caspase-8 [28]. Translocation of activated BAX to the mitochondria is mediated by mitochondrial receptors. These can be proteins, e.g., components of the

translocase of outer membrane (TOM) [29, 30] or lipids such as cardiolipin [31]. Cardiolipin is an anionic phospholipid located predominantly in the inner membrane of mitochondria and at sites of contact between the mitochondrial inner and outer membranes, the so-called microdomains [32]. Recently it has been found that cardiolipin in these microdomains can form a procaspase-8 activation platform to generate tBID at the mitochondria. Anti-apoptotic BCL-X_L prevents membrane-bound tBID from binding BAX but the "derepressor/sensitiser" BAD can displace tBID from BCL-X_L. This restores the binding of tBID to BAX and induces oligomerization of BAX [33, 34]. How BAX oligomerizes remains unclear, but its insertion in the mitochondrial outer membrane is required to induce pore formation leading to mitochondrial outer membrane permeability (MOMP) [34]. MOMP induces the release of cytochrome *c* and other soluble proteins of the mitochondrial intermembrane space (IMS). The release of mitochondrial factors is sensed by apoptotic peptidase activating factor 1 (APAF-1), which consists of three functional units: an N-terminal caspase-recruitment domain (CARD) responsible for recruiting caspase-9, an NB-ARC region that binds ATP or dATP and is responsible for oligomerization, and a C-terminal region with two WD40 domains that binds cytochrome *c* [35]. In the absence of an apoptotic signal, APAF-1 exists in a compactly folded and autoinhibited form. When cytochrome *c* is released from the mitochondria, it binds to the WD-40 domains, moving the WD-40 repeats away from the CARD and NB-ARC region, causing hydrolysis of (d)ATP to (d)ADP and inducing a conformational change of APAF-1. At this stage, APAF-1 is partially unfolded but still autoinhibited [35]. The APAF-1 bound (d)ADP is exchanged for (d)ATP when sufficient amounts of exogenous (d)ATP is available [36]. When the exchange is successful, the NB-ARC region starts to oligomerize into a wheel-shaped complex, the apoptosome, which consists of seven APAF-1 molecules, exposing their CARD domains. Caspase-9 is produced as an inactive monomer. Like the initiator caspase-8 and -10, its activation requires dimerization but not cleavage [37]. The apoptosome brings together several procaspase-9 molecules through CARD–CARD interactions, inducing proximity activation [38]. Then, caspase-9 proteolytically activates executioner caspase-3 and -7. This is the final step in the apoptotic signaling cascade; these activated proteases cleave many proteins from different cellular compartments, leading to apoptosis and ordered cellular disintegration.

TNF-R1-mediated NF-κB activation and induction of anti-apoptotic genes

TNF-R1 activation leads to rapid recruitment of TRAF2 to the intermediate domain of RIP1. The E3 ubiquitin ligase activity of TRAF2 has been suggested [39] to be responsible for the Lys-63 ubiquitination on a critical Lys in the intermediate domain of RIP1 (Lys 377 in hRIP1 and Lys376 in mRIP1) [40]. Ubiquitination of RIP1 does not require autophosphorylation [39], and the RIP1/TRAF2 interaction is stabilized by TRADD, at least in some cell lines [41]. TNF-induced NF-κB activation is only completely blocked in TRAF2$^{-/-}$/TRAF5$^{-/-}$ double knock-out mice, pointing to functional redundancy of TRAF2 and TRAF5 [42]. Cellular inhibitor of apoptosis proteins cIAP1 and cIAP2 have E3-ubiquitin ligase activity, functionally interact with TRAF2 [43] and RIP1 [13], and induce polyubiquitination of RIP1 upon TNF-stimulation [44, 45]. Consequently, loss of both cIAP1 and cIAP2 greatly attenuates TNF-induced NF-κB activation [44]. Others show that SMAC mimetic-induced degradation of cIAPs does not impair TNF-induced NF-κB activation [13]. The TAK1-associated binding proteins TAB2 and TAB 3 contain a conserved C-terminal zinc-finger domain that binds preferentially to the Lys-63 polyubiquitin chain of RIP1. The recruited TAB 2/TAB 3 facilitates the dimerization or oligomerization of TAK1, thereby promoting the trans-autophosphorylation and activation of TAK1 [46]. The IKK complex, consisting of IKKα, IKKβ and IKKγ, is recruited to RIP1 through binding of IKKγ to the ubiquitin chain of RIP1 [40]. Activated TAK1 directly phosphorylates IKKβ within the activation loop, leading to activation of the IKK complex [47] and NK-κB [48].

Several proteins were shown to intercept TNF-induced NF-κB activation at the level of ubiquitinated RIP1 (see Fig. 1). First, A20, an NF-κB inhibitory protein recruited to the TNF-R1 complex, negatively regulates Lys-63 linked ubiquitination of RIP1. It removes the Lys 63-linked RIP1 ubiquitin chains and promotes Lys 48-linked ubiquitination of RIP1, which leads to its degradation by the 26S proteasome complex [49] and thereby terminates signaling to NF-κB. IKKγ stabilizes the bound polyubiquitinated RIP1 by inhibiting its degradation, most probably by impairing its interaction with A20 [50]. A20 activity is positively regulated through its association with ITCH and the hTLV TAX binding protein (TAX1BP) [51]. Second, CEZANNE (cellular zinc finger anti-NF-kappa B protein) is recruited to the activated TNF-R1 and promotes RIP1 deubiquitination, thereby attenuating NF-κB activation [52]. Third, at internalized TNF-receptosomes, RIP1 is ubiquitinated by endocytic vesicle associated caspase 8/10-associated ring protein 2 (CARP2), inducing RIP1 degradation, which terminates NF-κB activation [53]. Fourth, the cylindromatosis (CYLD) protein [54] efficiently binds RIP1 and blocks TNF-induced ubiquitination of RIP1, thereby counteracting NF-κB activation [55] and promoting apoptosis [13].

When successful, TNF-induced NF-κB activation induces transcription and expression of genes encoding

proinflammatory cytokines such as interleukin-6 (IL-6), anti-apoptotic factors such as XIAP, cIAP1, and cIAP2, the decoy caspase-8 c-FLIP, and the BCL-2 homologue BCL-X_L [56]. In this way, a cell remains inert to apoptotic stimuli [57].

Mechanisms controlling caspase activation

Proximity-induced activation of procaspase-8 at complex II [58] implies that activation can be prevented by interfering with procaspase-8 dimerization itself. This is achieved at different levels by cFLIPs. These proteins are expressed in three isoforms, two short splice variants ($cFLIP_s$ and $cFLIP_R$) and one long variant ($cFLIP_L$). All three isoforms contain two DEDs, which are structurally similar to the N-terminal part of procaspase-8. $cFLIP_L$ also contains catalytically inactive caspase-like domains. $cFLIP_S$ competes with procaspase-8 for binding to FADD through DED interactions and interferes with procaspase-8 oligomerization and activation, thus blocking apoptosis [8]. In high amounts, $cFLIP_L$ interferes with procaspase-8 recruitment at the DISC [8] in a way analogous to the action of $cFLIP_S$, and thereby blocks apoptosis. However, the low amounts of $cFLIP_L$ that remain in the cytosol heterodimerize with procaspase-8 to support activation of the caspase [59]. Heterodimers of activated caspase-8 and $cFLIP_L$ recruit RIP1 and TRAF2 and activate prosurvival NF-κB signaling [60]. Importantly, cFLIP levels are tightly controlled by rapid protein turnover and the balance between survival and stress signals [8]. Many tumor cells overexpress cFLIPs, inducing resistance to cell death ligands FasL and TRAIL on the one hand, while stimulating proliferation and invasiveness on the other hand [61].

Cells express another set of proteins that also effectively block aberrant caspase activation, namely the inhibitor of apoptosis proteins (IAPs). The mammalian genome contains at least eight different IAPs [62]. All of these proteins share a zinc-binding module, which is referred to as the baculoviral IAP repeat (BIR) domain. The IAP proteins cIAP1, cIAP2, and the X-linked IAP (XIAP) contain three BIR domains and a RING-motif with E3 ubiquitin ligase activity. XIAP is specifically involved in inhibition of apoptosis [63]. XIAP binds caspase-3 and -7 through BIR2 and a short 18-amino-acid N-terminal region of BIR2 [64]. Anchoring of XIAP to these caspases blocks substrate entry into their substrate-binding pockets [65]. XIAP also potently inhibits caspase-9 through an unrelated mechanism involving XIAP-BIR3. The distal helix of BIR3 forces caspase-9 into an inactive monomer conformation by interposing between the caspase dimerization interfaces [66]. Inhibition of caspases by XIAP must be relieved when an authentic cell death trigger is imposed. SMAC/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with low p*I*) and OMI/HTRA2 (high-temperature requirement protein A2) are mitochondrial IMS proteins released into the cytosol after MOMP. They contain an IAP binding motif (IBM) that binds XIAP-BIR3 and thereby displaces XIAP from the XIAP-caspase-9 complex [67]. The mature SMAC protein can also relieve XIAP-mediated inhibition of caspase-3 and -7 [68] because dimeric SMAC bound to XIAP-BIR3 also interacts with the region N-terminal of BIR2, causing steric hindrance and thus precluding XIAP from simultaneously binding to caspase-3 and -7 [69]. Hence, small-molecule XIAP inhibitors that can set interacting caspases free are now being tested for enhancement of TRAIL-induced antitumor therapy [70]. Because of its inhibitory effect on both initiator and executioner caspases, XIAP has become a promising therapeutic target, especially in cells in which the mitochondrial pathway cannot be invoked because of overexpressed BCL-2.

Two other IAP members, cIAP1 and cIAP2, interact also with caspase-7 and -9, but their BIR2 and BIR3 domains differ from the corresponding XIAP-BIRs in critical amino acids so that they cannot inhibit the caspases [71]. Although cIAP1 and cIAP2 are considered weak caspase inhibitors [63], they induce degradation of caspases [72]. Interestingly, binding of SMAC (or small molecules that mimic the IBM motif of SMAC) to cIAP1 and cIAP2 induces autoubiquitination and leads to degradation of both cIAPs [45, 73, 74], which potentiates TRAIL- and TNF-induced apoptosis [73]. In several cancer cell lines, cIAPs seem to ubiquitinate RIP1 constitutively, which increases the steady-state levels of NF-κB activation and raises the anti-apoptotic status of the cell. Thus, treatment of these cells with SMAC mimetics not only leads to degradation of cIAPs but also initiates deubiquitination of RIP1. This allows RIP1 to attract and activate caspase-8 to induce apoptosis [13, 45].

TNF-R1-mediated necrosis

It has become clear that many cell types that cannot initiate or propagate the apoptotic signaling cascade do not survive but die by necrosis [75, 76]. This type of cell death is typically not associated with activation of caspases and is characterized by cytoplasmic swelling, irreversible plasma membrane damage, and organelle breakdown [77]. Some pathophysiological processes, such as ischemia–reperfusion (I/R), inflammation, reactive oxygen species (ROS)-induced injury and glutamate excitotoxicity, induce necrotic cell death in vivo [78]. In addition, in some tumor cell lines, e.g., the fibrosarcoma cell line L929, TNF induces necrosis by default [77]. In contrast to apoptotic signaling, our knowledge of necrosis does not enable us to clearly distinguish between the different phases of

signaling during necrotic cell death due to lack of markers specific for the different phases of necrotic signaling. The work of Holler et al. [79] showed that the kinase activity of RIP1 is essential for initiating necrosis. In 2005, Degterev et al. [80] discovered necrostatin-1 (Nec-1) and recently they reported that it specifically blocks the kinase activity of RIP1 [17]. In vitro, Nec-1 inhibits TNF-induced necrosis in L929 cells and FasL-induced necrosis in Jurkat cells deficient in FADD or pretreated with zVAD-fmk [80]. These results confirm a fundamental role for RIP1 kinase activity in DR-induced necrotic signaling. Although necrotic cell death induced by DNA damage also depends on RIP1, there are no reports that this is due to its kinase activity [81]. In vivo, Nec-1 was shown to delay mouse ischemic brain injury [80], inhibit myocardial cell death, and reduce infarct size [82]. The identification of necrostatin not only provides us with a valuable therapeutic tool but also allows us to study the contribution of necrotic cell death to many experimentally induced pathologies, including ischemia reperfusion damage upon organ transplantation, cardiac infarction, stroke, and traumatic brain injury.

An important question is how RIP1 is activated and how it contributes to the propagation of necrotic signaling. Two different models in which RIP1 is essential for activating necrosis, namely DNA damage-mediated poly(ADP-ribose) polymerase-1 (PARP-1) overactivation and I/R [80, 81], display perturbation of cellular metabolism, which might account for triggering RIP1 activity. Activated PARP-1 catalyses the synthesis of polymeric poly(ADP-ribose) (PAR) on many target proteins using nicotinamide adenine dinucleotide (NAD) as a substrate, resulting in total deficit of NAD when PARP-1 is overactivated. This slows down or stops glycolysis, because NAD is an essential cofactor for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In response to this, cells activate other pathways to produce NAD, but this is associated with excessive ATP consumption [83]. The same accounts for the model of I/R, because this is also accompanied by overactivation of PARP-1 [84]. Moreover, metabolic perturbation in this model is even more clear because cells deprived of glucose during ischemia shift to glycogenolysis [85]. One hypothesis we find appealing is that these metabolic changes lead to activation of RIP1 and subsequent necrotic cell death. How RIP1 would sense metabolic changes is unknown, but the simplest explanation could be that RIP1 is activated by stressors like decreasing concentrations of NAD and ATP upon PARP-1 overactivation, or by lower intracellular pH due to lactate production in anaerobic conditions during ischemia. Alternatively, it is also conceivable that cellular stress leads to activation of a mechanism that can upregulate metabolism, for instance via autocrine production of TNF, because TNF can restore metabolism by activating glycolysis [86]. This autocrine TNF, however, will activate RIP1 and induce necrosis. This mechanism has been demonstrated in zVAD-fmk induced cellular stress, which results in TNF-mediated necrosis [87]. In view of the off-target effects of zVAD-fmk on the interaction between adenine nucleotide translocator (ANT) and cyclophylin-D (CyP-D) [88], the resulting energy crisis apparently leads to production of TNF [87].

It was recently shown that RIP3 is indispensable for TNF-induced necrotic cell death and RIP1 propagates necrotic signaling through association with RIP3 to form the so-called 'necrosome' [89–91]. Formation of this protein complex requires the kinase activity of RIP1 and it is stabilized through homotypic RHIM associations between the two proteins. In this complex, both kinases are subjected to reciprocal phosphorylation [92]. Under necrotic conditions, RIP3 also binds to several metabolic enzymes, including the cytosolic glycogen phosphorylase (PYGL) and the cytosolic glutamate-ammonia ligase (GLUL) [90], which regulate glycogenolysis and formation of glutamine, respectively. Furthermore, RIP3 positively regulates the activity of PYGL and GLUL [90], suggesting on the one hand that these enzymes could be direct substrates of RIP3 and on the other hand that the metabolic compound of necrosis signaling comes into play at the level of RIP3 and possibly from the moment of necrosome formation.

Despite recent progress in identifying new effectors, necrotic cell death is not yet confined to a clear pathway. However, in the next section, we will review some mediators that contribute to the necrotic signaling pathway (see Fig. 2).

Mitochondrial events during necrosis: ROS production and mPT regulation

Mitochondria-derived ROS are an absolute requirement in necrotic killing of L929 cells by TNF, and are also responsible for the ultrastructural changes in the mitochondria and endoplasmic reticulum (ER) during cell death [93]. In mitochondria, molecular oxygen is completely reduced by four electrons of the electron transport chain (ETC) to form water. However, at the respiratory chain complexes I and III, electrons leak from the ETC and reduce molecular oxygen partially by only one electron, yielding superoxide and hydrogen peroxide [94]. Calcium stimulates activity of nitric oxide synthase to generate NO inhibiting complex IV, which in turn leads to enhanced ROS production at complex III. It was reported that complex I is the main site for ROS production in TNF-induced necrosis in L929 cells [95, 96]. Importantly, it is not respiration itself that is important for ROS production and necrosis, but the substrate that feeds the ETC at this complex. It was found that glucose in these cells

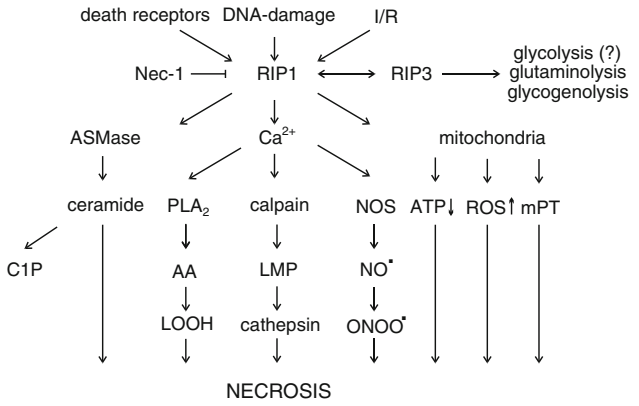

Fig. 2 Necrotic cell death is the result of interplay between several signaling cascades. Kinase activity of RIP1 is needed to induce necrosis in several in vitro and in vivo models. The main players in the propagation of necrosis are RIP3, calcium and mitochondria. RIP3 interacts with RIP1 and binds to several enzymes of the carbohydrate and glutamine metabolism. Calcium controls activation of PLA_2, calpains and NOS, which induce a series of events leading to necrotic cell death. Mitochondria contribute to necrosis by excessive ROS formation, mPT, and ATP depletion due to mitochondrial dysfunction. Several of these mediators are implicated in a self-amplifying loop. See text for details

is converted mainly to lactate, while glutamine is converted mainly to α-ketoglutarate to fuel the Krebs cycle and to maintain electron flow in complex I. Consequently, cells adapted to growing without glutamine and cells pretreated with inhibitors of key enzymes of glutaminolysis for production of α-ketoglutarate showed far less ROS production at complex I, oxidative stress and TNF-triggered necrotic cell death than control cells [97]. The involvement of glutaminolysis in the necrotic cell death process was recently confirmed by Zhang et al. They link this metabolic pathway to RIP3, by showing that GLUD1, the enzyme that initiates glutaminolysis, binds to RIP3 under necrotic conditions, and that RIP3 positively regulates its enzymatic activity [90]. Taken together, we speculate that RIP1 and RIP3 increase carbohydrate and glutamine metabolism of the cell, leading to increased ROS production and eventual necrosis.

In vivo, necrotic cell death is typically observed after I/R of heart and brain. The production of lactic acid through anaerobic glycolysis [98], with a consequent drop in intracellular pH, increases Ca^{2+} influx by activating acid-sensing ion channels in the cell membrane [99]. After reperfusion and replenishment of cells with oxygen, Ca^{2+} enters the re-energized mitochondria, stimulates Krebs cycle, and induces ROS production. This scenario is optimal for the opening of mitochondrial permeability transition pores (mPTP) [100, 101]. The immediate result of opening of mPTP is influx of water and efflux of glutathione and matrix pyridine nucleotides [NAD(P)H] from the mitochondria, causing inhibition of oxidative phosphorylation, and depolarization of the inner membrane [102], which induces hydrolysis of ATP by the mitochondrial ATPase followed by cell necrosis. The molecular structure of the mPTP is controversial [103]. A model portrays it as a pore that forms at sites of contact between the inner and outer mitochondrial membranes and spanning both membranes. It is believed to consist of VDAC located at the outer membrane, ANT located at the inner membrane, and CyP-D, a peptidyl-prolyl *cis–trans* isomerase located in the matrix [104]. However, knock-out studies prove that Cyp-D is essential for mPT but ANT and VDAC are not [103]. ANT should be considered an important regulator.

Membrane events during necrosis: phospholipases, lipoxygenases, and sphingomyelinases

Lipid hydroperoxidation might lead to disruption of organelle and plasma membranes [105], which are key features of necrosis. Lipid (hydro) peroxidation (LOOH) is achieved either nonenzymatically from unsaturated fatty acids, enzymatically through lipoxygenase (LOX) activity, or induced by hydroxyl radicals generated by dysfunctional mitochondria [106]. The main substrate of LOXs in mammalian cells is arachidonic acid (AA), either in esterified or free form depending on the type of LOX [106]. Phospholipase A_2 (PLA_2) encompasses a family of esterases that produce AA from arachidonate-containing phospholipids [107]. Several distinct mammalian PLA_2 enzymes have been identified and classified into three major subfamilies: Ca^{2+}-independent PLA_2 ($iPLA_2$), secretory PLA_2 ($sPLA_2$), and cytosolic PLA_2 ($cPLA_2$). The translocation of $cPLA_2$ to the membranes of the nucleus, ER and Golgi apparatus, where it interacts with its substrates, is essential for $cPLA_2$-mediated release of AA from membranes. Ca^{2+} is needed for the translocation of $cPLA_2$ but not for its activity [108] while phosphorylation is essential for both its translocation and activity [109]. Treatment of L929 cells with TNF led to activation of PLA_2, and overexpression of $cPLA_2$ sensitized TNF-resistant L929 variants to TNF-induced necrosis [110]. $cPLA_2$ was also shown to play a major role in TNF-induced necrosis of MCF7 cells [106] and in chemically induced and oxidant-induced renal epithelial cell necrosis [107]. Besides a role for $cPLA_2$ in necrosis, a contribution of $iPLA_2$ has been demonstrated in several caspase-independent cell death signaling pathways leading to nuclear shrinkage [108]. We recently showed that activation of $cPLA_2$/$iPLA_2$ and the LOX pathway contribute to TNFα-induced necrotic death of L929 cells [96]. Furthermore, a role for sPLA2 in TNF/zVAD induced necrosis in this model was suggested [109].

Sphingolipids are a family of membrane lipids that contribute to the regulation of the fluidity and the subdomain structure of the lipid bilayers. Sphingomyelins are

sphingolipids with a polar head group of phosphocholine or phosphoethanolamine and an apolar ceramide group. Ceramide can be synthesized de novo from palmitoyl CoA and serine or obtained by catabolism of sphingomyelins by sphingomyelinases (SMases). Ceramide is considered a lipid second messenger that binds to several target proteins and alters their functions. The several isoforms of SMases are distinguishable by different pH optima and subcellular localization. A neutral SMase (nSMase) is found at the plasma membrane and an acid SMase (aSMase) is localized in the endosomal-lysosomal compartments. A pronounced accumulation of ceramide is observed during TNF-induced caspase-independent cell death of L929 cells and NIH3T3 fibroblasts, as well as in human leukemic Jurkat T cells stimulated with TNF/zVAD-fmk, and in FADD-deficient Jurkat cells [110]. This increase in ceramide is mediated by aSMase activity, and specific pharmacological inhibition or knockdown of aSMAse by RNAi protects from caspase-independent cell death [110]. In addition, L929 clones overexpressing acid ceramidase (AC) [111], the enzyme that degrades ceramide generated by aSMase, as well as aSMAse-deficient fibroblasts [110], were more resistant to TNF/zVAD-fmk than parental cells, and treatment of NB16 neuroblastoma cells with ceramide analogues induced primarily necrotic cell death [112]. Ceramide production and cell death is even enhanced when caspases are inhibited [110, 113]. RIP1 seems indispensable for activating aSMAse, because depletion of RIP1 by RNAi or by radicol and geldanamycin-induced degradation of RIP1 conferred protection against TNF/zVAD-fmk-induced generation of ceramide and caspase-independent death in all types of cells studied [110]. Also, $cPLA_2$ activity seemed to be necessary for ceramide production [113].

Ceramide has many target proteins and elicits many different effects, including production of ROS in the mitochondria, stimulation of NOS and lipid peroxidation, inhibition of catalases, and regulation of NADPH oxidase activity [114]. Ceramide also activates calpains during caspase-independent cell death [115] and contributes to cell death through sustained JNK (Jun N-terminal kinase) activation during the reperfusion of ischemic liver [116]. Ceramide can be converted to ceramide-1-phosphate (C1P) in a single-step enzymatic reaction catalyzed by ceramide kinase. In turn, C1P activates $cPLA_2$ directly or through PKC signaling [117] and controls calcium homeostasis [118]. So C1P formation could be an important amplification loop during necrotic cell death.

Proteases in necrosis: calpains and cathepsins

Calpains are intracellular, non-lysosomal cysteine proteases that are ubiquitously and constitutively expressed in mammalian cells. They are kept inactivated by their physiological inhibitor, calpastatin, and become directly activated by increased cytosolic Ca^{2+} [119]. A moderate increase in cytosolic calcium is sufficient for calpain activation, because binding of calpains to phospholipids and interactions with other proteins decrease the Ca^{2+} requirement for calpain activation. These proteases are involved in different cell-death modalities and at different levels. Calpain cleaves the anti-apoptotic BCL-X_L [120] and BAX, and the truncated form of BAX is a more potent inducer of apoptosis than full-length BAX [121]. These proteases also cleave caspase-7, -8, and -9, but it is controversial whether this proteolysis inhibits or stimulates caspase activity [122, 123].

It has been suggested that calpains are important mediators in taxol-induced caspase-independent apoptosis in A549 non-small-cell lung carcinoma cells [124]. Calpains contribute to ROS-dependent, necrotic cell death by cleavage of the mitochondrial Na^+/Ca^{2+} exchanger, inducing Ca^{2+} overload in the mitochondria and thus leading to sustained ROS production by these organelles [119]. Likewise, glutamate receptor-induced excitotoxicity in neuronal cells is accompanied by calpain-mediated cleavage of the Na^+/Ca^{2+} exchanger of the plasma membrane, leading to increased cytosolic Ca^{2+} and neuronal death [125]. Calpains were shown to fulfil important roles in necrotic cell death in neurons of *C. elegans* [126] and in necrosis of LLC-PK1 cells induced by high concentrations of glucose [127]. Activated calpain translocates to the lysosomal compartment in post-ischemic CA1 neurons of primates [128] and cleaves a form of hsp70-1 (a chaperone protein that controls lysosomal membrane integrity) that is first oxidatively modified by carbonyl groups to induce lysosomal membrane permeabilization (LMP) [129]. Involvement of lysosomes is a good amplification loop for cell death signaling that requires proteolytic activity without involvement of caspases.

Lysosomes are involved in several in vitro and in vivo cell death models, and are engaged by many extrinsic and intrinsic pathways during the induction of cell death [130]. The involvement of lysosomes in a cell death pathway relies on lysosomal membrane permeability (LMP). Many factors induce LMP, including protease activity (caspases, cathepsins, and calpains), lipids, ROS, and BAX-induced pore formation [130]. LMP contributes to cell death in several ways. First, it might contribute to acidification of the cell, an absolute requirement for induction of necrosis in *C. elegans* [131]. Second, it induces ROS directly through massive release of iron so that Fenton-type reactions are accelerated. This reaction involves splitting of hydrogen peroxide into the extremely reactive hydroxyl radical. A sudden increase of free, cytosolic iron is pivotal for TNF-induced necrosis in L929 cells [132].

Alternatively, LMP induces ROS indirectly through activation of PLA_2 [133]. Third, it induces the release of proteases such as cathepsins into the cytosol, and these cathepsins target mitochondria, phospholipases, and the BCL-2 family members BID, BAX, and BAK [134]. Remarkably, the extent of lysosomal leakage is a determinant for cell death modality: a moderate lysosomal rupture induces apoptosis, whereas extensive lysosomal leakage results in necrosis [135]. TNF induces a moderate increase of intracellular $Ca^{2+,}$ which provokes an increase in both lysosome number and size [136]. These oversized lysosomes undergo LMP easily and induce plasma membrane collapse and cell death [137]. Cells depleted of the plasma membrane calcium ATPase 4 (PMCA4), a Ca^{2+}-channel that extrudes Ca^{2+} from cells, have very high intracellular concentrations of Ca^{2+}. This promotes exocytosis of lysosomes and prevents intracellular build-up of oversized lysosomes, thus attenuating cell death [136].

Conclusions and perspectives

In this review, we focused on two cell death modalities, apoptosis and necrosis, both of which can be induced by triggering the TNF death receptor. During the last decade, the apoptotic signaling pathways have been extensively characterized at the molecular level. Until recently, it seemed that necrosis is largely unregulated because no signaling molecules had been identified. Over the years, many mediators have been proposed as being required for necrotic cell death, mostly because inhibiting their activities inhibits membrane permeabilization, a hitherto often used read-out to score necrotic cell death. We should, however, remark that the activity of some of these mediators might in fact merely contribute to membrane permeability without contributing to the signaling pathway leading to necrosis. It is therefore also important to search for markers that are specific for the different signaling phases during necrosis. It has become clear that RIP1 and RIP3 have a central role in this cell death process. The recently discovered compound, necrostatin, which specifically inhibits the kinase activity of RIP1, allows in-depth analysis of the necrotic signaling pathways both in vitro and in vivo. In addition, it has become clear that ubiquitination of RIP1 is an important factor for signaling towards induction of apoptosis and NF-κB activation. Furthermore, it has become evident that in some cases the kinase activity of RIP1 is required for apoptotic signaling as well. These new findings raise several intriguing questions. What determines whether RIP1 activity will lead to necrosis or apoptosis? What are the necrosis associated substrates of RIP1 and RIP3? Will we be able to treat human diseases involving ischemia reperfusion damage that occurs upon organ transplantation, cardiac infarction, stroke, and traumatic brain injury with necrosis inhibitors such as necrostatins? Do necrotic stimuli such as Toll-like receptor 3 and Toll-like receptor 4 ligands also involve RIP1 and RIP3 kinase activity? The answer to these questions will boost our knowledge of necrotic signaling and how necrotic and apoptotic pathways are interconnected.

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