Review

RIP1, a kinase on the crossroads of a cell's decision to live or die

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Binding of inflammatory cytokines to their receptors, stimulation of pathogen recognition receptors by pathogen-associated molecular patterns, and DNA damage induce specific signalling events. A cell that is exposed to these signals can respond by activation of NF-κB, mitogen-activated protein kinases and interferon regulatory factors, resulting in the upregulation of antiapoptotic proteins and of several cytokines. The consequent survival may or may not be accompanied by an inflammatory response. Alternatively, a cell can also activate death-signalling pathways, resulting in apoptosis or alternative cell death such as necrosis or autophagic cell death. Interplay between survival and death-promoting complexes continues as they compete with each other until one eventually dominates and determines the cell's fate. RIP1 is a crucial adaptor kinase on the crossroad of these stress-induced signalling pathways and a cell's decision to live or die. Following different upstream signals, particular RIP1-containing complexes are formed; these initiate only a limited number of cellular responses. In this review, we describe how RIP1 acts as a key integrator of signalling pathways initiated by stimulation of death receptors, bacterial or viral infection, genotoxic stress and T-cell homeostasis.
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Receptor interacting protein (RIP) kinases constitute a family of seven members, all of which contain a kinase domain (KD) (Figure 1a). They are crucial regulators of cell survival and death[1] (Figure 2). Based on sequence similarities, mode of regulation and substrate specificities of their catalytic domain, RIP kinases are classified as serine/threonine kinases and are closely related to members of the interleukin-1-receptor-associated kinase (IRAK) family. RIP1 and RIP2 (CARDIAK/RICK) also bear a C-terminal domain belonging to the death domain (DD) superfamily, namely, a DD and a caspase recruitment domain (CARD), respectively, allowing recruitment to large protein complexes initiating different signalling pathways. The unique C-terminus of RIP3 bears a RIP homotypic interaction motif (RHIM), which is also present in the intermediate domain (ID) of RIP1. This RHIM domain is sufficient for interaction of RIP1 with RIP3.[1] RIP4 (DIK/PKK) and RIP5 (SgK288) are characterized by the presence of ankyrin repeats in their C-terminal domains.[1] RIP6 (LRRK1) and RIP7 (LRRK2) are RIP members containing a leucine-rich repeat (LRR) motif[1] that may be involved in recognition of pathogen-, damage- or stress-associated molecular patterns (PAMP, DAMP, SAMP). In addition, they harbor Roc/COR domains (Ras of complex proteins/C-terminal of Roc). Binding of GTP to the GTPase-like Roc domain leads to the stimulation of LRRK1 kinase activity.[2] Mutation analysis indicated that the COR domain might be important for transmitting the stimulating signal to the KD.[2] The function of RIP6 and RIP7 is yet unknown; however, mutations in the human LRRK2 gene are associated with both familial and sporadic Parkinson's disease.[3]
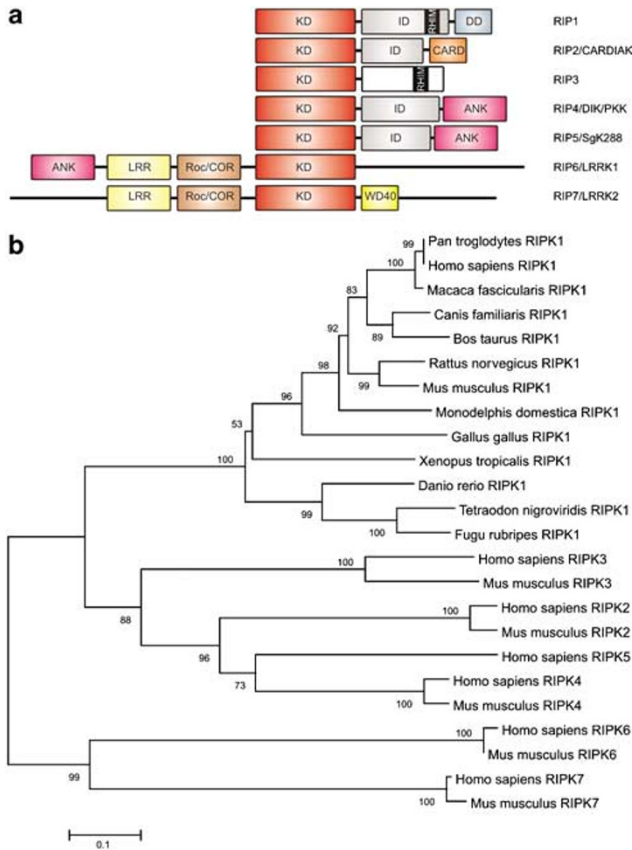
Figure 1 (a) Domain organization of the RIP kinases. All members share a homologous KD. RIP1 and RIP2 have C-terminal DD and CARD motifs, respectively, whereas the C-terminus of RIP3 lacks obvious sequence homology to known proteins. RIP4 and RIP5 have C-terminal ankyrin repeats. RIP6 and RIP7 are characterized by LRRs and a Roc/COR motif. (b) Phylogenetic tree of the KD of RIP kinases. Each RIP kinase (RIP1 to RIP7) evolved as a separate group. The conservation of RIP1 in evolution is depicted in the upper part of the phylogenetic tree. The sequences of the KD were aligned by Clustal W using Jalview version 2.08. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1. A neighbour-joining (NJ) tree was constructed and a bootstrap analysis was performed (cutoff value of 50%). *Pan troglodytes* (chimpanzee), *Macaca fascicularis* (monkey), *Canis familiaris* (dog), *Bos taurus* (cow), *Rattus norvegicus* (rat), *mus musculus* (mouse), *Monodelphis domestica* (opossum), *Gallus gallus* (chicken), *Xenopus tropicalis* (frog), *Danio rerio* (Zebra fish), *Tetraodon nigroviridis* (pufferfish), *Fugu rubripes* (pufferfish), *homo sapiens* (human)

RIP1, the first member of the family, was discovered more than 10 years ago.[4] It is highly conserved in vertebrate evolution (Figure 1b). RIP1 was initially discovered in a yeast two-hybrid screen as an interaction partner of the Fas death receptor[4] through a homotypic DD–DD interaction. Human RIP1 is 68% identical to mouse RIP1, and the greatest similarity is in the DD.[5] Later on, RIP1-DD was shown to be important for binding to other death receptors, such as tumor necrosis factor (TNF)-R1, TRAIL-R1 and TRAIL-R2, and to DD-containing adaptor proteins such as TNF-receptor-associated death domain (TRADD) and FADD.[1,4] Besides, RIP1 also interacts with a plethora of adaptor proteins through its ID, which is also used to recruit other kinases, such as MEKK1, MEKK3 and RIP3.[1] An active KD enables RIP1 to autophosphorylate.[5,6]
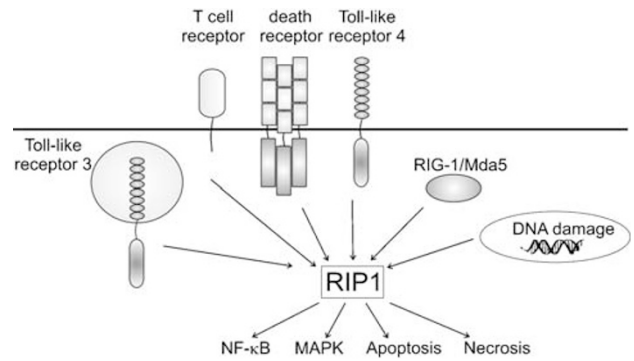

Figure 2 RIP1 is on the crossroads of the decision of cells to live or die upon exposure to several stress signals. Activation of the TcR, death receptors, TLR-3 and -4, and signalling pathways initiated upon detection of intracellular stress (dsRNA or DNA damage) all converge on RIP1. Depending on the cellular context, this will lead to the activation of NF-κB, MAPKs, apoptosis or necrosis. See text for details

RIP1 is constitutively expressed in many tissues.[4] However, TNFα treatment and T-cell activation can also induce RIP1 expression.[4,7] The importance of RIP1 in T-cell homeostasis is confirmed by the fact that RIP1$^{-/-}$ mice appear normal at birth but display extensive apoptosis in lymphoid tissue,[8] indicating that expression of RIP1 is required for T-cell survival. Also adipose tissue in RIP1$^{-/-}$ mice exhibits a high rate of apoptosis. RIP1 deficient mice die at the age of 1–3 days. Because RIP1 deficiency leads to severe phenotypic effects, this kinase must fulfil important roles in controlling the homeostasis of an organism.

In this review, we will systematically describe the role of RIP1 on the crossroads of the decision of cells to live or die upon exposure to several stress signals such as inflammatory cytokines, pathogen infections and genotoxic stress. In addition, we will discuss the important role of RIP1 in T-cell homeostasis. In all cells and under different conditions, RIP1 is apparently crucial for activating NF-κB. Moreover, in certain situations, RIP1 is also involved in activating mitogen-activated protein kinases (MAPKs) such as p38 MAPK, JNK and ERK. Recently, the role of RIP1 has been found to extend to necrotic cell death (Figure 2).

Role of RIP1 in Death Receptor Signalling

TNF-R1-induced apoptosis and activation of NF-κB and MAP kinases. Death receptors belong to the TNF receptor superfamily. When they bind their extracellular ligands, they aggregate and initiate a signalling pathway that results in either inflammatory signalling or cell death (Figure 3). Binding of TNFα to its receptor induces the sequential formation of two signalling complexes.[9] Initially, TNF-R1 recruits RIP1 and TNF receptor associated factor 2 (TRAF2) in the so-called complex I at the plasma membrane; recruitment of TRADD is controversial.[9,10] This leads to rapid activation of NF-κB[6,8] with subsequent induction of the expression of several antiapoptotic proteins, such as c-FLIP, cIAP1 and cIAP2. At this point, TRAF2 and RIP1 also mediate the activation of MAPKs such as p38 MAPK, JNK

and ERK. The activation of ERK depends on the kinase activity of RIP1, which is apparently not essential for the activation of other MAPKs and the activation of NF-κB. No differences in JNK activation were observed when wild-type and RIP1$^{-/-}$ cells were treated with TNFα,[8] restricting this function to TRAF2.[11] In contrast, RIP1 is essential for TNFα signalling to p38 MAPK.[12]
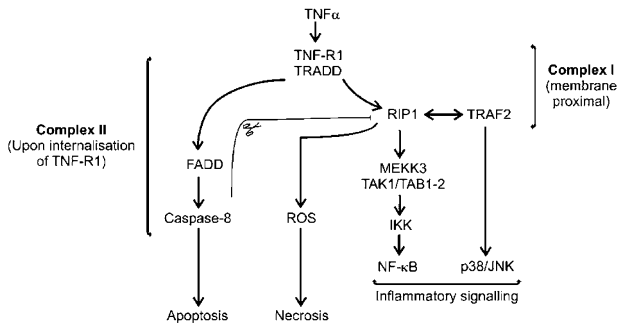

Figure 3 Schematic overview of TNFα-induced signalling pathways leading to the activation of NF-κB, MAPKs, apoptosis and necrosis and the role of RIP1 therein. Upon binding of TNFα to TNF-R1, RIP1 and TRAF2 are recruited to the TNF-R1 complex and as part of complex I, they are crucial adaptors in TNFα-induced NF-κB and MAPK activation. The induction of several NF-κB-dependent antiapoptotic genes prevents activation of apoptosis. However, under certain conditions, such as general inhibition of protein synthesis or specific blockade of NF-κB activation, TNFα can stimulate a strong proapoptotic signal. Upon internalization, FADD and caspase-8 are recruited, forming complex II, with consequent activation of the caspase cascade and apoptosis. In the absence of caspase activity, TNFα can also induce necrotic cell death. (See text for details)

Following receptor endocytosis, TNF-R1, TRADD, TRAF2 and RIP1 undergo extensive post-translational modifications.[9,13] Ubiquitination is one of the modifications of RIP1, TNF-R1 and TRAF2, whereas the nature of the extensive TRADD modifications is currently unknown. These modifications may promote the dissociation of TRAF2 and RIP1 from the cytosolically exposed complex I on endosome vesicles. Subsequently, a complex II is formed in which TRADD recruits FADD and procaspase-8 or -10, invoking conformational activation of caspase-8 and -10 initiating apoptosis.[9,10] Concomitant with the induction of apoptosis, NF-κB-mediated antiapoptotic signalling is blocked through the caspase-8-mediated cleavage of RIP1,[14] representing a crosstalk between signalling initiated by complexes I and II. Complex II only initiates apoptosis when complex I-mediated activation of NF-κB is too low to induce sufficient levels of antiapoptotic proteins such as XIAP and FLIP$_L$. This explains the high sensitivity of RIP1$^{-/-}$ cells to TNFα-induced cell death.[8]

Pathways leading to the activation of NF-κB are regulated by polyubiquitination of several signalling components. Upon TNF-R1 stimulation, RIP1 is autophosphorylated and poly-ubiquitinated in lipid rafts.[15] RIP1 ubiquitination can occur in both Lys48 and Lys63 linkages.[16] Lys63-linked polyubiquitination of RIP1 is required for TNFα-induced activation of NF-κB[17] (Figure 4). It has been suggested that TRAF2 mediates this Lys63-linked ubiquitination following TNFα stimulation.[16] However, redundancy at this point cannot be excluded because TRAF2$^{-/-}$ mice still exhibited almost intact TNFα-induced NF-κB activation but severe reduction in JNK activation.[18] A role for TRAF5 seems plausible as it has been
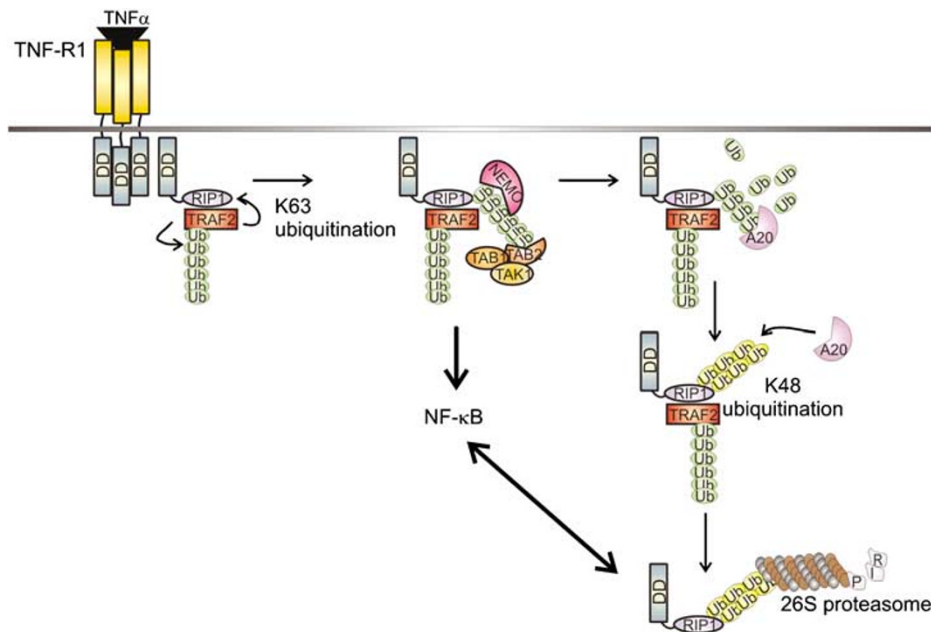
Figure 4 Lys63-linked ubiquitination of RIP1 is required for TNF-induced activation of NF-κB, a process that is negatively regulated by A20. Upon TNF-R1 stimulation, RIP1 is polyubiquitinated in lipid rafts. TRAF2 can mediate Lys63-linked ubiquitination, which may facilitate recruitment of TAB2 and thus activation of NF-κB through the TAK1-TAB1-TAB2 complex. Besides, binding of NEMO to Lys63-linked polyubiquitinated RIP1 is also an essential step in the propagation of signals from the occupied TNF-R1 to the activation of IKK and NF-κB. A20, an NF-κB inhibitory protein recruited to the TNF-R1 complex, negatively regulates this Lys63-linked ubiquitination of RIP1. It sequentially removes the Lys63-linked RIP1 ubiquitin chains, and promotes binding of Lys48-linked ubiquitin chains to RIP1. The latter causes RIP1 degradation by the 26S proteasome complex, terminating signalling to NF-κB. However, binding of NEMO to polyubiquitinated RIP1 stabilizes it by inhibiting its degradation, most probably by impairing the interaction of RIP1 with A20

observed that TNFα-induced NF-κB activation is completely absent in TRAF2^{−/−}/TRAF5^{−/−} mice.[19] Although Lys63-linked ubiquitination can occur on several Lys residues in the ID of RIP1, a critical Lys residue in the ID of RIP1 (Lys377 in hRIP1 and Lys376 in mRIP1) was shown to be the functional Lys63-linked ubiquitination site required for the recruitment of TAK1 and activation of NF-κB.[17] This ubiquitination most probably facilitates recruitment of TAK1-binding protein 2 (TAB2) and thus activation of NF-κB through the TAK1-TAB1-TAB2 complex. In addition, TAK1 can also directly bind and phosphorylate MEKK3, thus cooperating with MEKK3 to activate NF-κB.[20]

Besides, binding of NEMO to Lys63-linked polyubiquitinated RIP1 is also an essential step in the propagation of signals from the occupied TNF-R1 to the activation of IKK and NF-κB.[17,21] The NEMO–RIP1 interaction can be antagonized by ABIN2.[22] A20, an NF-κB inhibitory protein recruited to the TNF-R1 complex, negatively regulates Lys63-linked ubiquitination of RIP1. It removes the Lys63-linked RIP1 ubiquitin chains and promotes binding of Lys48-linked ubiquitin chains to RIP1, which causes RIP1 degradation by the 26S proteasome complex,[16] terminating signalling to NF-κB. However, binding of NEMO to polyubiquitinated RIP1 stabilizes the latter by inhibiting its degradation[21] most probably by impairing RIP1's interaction with A20.[23]

Fas-induced apoptosis and activation of NF-κB and MAP kinases. Whereas TNF primarily regulates gene transcription of pro-inflammatory and immunomodulatory genes, triggering of the Fas receptor mainly culminates in apoptotic cell death, through a pathway involving FADD and caspase-8 (Figure 5). In contrast to TNF-R1 signalling, Fas directly recruits FADD and caspase-8 to the plasma membrane, forming the death-inducing signalling complex (DISC),[24] enabling the activation of a caspase cascade within minutes. There is increasing evidence that Fas also fulfils apoptosis-independent functions.[25] When the proapoptotic Fas signal is impeded by high levels of FLIP$_L$ or FLIP$_S$, overexpression of Bcl-2 or addition of zVAD-fmk,
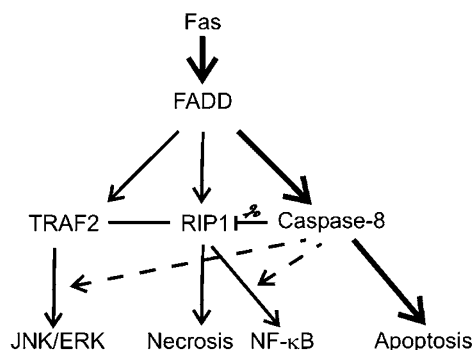

Figure 5 Schematic overview of Fas-induced signalling pathways leading to the activation of NF-κB, MAPKs, apoptosis and necrosis, and the role of RIP1 therein. Although the strongest signalling activity of FasL is induction of apoptosis in susceptible cells, there is accumulating evidence for the ability of this ligand to activate IKK and MAPK pathways. When caspases are inhibited, FasL can induce necrotic cell death. A dashed line represents the presence of zVAD-fmk or high levels of FLIP in these conditions. (See text for details)

then Fas induces the activation of NF-κB.[26] FasL-induced activation of NF-κB is impaired in RIP1^{−/−}, caspase 8[−/−] or FADD^{−/−} Jurkat cells, demonstrating that Fas-induced NF-κB signalling depends on FADD, caspase-8 and RIP1[26] (Figure 5). Whereas active caspase-8 counteracts NF-κB activation by cleaving RIP1,[14] inhibition of caspase-8 by zVAD-fmk prevents RIP1 cleavage, thereby augmenting the activation of NF-κB. Fas-induced NF-κB activation is thus independent of the enzymatic activity of caspase-8, and recruitment of RIP1 via the death effector domain (DED) of caspase-8 may be involved.[27] Fas-mediated upregulation of cFos and cJun, which most likely occurs via the ERK and JNK pathway, respectively, still occurs in RIP1-deficient Jurkat cells, indicating that this kinase is dispensable in these pathways.[26]

TRAIL-R-induced apoptosis and activation of NF-κB and MAP kinases. TRAIL forms a homotrimer, and upon binding to the receptor, the TRAIL receptor/ligand complex is internalized through the endosomal pathway.[28] TRAIL-R1 (DR4) and TRAIL-R2 (DR5) trigger at least two distinct signalling pathways, leading either to cell death or to NF-κB activation. The key signalling activity of TRAIL-R1 and TRAIL-R2 is induction of apoptosis. The composition of the TRAIL DISC appears to be more similar to that of Fas than to that of TNF-R1, as FADD is recruited but TRADD and RIP1 are not.[29]

Upon recruitment to the DISC, caspase-8 gets activated and apoptosis ensues.[30] In contrast to FasL, however, despite the widespread expression of TRAIL-R1 and -R2, TRAIL is not cytotoxic to normal tissues due to the presence of two decoy receptors. TRAIL is also capable of stimulating JNK, p38 MAPK and IKK kinase pathways, albeit less rapidly and much less potently than TNFα.[29] This occurs through the formation of a secondary signalling complex, which lacks TRAIL-R but retains FADD and caspase-8, and recruits RIP1, TRAF2 and/or NEMO.[29] The enzymatic activity of caspase-8 is necessary to induce the dissociation of FADD from TRAIL-R. When caspase-8 activity is blocked by zVAD-fmk, complex I is stabilized and the formation of the secondary complex is impeded. Lin et al.[30] reported that TRAIL-induced IKK activation depends on RIP1, and that both RIP1 and TRAF2 are required for TRAIL-mediated JNK activation. However, a recent study shows that knockdown of RIP1 does not influence TRAIL-induced JNK stimulation, whereas TRAIL-induced p38 MAPK and IKK activation are substantially inhibited[29] (Figure 6).

Death receptor-induced necrosis. In L929 fibrosarcoma cells, TNF alone or Fas ligand in the presence of zVAD-fmk, are both capable of inducing necrotic cell death.[31,32] Similarly, TNF-R1, Fas and TRAIL-R triggering in Jurkat cells in the presence of zVAD-fmk or in Jurkat cells deficient in caspase-8 results in necrotic cell death.[33,34] In contrast, the same stimuli in wild-type Jurkat cells in the absence of caspase-inhibitors lead to apoptosis (Figures 3, 5 and 6). TNF also mediates a caspase-independent necrotic cell death in MEF cells.[35] A crucial adaptor molecule at the crossroads of apoptotic and necrotic signalling is FADD, which contains both a DD and a DED. Whereas the DED of
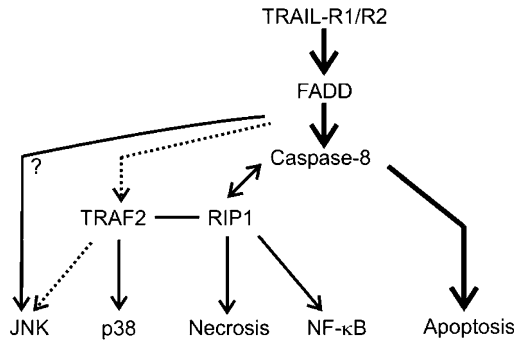
Figure 6 Schematic overview of TRAIL-R-induced signalling pathways leading to the activation of NF-κB, MAPKs, apoptosis and necrosis, and the role of RIP1 therein. Although the strongest signalling activity of TRAIL is induction of apoptosis in susceptible cells, there is accumulating evidence for the ability of this ligand to activate IKK and MAPK pathways. When caspases are inhibited, TRAIL can induce necrotic cell death. A dotted line delineates that TRAIL-induced TRAF-2-mediated activation of JNK can also occur independently of RIP1. (See text for details)

FADD is able to propagate apoptotic cell death, its DD seems to be crucial to initiate necrotic signalling.[36,37] In the case of Fas and TRAIL-R-induced signalling, FADD is recruited to the receptor and can directly initiate downstream signalling cascades among which is necrosis. The role of FADD in TNF-R1-induced necrosis is still unclear.

FADD and RIP1 are able to interact via either homo- or heterotypic interactions.[37] Studies in RIP1-deficient Jurkat cells showed that propagation of TNF-R1, Fas and TRAIL-R-induced caspase-independent death depends on the presence of kinase active RIP1.[33] The involvement of RIP1 in death receptor-induced necrosis was confirmed in studies on heat shock protein 90 (Hsp90), a cytosolic chaperone for many kinases, including RIP1. Inhibition of Hsp90 by geldanamycin or radicicol, a structurally unrelated inhibitor, leads to a 10-fold downregulation of RIP1 levels and inhibits Fas- and TNF-R1-induced necrosis.[33] Likewise, caspase-8 mediated cleavage of RIP1 during TNF-R1, Fas and TRAIL receptor-mediated apoptosis suppresses necrotic and anti-apoptotic pathways.[14] Pretreating cells with zVAD-fmk or overexpressing CrmA blocks caspase-8 activity and sensitizes to necrosis.[32] Notably, RIP1-induced activation of NF-κB and MAPKs is not required for TNF-R1-induced necrosis.[35] Moreover, TNF-R2 is not essential for TNF-induced necrosis but it seems to potentiate the process.[35]

Neither the precise role of the kinase activity of RIP1 nor its downstream targets are known. Previously, it was shown that mitochondria-produced reactive oxygen species (ROS) are important players in the execution of necrotic cell death.[38] Therefore, it is conceivable that RIP1 directly or indirectly targets mitochondria. Recently, it was demonstrated that TNF is able to reduce the interaction between adenine nucleotide translocase (ANT) and cyclophilin D (CypD) in a RIP1-dependent manner. Moreover, in TNF-stimulated cells, RIP1 translocates to the mitochondria, suggesting a possible role of this DD kinase in the displacement of CypD from ANT.[38] The direct consequence of improper functioning of ANT is enhanced ROS production and diminished ATP production. This crucial role of CypD in necrotic cell death has been confirmed in cells from CypD-deficient mice. Besides, RIP1 has also been shown to be essential for TNFα-induced production of ceramide, the latter mediating TNFα-induced caspase-independent cell death.[39] As cPLA$_2$ contributes to TNFα-induced necrosis,[40] it is conceivable that a RIP1-cPLA$_2$-acid sphingomyelinase pathway may lead to necrotic cell death. Indeed, cPLA$_2$-mediated production of arachidonic acid activates acid sphingomyelinase,[41] promoting enhanced levels of ceramide. Because inhibition of ceramide accumulation clearly diminished caspase-independent cell death but not as completely as inhibition of RIP1,[38] ceramide obviously may represent a central factor, but most likely not the only one, transmitting the death signals generated by RIP1 in response to TNFα.

We should remark that in some studies, following addition of zVAD-fmk or specific RNAi-mediated caspase-8 knockdown in L929 cells, RIP1-dependent autophagic cell death instead of necrosis was observed.[42,43] However, the induction of autophagic cell death in L929 cells is much slower than the induction of death receptor-induced necrotic cell death. Thus whether necrosis or autophagy ensues when apoptosis is inhibited will surely depend on cells and circumstances. Due to the lack of specific markers and clearcut definitions of necrotic and autophagic cell death, the two types of death may frequently get entangled.

Role of RIP1 in Signalling by Toll-like Receptors 3 and 4

TLR3- and TLR4-induced activation of NF-κB and MAP kinases. Toll-like receptors (TLRs) are expressed on sentinel cells in the immune system, most notably dendritic cells and macrophages, where they sense a wide range of PAMPs produced by bacteria, viruses, fungi and protozoa. RIP1 is involved in TLR3 and TLR4 signalling pathways. Two main pathways are activated by TLRs, one culminating in the activation of NF-κB and another in the activation of MAPKs. With the exception of TLR3, all TLRs activate NF-κB and MAPKs through a pathway involving the adaptor protein MyD88 and the kinases IRAK-4 and IRAK-1 (Figure 7). Additionally, stimulation of TLR3 and TLR4 also leads to the activation of the transcription factor known as interferon regulatory factor 3 (IRF3) and induction of type I interferons (IFNs), including IFNα and IFNβ, through a MyD88-independent pathway involving the TIR-related adaptor protein inducing IFN (TRIF) (Figure 7). TLR3 and TLR4 recruit TRIF (also called TICAM-1) either directly or indirectly through the adaptor protein TRIF-related adaptor molecule (TRAM, TICAM-2), respectively. Activation of NF-κB and MAPK, induced by TLR3 and TLR4 and mediated by TRIF, depends on RIP1.[44] The RHIM domain in the C-terminus of TRIF can activate NF-κB by binding with RIP1.[44] TRIF-induced activation of NF-κB can also proceed independently of the RHIM domain, through N-terminal binding of TRAF6.[45] RIP1 is dispensable for TRIF-dependent activation of IRF3,[44] which occurs in a TRIF/TRAF3/TBK1/IKKε-dependent manner.[45–47] IRF3 and NF-κB can activate the transcription of IFNβ, an antiviral cytokine.

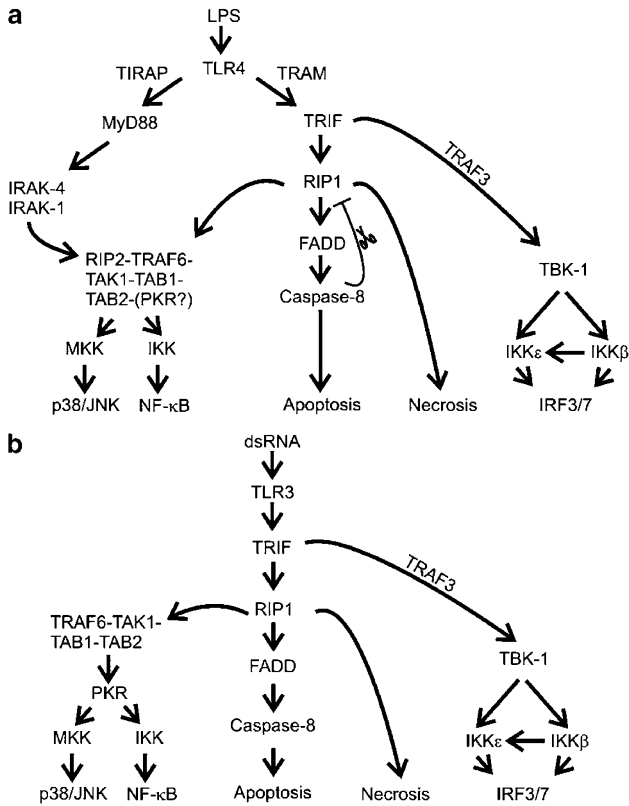Upon TLR3 and TLR4 activation, RIP1 is recruited to the receptor by binding to TRIF via its RHIM motif, and it is then

Figure 7 Schematic overview of signalling pathways induced by TLR4 (**a**) and TLR3 (**b**) and leading to the activation of NF-κB, MAPKs, IRFs, and induction of apoptosis and necrosis, along with the role of RIP1 therein. TLR4 can activate NF-κB and MAPKs either through a pathway involving the adaptor protein MyD88 and the kinases IRAK-4 and IRAK-1, or by a TRIF-dependent pathway. MyD88 is dispensable for TLR3 signalling cascades, which depend only on TRIF. Additionally, stimulation of TLR3 and TLR4 also leads to activation of IRF3 and induction of type I IFNs, through a MyD88-independent pathway involving TRIF. TLR3 recruits TRIF directly, whereas TRAM (TICAM-2) mediates recruitment of TRIF to TLR4. TRIF-dependent apoptosis has been confirmed for TLR4 and TLR3 agonists. When caspase activity is inhibited, triggering of TLR3 and TLR4 can lead to necrosis. (See text for details)

phosphorylated and polyubiquitinated.[44] TLR3 and TLR4 responses are reduced in TRAF6^{−/−} cells and a ligand-dependent recruitment of RIP1, TRAF6 and TAK1 to TLR3 has been reported.[44] RIP1 and TRAF6 recruitment to TLR3 appears to precede TAK1 recruitment, indicating that TRAF6 may modify RIP1 and thereby signal TAK1 recruitment. TAK1 in turn can be ubiquitinated and activated by TRAF6, promoting the binding of TAK1 to TAB2. This TRIF/TRAF6/RIP1/TAK1-TAB1-TAB2 complex can activate IKKβ and eventually NF-κB.[48] The dsRNA-activated protein kinase PKR has also been implicated in polyIC-induced NF-κB activation,[49] raising the possibility that dsRNA-induced NF-κB activation may involve PKR and RIP1. Consistent with this model, Jiang et al.[50] reported that PKR was recruited to a TRAF6/TAK1/TAB2 complex upon polyIC stimulation. Besides activating NF-κB, this TRAF6/TAK1/TAB2/PKR complex could also activate MKK6 and consequently MAPKs.[50] Another RIP member, RIP2, also contributes to immune responses upon TLR3 and TLR4 activation.[51]

TLR3- and TLR4-induced cell death. Viral infection frequently leads to apoptosis of host cells. The MyD88 pathway is most probably not involved in the induction of cell death, as overexpression of MyD88 induces only very low levels of apoptosis. In contrast, overexpression of TRIF potently induces apoptosis in HEK293 T cells.[52] The RHIM domain in the C-terminus of TRIF is required for the induction of apoptosis.[53] TRIF-induced apoptosis was blocked by dominant-negative FADD and caspase-8 and by the caspase inhibitors zVAD-fmk and CrmA, indicating that TRIF induces an apoptosis pathway that is dependent on RIP1/FADD/caspase-8. TRIF-dependent apoptosis has been confirmed for TLR4 and TLR3 agonists.[54] In contrast to its contribution to DD receptor pathways, RIP1 acts upstream of FADD and caspase-8 in TLR3/4-induced apoptotic signalling cascades (Figures 3, 5 and 6).

The interaction between RIP1 and RIP3 through their RHIM domains potentiates TLR-induced cell death by blocking RIP1-induced NF-κB activation.[53,55] When NF-κB activation is impeded, LPS induces apoptosis of macrophages, which can be prevented by ectopically expressed Bcl-x$_L$. When caspase-8 activation is blocked by addition of zIETD-fmk, CrmA or zVAD-fmk, the type of cell death induced by LPS switches from apoptosis to necrosis, and is RIP1 dependent. This caspase-independent cell death of macrophages involves the loss of mitochondrial membrane potential, and Bcl-x$_L$ is no longer protective.[56] Taken together, the following scenario can be conceived. Following TLR4 ligation, TRIF may interact with RIP1, promoting apoptosis by recruiting FADD and activating caspase-8.[56] Cleavage of RIP1 by caspase-8 can generate RIP$_c$, containing the DD, which can continue promoting apoptosis.[14] When caspase activity and NF-κB are blocked, full-length RIP1 is responsible for inducing TLR4-induced necrosis[56] (Figure 7a). More recently, Xu et al.[57] demonstrated autophagy in macrophages following treatment with LPS/zVAD-fmk. TRIF, RIP1 and ROS production, as well as poly-(ADP-ribose) polymerase-1 (PARP) activation, seem to be involved in inducing autophagy, which contributes to caspase-independent macrophage cell death. Previously, we demonstrated that activation of TLR3 in FADD- or caspase-8-deficient Jurkat cells results in a RIP1-dependent necrotic cell death[58] (Figure 7b).

Role of RIP1 in PARP-1-mediated Cell Death

Some pathophysiological processes, such as ischemia-reperfusion, inflammation, ROS-induced injury and glutamate excitotoxicity, are accompanied by PARP-1-mediated cell death.[38] Stimuli that directly or indirectly affect mitochondria, such as H_2O_2 and the DNA-alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), also induce cell death mediated by PARP-1.[38] Activation of PARP-1 catalyzes the hydrolysis of NAD$^+$ into nicotinamide and poly-ADP ribose, causing depletion of NAD$^+$. This results in cellular energy failure and caspase-independent death of different cell types.[38] MNNG-induced cell death depends on RIP1 and TRAF2, which function downstream of PARP-1 and are crucial for sustained JNK activation. JNK in turn affects mitochondrial membrane integrity, with consequent release of proteins of the mitochondrial intermembrane space, and
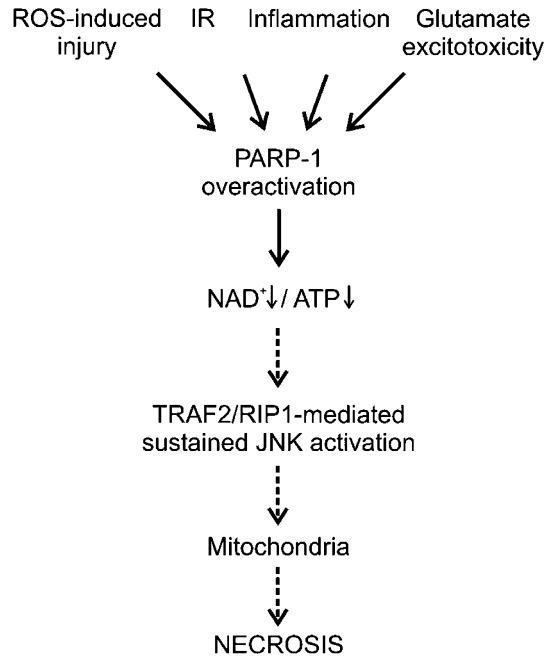
ROS-induced IR Inflammation Glutamate
injury excitotoxicity

↘ ↓ ↙ ↙

PARP-1
overactivation

↓

NAD⁺↓/ ATP↓

⇣

TRAF2/RIP1-mediated
sustained JNK activation

⇣

Mitochondria

⇣

NECROSIS

Figure 8 Schematic overview of PARP-1-mediated necrosis, along with the role of RIP1 therein. ROS-induced injury, ischemia-reperfusion (IR), inflammation and glutamate excitotoxicity can result in the overactivation of PARP-1, which catalyzes the hydrolysis of NAD^+ into nicotinamide and poly-ADP ribose, causing depletion of NAD^+ and resulting in a profound drop of ATP. MNNG and H_2O_2-induced cell death are shown to depend on RIP1 and TRAF2, which function downstream of PARP-1 and are crucial for sustained activation of JNK. This kinase then impairs mitochondrial membrane integrity, causing release of mitochondrial intermembrane space proteins, and consequent necrosis. Detailed molecular mechanisms downstream of NAD^+ and ATP drop are not known today and are therefore represented by dashed arrows. (See text for details)

necrosis[59] (Figure 8). It is not clear how JNK induces mitochondrial membrane depolarization, but it is plausible that it occurs through modifications of Bcl-2 family members,[60] or via caspase-independent JNK-mediated processing of Bid.[61] PARP-mediated cell death induced by H_2O_2 also depends on a TRAF2/RIP1/JNK-mediated signalling cascade.[62] How intracellular molecules such as RIP1 and TRAF2 sense PARP-1 activation remains elusive.

Role of RIP1 in the Innateosome Complex

Viral replication intermediates, including dsRNA, are potent stimuli that trigger host responses when they are recognized and engaged by specific pathogen recognition receptors (PRRs). TLRs are transmembrane receptors that survey extracellular fluids, including endosomal compartments, whereas NACHT-LRRs are intracellular PRRs whose function seems to be to detect intracellular PAMPs or danger signals in general. TLR3, which resides in an intracellular vesicular compartment in dendritic cells, is a known PRR for viral dsRNA.[63] In addition, Balachandran *et al*.[64] suggested the formation of a cytoplasmic signalling complex upon viral infection. DsRNA would be recognized by a cytoplasmic receptor molecule, which may recruit FADD and RIP1 into an 'innateosome' complex to regulate TBK1-mediated activation
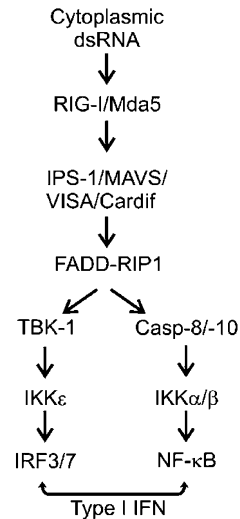
Cytoplasmic
dsRNA

↓

RIG-I/Mda5

↓

IPS-1/MAVS/
VISA/Cardif

↓

FADD-RIP1

↙ ↘

TBK-1 Casp-8/-10

↓ ↓

IKKε IKKα/β

↓ ↓

IRF3/7 NF-κB

⟍___↓___⟋

Type I IFN

Figure 9 Schematic overview of signalling pathways initiated following cytoplasmic dsRNA recognition. The role of RIP1 in the formation of the so-called innateosome complex is depicted. DsRNA can bind intracellularly to RIG-1 or Mda5, which contain a C-terminal helicase domain and two N-terminal CARDs. The latter transmit the downstream activation of IRF3 and NF-κB, through interaction with IPS-1/MAVS/VISA/Cardif. IPS-1 was shown to interact with FADD and RIP1 in a CARD-independent manner. Caspase-8 and caspase-10 are essential components mediating the NF-κB-dependent inflammatory responses, whereas they seem dispensable for activation of IRF3. (See text for details)

of IRF3, a process that is apparently independent of caspase-8, TLR3, PKR, TRIF and TRAF6.[64] The receptor that binds cytoplasmic dsRNA might be the product of retinoic acid inducible gene-1 (RIG-1) or melanoma differentiation-associated gene 5 (Mda5).[65] Both proteins sense viral RNA via a C-terminal helicase domain, and two N-terminal CARDs transmit the downstream activation of IRF3 and NF-κB.[65]

The latter depends on a CARD-containing adaptor protein IFNβ promoter stimulator 1 (IPS-1),[66] also called mitochondrial antiviral signalling protein (MAVS), virus-induced signalling adaptor (VISA) and Cardif, which binds to RIG-1. IPS-1 was also shown to interact with FADD and RIP1 in a CARD-independent manner[66] and to recruit effectors of NF-κB and IRF3 activation, such as IKKα, IKKβ and IKKε. Recently, Takahashi *et al*.[67] demonstrated that caspase-8 and caspase-10 are also essential components mediating the NF-κB-dependent inflammatory responses in antiviral signalling. Besides its N-terminal CARD, IPS-1 also contains a proline-rich region and a C-terminal transmembrane domain that targets it to the outer mitochondrial membrane.[68] These data argue that RIG-1 has a role in 'innateosome' complex formation (Figure 9). However, Newcastle disease virus infection seems to activate IRF3 in a RIG-1-dependent but FADD-independent way, demonstrating that partially shared but distinct signalling cascades exist.[65] This hypothesis is supported by the observation that Mda-5 appears to predominate over RIG-1 in type I IFN responses to polyIC, as these responses are abrogated in Mda-5$^{-/-}$ dendritic cells and macrophages.[69] This can be due either to the fact that responses to polyIC require the concerted activation of RIG-1 and Mda-5, or to the difference between the affinities or specificities of RIG-1 and Mda-5 for polyIC. The observation

that Mda-5 is selectively required for cytokine responses to encephalomyocarditis picornavirus but not to other viruses supports the hypothesis that Mda-5 and RIG-1 have different specificities for dsRNA.[69]

Role of RIP1 upon Genotoxic Stress

RIP1, independent of its kinase activity, also fulfils as part of a PIDD-containing complex an essential role in DNA damage-induced NF-κB activation. Apparently, two sequential PIDD-containing complexes are formed following DNA-damaging treatment. First a survival complex is formed, consisting of NF-κB signalling proteins RIP1 and NEMO, followed by the formation of a proapoptotic complex consisting of RAIDD and caspase-2. Caspase-2 activation is highly accelerated in RIP1$^{-/-}$ cells, indicating that the active NF-κB pathway blocks or delays the caspase-2-mediated death pathway[70] (Figure 10). In contrast to TNFα-mediated activation of NF-κB, the interaction between RIP1 and IKK induced by DNA damage does not require TRAF2.[71] However, the interaction between RIP1 and IKK and consecutive NF-κB activation do not occur in ATM$^{-/-}$ fibroblasts,[71] pointing to a stimulatory role for ATM in DNA damage-induced NF-κB activation (Figure 10). ATM, a member of the PI3K-like family, is a multifunctional protein kinase whose activity is stimulated by DNA double-strand breaks (DSBs). ATM was shown to be essential for NF-κB activation in response to DSBs, but not to proinflammatory stimuli, and this activity is mediated by the IKK complex.[72]

Role of RIP1 in T-cell Development and Homeostasis

The activation of NF-κB is essential for survival and proliferation of T cells. Members of the TNF-R superfamily play critical roles in lymphocyte apoptosis during immune regulation. Studies on RIP1$^{-/-}$ mice and lethally irradiated mice reconstituted with RIP1$^{-/-}$ hematopoietic precursors demonstrated that in the absence of RIP1, B-cell development is normal but thymic cell death is excessive.[8,73] It appears that RIP1 is required for CD4/CD8 double positive (DP) thymocyte survival.[73] RIP1$^{-/-}$ thymocytes, isolated from RIP1$^{-/-}$ mice at

embryonic day 18, have a normal anti-Fas response. However, RIP1$^{-/-}$ DP thymocyte loss may reflect sensitivity of RIP1$^{-/-}$ thymocytes to TNFα-induced cell death, consistent with the absence of DP thymocyte loss in neonatal RIP1$^{-/-}$/TNF$^{-/-}$ mice.[8,73] The decrease in DP thymocytes in the absence of RIP1 can be rescued by TNF-R2 but not TNF-R1

Figure 11 The role of RIP1 during T-cell development and homeostasis. RIP1 is required for thymocyte development and CD4/CD8 DP thymocyte survival through a TNF-R2-mediated mechanism, independently of the activation of NF-κB. Although RIP1 is constitutively expressed in many tissues, T-cell activation induces increased expression of RIP1. RIP1 levels are low early in activation, and both CD4 and CD8 cells are resistant to TNFα. TNF-R2 mediates the activation of NF-κB and survival, but it is not clear if RIP1 contributes to a TNF-R2-initiated NF-κB-independent survival mechanism. It is known that RIP1 is implicated in T-cell proliferation upon activation through binding with p43^{FLIP} and caspase-8/TRAF2, a signalling cascade initiated upon triggering of the TCR. After the immune response peaks, most of the activated antigen-specific T lymphocytes die via AICD. T cells transiently express and secrete FasL and TNFα, which engage the respective receptors on themselves or neighboring lymphocytes. Some studies suggested that Fas–FasL interaction promotes cell death early in AICD, whereas TNF-R2-TNFα functions at a later stage. Stimulation of Fas can either induce apoptosis or necrosis. The fact that caspase-8$^{-/-}$ T cells accumulate in an activated state might suggest that adaptor proteins that bind to caspase-8 are required for RIP1-induced necrotic cell death. Later in activation, RIP1 recruits TRAF2 to the TNF-R2, an event that is rapidly followed by recruitment of FADD and caspase-8, stimulating T-cell death. In contrast to what occurs following stimulation of TNF-R1, RIP1 acts upstream of FADD and cell death occurs in the presence of nuclear NF-κB. SP, single positive; CBM, CARMA-Bcl10-MALT1 complex; TcR, T-cell receptor
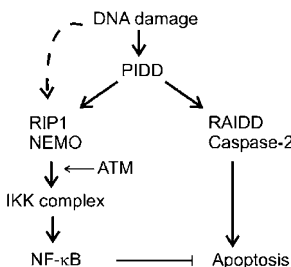
Figure 10 Schematic overview of signalling pathways initiated following genotoxic stress and the crucial role of RIP1 in these processes. Two PIDD-containing complexes are sequentially formed following DNA-damaging treatment. First a survival complex is formed, consisting of the NF-κB signalling proteins RIP1 and NEMO, followed by the formation of a proapoptotic complex consisting of RAIDD and caspase-2. Activation of caspase-2 is highly accelerated in RIP1$^{-/-}$ cells, suggesting that the active NF-κB pathway blocks or delays the caspase-2-mediated death pathway. The interaction between RIP1 and IKK induced by DNA damage does not require TRAF2, but it depends on ATM. (See text for details)
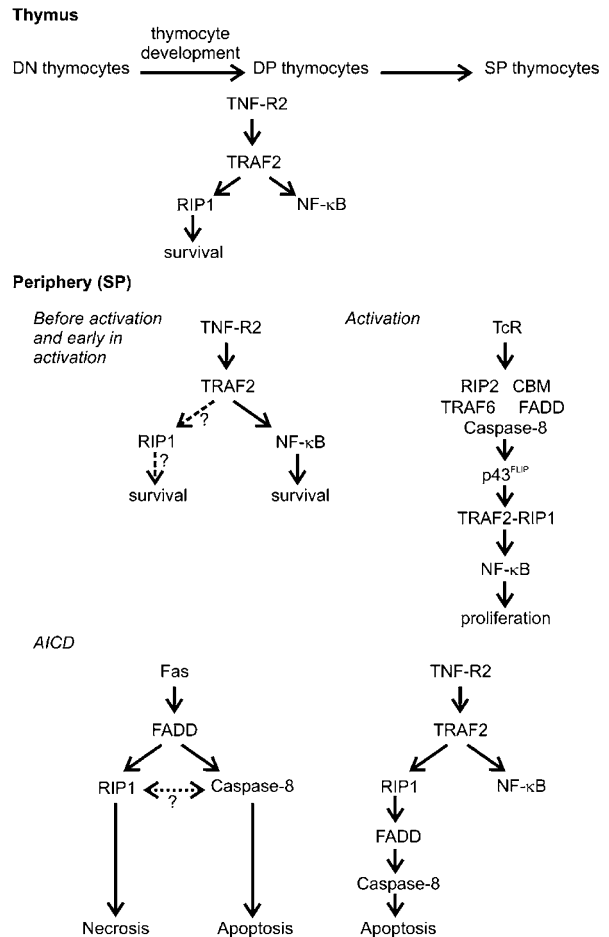
deficiency, demonstrating that the loss of DP thymocytes occurs through a TNF-R2-induced signalling pathway.[73] These results also suggest that RIP1 has a survival role in thymocytes during TNF-R2 triggering (Figure 11).

Although RIP1 is constitutively expressed in many tissues, T-cell activation induces increased expression of RIP1.[4,74] RIP1 is implicated in T-cell proliferation upon activation. Several reports have provided genetic evidence for the role of caspase-8 in the proliferation of immune cells.[75] It was recently shown that enzymatically active but unprocessed caspase-8 accomplishes this by mediating T-cell receptor (TcR)-induced NF-κB activation.[75] Activation upon TcR stimulation triggers recruitment of FADD and caspase-8 to the CARMA-Bcl10-MALT1 complex, which can in turn recruit the IKK complex, with consequent activation of NF-κB.[76] In addition to the CARMA-Bcl10-MALT1 complex,[77] TRAF2, TRAF6 and RIP2 were also shown to be essential for TcR-induced NF-κB activation.[51,78] Recruitment of caspase-8 to FADD is competed by FLIP$_L$, which can also bind RIP1 and TRAF2. Increased expression of c-FLIP$_L$ in T-cell lines augmented IL-2 production. In transgenic (tg) mice, c-FLIP$_L$ expression causes the T cells to hyperproliferate upon activation.[79,80] Dohrman *et al.* showed that this enhanced T-cell proliferation in c-FLIP$_L$ tg CD8$^+$ T cells is due to increased expression of the high-affinity IL2Rα-chain (CD25). The proliferative response of c-FLIP$_L$ tg CD8$^+$ T cells is dependent on caspase activity, as both proliferation and CD25 expression are largely inhibited by zVAD-fmk and QVD-Oph. Additionally, an increase in NF-κB activity was observed 2 days after activation of the c-FLIP$_L$ tg CD8$^+$ T cells.[79] Active caspase-8 cleaves c-FLIP$_L$ to p43^{FLIP}.[81] p43^{FLIP} was shown to interact with TRAF2 and induce NF-κB activation via a p43^{FLIP}/caspase-8/TRAF2 complex.[82] The activation of NF-κB by this ternary complex could be mediated by RIP1, which is also able to bind to p43^{FLIP}.[79] A dominant-negative form of RIP1, containing only the DD, was able to inhibit p43^{FLIP}-induced NF-κB activity, attesting to the view that the functions of c-FLIP in inducing NF-κB and concomitantly enhancing proliferation are indeed working through RIP1.[79]

After the immune response peaks, most of the activated antigen-specific T lymphocytes die by a process called activation-induced cell death (AICD), preventing auto-immunity and ensuring T-cell homeostasis. During activation, T cells transiently express and secrete FasL and TNFα, which can engage the respective receptors on themselves or on neighboring lymphocytes. Some studies suggested that Fas–FasL interaction promotes cell death early in AICD, whereas TNF-R2-TNFα acts at a later stage.[83] In activated T cells, RIP1 recruits TRAF2 to the TNF-R2, and rapidly afterwards FADD and caspase-8 are recruited, stimulating T-cell death. In contrast to what occurs upon stimulation of TNF-R1, RIP1 acts upstream of FADD, and cell death occurs in the presence of nuclear NF-κB[74] (Figure 11). However, when caspase activity is blocked, removal of the activated T cells still proceeds, demonstrating the existence of caspase-independent mechanisms contributing to AICD.[84] It was suggested that Fas-induced RIP1-dependent necrosis may be important for the removal of activated T cells in AICD.[33] These observations may explain why transgenic mice expressing FLIP$_L$ or a viral inhibitor of caspase-1 and -8, such as CrmA,

do not develop lymphadenopathy and auto-immune disease, whereas lpr mice do. In contrast to what could be expected from the results of FLIP$_L$ and CrmA transgenic mice, caspase-8-deficient T cells also accumulate in an activated state. Whether this is the result of unusual lymphoproliferation or failure to undergo AICD is not yet clear.[85] If caspase-8$^{-/-}$ T cells are indeed impaired in AICD, one can suggest that other adaptor proteins that bind to caspase-8 might be required for RIP1-induced necrotic cell death.[85]

Conclusion

Binding of inflammatory cytokines to their receptors, stimulation of either intra- or extracellular PRRs by PAMPs, SAMPs and DNA damage all induce specific signalling events. A cell can respond by activation of NF-κB, MAPKs and IRFs resulting in the upregulation of antiapoptotic proteins and several cytokines. The ensuing survival may or may not be accompanied by an inflammatory response. Additionally, a cell can also activate death-signalling pathways resulting in apoptosis or necrosis. There is constant interplay between death and survival complexes, which compete with each other until one eventually dominates and determines the cell's fate. RIP1 is a crucial adaptor kinase in several of these stress-induced signalling pathways. Full-length RIP1 is important for signalling to NF-κB, MAPKs and necrosis, whereas caspase-8 generates a C-terminal RIP1 cleavage fragment, promoting apoptosis as a positive feedback loop.[14] The kinase activity of RIP1 seems to be essential only for interceding necrotic cell death and ERK activation. This property is dispensable for RIP1-induced activation of NF-κB and other MAPKs.

RIP1 thus integrates several different upstream signals to initiate a limited number of cellular responses. In view of the necessity of kinase active RIP1 for inducing necrosis, abrogating the kinase function of RIP1 would allow interference with necrotic signalling pathways. It is also important that new studies define the composition of the various RIP1-containing complexes, their subcellular localization and the eventual kinase substrates. This information will help us to delineate adaptor and enzymatic functions of RIP1, and to define the molecular actions of RIP1 in the variety of cellular functions it is involved in. This knowledge will permit us to develop tools to specifically interfere with the formation of these complexes or to block crucial mediators downstream of RIP1. In the view of the high incidence of inflammatory disorders and the role of RIP1 in inflammatory signalling pathways, interfering with these RIP1-mediated pathways may have strong therapeutic potential.

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