Cell Death and Differentiation (2015) 22, 225–236

www.nature.com/cdd

Review

RIP kinases: key decision makers in cell death and innate immunity

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Innate immunity represents the first line of defence against invading pathogens. It consists of an initial inflammatory response that recruits white blood cells to the site of infection in an effort to destroy and eliminate the pathogen. Some pathogens replicate within host cells, and cell death by apoptosis is an important effector mechanism to remove the replication niche for such microbes. However, some microbes have evolved evasive strategies to block apoptosis, and in these cases host cells may employ further countermeasures, including an inflammatory form of cell death know as necroptosis. This review aims to highlight the importance of the RIP kinase family in controlling these various defence strategies. RIP1 is initially discussed as a key component of death receptor signalling and in the context of dictating whether a cell triggers a pathway of pro-inflammatory gene expression or cell death by apoptosis. The molecular and functional interplay of RIP1 and RIP3 is described, especially with respect to mediating necroptosis and as key mediators of inflammation. The function of RIP2, with particular emphasis on its role in NOD signalling, is also explored. Special attention is given to emphasizing the physiological and pathophysiological contexts for these various functions of RIP kinases.
Cell Death and Differentiation (2015) **22,** 225–236; doi:10.1038/cdd.2014.126; published online 22 August 2014

Facts

- RIP1 mediates the signalling switch between inflammatory gene expression and apoptosis.
- RIP1 and RIP3 form amyloid filaments to trigger necroptosis.
- RIP1/RIP3-mediated necroptosis is a defence mechanism but can cause inflammatory disease.
- RIP1 and RIP3 are important mediators of pattern-recognition receptor (PRR) signalling.
- RIP2 is a critical mediator of NOD signalling and mucosal immunity.

Open Questions

- How are the kinase activities of RIP1 and RIP3 regulated to control formation of the necrosome complex?
- How is RIP3 activated in those pathways that use RIP3 but not RIP1 to induce necroptosis?
- Apart from virally encoded caspase inhibitors, how is caspase 8 inhibited to promote RIP3-mediated necroptosis and inflammation?
- How does RIP3 regulate the NLRP3 inflammasome?
- Can RIP1/RIP3-mediated necroptosis and RIP2 signalling be targeted to treat inflammatory diseases?

The innate immune system is equipped with PRRs that act as the primary sensing systems for invading pathogens by recognizing molecular structures known as pathogen-associated molecular patterns (PAMPs). PRRs include transmembrane Toll-like receptors (TLRs),[1] cytosolic NOD-like receptors (NLRs),[2] RIG-I-like receptors[3] and DNA sensors.[4] When engaged by relevant PAMPs, PRRs trigger signal transduction cascades resulting in activation of transcription factors such as NFκB and induction of a plethora of pro-inflammatory genes. Tumour necrosis factor (TNF) and interleukin-1β (IL-1β) are two of the most critical pro-inflammatory cytokines, and their receptors can also activate NFκB to promote further expression of inflammatory genes. This facilitates infiltration of leukocytes into the infected tissue resulting in removal of the pathogen. Cell death can also be an important part of host defence by destroying the replication niche for some invading microbes. Indeed cell killing can integrate closely with inflammation by acting as a potent driving force behind the inflammatory response.[5] Although programmed cell death by apoptosis is generally regarded as silent in an inflammatory sense, regulated forms of necrosis result in membrane rupture and release of endogenous danger signals that can act like foreign PAMPS to amplify the inflammatory response.[6] It is vitally important that the pathways underlying these inflammatory responses and different types of cell death are tightly controlled and balanced as an

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Abbreviations: PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; TLR, Toll-like receptor; NOD, nucleotide-binding oligomerization domain-containing protein; NLR, NOD-like receptor; TNF, tumour necrosis factor; IL-1β, interleukin-1β; RIP, receptor interacting protein; DD, death domain; ID, intermediate domain; RHIM, RIP homotypic interaction motif; CARD, C-terminal caspase activation and recruitment domain; TNF-R1, TNF-receptor 1; TRAIL, TNF-related apoptosis-inducing ligand receptor; TRADD, TNF-R1-associated death domain protein; cIAP, cellular inhibitor of apoptosis protein; MLKL, mixed lineage kinase domain-like protein; MDP, muramyl dipeptide
Received 22.6.14; revised 17.7.14; accepted 21.7.14; Edited by H-U Simon; published online 22.8.14

exaggerated inflammatory response forms the basis to many inflammatory diseases, and excessive cell death can lead to depletion of protective immune cells and tissue damage. Given the close interplay of inflammation and cell death, it is not surprising that the signalling pathways controlling both processes are highly interconnected and co-ordinated. These pathways can dictate the magnitude and duration of the inflammatory response while also controlling cell fate and deciding on whether cells survive or die. In the latter case, the form of cell death is a critical decision. Significant progress has been made in delineating the components of signalling pathways that underpin the interplay of inflammation and cell death. This review will focus on the emerging importance of the kinase family of receptor interacting proteins (RIPs) as especially critical players in these signalling networks.

The RIP Kinase Family

The RIP kinase family contains seven members with each containing a homologous kinase domain (KD) that is the signature of the family (Figure 1).[7] In addition to its N-terminal KD, RIP1 contains a C-terminal death domain (DD) and a bridging intermediate domain (ID) that also harbours a RIP homotypic interaction motif (RHIM). RIP2 also contains the N-terminal KD, an ID (lacking a RHIM) and a C-terminal caspase activation and recruitment domain (CARD). Although RIP3 contains the N-terminal KD, it lacks the ID and instead has a unique C-terminal sequence that contains a RHIM. RIP4 and RIP5 have the KD and ID with both also sharing C-terminal ankyrin domains. RIP6 and RIP7 are less related in structure to the other members, and although both contain the homologous KD, they contain a number of additional and diverse domain structures, such as leucine-rich repeat regions. The functions of RIP 4–7 are poorly understood and are well reviewed elsewhere.[7] Briefly, RIP4 was initially identified as a PKCδ-interacting protein[8] and was subsequently shown to activate NFκB.[9] It has a key role in keratinocyte differentiation[10] and cutaneous inflammation.[11] Overexpression of RIP5 drives cell apoptosis,[12] but its physiological role remains to be delineated. Similarly, the functions of RIP6 and RIP7 (also known as leucine-rich repeat kinases 1 and 2) are unknown although both have been associated with the pathogenesis of Parkinson's disease.[13,14]

Although our understanding of the biology of RIP4–7 is still in its infancy, intensive research has clarified important molecular and physiological roles of RIP1–3 in inflammation and cell death, the core focus of the remainder of this review.

RIP1 and TNF Signalling: Inflammation *Versus* Apoptosis

Although RIP1 mediates the activation of NFκB in response to a number of death receptors, including TNF-receptor 1 (TNF-R1),[15,16] TNF-related apoptosis-inducing ligand receptor 1 (TRAIL1)[17] and Fas,[18] the greatest appreciation of the function of RIP1 has emerged from exploring its role in TNF-mediated inflammation and cell death. The stimulation of TNF-R1 with TNF leads to the interaction of TNF-R1-associated death domain protein (TRADD)[19] and RIP1[20] with the TNF-R1 signalling complex (Figure 2). This is followed by the recruitment of a number of E3 ubiquitin ligases to RIP1, including TNF receptor-associated factor 2 (TRAF2) or TRAF5 and the cellular inhibitor of apoptosis proteins (cIAPs) cIAP1 and cIAP2 resulting in the formation of Complex I.[21–24] TRAF2[25,26] and cIAPs[27–30] catalyse the polyubiquitination of RIP1. The ubiquitin-decorated RIP1 is recognized by ubiquitin-binding domain containing proteins in the IκB kinase (IKK)[31] and TAK1 kinase complexes[32–34] thus facilitating TAK-1-mediated phosphorylation and activation of IKKs. The latter subsequently phosphorylate the IκB proteins, which normally sequester NFκB in an inactive state in the cytoplasm, resulting in ubiquitination and proteasomal degradation of IκB and allowing for nuclear translocation of the liberated NFκB.[35–37] NFκB then drives the transcription of many pro-inflammatory genes that will mediate the inflammatory response. NFκB can also induce anti-apoptotic genes such as cellular FLICE inhibitory protein (c-FLIP) and cIAPs that prevent cell death.[38–40] RIP1 can also mediate TNF-induced activation of the mitogen-activated protein kinases (MAPKs) ERK, p38 and JNK and, interestingly, although the kinase activity of RIP1 is dispensable for activating NFκB, p38 and JNK, it is required to stimulate ERK activity.[41]

Although ubiquitinated RIP1 serves to promote downstream activation of NFκB and gene expression that drives inflammation and protects cells from death by apoptosis,[42] NFκB can also induce the deubiquitinating enzymes CYLD

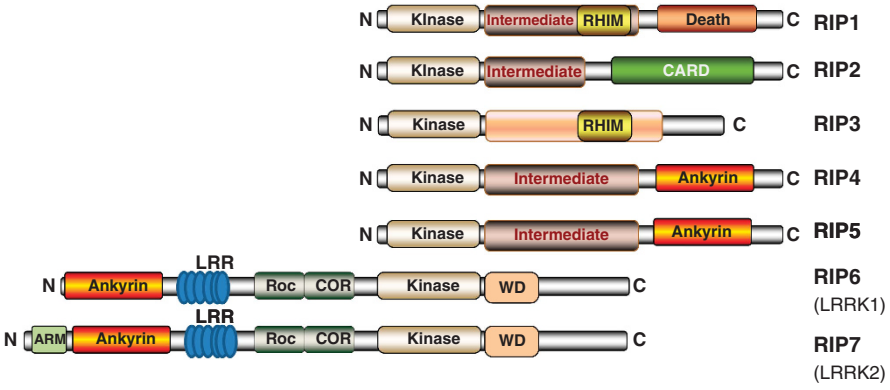

Figure 1 The RIP kinase family. The domain structures of members of the RIP kinase family are indicated. Roc, Ras of complex proteins; COR, C-terminal of Roc; WD, WD40 repeats; and ARM, Armadillo

and A20 that can remove the ubiquitin chains from RIP1 and terminate its ability to activate NFκB.[43–46] In this unmodified form, RIP can leave the TNF-R1 complex to associate with Fas-associated death domain (FADD) and procaspase 8 and form death-inducing signalling complex also known as Complex II.[27,43,47–49] The use of IAP antagonists or loss of cIAP proteins generates a similar ripoptosome complex consisting of RIP1, FADD and caspase 8, with components of this complex being subject to ubiquitination and inactivation by cIAPs.[49–51] Complex II and the ripoptosome can promote processing of procaspase 8 to its active form resulting in triggering of the caspase cascade that culminates in cell death by apoptosis.[45] Recently, we have demonstrated that a member of the Pellino E3 ubiquitin ligase family, Pellino3, targets RIP1 and impairs Complex II formation in the TNF signalling pathway to suppress cell apoptosis.[52] The kinase activity of RIP1 is required for ripoptosome assembly and its downstream triggering of apoptosis.[49,51] Thus the ubiquitination and kinase activity status of RIP1 dictates whether TNF signalling goes down the road of inflammatory gene expression or the terminal path to cell death. Intriguingly, the two pathways counter-regulate each other with NFκB driving anti-apoptotic gene expression, whereas caspase 8 can cleave RIP1 to suppress its ability to activate NFκB,[53,54] with one of the processed forms of RIP1 enhancing the interaction of TRADD and FADD to sensitize cells to the pro-apoptotic effects of TNF.[54]

RIP1 and RIP3 as Drivers of Necroptosis

Although the induction of apoptosis in virally infected cells represents an important defense system to curtail viral replication and dissemination, cytomegaloviruses are examples of microbes that have evolved evasive strategies to this defense system by encoding inhibitors of caspase 8-mediated apoptosis.[55] However, the host has developed further countermeasures to this escape mechanism, including a form of cell death known as necroptosis that is triggered by death

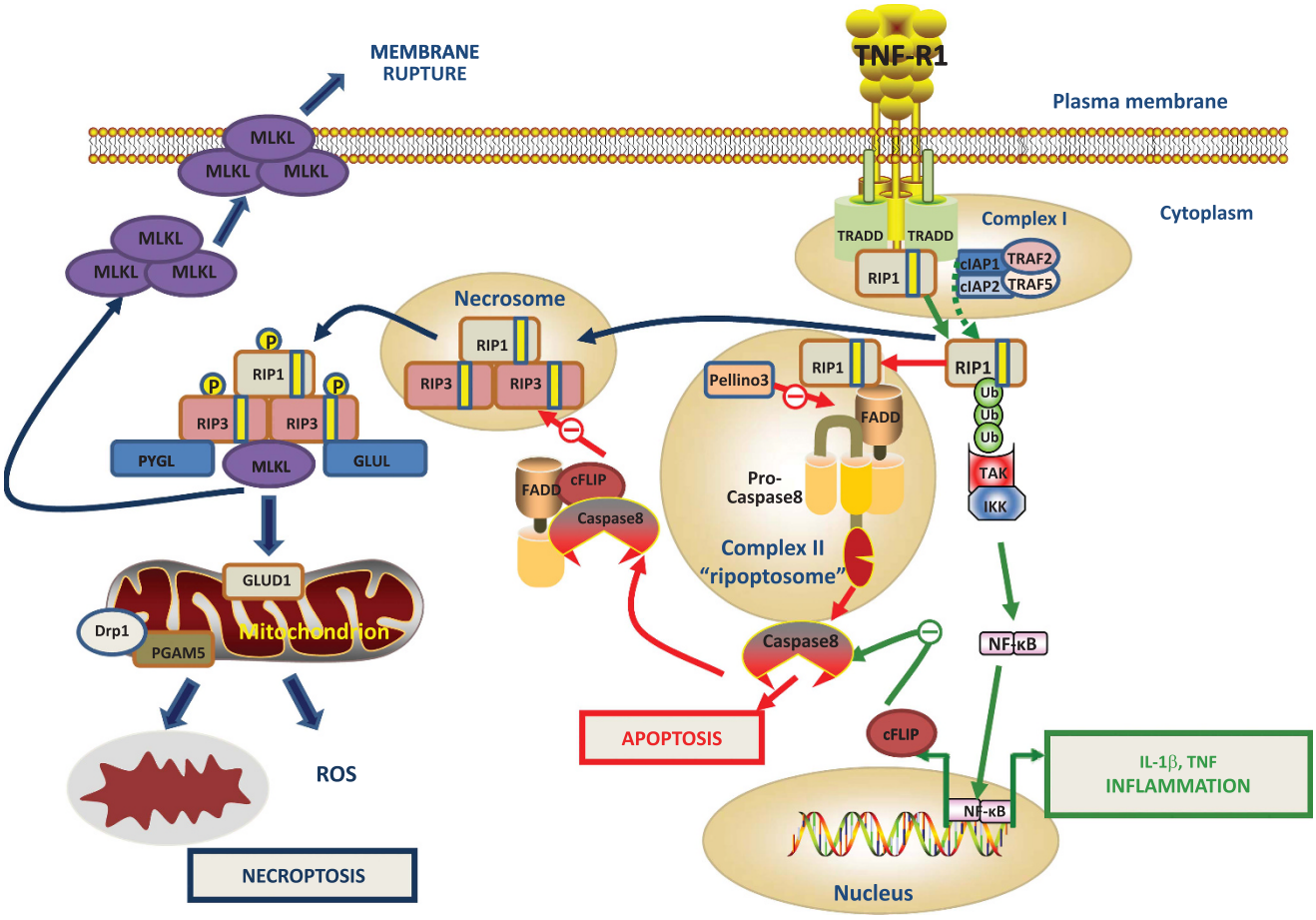

Figure 2 Regulatory roles of RIP1 and RIP3 in TNF signalling. Stimulation of cells with TNF leads to recruitment of TRADD and RIP1 to TNF-R1. RIP1 is ubiquitinated in a complex I containing TRAF2, TRAF5, cIAP1 and cIAP2 leading to TAK1/IKK-mediated activation of NFκB. The latter induces inflammation (by pro-inflammatory gene expression (e.g., IL-1β, TNF)) and anti-apoptotic proteins such as cFLIP. De-ubiquitination of RIP1 results in the formation of Complex II (or 'ripoptosome' in the presence of IAP antagonists) with FADD and procaspase 8. Auto-processing of caspase 8 triggers a downstream caspase cascade and cell death by apoptosis. Pellino3 targets RIP1 to block formation of Complex II and apoptosis. Under the conditions of caspase 8 inhibition, RIP interacts with RIP3 (via their RHIM motifs, indicated in yellow) followed by RIP1/RIP3 phosphorylation (P) and formation of an amyloid filamentous structure known as the necrosome. RIP3 then interacts with MLKL, PYGEL, GLUL and GLUD1 resulting in mitochondrial ROS production. PGAM5 can also be stimulated to interact with the mitochondrial fission factor Drp1 leading to mitochondrial fragmentation and necroptosis, but this may be cell and species dependent. MLKL can also form oligomers that bind to membrane phospholipids resulting in membrane rupture. A FADD/cFLIP/caspase 8 complex can cleave RIP1 and RIP3 to prevent RIP1/RIP3-mediated necroptosis

receptors under conditions of caspase inhibition. This form of cell death was initially observed when caspase 8 inhibition increased the sensitivity of cells to TNF-induced necrosis.[56,57] Furthermore, targeted deletion of the murine *caspase 8* gene resulted in prenatal lethality due to impaired heart muscle development.[58] Other death receptor ligands, such as TRAIL and Fas ligand, were also shown to induce caspase-independent cell death.[59] This necrotic form of cell death that is induced by death receptors is mediated by RIP1 and is dependent on its kinase activity.[59] RIP3 was subsequently shown to be also required for RIP1-induced necrosis,[60–62] with the kinase activity of RIP3 being essential for mediating cell necrosis.[61] Interestingly, RIP3 and its catalytic activity facilitate a switch between TNF-induced apoptosis and necrosis,[60] with embryonic fibroblasts from RIP3-deficient mice being resistant to TNF-induced necrosis[61] and RIP3 kinase dead knock-in mice displaying developmental lethality due to RIP1- and caspase 8-driven apoptosis.[63] RIP3 deficiency also rescues the prenatal lethality of caspase 8 knockout mice with double knockouts lacking both caspase 8 and RIP3 surviving and reaching maturity,[64,65] indicating that RIP3 mediates lethality in the absence of caspase 8. This is consistent with the ability of caspase 8 to cleave RIP3 resulting in loss of the kinase domain of RIP3 and abrogation of its ability to trigger caspase-independent cell death.[66] Caspase 8 has also been shown to repress necrosis by processing CYLD.[67] Interestingly, caspase 8 appears to act in a proteolytically active complex with FADD and cFLIP to block RIP1- and RIP3-mediated necrosis,[65,68] with c-FLIP-[69] and FADD-[70,71] deficient cells being highly sensitive to death by necrosis. This is consistent with the developmental lethality, due to cardiac failure, in FADD-deficient embryos,[72] with RIP1 deficiency rescuing the embryonic lethality associated with FADD deficiency.[71] These studies support a model in which the FADD–caspase 8–c-FLIP complex negatively regulates RIP-kinase-mediated necrosis. This raises the apparent paradox of c-FLIP interacting with caspase 8 to facilitate caspase-mediated processing of RIP kinases while c-FLIP also serves to inhibit caspase 8 in the apoptotic pathway. However, this may relate to auto-processing of caspase 8 being required to trigger apoptosis but not to repress necrosis.[73,74]

Many studies have probed the complex functional interplay between RIP1 and RIP3 in regulating cell necrosis. Under resting conditions, RIP1 is proposed to bind to RIP3 to prevent oligomerization of the latter and so prevent spontaneous RIP3 activation and necrosis.[75] This may, at least partly, underlie the perinatal lethality associated with RIP1 deficiency but would require that any such protective effects of RIP1 are independent of kinase activity as RIP1 kinase dead knockin mice survive to adulthood.[63,76,77] In addition, during development the physiological role of RIP1 in regulating RIP3-driven necroptosis appears to be highly dependent on the stage of development with RIP1 being required for TNF-induced necroptosis at E10.5[78] but inhibiting necroptosis and associated inflammation at later stages of development.[78,79] Although RIP3-driven necroptosis contributes to the perinatal defects associated with RIP1 deficiency, it is not the sole underlying mechanism.[63] This is supported by recent studies demonstrating an important role for RIP1 in protecting against TNF- and caspase 8-driven apoptosis.[76,79]

Under conditions of TNF stimulation, or during virus infection, that trigger RIP1-dependent necrosis, RIP3 promotes necrosis-specific phosphorylation of RIP1, thus forming a pro-necrotic necrosome complex.[62] Phosphorylation-induced activation of the necrosome is dependent on prior de-ubiquitination of RIP1 by CYLD, a step that is proposed to take place in the necrosome itself and not in Complex I.[80] De-ubiquitination of RIP1 is a prerequisite for TNF-induced necrosis as NEMO, a regulatory subunit in the IKK complex, can bind to ubiquitinated RIP1 and prevent its engagement with the necrosome.[81] Structural studies have shown the RHIMs of RIP1 and RIP3 to mediate their interaction[82] and facilitate assembly of heterodimeric filamentous structures, typical of beta-amyloids, and it is these amyloid structures that form the active necrosome complex.[83] The authors of the latter study propose that the RHIM sequences may be hidden in resting cells but that these cryptic motifs are revealed in response to RIP1-induced phosphorylation of RIP3 thus relaxing the auto-inhibited state and allowing for the formation of the RHIM-mediated amyloid filaments. In this process, the initial formation of a RIP1-RIP3 heterodimer is insufficient to trigger necroptosis and instead the RIP1-RIP3 amyloid structure must recruit more free RIP3 to the amyloid scaffold resulting in auto-phosphorylation of RIP3 and recruitment of mixed lineage kinase domain-like protein (MLKL) to trigger downstream necroptosis.[84] The recruitment of MLKL to the necrosome leads to RIP3-mediated phosphorylation of MLKL with the RIP3 inhibitor, necrosulfonamide, blocking necrosis downstream of RIP3 activation[85] and MLKL-deficient mice being resistant to necroptosis.[86,87] Various downstream effector mechanisms have been proposed to mediate necroptosis. Phosphorylation of MLKL promotes its oligomerization and translocation to the plasma membrane where it interacts with phospholipids and compromises membrane integrity ultimately resulting in cell rupture.[88–91] MLKL also promotes the generation of reactive oxygen species (ROS) and late phase activation of JNK.[92] The increased production of ROS, especially by the mitochondria, has been strongly linked with mediating TNF-induced necrosis.[93–95] Indeed cIAP1 and TAK1 has been shown to block TNF-induced necrosis by inhibiting RIP1/RIP3-mediated production of ROS.[96] RIP3 also interacts with and activates a number of metabolic enzymes, including glycogen phosphorylase (PYGEL), glutamate–ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1) that partly contribute to TNF-induced production of ROS and necroptosis[60,95] (Figure 2). In addition, the RIP1-RIP3 necrosome can interact with the mitochondrial protein phosphatase PGAM5 to drive downstream necrosis.[97] This study demonstrated that PGAM5 recruits the mitochondrial fission factor Drp1 to promote its GTPase activity by dephosphorylating serine residue 637 of Drp1. The activation of Drp1 triggers mitochondrial fragmentation, an essential driver of necrosis execution. However, a more recent study has questioned the role of PGAM5 in mediating TNF-induced necrosis, at least in murine fibroblasts.[87] Knockdown of PGAM5 expression in these cells failed to affect susceptibility to TNF-driven necroptosis, suggesting alternative or additional mediatory pathways. Such discrepancies may reflect varying effector mechanisms in different cells and species.[98]

RIP Kinases and TLRs

Although RIP1 and RIP3 have key roles in controlling the outcome of death receptor signalling pathways, they also have important roles in PRR pathways especially TLR3 and TLR4 (Figure 3). This is due to these pathways employing an adaptor protein termed TRIF that contains a RHIM that allows it to interact with and deploy RIP1 and RIP3. All TLRs, except TLR3, uses the MyD88 adaptor protein to promote downstream activation of NFκB.[99] TLR4 can also use TRIF to activate NFκB by a MyD88-independent pathway.[100,101] TLR3 is unique in the TLR family in that that it does not use Myd88 but instead exclusively employs TRIF to activate NFκB.[102] In the case of both TLR3 and TLR4 signalling, the RHIM motif of TRIF recruits RIP1 to mediate downstream activation of NFκB.[102,103] RIP3 is not required for activation of NFκB in TLR signalling pathways.[104] Similar to TNF-R1 signalling, RIP1 needs to be ubiquitinated in order to drive TRIF-induced activation of NFκB, and Pellino1 is a key E3 ligase that ubiquitinates RIP1 in the TLR3 and TLR4 pathways.[105]

In addition to activation of NFκB, TRIF can also stimulate the TBK1 and IKKi/IKKε kinases to activate interferon regulatory factor (IRF) transcription factors that drive expression of anti-viral type I interferons (IFNs)[99,106] (Figure 3). Interestingly, RIP1 is not used by TRIF in its activation of IRFs.[102,103] However, under circumstances of FADD depletion or its phosphorylation on serine residue 191 (during cell cycle arrest) or when caspases are inactivated (as occurs in virus-infected cells), IFNs can feed back on virally infected cells to activate the RNA-responsive protein kinase PKR, which then interacts with RIP1 and triggers RIP1/RIP3-mediated necroptosis.[107] When caspases are inhibited, TLR3 and TLR4 can also directly induce necroptosis by virtue of the RHIM motif of TRIF engaging RIP3 and MLKL to trigger downstream necrosis[108,109] (Figure 3). Interestingly, whereas RIP1 is required to mediate TNF-induced RIP3-dependent

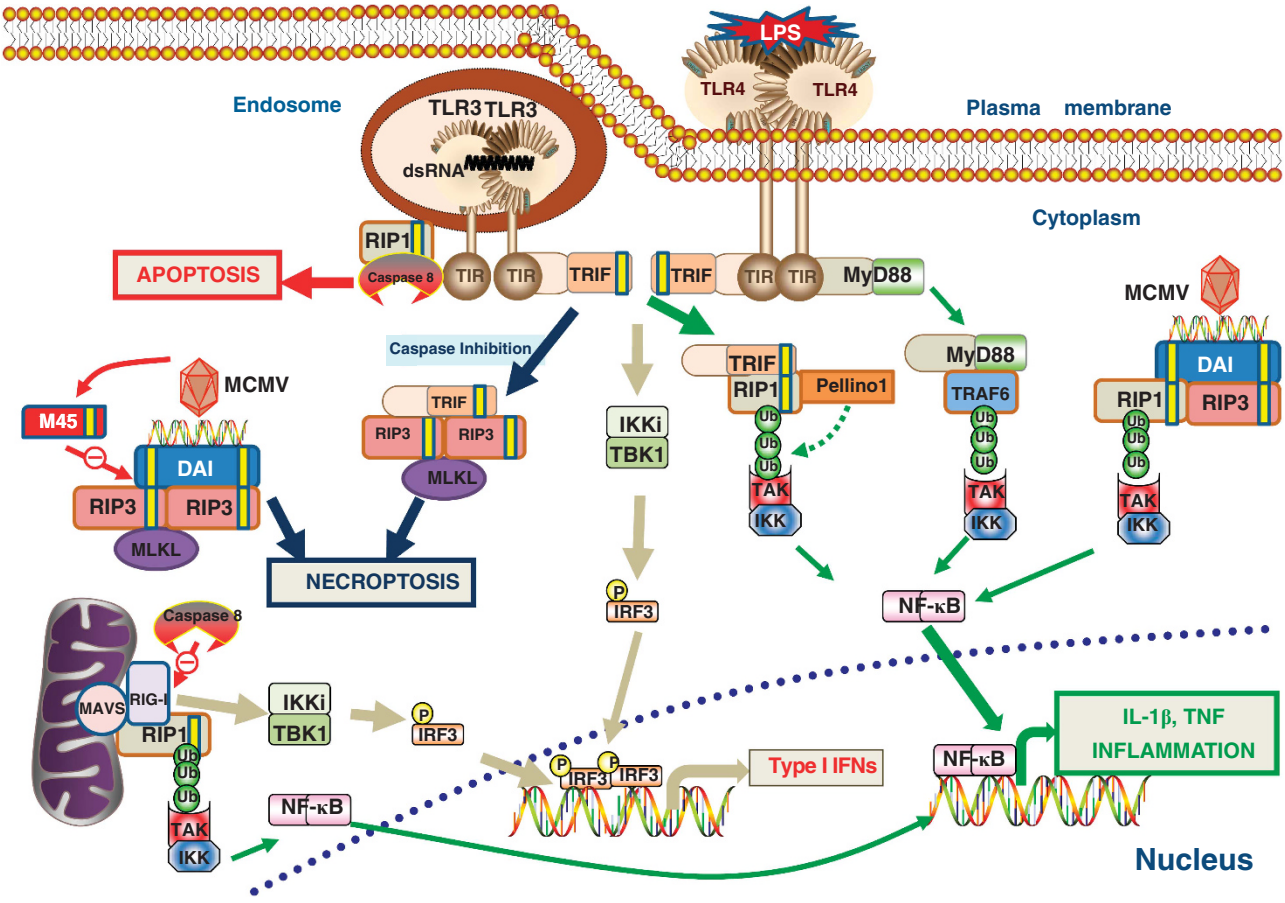

Figure 3 RIP1 and RIP3 in pattern recognition receptor signalling. LPS stimulates TLR4 to allow its Toll/IL-1 receptor (TIR) domain to interact with the TIR adaptor MyD88. This leads to ubiquitination of TRAF6, TAK1/IKK-mediated activation of NFκB and induction of pro-inflammatory genes such as IL-1β and TNF. The TIR domains of TLR3 and TLR4 can recruit another TIR adaptor TRIF, that contains a RHIM motif (in yellow), allowing TRIF to interact with RIP1. This is followed by Pellino1-mediated polyubiquitination of RIP1 allowing for TAK/IKK-induced activation of NFκB. TRIF (in a RIP1-independent manner) can also activate the TBK1/IKKi kinases to phosphorylate IRF3 and induce type I interferons (IFNs). Under conditions of caspase inhibition, the RHIM domain of TRIF can interact with the RHIM of RIP3 to trigger MLKL-mediated necroptosis. RIP1 can also facilitate the direct recruitment of caspase 8 to TLR3 leading to apoptosis. Murine cytomegalovirus (MCMV) can stimulate the DNA sensor DAI (containing two RHIMs) to interact with RIP1 and RIP3 to promote TAK/IKK-mediated activation of NFκB. DAI can also interact with RIP3 to promote MLKL-mediated necroptosis. The MCMV-encoded protein M45 contains a RHIM that allows it to target RIP3 and inhibit DAI- and RIP3-mediated necroptosis. The RNA helicase RIG-I is recruited to the mitochondria by MAVS followed by association with RIP1 and downstream activation of NFκB by TAK/IKK. RIG-1 also triggers TBK1/IKKi-mediated activation of IRF3 and induction of type I IFNs. RIP1 recruits caspase 8 to the RIG-1 complex resulting in RIP1 cleavage and termination of RIG-I signalling

necroptosis,[78] a recent report has indicated that RIP1 blocks TLR3-, TRIF- and IFN-driven necroptosis before birth.[79] TLRs that do not employ TRIF can also induce necroptosis in an indirect manner by inducing TNF to trigger necrosis via TNFR-1 as described above.[109] In addition, some of these pathways have been associated with cell apoptosis. Over-expression of TRIF results in interaction with RIP1 and RIP3 and induction of apoptosis,[110] and in the context of TLR3 signalling in lung cancer cells, the TLR3 ligand dsRNA can induce apoptosis by recruiting caspase 8 to TLR3 in a RIP1-dependent manner.[111] The ability of RIP kinases to orchestrate both apoptosis and necroptosis in response to triggering of viral-sensing TLR3 provides a major survival advantage to the host. Although TLR3-induced apoptosis can serve as the initial effort to eliminate virus-infected cells, some viruses encode caspase inhibitors to neutralize this defense system. However, in the absence of caspase activity, necroptosis will be strongly triggered as a contingency measure to deny the virus its home of replication.

RIP Kinases and Nucleic Acid Sensing

DNA-dependent activator of IRFs (DAI, also known as ZBP1 or DLM-1) is a cytosolic DNA sensor that can respond to immunostimulatory DNA to activate NFκB and IRFs and induce pro-inflammatory cytokines and IFNs.[112] DAI contains two RHIM motifs that allows it to interact with RIP1 and RIP3 and trigger downstream activation of NFκB[113,114] (Figure 3). The interaction of DAI with RIP3 also sensitizes cells to murine cytomegalovirus (MCMV)-induced necrosis with DAI- and RIP3-deficient cells being resistant to this form of death.[115] Intriguingly, MCMV encodes a M45 protein, that also contains a RHIM and targets the DAI–RIP3 interaction to suppress premature killing of endothelial cells during MCMV infection.[116,117] Such findings highlight the importance of RIP-mediated necroptosis to anti-viral immunity.[6,118]

The RNA helicase RIG-I also serves as a cytoplasmic viral sensor by recognizing viral RNA.[119] Engagement of RIG-I by RNA results in its recruitment by the MAVS adaptor protein to the outer membrane of the mitochondria.[3] The assembly of this complex triggers downstream activation of NFκB and IRF3 to induce pro-inflammatory cytokines and IFNs (Figure 3). A recent study has shown RIP1 to be recruited to the RIG-1 mitochondrial complex with ubiquitination of RIP1 serving to provide docking sites for key signalling molecules such as the IKK complex that activates NFκB.[120] However, RIP1 can also facilitate recruitment of caspase 8 to the complex, resulting in the cleavage of RIP1 and the generation of an inhibitory RIP1 fragment that represses RIG-I-induced activation of IRF3. Thus RIP1 is a key regulator of the temporal expression of virus-responsive genes.

RIP Kinases and the Inflammasome

IL-1β is one of the key pro-inflammatory cytokines that drives inflammation.[121] The secretion of mature IL-1β requires two signals. First, innate receptors, like TLR4, promote increased transcription of the gene encoding IL-1β, resulting in expression of an inactive pro-IL-1β precursor. A second signal requires the generation of a signalling platform termed the inflammasome consisting of a NLR protein such as NLRP3 that recruits the adaptor protein ASC and caspase 1 into a complex. Caspase 1 in this inflammasome complex will process pro-IL-1β precursor into the mature secreted form of IL-1β and will also effect an inflammatory form of cell death termed pyroptosis. A recent report has suggested that the inflammasome can be regulated by RIP1 and RIP3. Caspase 8 deficiency in dendritic cells enhanced TLR-4 induced formation and activation of the NLRP3 inflammasome by a mechanism that was dependent on RIP1, RIP3, MLKL and PGAM5.[122] This resulted in augmented LPS-induced expression of mature IL-1β and exacerbation of LPS-induced septic shock in mice with dendritic cell-specific deletion of the caspase 8 gene. Interestingly, these effects were proposed to be independent of necroptosis. Such findings suggest that caspase 8 has dualist roles in targeting RIP kinases to control inflammation. Caspase 8 acts to suppress RIP1/RIP3-driven necroptosis and the ensuing inflammatory fall-out from cell necrosis while also controlling RIP1/RIP3-mediated activation of the NLRP3 inflammasome and production of IL-1β. However, the role of caspase 8 is complex and context dependent as the causative agent of plague *Yersinia* pestis and its outer protein YopJ employs caspase 8, RIP1 and RIP3 to trigger cell death and caspase 1 activation.[123,124]

Interestingly, another study demonstrated that pharmacological or genetic depletion of the cIAP proteins in macrophages, in conjunction with TLR stimulation, resulted in augmented processing of pro-IL-1β into its mature form.[125] The processing of IL-1β was driven by two independent pathways involving NLRP3/caspase 1 and caspase 8. Both pathways were dependent on RIP3 and ROS. Thus under conditions of ripoptosome formation, as occurs with cIAP depletion, RIP3 can strongly drive IL-1β production further extending the pro-inflammatory potential of RIP3 beyond its ability to drive inflammatory cell death by necroptosis. However, the physiological circumstances under which cIAP proteins are depleted or inhibited in the presence of TLR stimuli remain to be characterized. These findings suggest that IAP proteins serve important regulatory roles in tempering the pro-inflammatory potential of RIP3. This is further supported by recent reports demonstrating that XIAP limits RIP3-dependent cell death and IL-1β expression in response to TNF[126] while cIAPs and XIAP control RIP1 and RIP3-dependent pro-inflammatory cytokine production in myeloid cells.[127]

RIP1 and RIP3 in a Pathophysiological Context

Given that necroptosis results in plasma membrane rupture and the release of endogenous danger signals that can activate PRRs, this form of cell death is regarded as being strongly pro-inflammatory in nature. Consequently, many studies have explored the potential contribution of RIP1/RIP3-mediated necroptosis to inflammatory diseases. To this end, necrostatins, inhibitors of the kinase activity of RIP1 and of necroptosis, have been evaluated in various disease models.[128] Necrostatins ameliorate pathology in a number of inflammatory disease models, including brain ischaemia,[129] mycocardial infarction[130] and head trauma.[131] Inhibition of RIP1 or RIP3 deficiency reduces mortality during

TNF-induced systemic inflammatory response syndrome and pathology in the caecal ligation and puncture model of polymicrobial sepsis highlighting the potential value of targeting RIP1/RIP3 in sepsis.[132] RIP3-deficient mice are also free of inflammation in an acute pancreatitis model[61] and show reduced macrophage necroptosis to ameliorate atherosclerosis development.[133] Keratinocyte-specific deletion of FADD results in serious inflammatory skin lesions via RIP3-mediated necroptosis.[134] Furthermore, conditional deletion of caspase 8 in the intestinal epithelium resulted in great levels of RIP3 and TNF-driven necroptosis and increased susceptibility to colitis.[135] Interestingly, the latter study also demonstrated high levels of RIP3 and necroptosis in the terminal ileum of patients with Crohn's disease, suggesting that RIP3-induced necroptosis may be a valuable therapeutic target in human disease. Absence or inhibition of RIP3 also reduces liver damage in response to ethanol[136] or acetaminophen.[137] Finally, RIP1 and RIP3 drive necrotic cell death in retinal pigment epithelial cells and photoreceptor cells, and these effects likely have key roles in vision problems, such as macular degeneration, retinitis pigmentosa and retinal detachment.[138–142] All of these studies emphasize the potential roles of RIP1 and RIP3 in driving diverse inflammatory diseases, and future research is faced with the challenge of exploiting these kinases as therapeutic targets.

RIP2 and NOD Signalling

RIP2 was initially identified as a RIP-like kinase that, when overexpressed, could activate NFκB and MAP kinases and augment caspase 8-mediated apoptosis.[143–145] RIP2 contains an N-terminal kinase domain and C-terminal CARD domain (Figure 1). The kinase activity of RIP2 is dispensable to manifest its activation of NFκB but is required to mediate the activation of ERK MAPK[145,146] and to stabilize RIP2 itself.[147,148] Early studies demonstrated that RIP2-deficient mice are viable but show impaired activation of NFκB in response to TLR signalling and are more resistant to LPS-induced lethal sepsis.[149,150] However, a more recent report, using synthetic and highly purified forms of TLR ligands, contend that TLR signalling is intact in cells from RIP2 null mice, but loss of RIP2 leads to abrogation of signalling in response to stimulation of nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 by their specific ligands or the intracellular pathogen *Listeria monocytogenes*.[151] These findings indicate that RIP2 mediates NOD1 and NOD2 signalling but not TLR signal transduction.

NOD1 and NOD2 are cytosolic receptors for bacterial peptidoglycan derivatives such as muramyl dipeptide (MDP) and are expressed highly in mucosal epithelium.[152–154] Loss-of-function mutations in NOD2 are associated with greatly increased susceptibility to Crohn's disease,[155–157] whereas gain-of-function mutations are linked to early onset sarcoidosis and Blau syndrome.[158,159] NOD2 contains an N-terminal CARD domain, a central NACHT region and C-terminal LRRs.[160] Upon binding of MDP to the LRRs of NOD2, the NACHT regions are exposed, allowing for self-oligomerization of NOD2 molecules, followed by homotypic interactions between the CARD domains of NOD2 and RIP2[161,162] (Figure 4). This results in ubiquitination of RIP2 followed by recruitment of the TAK1 and IKK complexes and downstream activation of NFκB and MAP kinase pathways by an analogous mechanism to that described above for RIP1 signalling in the TNFR-1 pathway.[148,163–165] This results in the expression of a range of inflammatory proteins, anti-bacterial proteins, activation of autophagy and antigen presentation.[166–168] Notably, RIP2 is required to mediate all of the *in vivo* host responses to MDP.[169]

The ubiquitination of RIP2 is a critical step in mediating activation of these NOD2 pathways, especially activation of NFκB,[164,165] and a number of E3 ubiquitin ligases, including TRAF6,[164] cIAP and XIAP proteins[170–172] and ITCH[173] have been proposed to catalyse ubiquitination of RIP2. However, other studies have questioned the importance of many of these E3 ligases in the context of ubiquitinating RIP2 and activating NFκB. Thus the ubiquitination of RIP2 is intact in TRAF6-deficient cells,[165] pharmacological depletion of cIAP1 and cIAP2 has no effect on RIP2 ubiquitination[171] and ITCH-mediated ubiquitination of RIP2 is associated with negative regulation of RIP2-mediated NFκB signalling.[173] We have recently described a key role for the E3 ubiquitin ligase Pellino3 in directly ubiquitinating RIP2 and mediating NOD2 downstream signalling, including its activation of NFκB and protective effects in colitis[174,175] (Figure 4). We also showed that Pellino3 protein expression is greatly reduced in the colons of Crohn's disease subjects consistent with a protective role in human disease.[174] We have proposed functional cooperation between Pellino3 and XIAP in that the former promotes the formation of polyubiquitin chains on RIP2 in which the isopeptide linkages between adjacent ubiquitin molecules are linked via lysine 63 of ubiquitin and XIAP facilitates linear ubiquitination of components of the RIP2 complex in which individual ubiquitin proteins are joined head to tail. This shows remarkable similarity to the RIP1-containing complex I in the TNFR-1 signalling pathway in which components of the complex are initially modified by lysine 63-linked chains followed by LUBAC-mediated linear ubiquitination that serves to stabilize the complex and further enhance downstream signalling pathways, such as NFκB and inflammatory gene expression.[176]

The ubiquitination pathway in NOD–RIP2 signalling is subject to various forms of regulation. Thus the inositol phosphatase SHIP-1 disrupts the interaction between XIAP and RIP2 to inhibit NOD2-induced NFκB activation.[177] In addition, free ubiquitin can compete with RIP2 for the binding of NOD1.[178] Furthermore, the autophagy protein ATG16L1, which has also been linked to Crohn's disease, interferes with the polyubiquitination of RIP2 and the recruitment of RIP2 into NOD-signalling complexes, resulting in impaired downstream signalling.[179] The allelic form of ATG16L1, which is associated with Crohn's disease, fails to regulate NOD-mediated inflammatory signalling, suggesting that the targeting of RIP2 is important in controlling intestinal pathogenesis. The LIM domain-containing protein TRIP can interact with RIP2 to positively regulate NOD1 signalling.[180] Finally, the MAP3K, MEKK4, interacts with RIP2 to preclude basal interaction of the latter with NOD2 while stimulation of cells with the NOD2 ligand MDP promotes dissociation of RIP2 from MEKK4 allowing for interaction of RIP2 with NOD2.[181]

The NOD–RIP2 pathway is also targeted by caspases, and this is especially interesting given that NOD proteins belong to

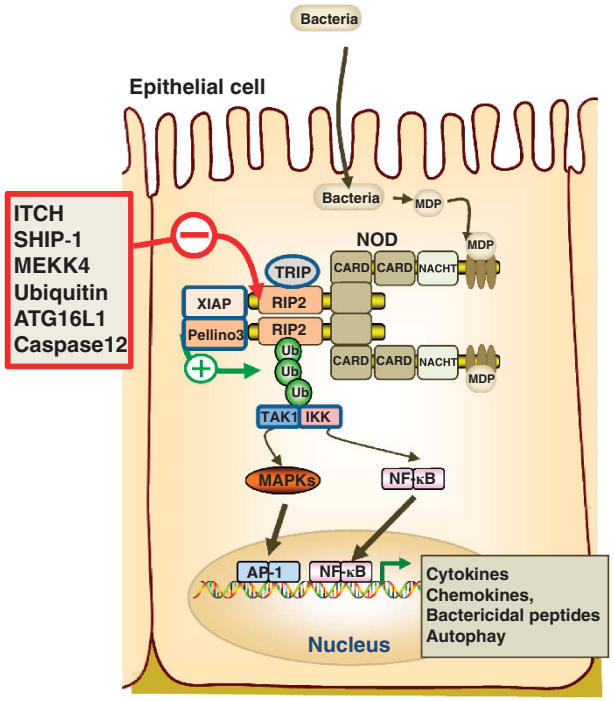

Figure 4 RIP2 and NOD signalling. Bacterial invasion of epithelial cells results in the stimulation of NOD proteins by peptidoglycan-derived peptides such as MDP. MDP binds to the leucine-rich repeat regions of NOD2 allowing for NACHT domains to mediate NOD oligomerization. The CARD domains of oligomerized NOD proteins interact with the CARD domains of RIP2 kinase molecules followed by binding of TRIP protein to RIP2 and XIAP and Pellino3-mediated polyubiquitination of RIP2. This facilitates recruitment of TAK1 and IKK complexes and downstream activation of NFκB, MAPKs and AP-1. The transcription factors drive expression of cytokines, chemokines and anti-bacterial peptides. NOD signalling can also result in cell autophagy. RIP2 is targeted by various negative regulatory proteins: ITCH catalyzes ubiquitination of RIP2 to inhibit NFκB activation; SHIP-1 disrupts the interaction between XIAP and RIP2; Free ubiquitin competes with RIP2 for the binding of NOD1; the autophagy protein ATG16L1 interferes with the polyubiquitination of RIP2 and the recruitment of RIP2 into NOD-signalling complexes; MEKK4 inhibits the basal interaction of RIP2 with NOD2; and Caspase 12 targets RIP2 and inhibits downstream signalling

the large NLR family, many members of which are components of caspase-containing inflammasomes. Caspase 12 has been shown to target RIP2 and inhibit downstream signalling in response to NOD2 stimulation.[182] This results in an impaired mucosal antimicrobial response to enteric pathogens due to reduced production of antimicrobial peptides, cytokine and chemokines. Intriguingly, some patients with variants of the caspase 1 gene, which encode for forms of procaspase 1 with greatly reduced or absent enzymatic activity, frequently exhibit fever even though levels of IL-1β are low.[183] The latter study demonstrated that the CARD domain of these procaspase 1 variants binds to the CARD of RIP2 to trigger activation of NFκB and presumably downstream inflammatory responses that underpin the regular febrile episodes.

RIP2 in Inflammation and Disease

Although the NOD–RIP2 signalling pathways are of particular relevance to the control of intestinal inflammation, RIP2 also has important roles in mucosal immunity in the respiratory system. Thus RIP2-deficient mice show impaired bacterial clearance in an *E. Coli* pneumonia infection model[184] and *Chlamydophila pneumoniae*-induced pneumonia.[185] In both models, the absence of RIP2 resulted in impaired expression of various pro-inflammatory mediators, reduced neutrophil infiltration and increased bacterial burden. However, under certain circumstances, the role of RIP2 in mediating an anti-bacterial response can be damaging to the host. This applies in the case of secondary bacterial infection following an initial viral infection.[186] In this case, viral challenge leads to production of type I IFNs that strongly upregulate NOD1, NOD2 and RIP2 resulting in an exaggerated inflammatory response to secondary infection with *E. Coli*. Thus the NOD–RIP2 pathway likely has key roles in the increased lethality and morbidity that is clinically observed in secondary bacterial infections.

RIP2 has been associated with other inflammatory disease states and models. Levels of RIP2 are elevated in the non-T-cell fraction of blood from multiple sclerosis (MS) subjects,[187] and pathogenesis in murine models of MS have been shown to be dependent on NOD1, NOD2 and RIP2 with the latter having an especially important role in activating CNS dendritic cells.[188] Intriguingly, peptidoglycan has been detected within antigen-presenting cells, including dendritic cells, in the brain of MS patients[189] suggesting that the peptidoglycan–NOD–RIP2 axis in CNS may contribute to MS pathogenesis. RIP2 has also been implicated as a driver in experimental allergic airway inflammation by activating NFκB and inflammatory gene expression.[190] Furthermore, RIP2-deficient macrophages, although showing weaker inflammatory signalling, display increased lipid accumulation that contributes to more severe atherosclerosis in recipient mice,[191] implicating a potential role for RIP2 in cardiovascular disease.

A complex picture thus emerges of the role of RIP2 in inflammation and immunity. Given its critical role in NOD pathways, RIP2 clearly has a protective role in mucosal immunity and homeostasis as deficiency in NOD signalling is linked to Crohn's disease and loss of RIP2 leads to increased bacterial burden in pulmonary infection models. However, high levels of RIP2 can lead to damaging inflammatory responses as indicated by pathogenesis in models of MS and secondary bacterial infections. Such opposing roles of RIP2 highlight the need for efficient regulatory mechanisms to avoid the potential damaging consequences of RIP2 action.

Conclusion and Perspective

Although much research has focused on the role of RIP proteins in cell death, it is clear that these kinases have many physiological and pathophysiological functions that are derived from their important roles in inflammation and innate immunity. Furthermore, many of the regulatory roles of RIP kinases in inflammation are mediated by their effects on cell death. Thus, while RIP1 is a key determinant in deciding whether a cell produces pro-inflammatory mediators or dies by apoptosis, RIP3 can direct a cell to die by the more inflammatory process of necroptosis. Although the latter provides a safeguard death mechanism against intracellular pathogens that encode for factors that interfere with

apoptosis, necroptosis is also emerging as a key player in a number of inflammatory diseases. Thus, like RIP2, RIP1and RIP3 must be tightly controlled, with loss of this control leading to hyper-inflammation and pathology. RIP kinase thus emerge as lead therapeutic targets in a number of diseases. The first inhibitors of RIP kinases have emerged over the past number of years.[128] The challenge and opportunity sit side by side to translate our increased understanding of RIP kinase biology into RIP-targeted therapeutics to treat inflammatory diseases.

Conflict of Interest

The authors declare no conflict to interest.

Acknowledgements. This work is supported by a grant from Science Foundation Ireland (12/IA/1736).

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