Molecular & Cellular Oncology 2:2, e965638; April/May/June 2015; Published with license by Taylor & Francis Group, LLC

Necroptosis: Fifty shades of RIPKs

Gabriel Ichim and Stephen WG Tait*

Cancer Research UK Beatson Institute; Institute of Cancer Sciences; University of Glasgow; Glasgow, UK

Apoptosis and necroptosis are 2 major, yet distinct, forms of regulated cell death. Whereas apoptosis requires caspase protease function, necroptosis requires activation of the receptor interacting protein kinases 1 (RIPK1) and RIPK3. Following activation, RIPK3 phosphorylates mixed-lineage kinase domain-like (MLKL), leading to cell death. Apoptosis and necroptosis are deeply intertwined such that a given death stimulus can often engage either form of cell death. Recent studies published in Cell Death and Differentiation by the Han, Oberst, and Vaux laboratories provide exciting new insights into necroptosis and how it interconnects with apoptosis. As we will discuss, their findings address key questions including: How does a cell choose between apoptosis or necroptosis? How can RIPK3 also induce apoptosis? What is the nature of the RIPK1–3 signaling cascade leading to necroptosis? Finally, data from the Oberst and Han groups strongly argue that RIPK1 is not only involved in executing necroptosis, but also protects against this process in some settings.

Keywords: necroptosis, apoptosis, RIPK1, RIPK3, MLKL, TNF

*Correspondence to: Stephen WG Tait; Email: stephen.tait@glasgow.ac.uk

Submitted: 08/05/2014

Revised: 08/13/2014

Accepted: 08/13/2014

http://dx.doi.org/10.4161/23723548.2014.965638

Introduction

Just like us, cells die in many ways; some mechanisms of cell death are regulated and involve engagement of dedicated molecular machinery whereas others are passive and occur as a result of overwhelming damage. The best understood form of regulated cell death is apoptosis, an evolutionarily conserved process that plays key roles in most areas of biology. Apoptosis requires the activation of caspase proteases that cleave hundreds of substrate proteins leading to rapid cell death.[1] Recently, intense interest has focused on another form of regulated cell death called necroptosis. Morphologically, the execution phase of necroptosis resembles necrosis, in which dying cells swell prior to lysis.[2] However, in stark contrast to necrosis, necroptosis is highly controlled and requires activity of the receptor interacting protein kinases 1 (RIPK1) and RIPK3.[3–6] In a simplified model, active RIPK1 phosphorylates RIPK3 leading to its activation.[7] In turn, RIPK3 phosphorylates the pseudokinase mixed-lineage kinase domain-like (MLKL) promoting its oligomerization and plasma membrane translocation.[8–11] Through poorly understood means, oligomerized MLKL ruptures the plasma membrane thereby killing the cell. Far from existing in isolation, extensive crosstalk occurs between apoptosis and necroptosis. For example, some apoptotic stimuli can also trigger necroptosis when caspase activity is inhibited. Here we discuss recent work by the Han, Oberst, and Vaux groups that provides exciting new insight into the regulation of necroptosis.[12–14] Specifically, these papers offer new understanding into how a cell decides to undergo apoptosis or necroptosis and how RIP kinases transduce the death signal, in addition to demonstrating that RIPK1—previously viewed solely as pro-necroptotic—can also exert antinecroptotic activity.

Pick Your Poison—RIPK3-Dependent Apoptosis or Necroptosis?

As discussed, various stimuli can trigger apoptosis and necroptosis. This crosstalk is best understood in the context of tumor necrosis factor (TNF) signaling. Binding of TNF to its receptor can trigger multiple outcomes that include prosurvival and

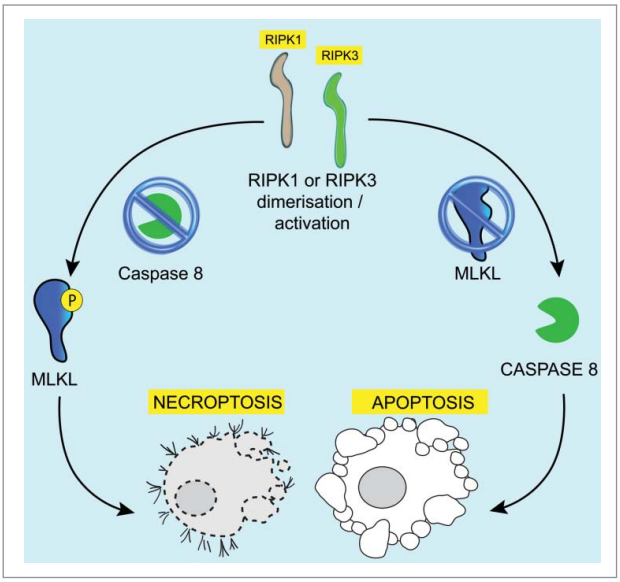

Figure 1. Availability of killer molecules determines the mode of RIPK1/RIPK3-driven cell death. Dimerization of RIPK1 or RIPK3 leads to kinase activation and cell death. Depending on the availability of downstream molecules, cells die via necroptosis (if caspase-8 is lacking) or apoptosis (if MLKL is lacking). If both molecules are available, as is often the case, apoptosis or necroptosis may not be mutually exclusive and might occur concurrently in the same cell. RIPK1, receptor interacting protein kinase; MKLK, mixed-lineage kinase domain-like.

proinflammatory NFκB activation, apoptosis, or necroptosis. The ability of TNF to induce necroptosis requires caspase-8 inhibition, among other factors. Caspase-8 prevents TNF-induced necroptosis through cleavage of multiple pronecroptotic proteins that include RIPK3, RIPK1, and cylindromatosis (CYLD).[15–17] RIPK3 is essential for TNF-induced necroptosis, and in most cases RIPK1 is also required. Importantly, besides their role in necroptosis, various reports have also implicated a proapoptotic role for RIPK1 and RIPK3. What dictates the ability of RIPK1 or RIPK3 to trigger either necroptosis or apoptosis? Cook and colleagues provide at least one explanation.[13] Our laboratory has previously shown that chemically induced oligomerization of RIPK3 suffices to trigger necroptosis.[18] Using an analogous method, Cook and co-workers show that dimerization of either RIPK1 or RIPK3 is sufficient to kill cells via necroptosis. This reductionist approach avoids potentially complicating effects of ancillary TNF signaling cascades. Importantly, the authors demonstrate that the ability of activated RIPK1 or RIPK3 to engage apoptosis or necroptosis is solely dictated by the availability of downstream target: if MLKL is lacking, RIPK1 or RIPK3 activation induces apoptosis, whereas necroptosis is induced if caspase 8 is absent (**Fig. 1**). As predicted from earlier studies, the kinase activity of RIPK3 is required for necroptosis but, intriguingly, is dispensable for apoptosis. Rather, the ability of kinase-inactive RIPK3 to induce apoptosis requires RIPK1, FAS-associated protein with death domain (FADD), and caspase-8. In this scenario, RIPK3 (probably dimerized) binds RIPK1, leading to recruitment of FADD and ultimately the recruitment and activation of caspase-8. Offering support for these findings *in vivo*, a recent study has shown that knock-in mice expressing a kinase-dead RIPK3 exhibit early embryonic lethality that can be rescued by concomitant loss of RIPK1 or caspase-8.[19] Additionally, Cook and colleagues found that kinase-dead RIPK3 can kill cells via apoptosis, albeit to a lesser degree, in the absence of RIPK1. How this occurs is unclear, but it mirrors earlier findings from the same group showing that elevated RIPK3 levels can bypass an absolute requirement for RIPK1 during TNF-induced necroptosis.[20] The important take-home message of this work is that the decision between apoptosis or necroptosis may not be an either/or switch mechanism, as often depicted. Undoubtedly, this situation will be more complicated in real-death situations, e.g., TNF-induced necroptosis, but these findings suggest that apoptosis and necroptosis may occur concurrently in the same cell, something that could be addressed by live-cell imaging.

How Letting One RIP Leads to Another

The necrosome is the necroptosis-initiating TNF-induced complex that contains FADD, caspase-8, RIPK1, and RIPK3. RIPK3 activation requires interaction with RIPK1 through domains present in both proteins called RIP homotypic interaction motifs (RHIMs). These motifs allow extensive RHIM-mediated polymerization to occur, forming amyloid fibrils in the cell that consist of RIK3–RIPK1 heterodimers and/or RIPK1 or RIPK3 homodimers.[21] How these interactions are coordinated to activate RIPK3 and trigger necroptosis is unclear. To explore this key question, Wu and colleagues used chemically dimerizable pairs of RIPK1 and RIPK3 that allow defined hetero- and homodimerization between the different kinases.[12] Using this method, they found that the RIPK1–RIPK3 interaction was required for necroptosis, as one would predict. However, their data show that the RIPK1–RIPK3 heterodimer lacks detectable RIPK3 activity and by itself is incapable of killing cells. Rather, it acts to seed necrosome formation by incorporating additional RIPK3 molecules that activate and kill the cell. Extending these findings, they found that RIPK3 homodimers are as effective as oligomers at inducing necroptosis. Collectively, these data argue that oligomerization of active RIPK3 dimers *per se* is not necessary for necroptosis; instead, the actual number of active RIPK3 dimeric units may be the key factor. With respect to this conclusion, it is important to note that the Oberst laboratory found the opposite—in their study oligomerization of RHIM-deficient RIPK3 was necessary for necroptosis whereas dimerization was insufficient.[14]

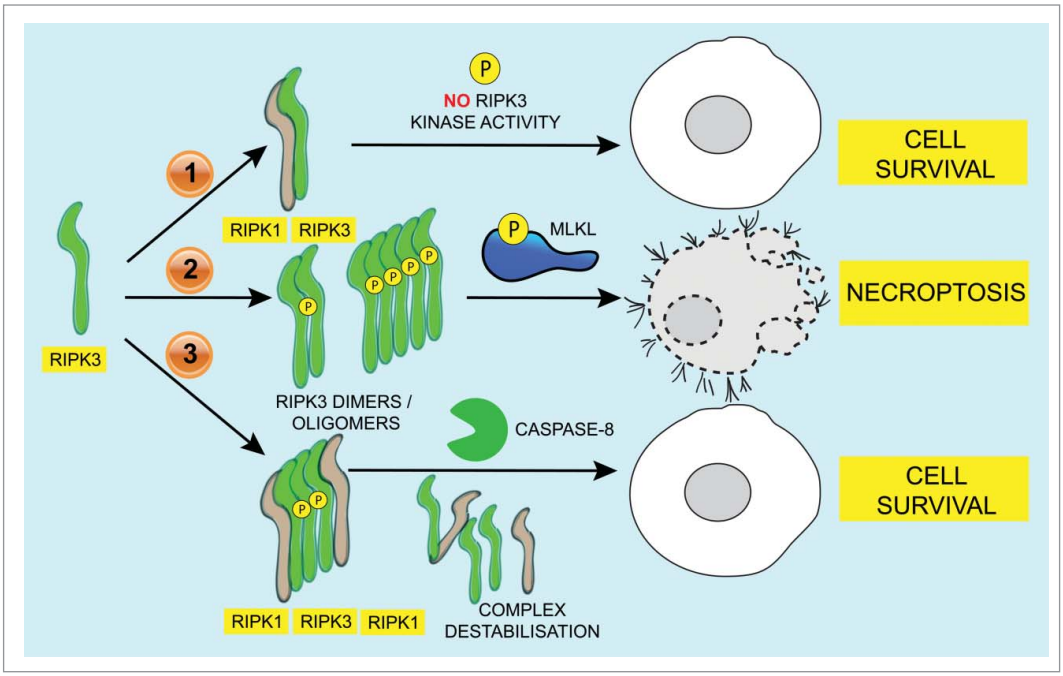

Figure 2. Antinecroptotic functions of RIPK1. (1) Dimerization of RIPK1 with RIPK3 prevents RIPK3 kinase activity, thus allowing cell survival; 2) Dimerization or oligomerization of RIPK3 leads to its activation and necroptosis; 3) Incorporation of RIPK1 into RIPK3 oligomers recruits caspase-8, which destabilizes the necrosome allowing cell survival. RIPK, receptor interacting protein kinase.

The disparity in these findings likely relates to the different location of the dimerization domain (N- or C-terminal to RIPK3) between the 2 studies. As such, although RIPK3 dimerization is sufficient to cause necroptosis, the exact requirements for dimerization *versus* oligomerization in an endogenous setting are currently unclear.

RIPK1—Killer or Savior?

RIPK1 is widely considered to be pronecroptotic and is often essential for necroptosis in response to different stimuli. Orozco and coworkers provide provocative new data strongly arguing that RIPK1 can also perform an antinecroptotic function.[14] Similar to other studies, they used chemical dimerizers to dissect the role of RIPK3 dimerization *versus* oligomerization upon its activation during necroptosis. In itself, RIPK3 dimerization was insufficient to trigger necroptosis but led to robust RIPK3 activity and necroptosis through recruitment of other RIPK3 molecules. Importantly, caspase-8 and RIPK1 were also recruited to the RIPK3 oligomeric complex and exerted an inhibitory effect. Strikingly, either RNA knockdown or chemical inhibition of caspase-8 potentiated RIPK3-mediated necroptosis. Most surprisingly, whereas chemical inhibition of RIPK1 activity inhibited RIPK3-mediated necroptosis, knockdown of RIPK1 actually promoted it. Together with the other data presented, these findings paint a picture whereby recruitment of RIPK1 to RIPK3 complexes inhibits necroptosis by recruiting caspase-8 (indirectly via FADD), leading to destabilization of the necrosome (**Fig. 2**). In a somewhat analogous manner the work by Wu and colleagues also supports an inhibitory role for RIPK1, showing that binding of RIPK1 to RIPK3 effectively blocks RIPK3 activity[12] (**Fig. 2**). These findings predict that the relative levels of RIPK1 and RIPK3 could regulate whether a cell can undergo necroptosis—the expectation being that high RIPK1 levels would exert an inhibitory effect on RIPK3 activation. Importantly, recent data offer *in vivo* support for an inhibitory role for RIPK1 in necroptosis because RIPK3 ablation (combined with FADD or caspase-8) is required to rescue the post-natal lethality of RIPK1-deficient mice.[22–24] Why does RIPK1 exert an inhibitory effect on RIPK3 function? As Orozco et al. speculate, one possibility is that RIPK1 serves to inhibit "accidental" RIPK3 activation and necroptosis mediated by spontaneous RIPK3 self-association. Beyond their interest with respect to basic biology, these findings also raise important clinical considerations; for example, when targeting RIPK1 kinase function in proinflammatory disease it may be prudent to avoid disrupting its antinecroptotic function.

In summary, these papers offer exciting new perspectives into various aspects of necroptosis and how it interconnects with apoptosis. Out of necessity, all of these studies have used stripped-down versions of necroptosis signaling. Undoubtedly the real-life situation is likely to be much more complicated but, as we have discussed, *in vivo* evidence supporting many of these findings is already emerging. Furthering our understanding of necroptosis will provide an improved rationale for targeting this process in disease, in addition to highlighting potential therapeutic pitfalls.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

Research in the Tait lab is supported by funding from the Royal Society, BBSRC, Cancer Research UK, and the European Union. ST is a Royal Society University Research Fellow. GI is supported by an EMBO long-term fellowship, ALTF 55–2013.

References

1. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harbor perspectives in biology 2013; 5:a008656; PMID:23545416; http://dx.doi.org/10.1101/cshperspect.a008656
2. Vanden Berghe T, Vanlangenakker N, Parthoens E, Deckers W, Devos M, Festjens N, Guerin CJ, Brunk UT, Declercq W, Vandenabeele P. Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell death and differentiation 2010; 17:922-30; PMID:20010783; http://dx.doi.org/10.1038/cdd.2009.184
3. He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-α. Cell 2009; 137:1100-11; PMID:19524512; http://dx.doi.org/10.1016/j.cell.2009.05.021
4. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 2009; 137:1112-23; PMID:19524513; http://dx.doi.org/10.1016/j.cell.2009.05.037
5. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 2009; 325:332-6; PMID:19498109; http://dx.doi.org/10.1126/science.1172308
6. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat immunol 2000; 1:489-95; PMID:11101870; http://dx.doi.org/10.1038/82732
7. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat Rev Mol Cell Biol 2014; 15:135-47; PMID:24452471; http://dx.doi.org/10.1038/nrm3737
8. Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, Wang FS, Wang X. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. Mol Cell 2014; 54:133-46; PMID:24703947; http://dx.doi.org/10.1016/j.molcel.2014.03.003
9. Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman I, Hulpiau P, Weber K, Sehon CA, Marquis RW, et al. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. Cell Rep 2014; 7:971-81; PMID:24813885; http://dx.doi.org/10.1016/j.celrep.2014.04.026
10. Cai Z, Jitkaew S, Zhao J, Chiang HC, Choksi S, Liu J, Ward Y, Wu LG, Liu ZG. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol 2014; 16:55-65; PMID:24316671; http://dx.doi.org/10.1038/ncb2883
11. Chen X, Li W, Ren J, Huang D, He WT, Song Y, Yang C, Li W, Zheng X, Chen P, et al. Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. Cell Res 2014; 24:105-21; PMID:24366341; http://dx.doi.org/10.1038/cr.2013.171
12. Wu XN, Yang ZH, Wang XK, Zhang Y, Wan H, Song Y, Chen X, Shao J, Han J. Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. Cell death and differentiation 2014; 21:1709-20; PMID:24902902; 10.1038/cdd.2014.77; http://dx.doi.org/10.1007/978-1-4614-9302-0
13. Cook WD, Moujalled DM, Ralph TJ, Lock P, Young SN, Murphy JM, Vaux DL. RIPK1- and RIPK3-induced cell death mode is determined by target availability. Cell death and differentiation 2014; 21:1600-12; PMID:24902899; http://dx.doi.org/10.1038/cdd.2014.70
14. Orozco S, Yatim N, Werner MR, Tran H, Gunja SY, Tait SW, Albert ML, Green DR, Oberst A. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. Cell Death Differ 2014;21:1511-21; PMID:24902904; http://dx.doi.org/10.1038/cdd.2014.76
15. O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT. Caspase 8 inhibits programmed necrosis by processing CYLD. Natu Cell Biol 2011; 13:1437-42; PMID:22037414; http://dx.doi.org/10.1038/ncb2362
16. Feng S, Yang Y, Mei Y, Ma L, Zhu DE, Hoti N, Castanares M, Wu M. Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signall 2007; 19:2056-67; PMID:17644308; http://dx.doi.org/10.1016/j.cellsig.2007.05.016
17. Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 1999; 13:2514-26; PMID:10521396; http://dx.doi.org/10.1101/gad.13.19.2514
18. Tait SW, Oberst A, Quarato G, Milasta S, Haller M, Wang R, Karvela M, Ichim G, Yatim N, Albert ML, et al. Widespread mitochondrial depletion via mitophagy does not compromise necroptosis. Cell Rep 2013; 5:878-85; PMID:24268776; http://dx.doi.org/10.1016/j.celrep.2013.10.034
19. Newton K, Dugger DL, Wickliffe KE, Kapoor N, de Almagro MC, Vucic D, Komuves L, Ferrando RE, French DM, Webster J, et al. Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. Science 2014; 343:1357-60; PMID:24557836; http://dx.doi.org/10.1126/science.1249361
20. Moujalled DM, Cook WD, Okamoto T, Murphy J, Lawlor KE, Vince JE, Vaux DL. TNF can activate RIPK3 and cause programmed necrosis in the absence of RIPK1. Cell DeathDis 2013; 4:e465; PMID:23328672; http://dx.doi.org/10.1038/cddis.2012.201
21. Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A, et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 2012; 150:339-50; PMID:22817896; http://dx.doi.org/10.1016/j.cell.2012.06.019
22. Dillon CP, Weinlich R, Rodriguez DA, Cripps JG, Quarato G, Gurung P, Verbist KC, Brewer TL, Llambi F, Gong YN, et al. RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. Cell 2014; 157:1189-202; PMID:24813850; http://dx.doi.org/10.1016/j.cell.2014.04.018
23. Kaiser WJ, Daley-Bauer LP, Thapa RJ, Mandal P, Berger SB, Huang C, Sundararajan A, Guo H, Roback L, Speck SH, et al. RIP1 suppresses innate immune necrotic as well as apoptotic cell death during mammalian parturition. Proc Natil Acad Sci U S A 2014; 111:7753-8; PMID:24821786; http://dx.doi.org/10.1073/pnas.1401857111
24. Rickard JA, O'Donnell JA, Evans JM, Lalaoui N, Poh AR, Rogers T, Vince JE, Lawlor KE, Ninnis RL, Anderton H, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. Cell 2014; 157:1175-88; PMID:24813849; http://dx.doi.org/10.1016/j.cell.2014.04.019