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MULTI-AUTHOR REVIEW

Necroptosis-independent signaling by the RIP kinases in inflammation

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Abstract Recent advances have identified a signaling cascade involving receptor interacting protein kinase 1 (RIPK1), RIPK3 and the pseudokinase mixed lineage kinase domain-like (MLKL) that is crucial for induction of necroptosis, a non-apoptotic form of cell death. RIPK1–RIPK3–MLKL-mediated necroptosis has been attributed to cause many inflammatory diseases through the release of cellular damage-associated molecular patterns (DAMPs). In addition to necroptosis, emerging evidence suggests that these necroptosis signal adaptors can also facilitate inflammation independent of cell death. In particular, the RIP kinases can drive NF-κB and inflammasome activation independent of cell death. In this review, we will discuss recent discoveries that led to this realization and present arguments why cell death-independent signaling by the RIP kinases may have a more important role in inflammation than necroptosis.

Keywords RIPK1 · RIPK3 · IL-1β · NF-κB · RelB

Introduction

Organismal homeostasis is achieved through an intricate balance of cellular proliferation, senescence and cell death. Apoptosis is an evolutionary conserved process for complex organisms to eliminate unwanted or damaged cells. Early during the process, apoptotic cells express the "eat-me" signal phosphatidyl serine (PS) on the cell surface, which prompts their clearance by macrophages or phagocytes. This process is normally efficient, which explains why apoptotic cells are hard to detect in situ. Immunologists have long recognized the anti-inflammatory nature of apoptosis. This is an important characteristic since apoptosis is prevalent during metazoan development and inflammation would not be a desired outcome. However, excessive apoptosis can occur in certain pathological conditions such as infections. In this situation, apoptosis can progress to secondary necrosis, leading to plasma membrane leakage, release of immunogenic cellular contents and inflammation. In mouse models, inhibition of apoptotic cell clearance often promotes autoinflammatory disease-like symptoms. These observations highlight the intimate link between cell death and inflammation.

In contrast to apoptosis, necrosis is generally considered to be pro-inflammatory and immunogenic. Although necrosis was thought to be the consequence of non-specific trauma to the cells, recent advances demonstrate that necrosis can also be executed in a regulated manner. The receptor interacting protein kinases (RIPKs) are key drivers for a form of regulated necrosis termed necroptosis. The regulation of necroptosis is extensively discussed in other reviews in the same issue and will not be the focus of this review. Instead, we will discuss the emerging evidence that points to a necroptosis-independent role for the RIPKs in inflammation. We present an alternative viewpoint that the RIPKs predominantly drive inflammation through necroptosis-independent mechanisms, and that necroptosis is a fallback option when the inflammatory process goes awry.

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The RIPK1 and NF-κB activation

The serine/threonine kinases RIPK1 and RIPK3 are essential adaptors for TNF-induced necroptosis. RIPK1 was originally identified in a yeast two-hybrid screen as a Fas/CD95-interacting adaptor [1]. Although early studies showed that overexpression of RIPK1 could lead to cell death, subsequent studies revealed that RIPK1 predominantly signals for NF-κB activation downstream of TNF receptor 1 (TNFR1) [2–4]. Because NF-κB is a transcription factor that drives expression of many inflammatory genes, it is clear from the early days that RIPK1 can promote cell death-independent inflammation. However, it is noteworthy that studies from different groups have not consistently detected defects in TNF-induced NF-κB activation in *Ripk1*$^{-/-}$ MEFs [5–7]. In the RIPK1-deficient T cell leukemia cell line Jurkat, early TNF-induced phosphorylation and degradation of IκBα (5 min) was abolished compared with wild-type Jurkat cells. However, by 15 min after TNF stimulation, phosphorylation of IκBα was comparable between wild-type and RIPK1-deficient Jurkat cells [8]. Given the multi-phasic nature of NF-κB-dependent gene expression [9], it is possible that the moderate delay in NF-κB activation in *Ripk1*$^{-/-}$ cells can result in altered gene expression pattern. Regardless of the extent of its impact on gene expression, it is safe to say that RIPK1 plays only an accessory role in NF-κB activation by modulating the kinetics of IκBα phosphorylation and degradation. Since the majority of signal adaptors of the TNFR1 pathway are ubiquitinated species, they may compensate for the loss of RIPK1 to facilitate IKK complex and NF-κB activation in *Ripk1*$^{-/-}$ cells [10].

The kinase activity of RIPK1, which is crucial for death receptor-mediated apoptosis and necroptosis, is dispensable for NF-κB activation [2, 3]. Interestingly, while germline *Ripk1*$^{-/-}$ mice suffer from post-natal lethality [4], knock-in mice expressing kinase-inactive RIPK1 are viable [11–13]. This indicates that RIPK1 has a unique function in organismal survival that is scaffold dependent, but kinase independent [14–17]. This is an emerging theme for RIPK1 and RIPK3 (see below): that they can promote inflammation through scaffold-dependent and necroptosis-independent mechanisms. In contrast to *Ripk1*$^{-/-}$ mice, mice lacking the canonical NF-κB subunit RelA/p65 die in utero on e15.5 [18], and deficiency of the non-canonical NF-κB subunit RelB did not compromise post-natal survival [19]. As such, RIPK1 mediates post-natal survival independent of NF-κB.

Scaffold-dependent signaling by RIPK1 is critical for survival of intestinal and skin epithelial cells and hematopoietic stem cells (HSCs) [6, 7, 16, 20]. Although RIPK1 is crucial for HSCs survival [16, 20], it is dispensable for survival of fully differentiated mature bone marrow-derived dendritic cells (BMDCs) ([21] and unpublished observation). Similarly, mature T cells from *Ripk1*$^{-/-}$*Fadd*$^{-/-}$ mice proliferated normally in response to viral pathogen challenge [22]. Hence, RIPK1 is dispensable once HSCs differentiate beyond a certain developmental checkpoint. A similar function for RIPK1 may also protect rapidly dividing tissues such as the skin and intestinal epithelium from cell death-induced inflammation.

RIPK3 and NF-κB activation

Because of its homology to RIPK1, early studies on RIPK3 also focused on its ability to induce apoptosis and NF-κB. Results from overexpression studies were confusing, with reports showing both an activating and inhibitory role for RIPK3 in NF-κB activation. For instance, RIPK3 inhibited NF-κB activation by the toll-like receptor 3 (TLR3) and TLR4 signal adaptor TRIF, TNFR1 and DNA activator of interferon (DAI) [23–26], but enhanced NF-κB activation in other studies [27, 28]. In mouse embryonic fibroblasts (MEFs) and bone marrow-derived macrophages (BMDMs), RIPK3 was reported to be dispensable for TNF- or TLR4-induced NF-κB activation [29, 30]. Hence, it was widely accepted that RIPK3 plays no major role in NF-κB activation. However, closer examination of the published results showed that although TNF, TLR2 and TLR4-induced IκBα phosphorylation and degradation was normal in *Ripk3*$^{-/-}$ cells, LPS-induced TNF, IL-6 and IL-1β expression and hypothermia were reduced in *Ripk3*$^{-/-}$ mice [30–32].

We re-evaluated the role of RIPK3 in NF-κB activation and found that RIPK3 expression in BMDCs is crucial for LPS-induced and NF-κB-dependent cytokine expression [33]. Consistent with results from MEFs and BMDMs, the initial LPS-induced IκBα phosphorylation and degradation was normal in *Ripk3*$^{-/-}$ BMDCs [30, 33]. However, LPS-induced nuclear translocation of the RelB-p50 heterodimer was severely impaired in *Ripk3*$^{-/-}$ BMDCs [33]. Strikingly, nuclear translocation of other NF-κB subunits was not affected. These results indicate that while RIPK1 facilitates the early phosphorylation and degradation of IκBα, RIPK3 regulates NF-κB activation downstream of IκBα in a cell type-specific manner. Thus, although RIPK1 and RIPK3 often act in synergy to promote cell death, they regulate NF-κB activation independently through distinct mechanisms.

How might RIPK3 regulate RelB-p50 nuclear translocation? Curiously, a recent report shows that RIPK1, RIPK3 and MLKL translocate to the nucleus during

necroptosis [34]. Nuclear RIPK3 has been detected in damaged neurons after ischemia–reperfusion-induced injury, a process believed to involve necroptosis [35]. This raises the tantalizing possibility that nuclear RIPK3 may control cell death. Several early studies showed that RIPK3 contains both nuclear localization and nuclear export signal sequences and could shuttle between the cytosol and the nucleus [36, 37]. Hence, RIPK3 may directly chaperone RelB-p50 dimer into the nucleus in response to TLR4 stimulation. Alternatively, RIPK3 may indirectly control RelB-p50 nuclear translocation through its molecular chaperone Hsp90, which binds to and regulates RIPK3-dependent necroptosis [38, 39]. In this regard, Hsp90 has been shown to regulate NF-κB nuclear translocation by stabilizing the upstream activators IKKs and IRAK-1 [40]. Hsp90 inhibitors are promising anti-cancer agents [41]. It will be interesting to determine whether Hsp90 inhibitors exert their anti-tumor effects by suppressing RIPK3-dependent necroptosis and NF-κB-dependent inflammatory gene expression.

Context is important: the mechanism of RIPK3-mediated inflammasome activation

Much of the work on necroptosis-independent signaling by RIPK3 has focused on its role in NLRP3 inflammasome activation. The inflammasome is a macro-molecular complex composed of the adaptor protein ASC, the IL-1β converting enzyme (ICE) caspase 1, and a sensor molecular such as NLRP3. Inflammasome activation results in caspase 1-mediated cleavage of pro-IL-1β and pro-IL-18, secretion of the mature cytokines, and a non-apoptotic form of cell death called pyroptosis [42, 43]. In contrast to LPS-primed BMDMs, which require a second inflammasome signal to secrete mature IL-1β, LPS alone is sufficient to induce IL-1β secretion in BMDCs [44]. This LPS-induced IL-1β secretion was completely abolished in *Ripk3*$^{-/-}$ BMDCs [21, 44]. Since pro-IL-1β protein expression was normal in LPS-primed *Ripk3*$^{-/-}$ BMDCs, RIPK3 regulates processing, but not de novo synthesis of IL-1β. RIPK3 promotes pro-IL-1β cleavage through the NLRP3 inflammasome as well as the ripoptosome, a macro-molecular apoptosis and necroptosis-inducing complex consisting of RIPK1, RIPK3, FADD and caspase 8 [45–47]. Importantly, kinase activities of RIPK1 and RIPK3 are dispensable and cell death was not detected under these conditions (Fig. 1a). Hence, in contrast to ripoptosome assembly during cell death, RIPK3 acts as a positive activator for caspase 8 during pro-IL-1β processing.

As in the case of necroptosis, RIPK3 and ripoptosome-mediated pro-IL-1β processing is tightly controlled by FADD, caspase 8 and the E3 ligase IAPs, cIAP1, cIAP2 and X-linked IAP (XIAP). Genetic inactivation or pharmacological depletion of the IAPs, especially XIAP, greatly enhanced IL-1β secretion in LPS-primed BMDMs [48, 49]. As in the case of LPS-induced BMDCs, an intact RIPK3, but not its kinase activity, is essential for IL-1β release under this condition (Fig. 1b). RIPK3 and the ripoptosome stimulate pro-IL-1β processing by turning on the NLRP3 inflammasome [50]. When caspase 8 activity is inhibited by caspase inhibitors, the ripoptosome recruits an additional component, the necroptosis effector MLKL, to promote IL-1β secretion (Fig. 1b). Strikingly, RIPK3 kinase activity is required for optimal IL-1β secretion when caspase 8 activity is compromised [50]. These results highlight the highly intertwined nature of the machineries that mediate necroptosis and NLRP3 inflammasome activation. However, *Asc*$^{-/-}$ and *Casp1*$^{-/-}$ macrophages are equally sensitive to LPS and zVAD-fmk-induced necroptosis as wild-type macrophages (unpublished observation). Thus, the ripoptosome and NLRP3 inflammasome are not interchangeable in function.

In addition to determining the mechanism of ripoptosome-induced NLRP3 inflammasome activation, caspase 8 also has a scaffolding function in inflammasome activation. In *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ and *Ripk3*$^{-/-}$*casp8*$^{-/-}$ BMDMs, ASC oligomerization, caspase 1 activation, and IL-1β secretion in response to the synthetic double-strand RNA poly(I:C), a TLR3 ligand, and ATP were abrogated [51]. This response was also abrogated in *Ripk1*$^{-/-}$, but not *Ripk3*$^{-/-}$ BMDMs. Hence, RIPK1, FADD and caspase 8 can drive NLRP3 inflammasome activation without RIPK3 (Fig. 1c). Several reports have indicated a role for RIPK3 in NLRP3 inflammasome activation in response to RNA virus infection [51, 52]. However, we have not been able to detect RIPK3-dependent IL-1β secretion in response to vesicular stomatitis virus infection (unpublished observation). Therefore, in BMDMs with intact caspase 8, RIPK3 appears to have minimal role in RNA-induced IL-1β release. When caspase 8 activity is inhibited by pharmacologic inhibitors, RIPK3 becomes an essential component for TLR3-induced NLRP3 inflammasome assembly (Fig. 1c). In addition to RIPK3, RIPK1 and MLKL are also required for this assembly. Although caspase 8 protease activity is dispensable for this activity, TLR3-induced NLRP3 inflammasome assembly was completely abolished in caspase 8-deficient cells. Hence, caspase 8 scaffold function is crucial for RIPK1–RIPK3–MLKL-mediated NLRP3 inflammasome activation (Fig. 1c).

In contrast to TLR3, TLR4-induced caspase 1 activation and IL-1β secretion were highly elevated in LPS-primed *Fadd*$^{-/-}$ or *caspase 8*$^{-/-}$ BMDCs [53, 54]. This is especially surprising given that FADD and caspase 8 have been implicated in transcriptional priming of pro-IL-1β and NLRP3 in *Fadd*$^{-/-}$*Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Ripk3*$^{-/-}$

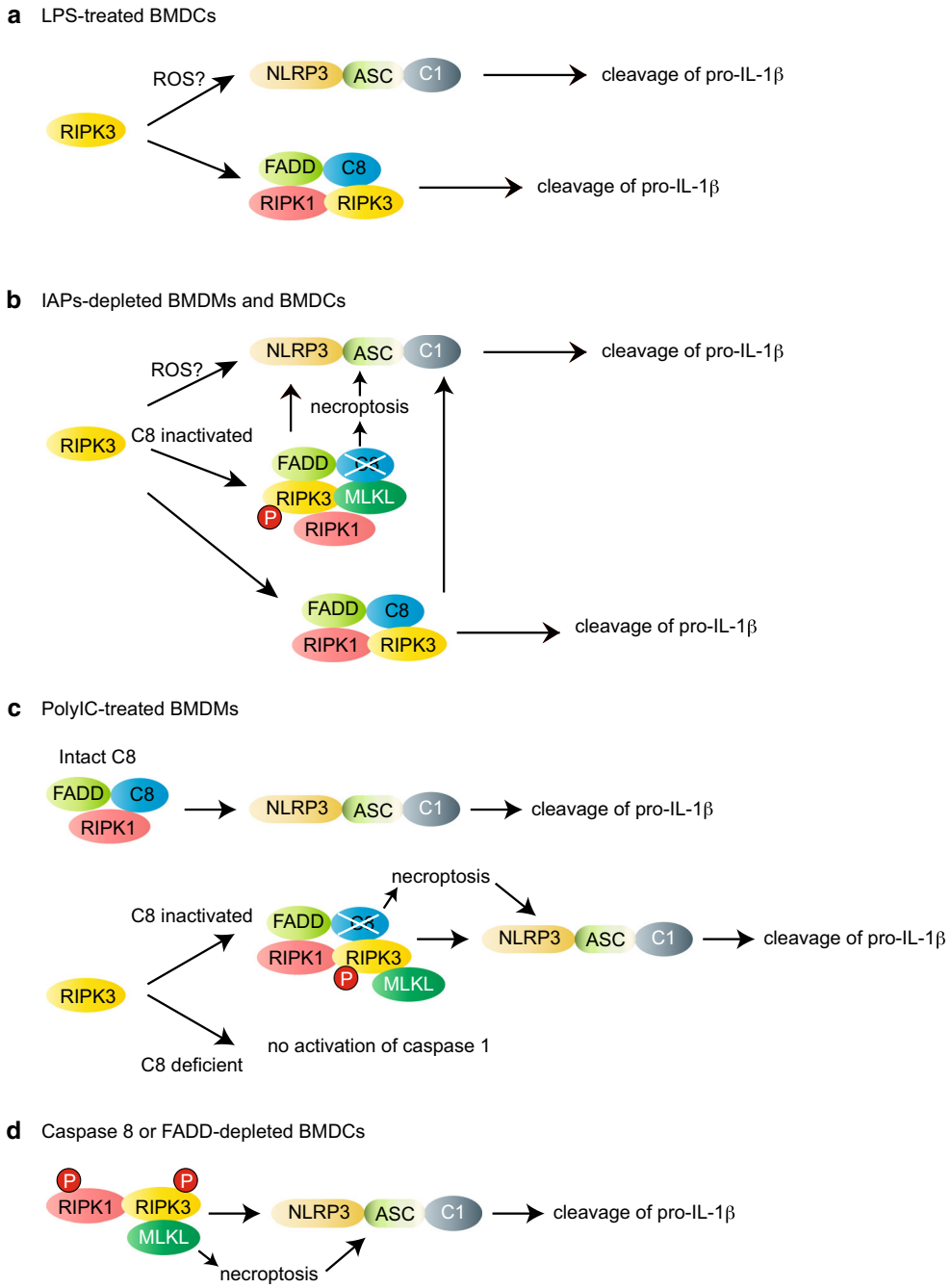

Fig. 1 The different modes of RIPK3-mediated NLRP3 inflammasome activation. **a** In BMDCs, RIPK3 promotes activation of caspase 1 (C1) in response to LPS alone, possibly through ROS production. In addition to caspase 1, RIPK3 promotes caspase 8 activation through the ripoptosome, which directly cleaves pro-IL-1β. **b** Depletion of IAP proteins induces IL-1β secretion through robust activation of caspase 1 and caspase 8 in LPS-primed BMDMs and BMDCs. In contrast to necroptosis, the kinase activities of RIPK1 and RIPK3 are dispensable for pro-IL-1β processing through caspase 1 and caspase 8. However, when caspase 8 (C8) activity is blocked, the RIPK3 kinase activity and MLKL becomes essential to stimulate the NLRP3 inflammasome activation. **c** In BMDMs, poly(I:C) treatment stimulates TLR3 and TRIF, leading to FADD, RIPK1 and caspase 8 (C8)-dependent NLRP3 inflammasome activation. RIPK3 is not required for this response. However, when caspase 8 activity is blocked, RIPK3 kinase activity and MLKL phosphorylation become essential for NLRP3 inflammasome activation. In addition, RIPK3-dependent NLRP3 inflammasome activation requires caspase 8 scaffold function, since TLR3 can no longer stimulate NLRP3 inflammasome when caspase 8 is missing. **d** LPS-primed caspase 8 or FADD-deficient BMDCs produce increased levels of IL-1β through enhanced caspase 1 activation. This response requires RIPK1 and RIPK3 kinase activities and MLKL. MLKL activation through the RIPK3 kinase activity may directly activate the NLRP3 inflammasome. Alternatively, MLKL might enhance necroptosis, leading to DAMPs release or K^+ efflux [102] and subsequent NLRP3 inflammasome activation. *P in a circle* indicates that the kinase activity of RIPK1 or RIPK3 is required

BMDMs [31, 55]. Increased IL-1β secretion by *Fadd*$^{-/-}$ and *casp8*$^{-/-}$ BMDCs requires RIPK1 kinase activity, RIPK3 and MLKL [53]. Thus, RIPK1–RIPK3–MLKL-driven inflammasome activation and IL-1β secretion can occur in the absence of FADD and caspase 8 in BMDCs (Fig. 1d). Taken together, these studies reveal the complex interplay between RIPK1/RIPK3 and FADD/caspase 8. In the context of necroptosis, caspase 8 acts as a natural inhibitor of RIPK1 and RIPK3 through its proteolytic activity. On the other hand, RIPK3 drives caspase 8 activation through assembly and activation of the ripoptosome in response to TLR3 and TLR4 stimulation [56]. The molecular basis that dictates cell type- and context-dependent ripoptosome and inflammasome activation is unknown at present, but is likely to be related to different expression and wiring of ripoptosome components in BMDMs versus BMDCs.

How does RIPK3 turn on the NLRP3 inflammasome?

Although the role of RIPK3 in NLRP3 inflammasome activation is well established, the underlying mechanism is undefined at present. Direct physical interaction between the ripoptosome and inflammasome components has not been reported, suggesting that RIPK3 regulates inflammasome activation in an indirect manner. Interestingly, reactive oxygen species (ROS) scavengers such as *N*-acetyl cysteine inhibit caspase 1, but not caspase 8 activation [33, 49]. Since mitochondrial ROS has been implicated in NLRP3 inflammasome activation [57], RIPK3 may indirectly promote NLRP3 inflammasome activation through stimulating mitochondrial ROS production.

As we have already discussed in previous sections, in the presence of intact FADD and caspase 8, RIPK1 and RIPK3 kinase activities are dispensable for ripoptosome-mediated pro-IL-1β processing. For example, BMDCs that express kinase-inactive RIPK1 or RIPK3 produced normal levels of IL-1β in response to LPS [21]. This is distinct from necroptosis, which critically depends on the kinase function of RIPK3. By contrast, an intact RHIM is required for RIPK3-dependent necroptosis, ripoptosome formation and NLRP3 inflammasome activation. The RHIM, or RIP homotypic interaction motif [58], is found in a select group of cell death/innate immune signal adaptors including TRIF, RIPK1, RIPK3, DAI, herpesvirus-encoded necroptosis inhibitors, and certain *Drosophila* immune deficiency (IMD) pathway adaptors [59–64]. During necroptosis, the RHIM mediates conformational change that leads to amyloid-like filament formation. This process is crucial for nucleating the ripoptosome complex [65]. Mutations in the tetra-peptide core of the RIPK3 RHIM domain abolished amyloid formation and TNF-induced necroptosis [29]. An intact RHIM is also required for LPS-induced ripoptosome activation and IL-1β secretion by BMDCs (unpublished observation), although it is not clear if amyloid conversion is also involved.

RIPK1 as an inhibitor of RIPK3

Although RIPK1 is widely known to act in synergy with RIPK3 to promote apoptosis, necroptosis and NLRP3 inflammasome activation, recent evidence indicates that RIPK1 can surprisingly inhibit RIPK3 activity in certain situations. Deletion of *Ripk1* in intestinal epithelium or skin epidermal tissues led to spontaneous cell death and inflammation [6, 7]. Inactivation of *Ripk3* in the skin epidermis rescued the *Ripk1* deficiency-induced inflammation, while dual inactivation of RIPK3 and FADD restored normal intestinal integrity in *Ripk1*-deficient mice [6, 7]. These results indicate that RIPK1 enforces barrier integrity by limiting RIPK3 activity. Germline *Ripk1*$^{-/-}$ mice suffer from post-natal mortality due to multi-organ cell injury and inflammation [4]. In contrast to the germline *Ripk1*$^{-/-}$ mice, knock-in mice expressing kinase-inactive RIPK1 are viable and do not exhibit increased cell death and inflammation [11–13]. Hence, while RIPK1 kinase activity is responsible for apoptosis and necroptosis, it is dispensable for its RIPK3 inhibitory effect. RIPK1-independent but RIPK3-dependent necroptosis has been observed in tissue culture experiments [66–68], although the precise mechanism by which RIPK1 inhibits RIPK3 activation is unknown at present.

Does necroptosis-independent signaling matter in tissue inflammation?

As discussed in other reviews in this issue, the current dogma predicates that RIPK3 drives tissue inflammation mainly through necroptosis-associated release of DAMPs, which subsequently trigger an inflammatory cytokine storm. Evidence that supports this model mainly comes from mouse studies in which multiple IAPs, FADD or caspase 8 are inactivated [69–72]. In these models, germline inactivation of RIPK3 was often sufficient to rescue the inflammatory conditions. With the realization that RIPK3 can promote inflammation through scaffold-dependent and kinase-independent mechanisms, it is high time for researchers to re-evaluate the relative contribution of these mechanisms to physiological inflammation [73].

One of the first examples of physiological/pathological necroptosis is found in vaccinia virus infection. Poxviruses such as vaccinia virus encode caspase inhibitors that can

skew the response from apoptosis to necroptosis (reviewed in [74]). Like other poxviruses, vaccinia virus causes tissue necrosis and inflammation that are eventually resolved in wild-type mice. Surprisingly, tissue necrosis and inflammation was significantly reduced in infected *Ripk3*$^{-/-}$ mice. This led to highly elevated levels of viral load and eventual death of *Ripk3*$^{-/-}$ mice [29]. In contrast to vaccinia virus, a growing number of studies show that herpes viruses actively suppress RIPK3-dependent necroptosis through the viral inhibitor of RIP kinase activation (vIRA) [59, 60, 75, 76]. The equine herpesvirus encoded inhibitor E8 and Kaposi sarcoma virus encoded K13, which are viral inhibitors of caspase 1 and caspase 8, also inhibited TNF-induced necroptosis [77]. Hence, herpesviruses have developed multiple strategies to counteract the anti-viral effects of necroptosis. Interestingly, the human poxvirus cell death inhibitor MC159 also potently inhibited TNF-induced necroptosis [77, 78]. Therefore, sensitization to necroptosis may not be a common feature for all poxviruses.

Inhibition of necroptosis is not restricted to virus infections. In tissue culture experiments, necroptosis induction requires inhibition of the cIAPs and FADD/caspase 8. In addition, the TNF receptor signal adaptors TRAF2, TAK1, IKKs and NEMO have also been shown to restrict necroptosis [79–83]. The multiple inhibitory mechanisms argue that physiological necroptosis requires the "perfect storm" in which all these regulatory checkpoints are compromised. This is certainly a high bar to reach under normal circumstances. Genetic studies tell us that these cellular necroptosis inhibitors are critical for organismal survival. In fact, deficiency of these molecules in specific tissues is often sufficient to cause deleterious inflammation. These observations argue that physiological necroptosis is a rare occurrence.

An examination of the role of RIPK1 and RIPK3 in lymphocytes also suggests that necroptosis may not always result in inflammation. Mice lacking FADD or caspase 8 and RIPK3 developed systemic autoimmune lymphoproliferation that resembles human lupus and mice with Fas or Fas ligand (FasL) mutations [22, 84–87]. Hence, caspase-dependent apoptosis and RIP kinase-mediated necroptosis cooperate to regulate lymphocyte homeostasis. By eliminating activated, cytokine-producing lymphocytes, one can consider RIPK1/RIPK3-dependent necroptosis as an anti-inflammatory response [88]. As such, we propose an alternative model in which the main mechanism by which RIPK3 promotes inflammation is through NF-κB-dependent cytokine gene transcription and ripoptosome/inflammasome-mediated pro-IL-1β processing. When key components of this pathway are disrupted, such as that found in certain virus infections and mice lacking FADD, caspase 8 or cIAPs, necroptosis is activated as a last resort to tamp down the collateral damage from a hyperactive inflammatory response. The concept that RIPK1 and RIPK3 have "day jobs" other than necroptosis is not new among cell death signal adaptors. Members of the Bcl-2 family, for instance, have been shown to regulate diverse cellular functions such as glucose and mitochondrial metabolism [89], regulation of calcium signaling [90] and autophagy [91].

Concluding remarks

To distinguish the contribution of necroptosis-dependent and independent signaling by RIPK1 and RIPK3 in inflammation, we need better knowledge on how these kinases are activated under different conditions. Phosphorylation sites on RIPK1 and RIPK3 that are important for necroptosis have been identified [8, 92–94]. In addition, phospho-MLKL and phospho-RIPK3 antibodies have been developed [94–97]. These reagents will be useful tools in distinguishing the mode of RIPK3 activation during necroptosis-independent signaling. In addition to benefiting basic science, a holistic understanding of the biology and mechanism of RIP kinase activation is important for potential therapeutic targeting of these molecules in inflammatory diseases. In this regard, RIPK1 and RIPK3 inhibitors have been developed [56, 95, 98–101]. Developing inhibitors that can target additional inflammatory pathways beyond necroptosis may magnify therapeutic potential of RIP kinase-targeted therapies.

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