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StIKKing it to a death kinase: IKKs prevent TNF-α-induced cell death by phosphorylating RIPK1

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ABSTRACT

Signaling pathways activated by the cytokine TNF-α are among the most intensively studied and well-understood in all mammalian biology. In a simplistic model, two primary signals emanate from the TNF-α receptor, one that activates cell survival via an NF-κB transcriptional response and a second that triggers cell death when cell survival signals are neutralized. The kinase RIPK1 participates in both these axes, and its poly-ubiquitylation was thought to represent the primary mechanism by which it toggles between survival versus death signaling. When RIPK1 is ubiquitylated, it acts non-enzymatically as an adaptor protein in IKK recruitment and subsequent NF-κB activation; when ubiquitylation of RIPK1 is prevented, it functions as a cell death kinase capable of triggering apoptosis or necroptosis. Bertrand and colleagues (Dondelinger et al., 2015) now demonstrate that phosphorylation of RIPK1 represents an additional mechanism by which this protein switches between its life and death duties. They show that both IKK-α and IKK-β phosphorylate RIPK1, dampening its capacity to assemble the death effectors FADD and caspase 8 into a functional pro-apoptotic signalsome. These IKKs also protect against RIPK1-mediated necroptosis. Importantly, IKK-α/β prevent RIPK1-driven cell death independently of NF-κB transcriptional responses. These findings identify phosphorylation of RIPK1 by IKKs as a new mechanism by which cell fate decisions downstream of TNFR1 are regulated.

RIPK1 was identified twenty years ago as a serine/threonine kinase that interacted with the cytoplasmic tails of death receptors Fas/CD95 and TNFR1 [1]. In initial overexpression studies, RIPK1 was found to be a potent inducer of apoptotic cell death; indeed, this protein was originally called RIP, for 'receptor-interacting protein', but also in acknowledgment of its cytotoxic properties in cell culture [1]. Subsequent work, however, revealed that RIPK1 can also regulate NF-κB and was likely required for NF-κB activation downstream of the TNFR1 [2]. Curiously, NF-κB activation was found not to require the kinase activity of RIPK1 [3]. Germline ablation of the *ripk1* gene in mice resulted in perinatal lethality and confirmed a role for this protein in TNF-α-induced NF-κB activation [4]. Moreover, *ripk1*-null cells were found to be more susceptible to TNF-α-induced apoptosis than wild type controls, indicating that a cell-survival role for RIPK1 was probably dominant over any cell death function this kinase may have in TNF-α-induced cell fate responses [4]. As a consequence, subsequent studies focused on how RIPK1 participated in transducing the TNFR1-initiated NF-κB signal, and investigations into its death-activating capacity fell by the wayside, with a few notable exceptions [5–7]. In these studies, RIPK1 was shown to mediate a novel caspase-independent form of programmed necrosis downstream of death receptors. Intriguingly, and in contrast to its role in NF-κB signaling where its enzymatic activity is dispensable, the kinase function of RIPK1 was required for induction of necrotic cell death.

But it was to be several years before the ramifications of these findings became apparent. The Yuan laboratory had identified a class of small-molecule inhibitors (the 'necrostatins') for death receptor-driven caspase-independent cell death, which they termed 'necroptosis' [8]. In 2008, they reported that RIPK1 was the kinase target of one of these inhibitors, necrostatin-1 [9]. Shortly thereafter, in 2009, the kinase RIPK3 was discovered as an essential mediator of necroptotic death [10–12], and a flurry of subsequent activity has helped lay bare the outline of necroptotic cell death signaling downstream of TNF-α. Under certain conditions, such as when caspase 8 or certain members of the cIAP class of E3 ubiquitin ligases are inhibited, TNF-α stimulation drives the assembly of RIPK1–RIPK3 phospho-complex called the 'necrosome'. Within the necrosome, RIPK3 then phosphorylates the pseudokinase MLKL. Once phosphorylated by RIPK3, MLKL oligomerizes, translocates to

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the plasma membrane and to other membranes within the cell, where it triggers necrotic sequelae, ostensibly by puncturing holes in these membranes and disrupting their integrity [13]. Necroptosis downstream of TNF-α is initiated by the kinase activity of RIPK1: at long last, the kinase function of RIPK1 had a role in TNF-α signal transduction and the cell death-inducing capacity of this molecule was back in the limelight, almost two decades after its discovery.

A current model of TNF-α signaling that builds on early observations by Goeddel and colleagues [14,15], later paradigmatic work by Micheau and Tschopp [16], and these new discoveries in necroptosis, reads as follows. TNF-α stimulation activates two sequential signaling complexes, the first of which is rapidly assembled at the receptor and mediates NF-κB activation. This complex, termed complex I, contains TRADD, TRAF2, RIPK1, the E3 ubiquitin ligases cIAP1, cIAP2 and LUBAC, and signals cell survival. The second complex, complex IIa, forms more slowly in the cytosol, contains TRADD, RIPK1, FADD and caspase 8, and can drive apoptosis. The NF-κB target cFLIP is a key determinant of whether or not complex II activates apoptosis: when NF-κB is transcriptionally active and cFLIP levels are high, caspase 8 in inhibited and apoptosis is blocked. When NF-κB activity is diminished and/or cFLIP levels are reduced, caspase 8 is active and the cell dies by apoptosis upon exposure to TNF-α. Curiously, RIPK1 is a member of both complexes; while its presence as an adaptor protein in complex I is essential for induction of NF-κB, it is entirely dispensable for the slow, NF-κB-inhibitable apoptosis triggered by complex IIa. So what then is RIPK1 doing in complex IIa? Under certain conditions, such as when cIAPs are depleted or the kinase TAK1 is inhibited, a cytosolic RIPK1/FADD/caspase 8 containing death complex is assembled that can activate rapid apoptosis dependent on the kinase activity of RIPK1. This complex is termed complex IIb, to differentiate it from the TRADD-containing complex IIa which induces slow apoptosis without need for RIPK1. And when caspase 8 or FADD is additionally neutralized in complex IIb, RIPK1 now associates with RIPK3 and MLKL to form the necrosome and drive necroptosis. Thus, RIPK1, when in complex I, is a promoter of cell survival, but when a part of complex IIb or the necrosome, is a death activating kinase [17,18].

Non-degradative ubiquitylation has emerged as a major determinant of whether RIPK1 functions as an NF-κB signaling adaptor protein in complex I or cell death kinase from complex IIb or the necrosome [18,19]. In complex I, the E3 ubiquitin ligases TRAF2, cIAP1 and cIAP2 attach K63-linked poly-ubiquitin chains onto RIPK1 to initiate the NF-κB activation signal. These poly-ubiquitin chains then serve as docking sites for the assembly of a signalsome containing TABs, NEMO, and the kinases TAK1, IKK-α, IKK-β. From within this signalsome, TAK1 stimulates the IKK complex by phosphorylating IKK-β, which then phosphorylates I-κBα to activate NF-κB [20]. When RIPK1 ubiquitylation is reversed or prevented, such as by the activity of deubiquitylases like CYLD, or by the depletion of cIAPs with SMAC mimetics, RIPK1 switches from an NF-κB activating scaffold protein to a death promoting kinase [17,18].

RIPK1 is ubiquitylated within seconds of TNFR1 activation, explaining why most cell types respond to TNF-α by activating a canonical NF-κB transcriptional response, rather than by inducing apoptosis or necroptosis. Indeed, it is very probable that additional rapid (i.e., non-transcriptional) mechanisms exist which allow the cell to survive TNF-α. After all, cell-survival via NF-κB activation requires gene *de novo* gene expression, while all cell death components are present *a priori*. So why then is cell death not the default outcome upon exposure to TNF-α? This conundrum, originally enunciated by Natoli and colleagues [21] and termed the 'NF-κB paradox' by O'Donnell and Ting [22], will have its likely resolution in the discovery of mechanisms (such as RIPK1 ubiquitylation) that rapidly inactivate death effectors before the NF-κB-mediated cell-survival program takes effect. In support of this idea, Ting and colleagues have demonstrated that binding of NEMO to ubiquitylated RIPK1 blocks its association with caspase 8 and subsequent assembly of complex IIb [23], while Bertrand and colleagues have previously shown that TAK1 similarly prevents premature assembly of complex IIb independently of its role in NF-κB activation [24].

During the course of their analysis of how TAK1 prevented assembly of complex IIb, Bertrand and colleagues observed that TAK1 blocked the shuttling of RIPK1 from complex 1 into complex IIb [24]. When TAK1 was inhibited, RIPK1 migrated into complex IIb but, surprisingly, did so without first becoming deubiquitylated in complex I. Realizing that ubiquitylation was not the sole means by which RIPK1 is prevented from inducing cell death, and that TAK1 likely protected cells from RIPK1-mediated cytotoxicity by mechanisms other than simply regulating its ubiquitylation, Bertrand and colleagues sought to identify additional mechanisms by which RIPK1 is held in check in complex I.

In the current study [25], Bertrand and colleagues examined TNF-α mediated cell death in cells from IKK-α/β- or NEMO-deficient mice, or in cells in which IKK-α/β activity was pharmacologically inhibited, and found that in each of these cases, TNF-α-treated cells underwent rapid RIPK1 dependent apoptosis. Cell death under these conditions required the kinase activity of RIPK1, but proceeded independently of downstream NF-κB pro-survival signaling. The authors noticed that RIPK1 was phosphorylated within complex I of TNF-α-treated control wild type cells, but not in cells lacking IKK-α and IKK-β. As the IKK complex physically associates with RIPK1, the authors hypothesized that IKK-α and IKK-β likely mediate their NF-κB-independent survival effects by directly phosphorylating RIPK1, and set about testing this hypothesis in a series of biochemical studies. Bertrand and colleagues demonstrate that both IKK-α and IKK-β can directly phosphorylate RIPK1 *in vitro*, and that phosphorylation of RIPK1 prevents it from integrating into cytosolic complex IIb. They also demonstrate that ubiquitylation of RIPK1 is required for its phosphorylation by IKKs, but that phosphorylation of RIPK1 is not a requisite for its ubiquitylation. Thus, ubiquitylation and phosphorylation appear to be sequential events that regulate the RIPK1 cell death checkpoint. In a model that integrates their findings with previous work, RIPK1 is first ubiquitylated in complex I, creating binding sites for TABs and NEMO. These ubiquitin binding proteins then recruit, respectively, TAK1 and IKK-α/β, following which TAK1 phosphorylates IKK-β to activate the IKK signalsome. Once activated, IKK-β and IKK-α then phosphorylate RIPK1 (Fig. 1).

To examine the physiological consequence of RIPK1 phosphorylation by IKKs, the authors treated wild type and RIPK1 kinase-dead mice with a combination of TNF-α and an IKK inhibitor. Remarkably, all wild type mice succumbed to this treatment, while RIPK1-kinase-dead animals were protected. Interestingly, treatment of wild type, but not RIPK1 kinase-dead mice, with this combination resulted in high levels of circulating LDH, a marker for necrosis. These observations led the authors to test if phosphorylation of RIPK1 by IKKs not only protected cells from apoptosis, but also from programmed necrosis (or necroptosis). They found that IKK blockade also sensitized cells and mice to RIPK1 kinase-driven necroptosis, again independently of NF-κB, suggesting that phosphorylation by IKKs is likely a universal repressor mechanism of RIPK1-mediated cell death (Fig. 1).

Bertrand and colleagues have identified phosphorylation by IKKs as a critical new mechanism for RIPK1 regulation, with significant implications to our understanding of TNF-α signaling. Their findings set the stage for a comprehensive analysis of which residues in RIPK1 are phosphorylated by either or both IKKα and IKKβ, studies that will be necessary to reveal the details of how IKKs control RIPK1, as well as the significance of RIPK1 phosphorylation in