Ripk1 and haematopoiesis: a case for LUBAC and Ripk3

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Although long recognized as a component of inflamed tissues, the potential role of cell death as an active component that contributes to tissue homeostasis, inflammation and disease pathogenesis has only recently gained attention (1). The notion that certain cell death regulatory components are hard-wired into inflammatory signalling pathways, such as those emanating from the TNF-receptor or Toll-related receptors, indicate that such death components act as positive regulators of tissue homeostasis, replacing malfunctioning or damaged tissues and enhancing the resilience of epithelia to tissue stress (1, 2).

A number of dedicated sensors have evolved to detect different stressors and induce appropriate adaptive responses. Receptor-interacting serine/threonine-protein kinase 1 (Ripk1) represents such sensor, functioning as key signalling node in regulating adaptive responses to tissue stress (1). Ripk1 is activated in response to a variety of input signals, including those generated in response to TNF family cytokines, ligands for some Toll-like receptors (TLRs), pattern recognition receptors for viral infection, and interferons. Once activated, Ripk1 can signal for activation of MAP kinases, NF-κB and apoptotic as well as necrotic cell death (necroptosis and pyroptosis) (1). Ripk1 mediates these effects in a kinase-dependent and/or scaffold-dependent manner. While the scaffold function of Ripk1 is necessary to maintain tissue homeostasis (1), the kinase activity of Ripk1 is required for the induction of necroptosis, and, depending on the cellular context, apoptosis (3, 4). The kinase and scaffolding functions of Ripk1 are tightly controlled by ubiquitylation, phosphorylation, and caspase-mediated cleavage that regulate the integration of Ripk1 into distinct multi-protein signalling complexes (1, 5, 6). Deregulation of Ripk1 has detrimental consequences as loss- or gain-of-function of Ripk1 is associated with organismal lethality, aberrant cell death or chronic inflammation. Understanding how Ripk1 is regulated and how it initiates and escalates cell death and inflammation has important implications for normal development and disease pathologies, such as chronic inflammation and

cancer. But it also offers novel opportunities for immuno-oncology as Ripk1 and NF-kB signalling is required for antigen cross-priming of CD8+ T cells (7).

In recent years, much has been learned about checkpoints of RIPK1 regulation, and the pathophysiological consequences of their deregulation. In the current issue of *Nature*, Peltzer and co-workers identified two new intricate pieces of the puzzle (2). They discovered that the LUBAC component Hoil-1 is essential to prevent aberrant Ripk1-dependent cell death. And more unexpectedly, they uncovered a novel cell death-independent signalling output of Ripk1 that disrupts haematopoiesis in the developing embryo, and which is negatively regulated by LUBAC and Ripk3.

LUBAC is a tripartite E3 ligase complex composed of Hoip, Hoil-1 and Sharpin, capable of synthesising linear ubiquitin chains on target substrates (2). The E3 ligase activity of LUBAC is required for optimal gene activation of pro-inflammatory genes downstream of innate immune receptors, such as TNFR1 and TLRs. Interfering with LUBAC activity not only delays the gene activation program but also unleashes the cytotoxic potential or Ripk1 kinase, which is thought to be one of the central LUBAC protein substrates. Genetic deletion of the LUBAC catalytic subunit *Hoip* was reported years back by Peltzer et al. to cause embryonic lethality at mid-gestation due to TNF-mediated cell death of endothelial cells, leading to vasculature defects (8). In the last issue of Nature, Peltzer et al. expand these studies and demonstrate that, similarly to *Hoip*, genetic deletion of *Hoil-1* also causes embryonic lethality at mid-gestation due to death of endothelial cells of the yolk sac (9). Although this phenocopies loss of *Hoip*, the lethality of *Hoil-1* deficient mice was unexpected, first because Hoil-1 E3 ligase activity is dispensable for LUBAC activity *in vitro* (10), and second because Tokunaga et al. previously showed that *Hoil-1* deficient mice are viable (11). Peltzer et al. demonstrated that while loss of Hoil-1 catalytic domain has no impact on LUBAC activity, the absence of the UBL domain thwarts LUBAC assembly and its subsequent recruitment into the Tnfr1 signalling complex. Consequently, in the absence of LUBAC, TNF stimulates Ripk1-mediated cell death (9).

The fact that *Ripk1* kinase inactive mutant mice rescued the early lethality of *Hoil-1* deficient mice is consistent with LUBAC being a negative regulator of RIPK1 kinase activity. However, this rescue was only partial, delaying death from mid- to late gestation. *Hoil-1*$^{-/-}$ embryos still died due to persistent endothelial cell death at late gestation. To address whether Ripk1 kinase-independent cell death could contribute to the lethality of *Hoil-1*$^{-/-}$ embryos at late gestation, the authors co-deleted *Ripk3* and *Caspase-8* in *Hoil-1* null mice to abrogate both apoptosis and necroptosis. Surprisingly, co-deletion of *Ripk3* and *Caspase-8* did not confer

any survival advantage when compared to Ripk1 kinase inactivation, although cell death was blocked. Even more surprisingly, co-deletion of *Caspase-8* and *Mlkl* allowed *Hoil-1* null mice to be born and survive post the age of weaning. This unexpected difference between *LUBAC/Ripk3* (lethal) and *LUBAC/Mlkl* (viable) double-mutants prompted the authors to initially postulate that Mlkl might be activated independently of Ripk3 when LUBAC is missing. However, ablation of *Mlkl* in *Hoil-1$^{-/-}$Caspase-8$^{-/-}$Ripk3$^{-/-}$* mice failed to prevent embryonic lethality. Therefore, the absence of Ripk3, rather than the presence of Mlkl, was detrimental to LUBAC mutant embryos. This led the authors to conclude that Ripk3 must possess a previously unrecognised, pro-survival function, which springs into action when both Hoil-1 and Caspase-8 are missing. Interestingly, *Hoil-1$^{-/-}$Caspase-8$^{-/-}$Ripk3$^{-/-}$* embryos had severe early haematopoietic defects, indicating that Ripk3 ensures proper haematopoiesis in the absence of LUBAC and Caspase-8. To elucidate the target of Ripk3's pro-survival action, Peltzer and co-workers focused on Ripk1, since Ripk1 and Ripk3 bind to one another via their respective RHIMs (RIP homotypic interaction motif), and because Ripk1 is a key substrate of LUBAC. The quadruple *Hoil-1$^{-/-}$Caspase-8$^{-/-}$Ripk3$^{-/-}$Ripk1$^{-/-}$* mouse finally solved the mystery. This quadruple KO mouse is indeed viable. This suggested that in the absence of Ripk3 and Hoil-1, Ripk1 exerts lethal functions during embryonic development by interfering with early haematopoiesis. Importantly, this lethal function seems to be independent of the kinase activity of Ripk1.

Peltzer et al. therefore discovered a novel function for Ripk1. In the absence of Hoil-1 and Ripk3, Ripk1 acts as a negative regulator of haematopoiesis during embryonic development. The fact that neither *Hoil-1* null mice nor *Caspase-8$^{-/-}$Ripk3$^{-/-}$* exhibit defects in haematopoiesis suggests that Hoil-1 and Ripk3, individually, are sufficient to suppress this function of Ripk1. Mechanistically, it is not entirely clear how this works. While it is clear that the kinase activity of Ripk1 is not required for the new signalling role of Ripk1, it remains to be determined how Ripk1 impairs haematopoiesis via its scaffolding function. One possibility put forward by the authors is that deregulated Ripk1 drives ectopic cytokine production, which in turn interferes with haematopoiesis. In agreement with such a hypothesis, *Hoil-1$^{-/-}$Caspase-8$^{-/-}$Ripk3$^{-/-}$* animals harbour elevated levels of various cytokines. This is also consistent with a recent report from David Wallach's group, indicating that *Caspase-8$^{-/-}$Ripk3$^{-/-}$* embryos exhibit aberrant Ripk1-mediated cytokine production, albeit this does not result in pathological changes (12). According to this scenario, the high levels of cytokines of *Caspase-8$^{-/-}$ Ripk3$^{-/-}$* embryos would even further increase if *Hoil-1* is co-deleted, removing the last inhibitory stop on Ripk1. This might ultimately lead to pathological levels of cytokines that could impair haematopoiesis. Clearly, further work is needed to test this hypothesis. Moreover, it remains enigmatic how LUBAC suppresses Ripk1 activity in the absence of Ripk3 and Caspase-8. While a direct regulation (LUBAC-

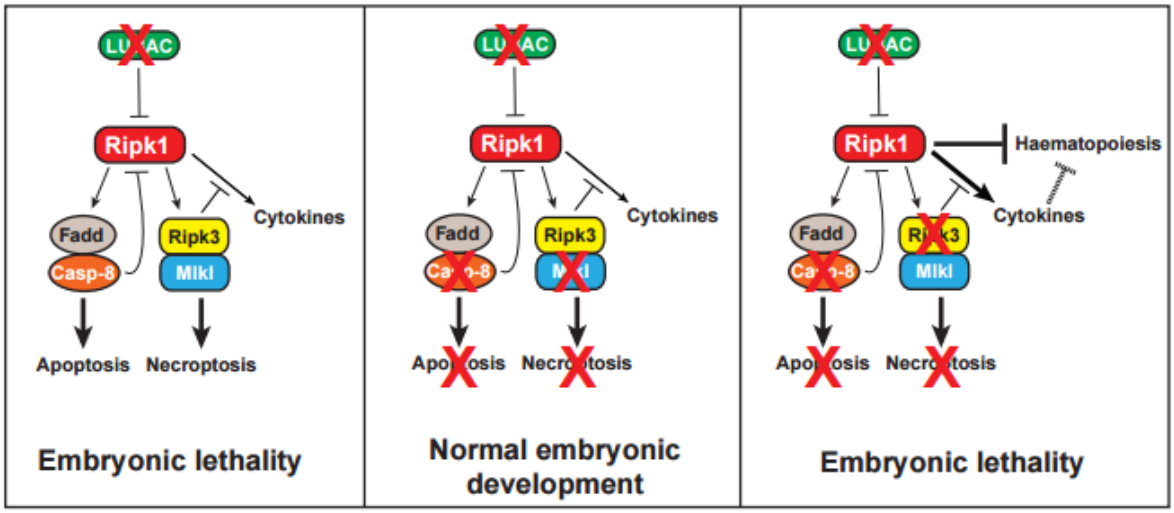
mediated ubiquitylation of Ripk1) is possible, given that LUBAC also controls optimal activation of IKKβ and NF-κB-mediated gene expression, it is equally likely that LUBAC regulates Ripk1 activity indirectly, e.g. in a phospho- or transcription-dependent manner. Further, it remains unclear how Ripk1 is activated under these settings. Despite these open questions, the findings of Peltzer and colleagues clearly shed new light on the possible functions of Ripk1.

Given that Ripk1 and NF-κB signalling are required for antigen-cross priming of CD8+ T cells (7), a better understanding of Ripk1 regulation might also help to harness Ripk1 function for therapeutic purpose, particularly in the field of immunotherapy. Various cancer types loose expression of Ripk3 and/or acquire Caspase-8 inactivating mutations (13-15). Therefore, inactivating LUBAC might drive Ripk1-mediated cytokine production. Combining LUBAC inhibition with cell death inducing treatments might be a potentially effective approach to induce immunogenic cell death of malignant cells lacking Ripk3 and/or Caspase-8 activity. This might in turn mobilise the immune system against malignant cells and lead to durable anti-tumour immune responses.

Figure 1

Models depicting LUBAC- and Ripk-mediated regulation of embryonic development. The absence of LUBAC results in Ripk1/Ripk3/Caspase-8-dependent death of the embryo. Co-deletion of Caspase-8 and Mlkl restores embryonic development. However, when LUBAC, Caspase-8 and Ripk3 are mutated, Ripk1 drives ectopic activation of cytokine production, which might impede early haematopoiesis, leading to Ripk1-mediated embryonic lethality.

Figure 1


Ripk1
Fadd
Casp-8
Ripk3
Mlkl
Cytokines
Apoptosis
Necroptosis
Embryonic lethality
Ripk1
Fadd
Ripk3
Cytokines
Normal embryonic development
Ripk1
Haematopoiesis
Fadd
Ripk3
Mlkl
Cytokines
Embryonic lethality

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