Checkpoints in TNF-induced cell death: implications in inflammation and cancer

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Summary

Tumor Necrosis Factor (TNF) is a pro-inflammatory cytokine that coordinates tissue homeostasis by regulating cytokine production, cell survival and cell death. However, how life and death decisions are made in response to TNF is poorly understood. It is now recognized that many inflammatory pathologies are driven by aberrant TNF-induced cell death, which, in most circumstances, depends on the kinase RIPK1. Recent advances have identified ubiquitin-mediated phosphorylation of RIPK1 as crucial checkpoints for cell fate in inflammation and infection. A better understanding of these checkpoints could lead to new approaches for the treatment of chronic inflammatory diseases that are fuelled by aberrant RIPK1-induced cell death, and/or reveal novel strategies for anti-cancer immunotherapies that harness RIPK1's ability to trigger immunogenic cell death.

The two faces of TNF

Mammalian tissue repair following injury or infection critically depends on the coordination of cell death and inflammation. Dying cells can release a broad spectrum of highly conserved patterns (molecules) that are sensed by specialized Pattern Recognition Receptors (PPRs), which subsequently trigger the induction of pro-inflammatory cytokines and chemokines [1]. Together, the release of cytokines, chemokines and damage associated molecular patterns (DAMPs) help to direct repair programs aimed at restoring tissue homeostasis [2].

Tumor Necrosis Factor (TNF) is one of the most important cytokines that contribute to such homeostatic responses as it acts as a master regulator of inflammation and is a key player in the cytokine network that ensures tissue homeostasis [3]. Originally, TNF was identified for its ability to induce rapid hemorrhagic necrosis of tumors in humans and mice [4]. The early hope that TNF could be harnessed as an anti-cancer cytokine was soon replaced with the realization that TNF is a major mediator of inflammation [5]. The key role of chronic TNF in mounting inflammatory responses has been demonstrated by the capacity of TNF-blocking agents, such as Infliximab, to ameliorate inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease and psoriasis [6-8]. Many of the pro-inflammatory diseases that are driven by TNF are not actually caused by its ability to induce NF-κB- and MAPK-dependent expression of pro-inflammatory genes, but rather, are due to chronic TNF-induced pro-inflammatory cell death [9]. This viewpoint is supported by the evidence that TNF-driven systemic inflammatory conditions in mice can be rescued by the genetic ablation of cell death components [10-12]. Cells that die in response to TNF are known to release a plethora of cytokines, chemokines, and damage associated molecular patterns. Consequently, death by TNF rapidly activates inflammatory receptors on, or in, surrounding cells, ultimately fuelling the vicious cycle of chronic inflammation [13].

There are a number of checkpoints that determine whether TNF can lead to cell killing or whether it stimulates transcriptional responses and cell survival [14] (Figure 1, Key Figure). The Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) represents a key node in the TNF signal transduction pathway, actively controlling

the balance between gene activation and cell death induction in the form of apoptosis and necroptosis [15], a recently discovered caspase-independent form of cell death. RIPK1 not only operates downstream of TNF, but also acts as a master regulator of cell fate under diverse cellular stress settings including cytokine-induced, chemical and metabolic stress [16] (Figure 2). It acts as molecular switch that can induce inflammation and cell survival, as well as apoptosis and necroptosis [17]. The activities of RIPK1 are tightly controlled by complex post-translational modifications that regulate the integration of RIPK1 into distinct multi-protein signalling complexes [15] (Figure 2). In recent years, much has been learned about checkpoints of RIPK1 regulation, and the pathophysiological consequences of their deregulation. Insight into these checkpoints has not only lead to an improved understanding of disease pathogenesis, but also has opened up new avenues for therapeutic interventions. Ubiquitylation, phosphorylation, and caspase-mediated cleavage are some of the post-translational mechanisms that are implicated in the control of the cytotoxic potential of RIPK1 (Figure 2). Here, we review current progress on the different checkpoints of RIPK1. Since ubiquitin (Ub)-mediated regulation of RIPK1 has been covered extensively in excellent reviews elsewhere [15], we predominantly focus on recent advances that reveal the phosphorylation-dependent regulation of RIPK1 kinase. Knowing how to activate and inhibit the killing potential of RIPK1 is clinically important as it offers new ways to overcome treatment resistant cancers and stop chronic inflammatory diseases, respectively.

RIPK1 is a key effector of TNF signalling

The serine/threonine kinase RIPK1 harbors an N-terminal kinase domain, a 250 amino acid long linker region or intermediate domain (ID), and a C-terminal RIP

homotypic interaction motif (RHIM) and Death Domain (DD) (Figure 2). RIPK1 has important kinase-dependent and scaffolding functions that can trigger or inhibit cell death. While the Ser/Thr kinase activity of RIPK1 is essential for necroptosis [18], RIPK1-dependent apoptosis displays stimulus- and cell type-specific requirements of its kinase activity [19, 20]. In contrast to its role in inducing cell death, RIPK1's kinase activity is entirely dispensable for the pro-survival function of RIPK1 [21]. Consistently, *Ripk1*-kinase dead knock-in animals are born and develop normally, while *Ripk1*-deficient mice die at birth due to spontaneous caspase-8-mediated apoptosis and RIPK3-dependent necroptosis [22-24]. Moreover, conditional deletion of RIPK1 in skin and intestinal epithelial cells results in homeostasis alteration as a consequent of aberrant cell death, causing premature death of the mice [25, 26]. This suggests that the scaffolding function of RIPK1, rather than its kinase activity, is required to curb aberrant cell death in mouse. RIPK1 promotes cell survival by two mechanisms: First, RIPK1 helps to recruit cellular FLICE-inhibitory protein (cFLIP) to caspase-8, which results in sub-lethal activation of caspase-8 and subsequent cleavage and inactivation of RIPK1 and RIPK3 [23, 27], therefore preventing apoptosis and necroptosis. Second, RIPK1 can contribute to timely activation of NF-κB and the production of pro-survival genes, such as cFLIP, A20, cellular inhibitor of apoptosis 2 (cIAP2) and Bcl2 family members [28]. RIPK1-mediated activation of NF-κB also does not require RIPK1 kinase activity, but rather relies on ubiquitylation of the ID of RIPK1 [29]. Accordingly, loss of RIPK1 ubiquitylation at lysine 377, located in the ID domain, abolishes NF-κB activation in several cell types such as Jurkat and L929 [29, 30]

RIPK1-mediated death of tumor cells

Recent studies have revealed important functions for RIPK1 kinase-mediated necroptosis in inflammation, and suggested that it could be implicated in the pathogenesis of many human inflammatory diseases. For instance, the pathological features of experimental inflammatory diseases in mice, such as psoriasis, dermatitis, septic shock, kidney and heart ischemia reperfusion injury, are ameliorated or even prevented using pharmacological inhibitors or genetic inactivation of the kinase activity of RIPK1 [21, 31]. Influenced by these studies, a selective pharmacological inhibitor of RIPK1, GSK2982772, has been developed and is currently undergoing Phase 2a clinical trials for some of these inflammatory diseases, such as psoriasis and inflammatory bowel disease [32]. While inhibition of RIPK1 activity appeared to be beneficial in the above-mentioned diseases, boosting RIPK1's cytotoxic potential might be useful in conditions where immunogenic cell death is a desired outcome, as in the case of cancer. Indeed, recent mouse tumor studies of leukemia have shown great promise in harnessing RIPK1 to kill apoptosis resistant tumor cells, by using SMAC mimetic compounds (SMs) (Box 2) [33, 34]. It is now recognized that RIPK1 and NF-κB signalling in dying cells can determine cross priming of CD8+ T lymphocytes [35]. Decoupling NF-κB signalling from necroptosis or inflammatory apoptosis reduces priming efficiency and tumor immunity [35, 36]. Therefore, gaining a better understanding of the mechanisms that regulate RIPK1 activity may not only be beneficial for the treatment of inflammatory and autoimmune diseases, but may also present exciting new opportunities for cancer therapy. Indeed, engagement of RIPK1 and RIPK1-induced cell death may be used to induce a type of tumor cell death that is immunogenic, meaning that the patient's dying cancer cells stimulate a specific anti-tumor response (Box 3).

Checkpoints in TNF signaling

In mice and human, RIPK1 can be activated by a plethora of different death-inducing stimuli, such as TNF superfamily receptors (TNFR-1/2, Fas/CD95, TRAIL-R1/2, DR3 and DR6, Fn14), T cell receptors, interferons (IFNs), Toll-like receptors (TLR3, TLR4) [37-42], intra-cellular RNA and DNA sensors, as well as inhibition of the proteasome, and by stressors such as calcium overload, ER stress, DNA damage, and ischemia-reperfusion injury [31, 43] (Figure 2). In the case of TNF-R1 signaling, which is currently the best system for studying RIPK1 in human and mouse, its activation is initiated via the DD domain of RIPK1, allowing DD-mediated recruitment to TRADD and TNF-R1 [44], which in turn results in activation of RIPK1 and formation of the TNF-R1 signalling complex, frequently referred to as complex-I [28] (Figure 1). Recruitment of RIPK1 to complex-I liberates it from HSP90, which is predicted to lock RIPK1 in an inactive conformation in the cytosol [45]. Binding of TRADD and RIPK1 to TNF-R1 allows the subsequent recruitment of the adaptor TRAF2, and the E3 Ub-ligases cIAP1, cIAP2 and the Linear Ub chain Assembly Complex (LUBAC, composed of HOIL-1/HOIP/SHARPIN) [28, 46-50]. CIAPs and LUBAC synthesize Ub chains of various linkage types (K63, K48, K11, M1 and quite possibly branched chains) to RIPK1 and other components of complex-I, both in human and mouse [51-54] (Figure 1). These Ub chains serve as scaffolds that allow the recruitment of kinase complexes, such as TAK1/TAB2/TAB3 [55] and IKK1/2/NEMO [56], with TAK1 being able to phosphorylate and trans-activate the IKK complex [57, 58]. Formation of complex-I determines activation of NF-κB and Mitogen Activated Protein Kinases (MAPKs), which ultimately results in the production of cytokines and

pro-survival proteins, such as cFLIP, that are necessary for a coordinated inflammatory response [59] (Figure 1). TNF also leads to the formation of a secondary cytoplasmic complex (complex-II), whose core components consists of RIPK1, FADD, caspase-8 and cFLIP (Figure 1). This complex can also include caspase-10 and RIPK3. While caspase-10 is human specific, RIPK3 is present in both mouse and human but its expression is tissue-specific. Depending on how much caspase-8, and/or RIPK3 are recruited, complex-II can kill cells via apoptosis and/or necroptosis [20, 60]. In general, activation of caspase-8 inhibits necroptosis because caspase-8 cleaves and inactivates RIPK3 [27]. Although TNF can drive formation of complex-II, the predominant response to TNF is survival in most human and mouse cells [28], demonstrating that cell death is normally actively repressed. It is now clear that TNF's killing potential is safe-guarded by multiple checkpoints, some being transcription-dependent while others are transcription-independent [14].

Transcription-dependent TNF checkpoint

Historically, the prevailing model has been that TNF-mediated activation of NF-κB, and the subsequent production of pro-survival genes, such as cFLIP, cIAP2, and A20, inhibits the cell death pathway that is simultaneously triggered, both in human and mouse [61]. According to this scenario, functional activation of NF-κB results in cell survival, while its defect leads to death [28]. Particularly, NF-κB-mediated induction of cFLIP expression allows the integration of cFLIP into complex-II where it can form heterodimers with caspase-8 [61, 62]. Subsequently, cFLIP-caspase-8-hetero-dimerisation results in an active protease that cleaves RIPK1, RIPK3 and the deubiquitylase enzyme (DUB) CYLD, thereby suppressing lethal activation of complex-II (Figure 1), as shown by genetic data in mice [27, 63-66]. The observation

that skin-specific inducible deletion of cFLIP results in TNF-mediated, caspase-8-dependent cell death seems to demonstrate that survival of skin cells is an active process that relies on the ability of cFLIP to neutralize the killing potential of complex-II [63, 67]. Together, this evidence is consistent with the notion that NF-κB-dependent induction of pro-survival genes, such as cFLIP, represses cell death.

Ubiquitylation-dependent TNF checkpoint

A key event in TNF-induced activation of NF-κB is the conjugation of various Ub linkage types to components of complex-I in human and mouse cells (Box 1). RIPK1 is one of the most prominent ubiquitylation targets in complex-I (Figure 1) [68]. While the dogma dictates that ubiquitylation of RIPK1 is indispensable for TNF-induced activation of NF-κB [28, 29], based on the evidence that abrogating RIPK1 ubiquitylation in Jurkat and L929 cells abolishes NF-κB activation [29], several recent studies have refined this viewpoint, demonstrating that the requirement of RIPK1 for NF-κB activation is cell type dependent [41]. Accordingly, primary mouse embryonic fibroblasts, hepatocytes (E18), lung cells, and other human and mouse cell types do not require RIPK1 for TNF-induced activation of NF-κB [41].

Although ubiquitylation of RIPK1 not always contributes to NF-κB activation, genetic mouse models indicate that cIAP and LUBAC-mediated ubiquitylation of RIPK1 is almost always necessary to prevent TNF-induced cell death. Accordingly, cIAP1/2 double deficient animals die at early embryonic gestation due to vascularization defects, and the deletion of one copy of RIPK1 is sufficient to prolong survival of cIAP1/2 double deleted mice until birth [69]. Additionally, depletion of cIAPs with SM dramatically impairs RIPK1 ubiquitylation and boosts RIPK1-mediated complex-II

formation and cell demise in most human and murine cell types [68, 70]. Moreover, cIAP-mediated ubiquitylation of RIPK1 enables the recruitment of LUBAC, which in turn can linearly ubiquitylate RIPK1, and other components of complex-I in most human and murine cellular systems [71]. Similar to findings documenting the deletion of *cIAPs*, loss of the LUBAC component *HOIP* also results in early embryonic lethality (E10.5) in genetically engineered mice due to aberrant RIPK1-dependent cell death of endothelial cells, reported to ultimately lead to severe vascularization defects [72]. Mechanistically, ubiquitylation may suppress RIPK1's cytotoxic potential by tethering RIPK1 to complex-I, thereby delaying the flux of RIPK1 to complex-II [73], and/or allowing activation of downstream kinases that repress RIPK1-mediated cytotoxicity by direct phosphorylation [74-77], therefore preventing cell death. Consistent with a role of Ub in negatively regulating RIPK1's killing potential, de-ubiquitylation of RIPK1 by DUBs, such as CYLD, can enhance complex-II formation and caspase-8-mediated apoptosis or necroptosis in mouse and human [78].

Kinase-mediated regulation of RIPK1

In recent years, phosphorylation-dependent regulation of RIPK1 has emerged as central mechanism that thwarts TNF-mediated cell killing [79]. The first evidence of the importance of kinases for the outcome of TNF signaling came from studies on TAK1, as evidenced from a study where pharmacological inhibition of TAK1 compromised cell survival following TNF stimulation in human and mouse cells [19]. Once recruited, TAK1 phosphorylates and activates the IKK complex, which in turn triggers NF-κB activation [57]. In addition, TAK1 activates the MAPKs p38 and JNK via phosphorylation of MKK3/6 and MKK4/7, respectively [58]. p38 and its downstream effector MAPK-activated protein kinase-2 (MK2) positively regulate

expression of pro-inflammatory genes, such as *TNF* [80-82]. Moreover, genetic deletion of *Tak1* in mice results in vascularization defects and early embryonic lethality [83], which phenocopies loss of *cIAPs*, *caspase-8* and *HOIP* in mice [69, 72, 84]. Furthermore, pharmacological inhibition of TAK1 with a specific inhibitor can switch the TNF response from pro-inflammatory to pro-death, even though ubiquitylation of RIPK1 in complex-I occurs normally in human and murine cells [19]. Under these conditions, TNF-mediated cell death appears to be strictly RIPK1 kinase-dependent, because it can be inhibited by specific RIPK1 kinase inhibitors [19]. In addition, TAK1 appears to control RIPK1-mediated cell death independent of its role in regulating gene transcription. This is evident because blocking TAK1 enhances TNF-induced death under conditions where NF-κB is inhibited, both in human and mouse [19]. Given the number of kinases activated by TAK1, it remains unclear whether TAK1 suppresses RIPK1 kinase activity directly, or indirectly via downstream kinases. Identifying the kinases that directly control RIPK1's cytotoxic potential is important because it would allow more selective intervention strategies that might not interfere with NF-κB signaling in general, and hence might reduce potential side effects.

IKK-mediated regulation of RIPK1

The TNF signaling cascade branches out at the level of TAK1, with TAK1 promoting activation of IKK, as well as the JNK and p38/MK2 kinase pathways [85]. Simultaneous activation of these signal transduction cascades co-operates to induce pro-inflammatory cytokines, such as TNF and IL-6 [86]. The IKK complex comprises the kinases IKK1 (IKKα) and IKK2 (IKKβ), as well as the regulatory subunit and Ub-adaptor NEMO, which allows recruitment of IKK to complex-I via its binding to M1-

linked Ub chains [56]. Genetic data in mice show that the activity of the IKK complex is crucial to ensure embryonic development and proper maintenance of tissue homeostasis in different organs. Specifically, genetic deletion of *Nemo*, *Ikk1*, *Ikk2*, or *Ikk1*/*Ikk2* in mice results in early lethality with aberrant cell death in several organs, such as skin, liver, and in the case of *Ikk1/Ikk2* knockout (KO) mice, the neural tube [87-96]. While deletion of *Nemo* completely blocks activation of NF-κB following TNF stimulation, loss of *Ikk2* does not prevent NF-κB activation [96]. These findings reflect the non-redundant role of NEMO in the signal transduction cascade leading to NF-κB activation downstream of TNF-R1, whereas IKK1 and IKK2 appear to play a more redundant role in activating NF-κB. This indicates that while IKK1 and IKK2 can substitute for each other to activate NF-κB, NEMO plays a non-redundant role in NF-κB activation. Accordingly, human or murine *Nemo-/-* cells are highly sensitive to TNF-induced cell death, while *Ikk1* and *Ikk2* single mutant cells are not [96]. This might be due to *Nemo-/-* cells not being able to activate the transcriptional, NF-κB-dependent checkpoint described earlier. Therefore, in the absence of NEMO cells might die in response to TNF because cFLIP expression is lost [28], leading to lethal activation of complex-II. In contrast, loss of either *Ikk1* or *Ikk2* would not turn off the transcriptional checkpoint, resulting in cell survival. However, later studies demonstrated that the IKK complex could also control cell survival in an NF-κB-independent fashion [74]. For example, in mouse colonocytes and hepatocytes, genetic deletion of *Nemo* prevents cell death by directly controlling RIPK1 activity, independent of its role in regulating NF-κB, while in the mouse small intestine genetic deletion of *Ikks* prevent the death of Paneth cells in a strictly NF-κB-dependent manner [97]. Consequently, the mechanism through which IKK suppresses TNF killing appears to be cell type specific. In TNF-R1 complex-I, IKK1 and IKK2 both

directly phosphorylate RIPK1 at multiple sites (Figure 2) [74]. IKK-mediated phosphorylation of RIPK1 reduces its ability to bind FADD/caspase-8, and to induce apoptosis [74], although the underlying mechanism remains unknown. Of note, IKK is not the only kinase that directly controls the cytotoxic potential of RIPK1; three recent studies, including ours, indicate that MK2-mediated phosphorylation of RIPK1 also inhibits the formation of complex-II [75-77]. This observation provides a strong rational of using MK2 inhibitors in clinical trials, particularly because MK2 inhibitors synergize with SMs in killing leukemia cells (see below) [33].

The p38/MK2 axis of cell death control

The p38/MK2 axis is activated by TAK1 and critically modulates the production of cytokines, such as TNF, via stabilization and transcriptional de-repression of mRNA transcripts in the cytoplasm [98]. Accordingly, genetic deletion of MK2 protects mice from LPS-induced endotoxin shock due to post-transcriptional defects in TNF production [80-82, 99]. Using an unbiased approach for the identification of compounds that boost the therapeutic potential of SM, one study identified the p38/MK2 axis as negative regulator of TNF-dependent cell death [33]. In this study, the authors showed that p38 and MK2 inhibitors enhance the capacity of SM compounds to kill human and mouse leukemia cells in a RIPK1 and TNF/TNF-R1-dependent manner. This finding was perplexing given the requirement of p38 and MK2 in TNF transcript stabilization and expression. Therefore, one would have expected p38 and MK2 inhibitors to suppress SM-induced cell death. However, the opposite seems to be the case. Three recent studies now shed new light into how MK2 might suppress TNF-mediated cell death [75-77]. Collectively, they report that MK2 can directly phosphorylate RIPK1 at serine (S) 320 and S335 (S321 and S336

in mice) (Figure 2), independently from the ability of MK2 to regulate TNF transcript stability and expression [75-77]. Accordingly, RIPK1 phosphorylation at S320/321 is lost in MK2 deficient human and mouse cells upon addition of exogenous TNF, and MK2 can phosphorylate RIPK1 in an *in vitro* kinase assay. MK2-mediated phosphorylation of RIPK1 suppresses complex-II formation and cell death during normal inflammatory signaling [75-77]. Of note, these two sites are embedded in phosphorylation consensus motifs for MK2, defined as F-X-R-X-(L/N)-pS/T-(I/V/F/L)-X [100] where F is a bulky hydrophobic residue [100], suggesting that S320 and S335 are indeed *bona fide* MK2 phosphorylation sites. While S320 and S335 are transiently phosphorylated in response to TNF, stress or infection, there is some controversy about the kinase involved. While the three reports unambiguously point to MK2 as the culprit kinase [75-77], two additional reports suggest that these sites are phosphorylated by IKK and TAK1 [101, 102], respectively. Genetic deletion of *Ikk1/2* and *Nemo* in mouse cells, or pharmacological inhibition of IKK kinases by the IKK-specific inhibitor TPCA-1 in both human and mouse cells, indicates that the IKK kinase complex is not required for TNF-induced phosphorylation of S320 [75-77]. This further strengthens the evidence that TAK1 is indispensable for the phosphorylation of RIPK1 on S320 and S335 downstream of TNF-R1, because of its ability to activate p38 and MK2 rather than IKK. While it is formally possible that TAK1 can directly phosphorylate S320/335 under *in vitro* conditions, genetic deletion and pharmacological inhibition of p38 and MK2 in human and mouse cells prevent RIPK1 phosphorylation on S320 and S335 even though TAK1 is fully active under these conditions. This clearly demonstrates that RIPK1 phosphorylation of S320/355 is indeed mediated by MK2, [75, 76]. These observations open up new opportunities

of manipulating RIPK1 activity for therapeutic purpose.

In both mice and man, phosphorylation of RIPK1 by MK2 is an early and transient event in TNF signaling, as it occurs within 5 minutes and is lost after 30 minutes [75-77]. The short kinetics of RIPK1 phosphorylation by MK2 suggests that MK2 can act as a transcriptional-independent checkpoint in the TNF signaling pathway, limiting RIPK1-induced killing potential during normal inflammatory signaling [76]. Consistent with this, MK2 inhibition does not impair the TNF-induced NF-κB response [75-77], based on the fact that upon MK2 inhibition NF-κB activation occurs normally in human and mouse cells. Instead, MK2 inhibition appears to selectively enhance RIPK1-mediated complex-II formation and cell death [75, 76]. In both human and mouse cells MK2 inhibition switches the TNF response from survival to death only in conditions that already induce RIPK1-dependent cell death, such as following SM-induced cIAP1/2 depletion or inhibition of IKK1/2 [75, 76], while having no effect on cell death when combined with TNF alone [75, 76]. However, *in vivo* mere inactivation of MK2 alone is sufficient to sensitize to TNF-induced cell death, [75]. Similarly, cells also become acutely sensitive to MK2 inactivation alone when exposed to bacterial infection [75, 77]. Mechanistically, MK2-mediated phosphorylation of RIPK1 can inhibit its auto-phosphorylation at S166, which is required for RIPK1 kinase activation, complex-II recruitment and cell death in human and mouse cells [103, 104]. Likewise, IKKs also suppress RIPK1 kinase auto-activation [74]. However, IKKs and MK2 phosphorylate distinct sites within RIPK1 [74-77]. Although having a similar inhibitory effect on RIPK1 auto-activation, IKKs and MK2 do not depend on each other to regulate RIPK1, but operate additively to suppress complex-II formation and cell death. This is based on the evidence that IKK

deletion or inhibition does not suppress MK2-mediated phosphorylation of RIPK1 at S320 and S335 [75-77]. At present, it is unclear whether IKKs and MK2 can target the same molecule of RIPK1 or different pools. Given their distinct compartmentalization, it is highly likely that IKKs and MK2 phosphorylate different RIPK1 proteins, but this has not been tested. IKKs are activated within complex-I, and hence they target RIPK1 within this plasma membrane bound complex [74]. In contrast, MK2, which is not a constituent of complex-I, binds and phosphorylates RIPK1 in the cytoplasm [75]. The current data are consistent with a model whereby a first pool of RIPK1 might be recruited to TNF-R1 following TNF ligation. RIPK1 might then be ubiquitylated by cIAPs and LUBAC. Indeed, the observation that ubiquitylated RIPK1 is phosphorylated at P-S166 [104] in mouse cells suggests that the ubiquitylated pool of RIPK1, at least in part, is catalytically active [104]. Ubiquitylation of RIPK1 and other components of complex-I may subsequently enable recruitment and activation of IKKs, which in turn would phosphorylate RIPK1 at multiple sites [74]. Of note, IKKs phosphorylate ubiquitylated RIPK1 in TNF-R1 complex-I [74]. Spatially separated, MK2 seem to phosphorylate RIPK1 in the cytoplasm, locking RIPK1 in an ‘inactive’ state that precludes auto-phosphorylation. Although, RIPK1-P-S320/335 is readily recruited to complex-I, this form of RIPK1 is refractory to ubiquitylation and auto-phosphorylation, as indicated by the fact that the anti-RIPK1-P-S321 antibody does not detect ubiquitylated RIPK1 in complex-I from mouse cells [76], suggesting that P-S321 negatively regulates RIPK1 kinase activity [75-77]. Consistent with this, time course analysis performed in both human and mouse cells using anti-RIPK1-P-S321 and anti-RIPK1-P-166 specific antibodies suggests that P-S321 and P-S166 are mutually exclusive, and that the phospho-adduct of S321 has to be removed before RIPK1’s auto-activation at P-S166 can

occur [76] (Figure 3). Knock-In mice bearing phospho-mimetic (S321D) or phospho-mutant (S321A) versions of RIPK1 were generated to corroborate the physiological role of MK2-mediated regulation of RIPK1. Specifically, primary fibroblast and macrophages from the RIPK1-S321D mutant mice are more resistant to TNF induced cell death, whereas primary fibroblast and macrophages from RIPK1-S321A mutant mice are more sensitive. This finding is consistent with the notion that MK2-mediated phosphorylation of RIPK1 serves a pro-survival role. [76]. Reconstitution of RIPK1-/- mouse cells with a double mutant RIPK1 (S321A, S336A) renders them even more sensitive than the S321 reconstituted cells, indicating that both these sites restrain RIPK1 kinase-dependent cell death [75, 77]. Although IKKs predominantly target RIPK1 in complex-I, and MK2 seems to mainly phosphorylate the cytoplasmic pool of RIPK1, IKK- and MK2-mediated phosphorylation events on RIPK1 do not appear to be mutually exclusive because mass spectrometry analysis revealed that phosphorylated S321 (MK2-mediated) and phosphorylated S332 (IKK-mediated) can be detected on the same peptide [77]. This indicates that two different regulatory layers limit full activation of RIPK1, and that simultaneous inactivation of both kinases (IKK and MK2) would be required to maximally engage RIPK1's cytotoxic potential for therapeutic purpose.

The origin of complex-II

Ever since the identification of TNF-R1 death-inducing complex-II in 2003 [28], its true origin has been highly debated. Since complex-II assembles several hours after the formation of complex-I, and is devoid of TNF-R1, complex-II may be assembled from RIPK1 that either (i) comes entirely from complex-I, (ii) is generated from the cytosolic pool of RIPK1, or (iii) is seeded by RIPK1 from complex-I and extended by

cytosolic RIPK1 that has never even gone through the route of complex-I. Recent data have now clarified this long-standing issue, demonstrating that the death-inducing complex-II is not built entirely of RIPK1 from complex-I. Instead, complex-II is started from RIPK1 of complex-I that then serves as seeding molecule that recruits RIPK1 directly from the cytosol. The identification of the origin of complex-II was made possible with the help of RIPK1-ΔDD [76]. This RIPK1 mutant lacks the DD (Figure 2), and hence cannot be recruited to complex-I. However, since it retains the homotypic RHIM oligomerization domain RIPK1-ΔDD can be recruited into complex-II [76]. Even though RIPK1-ΔDD cannot be recruited to complex-I, it readily exacerbated TNF killing as long as endogenous WT RIPK1 was present [76]. Moreover, RIPK1-ΔDD also co-purified with components of complex-II, demonstrating that it can be recruited straight from the cytosol. Polymerization of RIPK1 in complex-II allows cooperative and hierarchical binding of cFLIP and caspase-8 [62], and, under conditions of low NF-κB activation and cFLIP expression, homo-oligomerization and lethal activation of caspase-8. This will then result in cellular demise. Together, these recent reports [75-77] shed new light on the mechanisms that control the ability of RIPK1 to assemble the death-inducing complex-II. By phosphorylating the cytosolic pool of RIPK1 following TNF stimulation, MK2 limits the recruitment of RIPK1 into complex-II, therefore skewing the TNF responses in favor of cell survival.

Why so many checkpoints?

RIPK1-based secondary complexes are formed every time a cytokine receptor of the TNF superfamily, or Toll-like receptor family is engaged, yet cells rarely respond with self-destruction [39]. The balance between life and death is delicately poised in favor

of life. Hence, TNF, TRAIL, and LPS rarely trigger cell death under steady-state conditions, and the various TNF signaling checkpoints ensure that life is the predominant outcome. But these ligands and danger signals can potently destruct cells and even erode entire tissues if the system is under strain, particularly under conditions where cells are exposed to multiple pro-inflammatory cytokines, such TWEAK and LIGHT that are able to drive non-canonical NF-κB signalling in both human and mouse cells. [105]. While TNF, TWEAK and LIGHT alone do not induce cell death, combined TNF and TWEAK or LIGHT switches the signaling cascade in favor of death [75, 105, 106]. We speculate that this may reflect settings of severe tissue stress and malfunction, occurring during infection or injury, when it might be beneficial to remove damaged cells, and replace them with new ones. Under such 'stress' conditions, the survival checkpoints are turned off for cell death to proceed. How tissue stress is sensed by cells, and how the survival checkpoints of the TNF signal transduction cascade are turned off to initiate a cell death program remains an open and pressing question. Mechanistically, one could speculate that that IKKs preferentially phosphorylate the pool of RIPK1 in complex-I, thereby ensuring that complex-I assembly and NF-κB activation occurs normally. MK2 on the other hand phosphorylates and safeguards the cytosolic pool of RIPK1. Since both pools of RIPK1 contribute to complex-II formation and cell death, it could well be that MK2 and IKK represent separate brakes for RIPK1 activation. The degree of environmental stress might determine whether either or both of these brakes are released, impacting on the extent of RIPK1 activation, cell death and tissue repair. However, future testing is warranted to validate this hypothesis.

Breaching the barricades

Many microbial pathogens have evolved mechanisms to inhibit innate and acquired host immune responses to prevent their immune recognition and elimination [107]. Equally, in an arm's race, eukaryotic cells can adopt strategies to circumvent pathogen adaptation mechanisms, preventing their replication and spreading (Figure 4). A striking example of this host/pathogen co-evolution is exemplified by the bacterium *Yersinia Enterocolitica*. *Yersinia Enterocolitica* causes RIPK1 kinase activity-dependent macrophage apoptosis through its virulence factor YopP [77, 108-110]. YopP directly prevents activation of TAK1 in mouse cells, and therefore of the IKK2 and p38/MK2 axis [77, 111-113]. This disables kinases that are important for NF-κB and MAPK-mediated immune responses, allowing, in principle, immune evasion by *Yersinia Enterocolitica*. However, due to the cytoprotective roles of IKK2 and MK2-mediated phosphorylation of RIPK1, YopP-dependent inhibition of NF-κB and MAPK can result in death of the infected cell, and, consequently, favoring host survival [77]. Therefore, while in the case of inflammatory diseases such as psoriasis and inflammatory bowel disease the inhibition of RIPK1 kinase activity is beneficial, in the case of some bacterial infections, RIPK1 kinase activity is required to kill off those cells that are infected, thereby clearing the infection.

Concluding Remarks

Inflammation is an essential defense response induced by infection or injury. Most if not all cellular stress responses, in addition to cell–autonomous adaptive changes, produce secreted factors that affect other cells tissues. This coordinates compensatory cell proliferation and tissue remodeling to replaces malfunctioning or damaged tissues [114]. Inflammation presumably evolved as an adaptive response for restoring homeostasis [115]. While inflammatory cytokines can coordinate tissue

homeostasis via activation of NF-κB and MAPKs, they can also achieve this through inducing cell death. Recent studies in genetic mouse models provide clear evidence that aberrant cell death can cause inflammation in different tissues. Consistently, inhibition of RIPK1-dependent cell death prevents inflammation in epithelial barriers. RIPK1 has been recognized as a fundamental signaling node downstream of important pro-inflammatory molecules, such as TNF, that mediates their effects. It can regulate NF-κB activation, apoptosis and necroptosis. Given that RIPK1 is essential for preventing cell death, maintaining tissue architecture and inhibiting inflammation, it is not surprising that its signalling potential is subject to tight regulation. Ubiquitylation and phosphorylation events combined with complex transcriptional responses have been identified as key mechanisms in preventing chronic tissue injury and inflammation in the skin, intestine and the liver. The recent studies on TNF and RIPK1 have demonstrated that regulation of cell death signaling plays a fundamental role in the maintenance of tissue homeostasis, and suggested that many inflammatory diseases could be caused by aberrant cytokine-driven and RIPK1-mediated cell death. Understanding the checkpoints that hold RIPK1 in control will undoubtedly be of significant clinical relevance as it may offer new solutions for the treatment of chronic inflammatory diseases such as psoriasis and rheumatoid arthritis. It may also indicate how to exploit the cytotoxic potential of RIPK1 to kill tumor cells, particularly those that are driven by cancer-related inflammation or have high numbers of infiltrating immune cells (immunologically hot tumors). Although many questions remain (see outstanding questions and Box 3), targeting molecules at the crossroad of cell death and host defense pathways, such RIPK1, may enable conceptualizing newer or improved approaches to treat a variety of inflammation-associated pathologies.

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Figure Legends

Figure 1. Checkpoints of TNF-mediated cell death in mammals. Binding of TNF to TNF-R1 leads to receptor trimerization and formation of TNF-R1 complexes, referred to as complex-I, whose ultimate function is to trigger inflammatory and pro-survival gene induction. RIPK1 in complex-I can dissociate form the plasma membrane bound TNF-R1 complex and nucleate a secondary cytoplasmic complex, complex-II, that has the potential to trigger apoptosis or necroptosis. A number of different checkpoints exist that control the formation and cytotoxic potential of complex-II: (1) Ub-dependent checkpoint, mediated by the E3 ligases cIAPs and LUBAC; (2) Phosphorylation-dependent checkpoint that depends on the activity of IKK1/2 and MK2 kinases; (3) Transcription-dependent checkpoint, based on NF-κB-mediated upregulation of pro-survival genes such as cFLIP, A20 and cIAP2; and (4) caspase-mediated cleavage and inactivation of RIPK1, RIPK3 and CYLD, mediated by a cFLIP/caspase-8 heterodimer, which ultimately prevents apoptosis as well as necroptosis. Dashed lines highlight the spatial distribution of the 4 checkpoints. The dashed arrow indicates that the activation of p38 by TAK1 is not direct.

Figure 2. Post-translational regulation of the cytotoxic potential of mammalian RIPK1. RIPK1 undergoes activation downstream of a vast array of receptors, including Death Receptors, Pattern Recognition Receptors (PRR), cytokine receptors and following ER stress. RIPK1 kinase and scaffold functions are tightly regulated by post-translational modifications such as ubiquitylation, phosphorylation and caspase-mediated cleavage. Depicted are the currently known sites that are modified by Ub (yellow), IKK1/2 (grey), MK2 (purple), caspase-8 (red) and that are modified as a consequence of RIPK1 auto-phosphorylation (blue). The enlarged circles depict sites

for which a functional role has been reported. The dashed line separates the ubiquitylation sites from the one that are controlled by phosphorylation.

Figure 3. Model of IKK1/2- and MK2-mediated regulation of RIPK1 cytotoxic potential. The kinase activity of RIPK1 is tightly regulated by IKK1/2- and MK2-mediated phosphorylation. While IKK1/2 phosphorylate ubiquitylated RIPK1, MK2 phosphorylates the cytosolic pool of RIPK1. Of note, ubiquitylated RIPK1 in complex-I is auto-phosphorylated, and hence is in its active state. Phosphorylation by IKK1/2 suppresses the binding of RIPK1 to FADD and caspase-8 through an unknown mechanism, possibly by preventing full activation of RIPK1. MK2-mediated phosphorylation of RIPK1 occurs in the cytosol and locks RIPK1 in an “inactive” state. While this does not prevent the recruitment of RIPK1 to complex-I, it interferes with RIPK1 ubiquitylation. Moreover, MK2-mediated phosphorylation of RIPK1 blocks RIPK1 auto-phosphorylation and might directly interfere with its recruitment to complex-II. Hence, IKK and MK2 target different pools of RIPK1, together controlling RIPK1 kinase activity and cell death. Red circles highlight the kinases that directly regulate RIPK1 activity. The fine dashed arrows indicate potential mechanisms by which MK2-mediated phosphorylation prevents RIPK1 from forming complex-II. The larger dashed arrow indicates that activation of p38 by TAK1 is not direct.

Figure 4. Mammalian RIPK1-dependent host adaptation to pathogens. Many pathogens attempt to evade recognition by blocking host innate immune responses. However, tampering with NF-κB activation removes survival checkpoints, thereby

unleashing the cytotoxic potential of RIPK1 and killing the infected cell. Bacterial pathogens are frequently sensed by the Toll-Like Receptor 4 (TLR4). This leads to TLR4-mediated activation of NF-κB and the stimulation of an effective innate and adaptive immune responses that helps to clear the pathogen. However, some pathogens have developed strategies to dampen inflammatory responses allowing their propagation. In the situation of *Yersinia Enterocolitica*, a bacteria effector molecule, YopP, specifically blocks kinases downstream of LPS such as TAK1 and IKK, thereby shutting off NF-κB activation. Interference with TAK1 and IKK, removes inhibition of RIPK1's cytotoxic potential. Thus, infected host cells are killed in a RIPK1-dependent fashion because IKK and MK2 no longer block auto-phosphorylation and activation of RIPK1. The dashed arrows indicate indirect activation events.

Glossary

Apoptosis: process of programmed cell death executed by proteases of the caspase superfamily.

Caspases: family of cysteine proteases involved in the initiation and execution of apoptosis. Caspases are not only involved in the regulation of cell death but also play important roles in many other biological processes, such as cell migration, innate immune signaling, and cell differentiation.

Cellular inhibitor of apoptosis (cIAPs): are the E3 Ub-ligases cIAP1 and cIAP2. These E3 ligases play crucial roles in ubiquitin-mediated regulation of canonical and non-canonical NF-κB signaling.

Damage-Associated Molecular Patterns (DAMPs): endogenous molecules that are exposed or released by dying or malfunctioning cells. These molecules are

recognized by pattern recognition receptors that subsequently trigger an innate immune response.

Deubiquitylase (DUB): enzyme that catalyzes the removal of ubiquitin from protein substrates.

E3 ubiquitin ligase: a protein that recruits an E2 conjugating enzyme and promotes the transfer of ubiquitin from the E2 to a target protein.

LIGHT: also known as Tumor necrosis factor superfamily member 14, is a pro-inflammatory ligand that belongs to the TNF superfamily.

Linear Ubiquitin Chain Assembly Complex (**LUBAC)**: is an E3 ubiquitin ligase complex, composed of SHARPIN, HOIL1 and HOIP, that generates M1-linked poly-ubiquitin chains.

Immunogenic cell death: any form of programmed cell death that is capable of stimulating an immune response.

Inflammatory Bowel Disease: is a term used to describe Crohn's disease and ulcerative colitis that are the principal types of inflammatory bowel diseases.

M1-linked ubiquitin chains: poly-ubiquitin chains synthesized by the E3 ligase LUBAC where two or more ubiquitin molecules are connected with one another via ubiquitin's first N-terminal methionine (M1).

Necroptosis: caspase-independent form of cell death.

Pathogen-Associated Molecular Patterns (PAMPs): pathogen-derived molecules that are recognized by cells of the innate immune system.

Pathogen Recognition Receptors (PRRs): host-derived surface and intracellular sensors that detect DAMPs and PAMPs and in response initiate an innate immune response. They are divided into different families on the basis of subcellular localization, structure and ligand specificity. They are predominantly expressed in

cells of the innate immune system, such as dendritic cells, macrophages, monocytes, neutrophils and epithelial cells.

Proteasome: large protein complex that degrades ubiquitylated proteins.

Psoriasis: long-lasting chronic inflammatory disease that affects the skin.

Receptor-Interacting Serine/Threonine Protein Kinase 1 (RIPK1): the protein kinase RIPK1 sits at the crossroad of inflammation and cell death. RIPK1 has scaffold as well as kinase functions. While the scaffold function is required to maintain tissue homeostasis and integrates various environmental stresses to inflammatory programs, the kinase activity is required for the pro-death functions.

Rheumatoid Arthritis: inflammatory condition that causes pain, swelling and stiffness in the joints.

SMAC mimetic (SM): small molecule inhibitor of IAPs. SM targets cIAPs for degradation.

TWEAK: also known as tumor necrosis factor ligand superfamily member 12 or TNF-related weak inducer of apoptosis, is a pro-inflammatory ligand that belongs to the TNF superfamily.

Toll-like receptors (TLRs): pathogen recognition receptors (PRR) that play a key role in the innate immune system.

Tumor Necrosis Factor (TNF): TNF is a pleiotropic cytokine, belonging to the TNF superfamily, that upon binding to its cognate receptor, TNF-R1, induces a signaling cascade that can result in the upregulation of pro-inflammatory genes or cell death. The TNF-signaling outcome is determined by the activity of two spatio-temporally distinct complexes: a TNF-R1-bound complex called **complex-I,** that drives NF-κB activation and an inflammatory response**,** and a secondary cytoplasmic complex called **complex-II,** that can trigger cell death**.**

Text Boxes

Box 1. The Ubiquitin system

The covalent attachment of ubiquitin (Ub) onto Lysine (K) residues of target proteins is one of the most common post-translational modifications [116]. Ubiquitylation occurs via a three-step process that involves Ub-activating enzymes (E1), Ub-conjugating enzymes (E2) and Ub-protein ligases (E3) [116]. The E1 Ub-activating enzyme binds to Ub in an ATP dependent manner via the formation of a labile thioester bond. Ub is subsequently transferred to the active site cysteine of the E2 Ub- conjugating enzyme. An E3 ligase enzyme ultimately recruits the substrate to the E2 conjugating enzyme, which allows the transfer of Ub from the E2 onto the substrate [116]. This occurs through the formation of an isopeptide bond between the carboxy-terminus group of Ub (Glycine (G)76) and the amino group of a K residue of the substrate. Ub can be conjugated to substrates as a single moiety to one (mono-Ub) or several K residues (multi-mono-Ub). Moreover, Ub can be conjugated as chains of variable length since Ub itself has seven Ks (K6, K11, K27, K29, K33, K48 and K63) as well as an amino-terminal Methionine (M1) that can all serve as Ub acceptors [117]. This results in the formation of poly-Ub chains of various linkage types that contain different biological information [117]. This information is deciphered and translated into biological outcomes by Ub receptors that are able to selectively recognize the distinct types of Ub chains [118].

Box 2. SMAC Mimetic

SMAC mimetics (SMs) are a class of small pharmacological antagonists of Inhibitor of APoptosis (IAP) proteins that are being developed for the treatment of solid tumors and hematologic cancers [119]. This new class of targeted agents bind to the

baculovirus inhibitory region (BIR) of IAPs [120]. Binding of a SM releases the BIR3-mediated inhibition on RING dimerization of cIAPs, resulting in activation of the E3 ligase function, auto-K48 ubiquitylation and proteasomal degradation of cIAPs [120]. SMs also bind to XIAP. Although they do not target XIAP for degradation, SM prevent the ability of XIAP to bind and inhibit caspases [121]. SM-induced degradation of cIAPs triggers activation of non-canonical NF-κB, and expression of NF-κB target genes, such as TNF [120]. TNF in turn acts in an auto/paracrine manner on cIAP-depleted cells causing RIPK1-mediated cell death. As a single agent, the cytotoxic effect of SM depends on the cell's ability to produce autocrine TNF [120]. Cells that do not produce autocrine TNF are insensitive to SMs, but can be sensitized to RIPK1–dependent cell death following exposure to exogenous TNF administration [120].

Box 3. Clinician's corner

Mobilizing and stimulating the immune system against tumor cells is one of the most effective ways to protect against cancers that recur and or metastasis. However, how best to trigger cell death, and most effectively activate tumor specific cytotoxic $CD8^+$ T lymphocytes is an open and pressing question in the field of immune-oncology.

Cell death pathways that engage RIPK1 kinase activity result in an immunogenic type of cell death that is capable of eliciting a specific anti-tumor immune response, which in turn can control (and sometimes eradicate) residual cancer cells. Molecularly, RIPK1- and NF-κB-mediated signaling in dying cells robustly activates $CD8^+$ T cells through a process called antigen cross-priming. Hence, insights into

mechanisms that boost activation of RIPK1 and NF-κB in dying tumor cells are likely to improve anti-cancer therapies aimed at activating a patient's own immune system.

Various checkpoints actively repress RIPK1's cytotoxic potential. Manipulating such checkpoints to unleash RIPK1's full killing potential in cancer cells is likely to improve treatment response and trigger long-lasting immunological protection.

The recent discoveries of IKK and MK2 as kinases that directly phosphorylate and inactivate RIPK1, provide new therapeutic opportunities to overcome treatment resistant cancers. Inhibitors of IKK and MK2 in combination with SMAC mimetics could be used to unleash RIPK1's killing potential, triggering immunogenic cell death, and robust antigen-cross priming of CD8+ T cells.

While regulated cell death pathways play important roles in host defense against invading microbes and tissue homeostasis, chronic and unscheduled cell death can cause tissue injury and inflammation in barrier epithelia. Understanding the checkpoints that safeguard RIPK1's killing potential has significant clinical relevance as chronic RIPK1-induced cell death appears to be causally involved in the pathogenesis of inflammatory diseases. Therefore, blocking the cytotoxic activity of RIPK1 may offer new solutions for the treatment of chronic inflammatory diseases such as psoriasis and rheumatoid arthritis.

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Trends Box

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.

Tumor Necrosis Factor (TNF) is a pleiotropic cytokine that plays key roles in inflammation, triggering either NF-κB activation or RIPK1 kinase dependent cell death.

Suppression of TNF-induced cell death is an active process that is controlled at multiple levels by diverse checkpoints, operating at both transcriptional as well as post-translational levels.

Ubiquitin-dependent phosphorylation of RIPK1 by IKK2 and MK2 has recently emerged as key checkpoints, limiting RIPK1 kinase activity and foiling TNF-mediated cytotoxicity, thereby licensing TNF-induced cytokine production necessary for a coordinated inflammatory response.

Accurate checkpoint control is vital as many pathogens target NF-κB and MAPK signaling to evade detection; however, since this removes critical survival checkpoints, RIPK1's cytotoxic potential is unleashed, killing the infected cell and safeguarding host survival.

Outstanding Questions

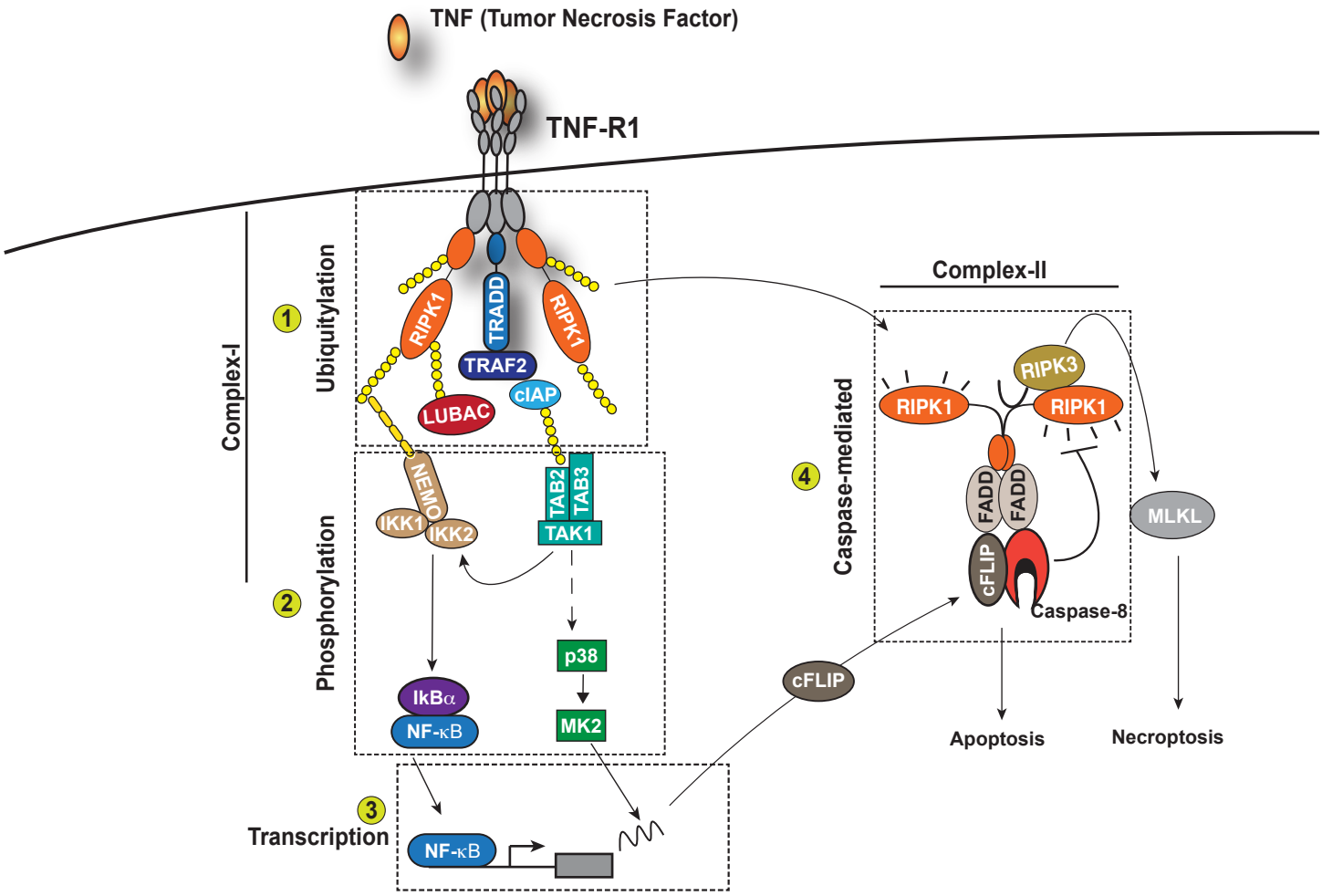
Under what conditions does TNF kill cells? How do micro-environmental conditions, such cellular stress upon pathogen infection or cellular malfunction, influence the threshold on the survival checkpoints that determine whether cytokines of the TNF superfamily either instruct an inflammatory response or trigger cell death?

Is it possible to identify biomarkers and treatment options for inflammatory diseases, such as psoriasis and inflammatory bowel disease, driven by excessive RIPK1-induced cell death, rather than exuberant activation of NF-κB? Could this potentially lead to better treatment options?

How can we harness the tumor necrosis function of TNF to target a variety of cancers, and drive long-lasting immunological protection?

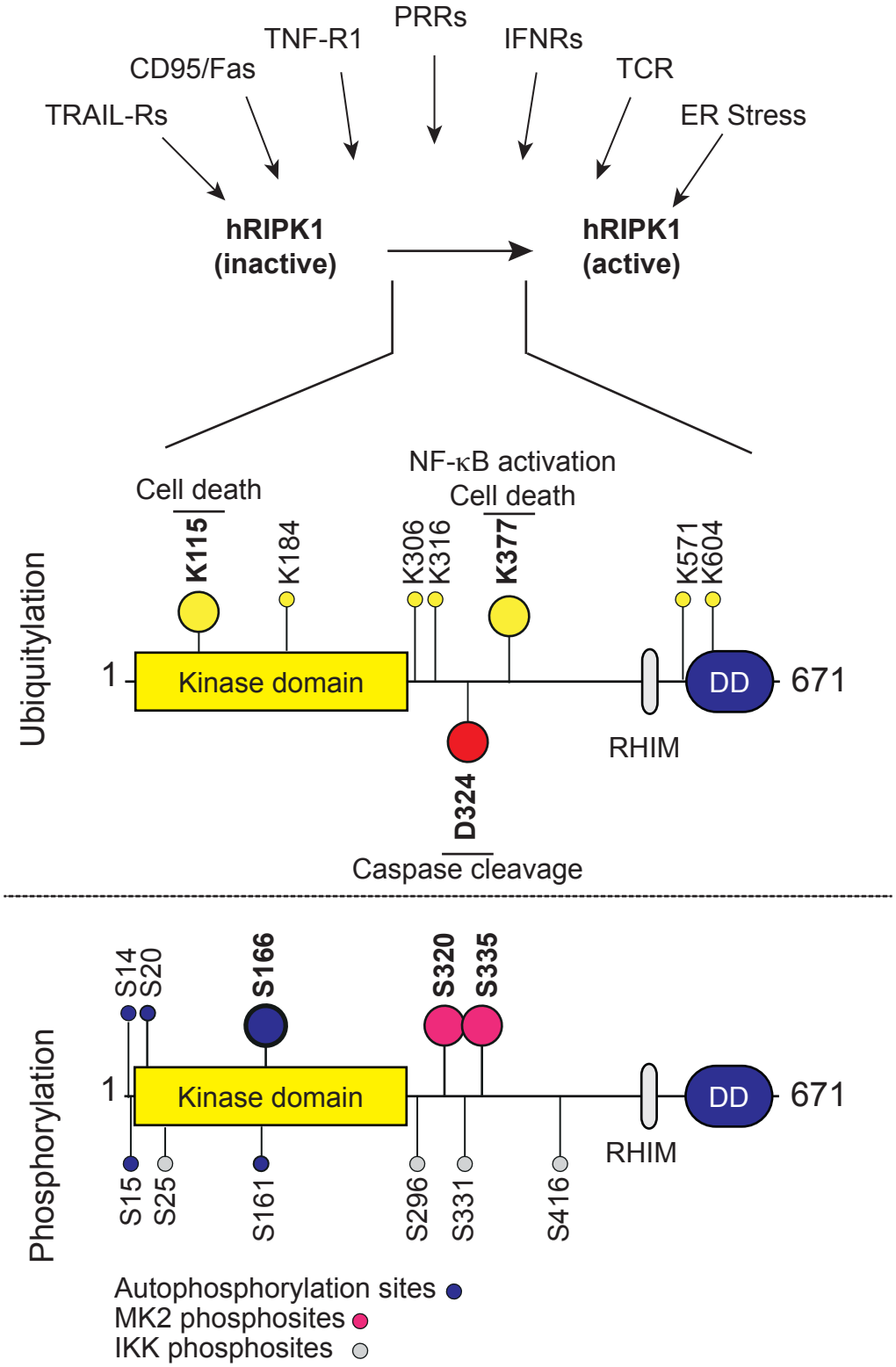
How do the different TNF signaling checkpoints interconnect to regulate RIPK1 signaling in health and disease?

Figure1



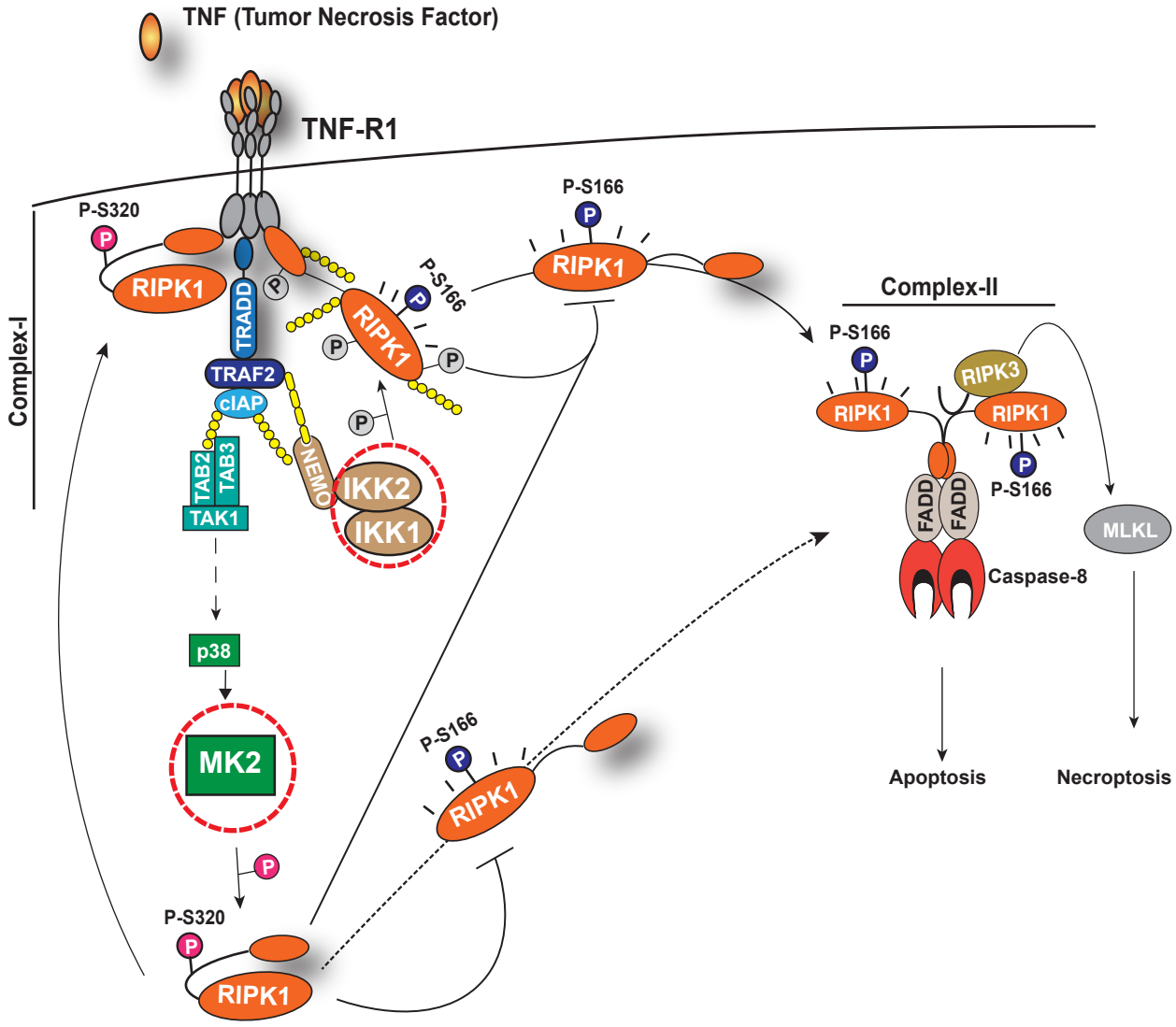
Figure

Figure2



Figure

Figure3



Figure

Figure4