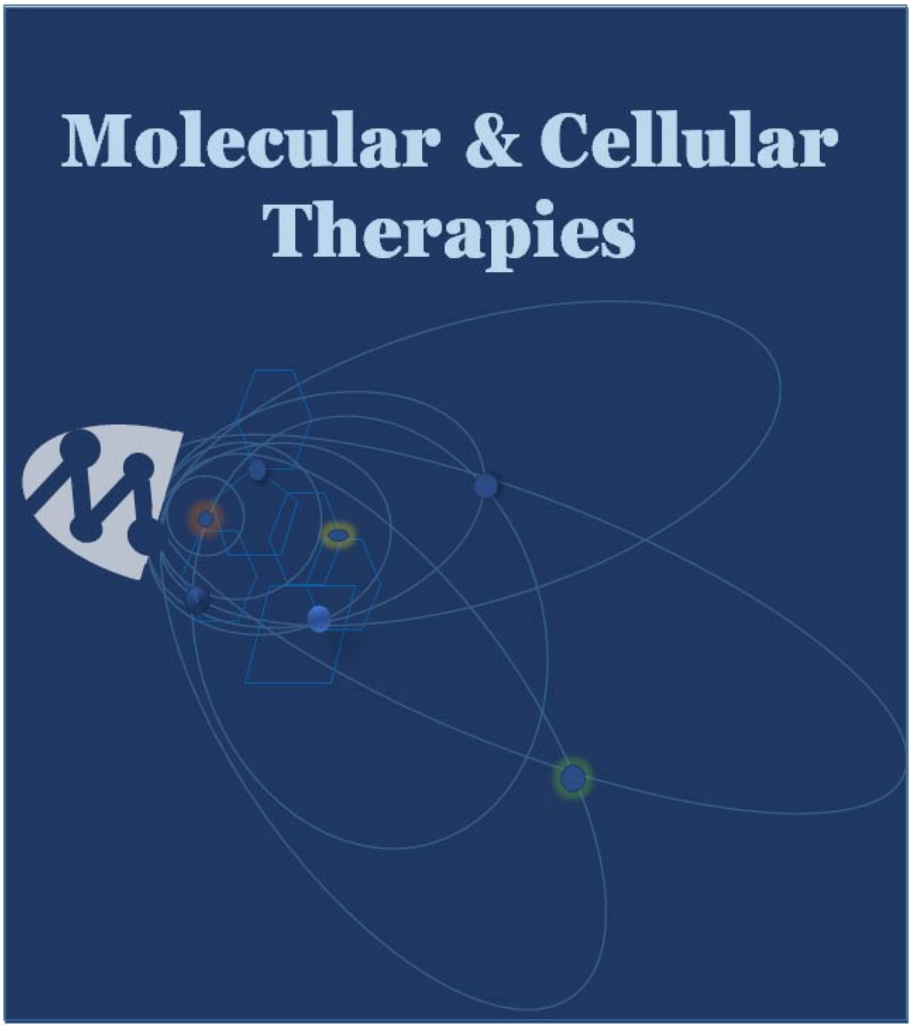

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Review

RIPK1 and RIPK3 – emerging targets in cancer?

Kerem Gurol[1], Suraj Shah[2], Alexei Degterev[2*]

[1]Master Program in Pharmacology and Experimental Therapeutics, Tufts University, Sackler School of Graduate Biomedical Sciences, 136 Harrison Ave, Boston, MA 02111, USA
[2]Department of Developmental, Molecular and Chemical Biology, Tufts University, Sackler School of Graduate Biomedical Sciences, 136 Harrison Ave, Boston, MA 02111, USA

*Correspondence to: alexei.degterev@tufts.edu

Abstract

RIPK1 and RIPK3 are homologous Ser/Thr kinases, which act in concert within the necrosome complexes to initiate a sub-type of regulated necrosis, termed necroptosis. Necroptosis has gradually emerged as a highly clinically relevant form of necrosis, which can be targeted therapeutically. Besides necroptosis, RIPK1 and RIPK3 have been implicated in other pathophysiologically-relevant responses, including regulation of apoptosis and inflammation. More recently, it became evident that RIPK1/RIPK3 pathways may be systematically altered in cancers. Status of these pathways may provide a prognostic value, and therapeutic modulation of RIPK1/RIPK3 signaling may represent a new strategy against various forms of human cancer.

Introduction – overview mechanisms of RIPK1/RIPK3-dependent responses

RIPK1 and RIPK3 are multidomain proteins, containing homologous N-terminal kinase domains and internal RIP Homotypic Interaction Motif (RHIM) dimerization domains. These factors mediate a wide range of physiologically and pathophysiologically important signaling responses, which have been extensively described in a number of recent reviews (1-11). Briefly, these factors came to prominence due to their critical roles in the process of regulated necrosis, termed necroptosis (12-14) (Fig. 1). Necroptosis, which can be activated by stimuli such as tumor necrosis factor alpha (TNFα), Toll-like receptor agonists, and Type I/II interferons (15, 16) requires catalytic kinase activities of both RIPK1 and RIPK3 and proceeds through the formation of large cytosolic detergent insoluble "necrosome" aggregates, formed through homotypic RHIM domain interactions of RIPK1 and RIPK3 (17). Necrosome formation enables RIPK3 to directly phosphorylate an effector pseudokinase MLKL, with the latter being both indispensable and sufficient for the activation of necroptosis(18).

Necrosome functions are tightly controlled by an ever-expanding range of positive and negative regulators, which have been extensively discussed elsewhere. The two critical negative modulators, which are particularly relevant for our review, are: the heterodimer of apoptotic caspase-8 with adaptor $FLIP_L$(19), and linear and K63 ubiquitinases-Linear Ubiquitin Assembly Complex (LUBAC) and inhibitor of apoptosis (IAP) proteins cIAP1/2, respectively (20). In response to TNFα, the latter class operates in the initial receptor-bound complex, preventing transition to the secondary necrosome complex.

Necroptosis represents just one of many facets of RIPK1 and RIPK3. Catalytic activities of RIPK1 and RIPK3 promote expression of a large set of cytokines and chemokines, which occurs through regulation of transcription factors including IRF3/7, NFkB, AP1 and Sp1 independently of cell death or MLKL (21-23). RIPK1 kinase activity has also been implicated in an alternative activation of caspase-8-dependent apoptosis (Fig. 1), which occurs independently from the catalytic activity of RIPK3 (24, 25). Conversely, RHIM-containing adaptors ZBP1/DAI and TRIF can bypass RIPK1 and directly activate RIPK3 kinase and necroptosis (26). Apart from necroptotic cell death functions, RIPK3 possesses kinase-dependent and -independent pro-inflammatory functions, which are exerted either through activation of NLRP3 inflammasomes or non-canonical caspase-8-dependent IL1β processing (10). All of these mechanisms may contribute to the development of an extensive range of inflammatory, degenerative and auto-immune conditions individually, additively or synergistically (27-29). In our review, the various emerging contributions of these factors in cancer will be discussed.

RIPK1/RIPK3 pathways are altered in human cancer

Emerging evidence clearly indicates that RIPK1, RIPK3 and other critical pathway components, such as caspase-8, IAPs, and MLKL are altered in human cancers. Genetic polymorphisms associated with high risk of chronic myelogenous leukemia (CML) and non-

Hodgkin lymphoma have been observed in RIPK1 and RIPK3 (30, 31). Loss of RIPK3 expression was detected in many commonly studied cancer cell lines(32-35), and in primary breast, colorectal, acute myelogenous leukemia (AML) and chronic lymphocytic leukemia (CLL) samples (33, 34, 36-40). In the latter case, the loss of expression of necroptotic deubiquitinase CYLD was also reported(40).MLKL, a key effector of RIPK3 in necroptosis, was also downregulated in pancreatic adenocarcinoma, colon and ovarian cancer (41-43).Importantly, the loss of RIPK3 expression has been shown to be associated with poor outcomes in patients with breast and colorectal cancers(33, 38, 39). Similarly, reduced levels of MLKL were found to be detrimental in patients with pancreatic, colorectal, ovarian, gastric and cervical cancers (41-45).

The mechanisms controlling the loss of these factors are starting to be elucidated. Hypermethylation of *Ripk3* promoter appears to be one prominent mechanism responsible for the decrease in the expression (33). The downregulation of epigenetic regulator UHRF1's expression in RIPK3-null colon carcinoma RKO cell line restored RIPK3 expression through the activity of the transcription factor Sp1 (46), which is also an important factor in TNFα upregulation downstream from RIPK1 activation and feed-forward regulation of necroptosis (21, 47, 48).In cells harboring mutations in isocitrate dehydrogenase enzymes (*IDH1/2*), aberrant metabolic product 2-hydroxyglutarate (2-HG) induced *Ripk3* promoter hypermethylation through DNMT1. Notably, loss of RIPK3 was found to promote tumorigenesis in human malignant gliomas harboring IDH1 R132H mutation (49). Overall, these data suggest that use of epigenetic inhibitors may be a productive approach to restoring necroptosis sensitivity in some tumors.

Other cancer-associated mechanisms controlling necroptosis resistance have been described. Stress-inducible antioxidant thioredoxin1 (Trx1) was shown to prevent disulfide bond formation through Cys32 residue of human MLKL, attenuating MLKL oligomerization and necroptosis (50). Trx1 was also proposed to restrict activation of RIPK1/caspase-8-dependent apoptosis (51). Hypoxia has been proposed to play an important role in downregulation of RIPK1 and RIPK3 expression in colon cancer cells (37). Certain clinical anti-cancer kinase inhibitors, such as pazopanib, dabrafenib, sorafenib, and ponatinib, display off-target activity against RIPK1 or RIPK3 kinases and prominently inhibit RIPK1 and/or RIPK3-dependent responses (52-55).

cIAP1/2 and XIAP IAPs are frequently upregulated in human cancer and their expression correlates with poor patient outcomes (56). These proteins attracted major interest as potential anti-cancer targets, resulting in the development of a number of monovalent and divalent clinical candidate inhibitors, termed SMAC mimetics (57). In case of XIAP, SMAC mimetics counter inhibition of active caspase-3, -7 and -9. With cIAPs, SMAC mimetics promote proteasomal degradation of cIAP1 and, to a lesser extent, cIAP2, leading to the synthesis of TNFα

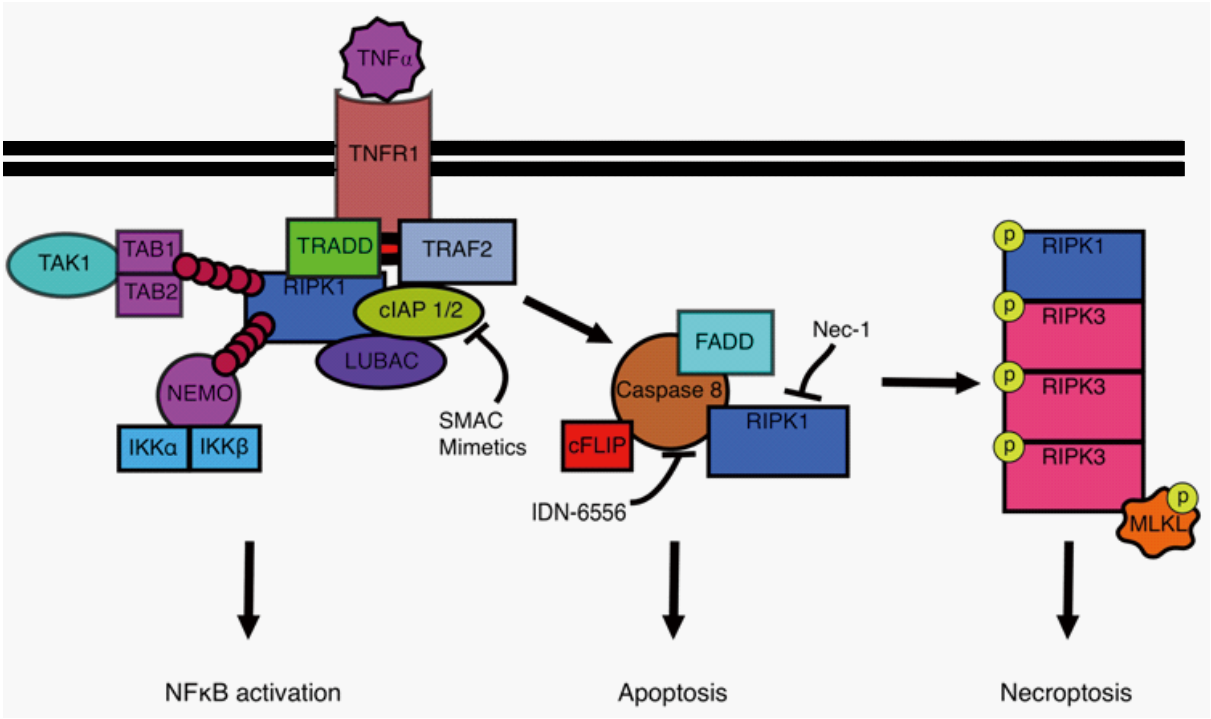

Figure 1. Mechanisms of TNF-induced NFkB activation, apoptosis, and necroptosis. TNF binding to TNFR1 results in the recruitment of TRAF2, TRADD, RIPK1, LUBAC, and cIAP1/2 to a membrane-bound Complex I. Ubiquitination of RIPK1 by LUBAC and cIAP1/2 leads to recruitment of TAK1 and IKK complexes and NFkB activation. Inhibition of cIAP activity by SMAC mimetics promotes formation of a pro-apoptotic cytoplasmic complex promoting RIPK1 kinase-dependent activation of apoptosis through Caspase-8. If Caspase 8 is inhibited, phosphorylated RIPK1 and RIPK3 form a necrosome complex to initiate necroptosis by recruitment and phosphorylation of MLKL.

due to the activation of the non-canonical NFkB pathway. This, in turn, creates an autocrine feed-forward loop for the activation of the TNFα-driven cell death due to the lack of RIPK1 ubiquitination by cIAP1/2 (58-61). However, because only a small subset of cancer cells is able to efficiently produce TNFα upon cIAP inhibition, SMAC mimetics display limited efficacy as monotherapeutic agents. Instead these molecules are undergoing a variety of clinical trials as potentiators of conventional chemotherapy-induced cell death (summarized in (62)).

Conversely, factors that promote sensitivity to necroptosis in cancer cells have also been described. In particular, activity of caspase-8 is frequently downregulated in cancers (63), making induction of necroptosis an interesting possibility for such apoptosis-resistant tumor cells. For example, mutations in *Casp8* were observed in ~30% of the hypermutated colorectal cancer (CRC) samples (64). He *et al*. directly tested the consequences of necroptosis in these cells by crossing ApcMin/+ CRC-prone mice to intestinal epithelium-deleted Casp8$^{-/-}$ mice (Casp8ΔIEC)(64) or by using xenografts of HT29 cells with CRISPR-deleted *Casp8*. In both cases, unbridled activation of RIPK1 following administration of a clinical SMAC mimetic LCL161 led to massive activation of cell death *in vivo*, presumably through necroptosis, and tumor regression. Another interesting mechanism controlling caspase-8 activation in response to chemotherapeutic drugs has been described in lung adenocarcinoma cells (65). cSrc dependent phosphorylation of caspase-8 on Tyr380 was observed in taxol-resistant lines, limiting caspase-8/apoptosis activation, but instead promoting induction of necroptosis, albeit inefficiently. Combination of cSrc inhibitor dasatinib with taxol led to both restoration of caspase-8 processing and potentiation of necroptosis, although mechanism of this dual regulation is currently unknown. Thus, pY380 casp-8 may represent an interesting biomarker of necroptosis susceptibility.

Activation of necroptosis may also be a fruitful strategy in apoptosis-competent cancer cells if caspase-8 activity is targeted pharmacologically. For example, combination of clinical SMAC mimetic Birinapant and clinical pan-caspase inhibitor IDN6556 led to an efficient extension of survival of animals with transplanted murine xenografts of AML subtypes that typically retain expression of RIPK3/MLKL - MLL-ENL and MLL-AF9 AML (66). Strikingly, this combination showed minimal toxicity towards normal hematopoietic cells, was well tolerated *in vivo*, was efficient even in the cells rendered resistant to birinapant alone, and efficiently induced necroptosis in 4 out of 8 primary human AML specimens, including 2 that failed initial chemotherapy. Efficient cell death induced by birinapant/IDN6556 combination was the result of two mechanisms: the upregulation of TNFα synthesis, which is induced by SMAC mimetics, and cell-intrinsic sensitization to necroptosis following inhibition of caspase-8/FLIP$_L$ complex by IDN6556 and cIAP1/2 by birinapant. At the same time, the authors observed that other sub-types of AML displayed resistance to birinapant/IDN6556 in the absence of obvious changes in RIPK1, RIPK3, MLKL expression, suggesting that the factors controlling sensitivity of AML cells to necroptosis remain to be fully elucidated. Pan-caspase inhibitor zVAD.fmk was also shown to efficiently kill primary human cisplatin resistant ovarian cancer cells in combination with SMAC mimetic (67) and primary human colon cancer samples in combination with 5-fluoroacil (68), supporting the notion that necroptosis may represent an efficient mechanism for promoting cell death in tumors, which retain RIPK3/MLKL expression and may otherwise be resistant to chemotherapies due to the inefficient or defective activation of apoptosis.

Mechanisms of RIPK1/RIPK3-dependent tumor suppression (Fig. 2)

As discussed above, loss of RIPK3 and MLKL expression is observed rather frequently in different types of cancer, suggest that these factors may be *bona fide* tumor suppressors. Survey of a large set of primary patient AML samples revealed significant downregulation of both RIPK3 and MLKL expression in several specific subtypes, especially FLT3-ITD and AML1-ETO9a (36). Loss of RIPK3 in corresponding mouse AML lines greatly accelerated leukemogenesis and death of the mice. Notably, loss of these proteins was linked to two distinct tumorigenic events – decrease in TNF receptor-mediated cell death, and inflammasome-dependent IL1β synthesis, with the latter promoting differentiation of the leukemia-initiating precursor cells. Another clear example of tumor suppressive role of RIPK3 has been described in CRC, including tumors associated with inflammatory bowel disease (IBD) where the decrease in RIPK3 mRNA was observed in cancerous tissue and has been found to correlate with poor prognosis(38, 39). Experiments in DSS/AOM model of colitis-associated CRC development showed significantly increased tumorigenesis and lower survival of *Ripk3*$^{-/-}$ animals (38, 69). In this case, increased tumorigenesis was associated with elevated intestinal inflammation, which may reflect a kinase-independent role of RIPK3 in promoting intestinal tissue repair (70).

In case of hepatocellular carcinoma, loss of RIPK1 and RIPK1-associated factor TRAF2, involved in TNFα-dependent NFkB activation, were found to be associated with poor prognosis (71). In mice, deletion of RIPK1 in liver parenchymal cells (LPC) promoted TNFα-dependent apoptosis in a kinase-independent manner. Combined loss of RIPK1 with NFkB pathway components in LPC cells, including TRAF2, IKKβ and RelA, led to a sustained injury, inflammation and HCC development (71, 72).

Overall, the loss of RIPK1 and RIPK3 in these *in vivo* examples recapitulated tumor suppressive properties of these factors. However, tumor suppression was attributed to a range of distinct actions of these factors, illustrating complex connections of the RIPK1/RIPK3 pathway components to the tumorigenesis mechanisms.

Roles of RIPK1/RIPK3 in immunogenic cell death responses to anti-cancer therapeutic agents

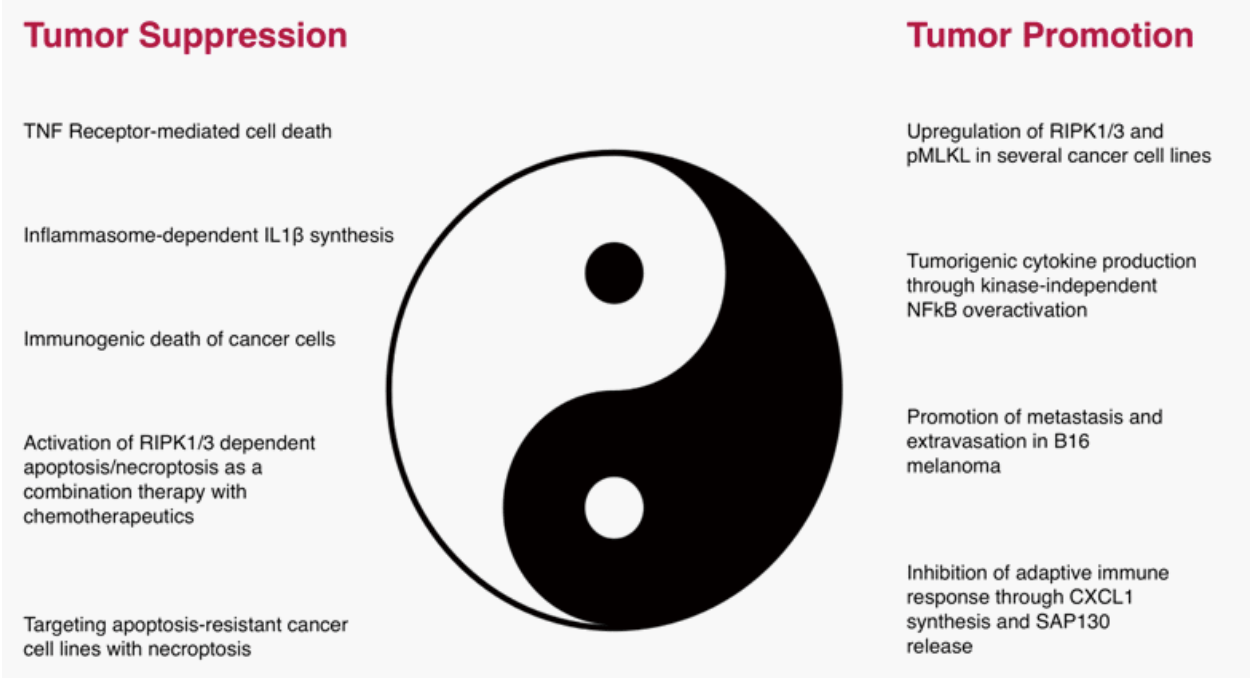

Figure 2. Proposed mechanisms of tumor suppression and promotion by RIPK1, RIPK3 and MLKL.

The roles of RIPK1/RIPK3 pathway components in the responses to anti-cancer therapeutics have also attracted significant interest. Activation of RIPK1/RIPK3-dependent apoptosis or necroptosis typically requires additional modifiers, such as inhibition of cIAPs for activation of RIPK1 kinase-dependent apoptosis (73, 74) and/or inhibition of caspase-8 for RIPK1/RIPK3 kinase-dependent necroptosis (64, 66, 75, 76). Consequently, Moriwaki *et al*. examined cell death in response to a panel of commonly used chemotherapeutic agents and did not observe any changes in cell death upon knockdown of either RIPK1 or RIPK3 in RIPK3-expressing cells or upon overexpression of RIPK3 in cells that lost RIPK3 expression(37), suggesting that RIPK1/RIPK3 may not be generally involved in chemotherapy-induced cell death. These data contrast other observations suggesting that RIPK1/caspase-8 dependent apoptosis is a mechanism of genotoxic stress-induced cell death (74, 77). Mechanistically, genotoxic stress-induced RIPK1-mediated apoptosis required autocrine TNFα synthesis and may depend on the loss of endogenous cIAP1/2 in response to the genotoxic stress. Koo *et al*. also reported a striking significance of RIPK3 for cell death induced by a range of chemotherapeutic agents in RIPK3-expressing Hela and HT29 cells(33). Furthermore, cell death induced by doxorubicin and etoposide was also associated with phosphorylation of MLKL and was inhibited by MLKL shRNAs, strongly indicating activation of necroptosis by these genotoxic agents even in the absence of caspase inhibition. Importantly, the authors also showed that restoration of RIPK3 expression by DNA methylation inhibitor Decitabine (5-aza-2'-deoxycytidine) sensitized breast cancer MDA-MB-231 cell line to doxorubicin *in vitro* and *in vivo*. Overall, these data suggest that RIPK1/RIPK3-dependent apoptosis and/or necroptosis may contribute to responses to chemotherapeutic agents, but their roles appear to be highly variable.

Recent data suggest that necroptosis may be an immunogenic form of cancer cell death, associated with: the release of key immunogenic danger associated molecular patterns (DAMPs)- ATP and HMGB1; upregulation of expression of critical immune-stimulating cytokines, such as Type I interferon pathway components; maturation of dendritic cells exposed to dying/dead necroptotic cells; tumor-associated antigen cross-presentation to $CD8^+$ cytotoxic lymphocytes (CTLs);and generation of long term adaptive anti-cancer immunity in mice exposed to necroptotic cancer cells (35, 78-81). These findings may add additional relevance to the attempts to elicit necroptosis as an anti-cancer strategy.

Immunogenicity of necroptosis may also contribute to and partially explain the roles of RIPK1/RIPK3/MLKL in anti-cancer drug responses. Yang *et al*.(35) recently showed that CRISPR/Cas9 knockout of *Ripk3* or *Mlkl* in TC-1 lung carcinoma and EL4 thyoma mouse lines did not significantly change the degree of cell death induced by methotrexate (MTX) either *in vitro* or *in vivo*. However, release of HMGB1 and ATP was reduced *in vitro* and so was the degree of necrosis observed in MTX-treated xenografts *in vivo*. Correspondingly, accumulation of dendritic cells and $CD8^+$ CTLs in *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ xenografts was markedly decreased as well as regression of the xenografts in response to MTX. Thus, necroptosis may be critical for the immunogenicity, rather than the extent, of cancer cell death. Notably, the efficacy of Newcastle virotherapy in promoting tumor-specific immune

memory to orthotopic glioma was also recently proposed to be in part mediated by the induction of necroptosis by this virus (82).Further details of necroptosis-associated immune regulation can be found in several comprehensive recent reviews (83-86).

Strategies to induce RIPK1/RIPK3-dependent responses in cancer cells

As discussed above, activation of RIPK1/RIPK3 responses, including but not limited to necroptosis, may be an attractive option for cancers which retain expression of the key elements of the pathway. SMAC mimetic compounds attracted major interest as activators of these responses. Finding conditions to drive necroptosis activation by these molecules emerged as one productive approach to achieve excellent anti-tumor activity. As mentioned above, Brumatti *et al*.(66) showed that combining birinapant with pan-caspase inhibitor IDN6556 resulted in greatly increased efficiency of cell death.This combination induced necroptosis, compared to apoptosis induced by birinapant alone, and was effective even in the cells specifically selected *in vitro* to be resistant to birinapant. This also translated into birinapant+IDN combination causing improved tumor regression and survival of animals transplanted with MLL-ENL murine leukemia cells. Similarly, pancreatic carcinoma (PC) cells frequently display resistance to apoptosis but can be efficiently killed through necroptosis by the combination of SMAC mimetic BV6 and pan-caspase inhibitor zVAD. fmk (75). Use of SMAC mimetic LCL161 induced massive necroptosis and tumor regression in caspase-8-deficient CRC xenografts (64). Efficacy of necroptosis can be further enhanced by co-delivery of SMAC mimetic and zVAD. fmk in cationic liposomes. This approach, additionally including delivery of MLKL expression vector, has been tested against mouse CT26 colon carcinoma xenografts and showed improved tumor growth suppression compared to the administration of the individual agents (87). Other co-agents promoting activation of necroptosis by SMAC mimetics may be proteasome inhibitors, such as bortezomib, or glucocorticoids, which have been recently shown to promote necroptosis in several non-Hodgkin lymphoma and acute lymphoblastic leukemia cell lines expressing low levels of caspase-8 (88, 89).

Pro-death activity of SMAC mimetics can also be greatly enhanced by the combination with IFNγ, importantly rendering cell death independent of autocrine TNFα signaling (90, 91). In the absence of caspase inhibition, SMAC mimetics and IFNγ induce RIPK1 dependent apoptosis, which is meditated by both caspase-8 and caspase-10. However, necroptosis occurs if the cells are caspase-deficient.

Resistance to SMAC mimetics due to the deficiency in TNFα synthesis can also be overcome by tapping into alternative sources of TNFα production through combining SMAC mimetics with innate immune inducers. For example, intravesical instillation of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is used for the treatment of early stage bladder cancer. Recent work suggests that BCG can synergize with SMAC mimetic to induce cancer cell death by eliciting TNFα production by neutrophils (92). TNFα synthesis can also be elicited by creating hyperosmotic conditions (93), or use of synthetic innate immune activators (such as poly(I:C) or CpG), oncolytic viruses or immune checkpoint inhibitors (ICIs)(94, 95). In the latter case, synergism between SMAC mimetics and ICIs in eliminating glioblastoma cells *in vivo* reflected immune-mediated tumor regression, which, in turn, required activity of $CD8^+$CTLs and SMAC/ICI-elicited inflammatory responses including type I interferon signature and TNFα synthesis. Even though the parameters of this response are highly reminiscent of the activation of adaptive immune responses to necroptotic cancer cell corpses (35, 78-81), the roles of RIPK1/RIPK3 pathway components in SMAC mimetic/ICI-mediated tumor regression have not yet been established.

Conversely, SMAC mimetics were shown to promote bone metastasis (96), which may be a concern for their use. This activity was independent from the effects of SMAC mimetics on cancer cell death, but rather reflected osteoclast activation, osteoporosis and enhanced tumor-associated osteolysis.

Lastly, a variety of other approaches have also been described recently as leading to activation of necroptosis in cancer cells. These include, for example, disruption of the activity of the members of HOX family of transcription factors in AML (97); inhibition of Aurora kinase A in pancreatic carcinoma cells (98); PPARγ-induced Annexin A1 expression in triple negative breast cancer cells, promoting cIAP1-degradation and, thus, activation of RIPK1-dependent cell death (99); activation of MLKL by ceramide liposomes in ovarian carcinoma cells (100); induction of ROS-dependent TNFα synthesis and necroptosis by selenium nanoparticles in PC-3 human prostate cancer cells (101); and activation of mitochondrial stress and autophagy-dependent necroptosis by BH3 mimetic drug Obatoclax (GX15-070) in human oral cancer cells(102). These and other recent findings indicate that exciting new strategies to therapeutically activate RIPK1/RIPK3-dependent pathways in human cancers may be possible in the near future, but further effort to translate these initial findings into clinically relevant approaches is required.

The dark side: RIPK1/RIPK3/MLKL as tumor promoters (Fig. 2)

While extensive evidence points to the loss of RIPK1, RIPK3, MLKL responses contributing to tumorigenesis, a number of counter-observations have also been made, suggesting that these factors may sometimes promote various functions of tumor cells. Therefore, inhibition of RIPK1/RIPK3 may be a therapeutic anti-cancer strategy in some cases. Along these lines, RIPK1 was found to be upregulated in glioblastomas (103) and lung cancers (104), RIPK3 was increased in serous ovarian cancers (67), and high levels of phosphorylated MLKL correlated with poor

survival of patients with esophagus and colon cancers (105). RIPK1, RIPK3 and MLKL were all highly expressed in pancreatic cancer (41, 106), and highly phosphorylated in M4/M5 sub-types of AML (107).

A number of distinct mechanisms explaining pro-tumorigenic activities of RIPK1/RIPK3/MLKL have been proposed. RIPK1 serves as an important scaffold for NFkB activation (108), which may explain induction of its expression in some cancer types. It is important to note, that NFkB activation likely represents a kinase-independent function of RIPK1 (Fig. 1). Thus, careful consideration of the mechanisms of regulation is required in determining whether inhibition of the catalytic activities of RIPK1/RIPK3 may be beneficial. Liu *et al*. reported that lentiCRISPR knockout of *Ripk1*, *Ripk3* and *Mlkl* in breast cancer MDA-MB-231 cell line drastically reduced anchorage independent growth of the cells *in vitro* and xenograft formation *in vivo* due to the defective NFkB-dependent synthesis of pro-tumorigenic cytokines, such as IL6, IL8, LCN2, MCP1, and CCL5(105). In AML, RIPK1/RIPK3/MLKL activation was required for TNFα-dependent stabilization of SOCS1 and inhibition of IFNγ-induced differentiation (107). Notably, using murine MLL-AF9 AML model, the authors showed that pharmacological inhibition of RIPK1 or genetic deletion of *Ripk1* or *Ripk3* rendered leukemogenesis highly sensitive to inhibition by IFNγ. In case of pancreatic cancer cells, two independent mechanisms of RIPK1/RIPK3 pro-tumorigenic activities were proposed – synthesis of pro-tumorigenic chemokine CXCL1 and release of the SAP130, which activated Mincle receptor on antigen-presenting cells (106). These two pathways cooperate to promote accumulation of myeloid derived suppressor cells (MDSC) in tumor microenvironment, M2-polarization of tumor associated macrophages (TAMs) and inhibition of adaptive immune response.

In a separate set of findings, RIPK1 and RIPK3 were also proposed to promote metastasis. Strilic *et al.* showed that a number of mouse and human cancer cell lines can trigger necroptosis in endothelial cells through cell-cell contacts (109). Necroptosis is activated by the binding of amyloid precursor protein (APP) on the surface of cancer cells to DR6 receptor on endothelial cells. Using genetic and pharmacologic tools, the authors established that this mechanism plays a major role in B16 melanoma extravasation and metastasis *in vivo*. Hanngi *et al*.(110) reported that RIPK1 kinase activity and RIPK3 (primarily as a scaffold) were required for activation of p38/Hsp27 by vascular permeability factors, such as VEGF-A, providing an alternative explanation for the role of these factors in melanoma extravasation.

Concluding remarks

Discovery of regulated cell death and inflammatory mechanisms controlled by RIPK1 and RIPK3 created a lot of excitement because these kinases represent potential druggable targets for a variety of human diseases, representing areas of major unmet medical need. Naturally, given the importance of these mechanisms in cancer, the roles of these factors in cancer have been investigated by multiple laboratories. While the initial findings reflect complex roles and regulation of these kinases that requires further research, the potential of RIPK1 and RIPK3 as therapeutic targets is promising.

RIPK1/RIPK3 and other critical pathway components appear to be clearly perturbed in different types of tumor. Polymorphisms, loss of expression, and pathway activation were all observed, although to our knowledge *bona fide* cancer-associated mutations affecting the known functions, e.g. catalytic activities, of these proteins are yet to be reported. These alterations suggest that both activation and suppression of RIPK1/RIPK3 signaling may take place in cancer cells, likely depending on the specific roles of different mechanisms mediated by these factors in tumorigenesis or drug resistance under particular settings. Furthermore, these alterations may reflect changes in particular modalities of the response, e.g. apoptosis *vs*. necroptosis *vs*. cell death-independent mechanisms, rather than global regulation of all RIPK1/RIPK3 pathways. Along these lines, loss of RIPK3 and MLKL is frequently observed, which may denote cancer types that retain sensitivity to RIPK1-dependent apoptosis. Conversely, while caspase-8 inactivation is another frequent event, it may provide conditions for activation of necroptosis as an efficient and immunogenic form of cancer cell death. However, it is clear that a “one size fits all” approach will not be useful in interpreting the roles of these mechanisms in cancer and finding ways to take advantage of them therapeutically. Rather, precision medicine approaches that take genetic and epigenetic landscapes in individual patient cancers into account will be obligatory.

Strikingly, while in many cases, loss of RIPK1/RIPK3 pro-death and, sometimes, inflammatory responses have been proposed to contribute to more aggressive and poorly treatable tumors, the opposite was also reported, where the same RIPK1/RIPK3 pathways promote or are required for tumor growth and metastasis. Thus, further understanding of the cell types where RIPK1/RIPK3 signaling is induced and cell-autonomous and/or non-cell autonomous, e.g. immunologic, consequences of these responses remains an important outstanding task. In particular, while changes in pathway components were observed in many studies, exact cell types *in vivo* where the changes are taking place, e.g. cancer cells or stroma or immune cells, have not been analyzed in most cases.

With respect to activation of RIPK1/RIPK3 responses as a new anti-cancer strategy, many questions remain. First, the repertoire of approaches that are currently available is limited. SMAC mimetics are the most exciting clinical candidates that have emerged. Conversely, clinical caspase inhibitor Emricasan is a liver-targeted agent(111, 112), probably limiting its general utility. New classes of caspase inhibitors (and especially caspase-8-specific molecules) will be undoubtedly very useful. Many other clinical and pre-clinical agents have been recently found to induce RIPK1/RIPK3 responses and/or restore the lost expression of key factors in cancer cells. However, the

data regarding these agents are currently very limited.

Excitingly, unlike tolerogenic sterile necrosis, necroptosis has emerged as a highly immunogenic form of cell death (78, 79). Necroptosis is associated with gene expression and DAMP release events recognized to be associated with immunogenicity of cell death (reviewed in detail in (83, 85, 86)). Furthermore, activation of these responses through RIPK1/RIPK3/MLKL may play a particular important role in the immunogenicity of common chemotherapeutic agents (35). On the other hand, activation of necroptosis may be no more attractive than other forms of immunogenic cancer cell death, i.e. apoptosis (reviewed in detail in (86)); rather, the availability/inactivation of particular pathways in a particular cancer may be the most critical factor (113). In addition, inflammatory gene expression appears to play differential roles in the immunogenicity of necroptosis in different reports (78, 79); whether necroptosis is pro-inflammatory or anti-inflammatory may depend on how quickly the cells lyse, die and/or are phagocytosed (22, 114-116); and necroptotic cells may expose not only immunogenic, but also tolerogenic signals, such as phosphatidylserine(114, 115). Thus, how necroptosis is activated may be equally important in defining outcomes. Interestingly, combination of SMAC mimetics, often used as RIPK1/RIPK3 pathway activators, with immune checkpoint inhibitors showed excellent promise (94). However, whether RIPK1/RIPK3 pathway components are responsible for the responses either in cancer or T cells has not yet been addressed.

The safety of RIPK1/RIPK3 pathway activation in cancer also remains an open question. Without a doubt, general activation of apoptosis or necroptosis in patients will be highly detrimental. On the one hand, there is little to suggest that oncogenic transformation *per se* renders cells significantly more sensitive to RIPK1/RIPK3 responses in a cell autonomous manner. On the other hand, data using combination of SMAC mimetics or TLR3 agonist poly(I:C) in combination with pan-caspase inhibitors suggest that such necroptosis-inducing stimuli may be well tolerated and efficacious(66, 80). One possibility is that only a very limited degree of RIPK1/RIPK3 cell death may be needed to get the immune system fired up to target the cancer, as some of the positive responses were not observed in immune-deficient animals (35, 80). This may limit the need to reach generally toxic conditions to achieve anti-cancer activity. Additionally, while most of the work thus far has focused on the RIPK1/RIPK3-dependent death of cancer cells, activation of necroptosis in other, tumor-associated lineages and its contribution to tumorigenesis or anti-cancer drug responses has not yet been studied.

Acknowledgements

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Conflict of Interest

A.D. is a consultant to Denali Therapeutics, focused on developing RIPK1 inhibitors for CNS indications.

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