CHAPTER

13

Targeting Necroptosis in Antitumor Therapy

*Francis Ka-Ming Chan**, *Himani Nailwal**, *Kenta Moriwaki***

***University of Massachusetts Medical School, Worcester, MA, USA**

****Osaka University, Japan**

Abstract

Necroptosis is an emerging mode of programmed cell death that is defined at the molecular level through involvement of receptor interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain-like (MLKL). In contrast to apoptosis, phosphorylation is a key event in necroptosis. Necroptosis can be triggered in response to various cell-surface receptors, such as death receptors in the tumor necrosis factor receptor family, Toll-like receptors, and interferon receptors. Because apoptotic machinery is often impaired in cancer cells, necroptosis has garnered attention as a promising strategy to treat apoptosis-resistant cancer. Moreover, since necroptosis leads to release of intracellular immunogenic contents and is therefore highly immunogenic, it has the potential to promote robust antitumor adaptive immune response. In this chapter, we describe current understanding of the role of necroptosis in cancer progression and discuss strategies through which we can harness the power of necroptosis in cancer therapy.

ABBREVIATIONS

CYLD	Cylindromatosis
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
LUBAC	Linear ubiquitin chain assembly complex
MHC	Major histocompatibility complex
MLKL	Mixed lineage kinase domain-like
MPT	Mitochondrial permeability transition
RHIM	RIP homotypic interaction motif
RIPK3	Receptor interacting protein kinase 3
RNAi	RNA interference
SNP	Single-nucleotide polymorphism
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor

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13.1 INTRODUCTION

Until recently, cell death research has primarily focused on studies of apoptosis. Apoptosis is characterized by rounding up of the cell and retraction of pseudopodia, followed by reduction in cell volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), plasma membrane blebbing, and *in vivo* engulfment by resident phagocytes. Apoptosis can be triggered via death receptors in the tumor necrosis factor receptor (TNFR) superfamily (extrinsic apoptosis), or through direct activation of mitochondrial apoptosis effectors (intrinsic apoptosis). The programmed and regulated nature of apoptosis stands in sharp contrast to necrosis induced by stress or trauma. Rapid clearance of apoptotic cells by professional phagocytes essentially limits inflammation. Hence, apoptosis is an immunologically silent form of cell death. On the other hand, necrosis is often marked by an increase in cell volume (oncosis) and swelling and rupture of the cell membrane. Rupture of the plasma membrane causes release of damage-associated molecular patterns (DAMPs), which elicit immune responses [1]. As such, lytic cell death is primarily linked to inflammation, infection, and trauma-induced cellular insults.

Besides accidental necrosis, recent evidence indicates that necrosis can also occur in a regulated manner. Regulated necrosis includes necroptosis, parthanatos, ferroptosis or oxytosis, mitochondrial permeability transition (MPT)-dependent necrosis, pyroptosis and pyronecrosis, and NETosis or ETosis [2]. Among these, necroptosis is the best characterized form of regulated necrosis. Similar to apoptosis, necroptosis is a tightly regulated process that can be induced in response to stimulation with death receptors in the TNF receptor family, as well as certain Toll-like receptors (TLRs). The serine/threonine kinase receptor interacting protein kinase 3 (RIPK3) is a key adaptor in necroptosis. One of the most well-known functions of necroptosis is to promote antimicrobial inflammation. In addition, deregulation of the necroptotic signaling pathway components is observed in a number of disease models such as TNF-mediated hypothermia and systemic inflammation [3], ischemic reperfusion injury [4], and Gaucher's disease [5]. In particular, there is growing evidence of dysfunction of necroptosis in different human cancers [6]. These findings highlight the tantalizing possibility of targeting necroptosis in therapies.

13.2 NECROPTOSIS SIGNALING PATHWAY

Death domain-containing receptors of the TNF receptor superfamily (i.e., TNFR1, Fas/CD95/APO-1, and TRAIL-R), Toll-like receptor 3 (TLR3), and TLR4 are the main receptors that trigger necroptosis. TNF/TNFR1 stimulation causes formation of a plasma membrane-associated complex termed Complex I [7], which contains the adaptor proteins TRADD, TRAF2, the E3 ubiquitin ligases cIAP1 and cIAP2, the serine/threonine kinase RIPK1, and the linear ubiquitin chain assembly complex (LUBAC). LUBAC promotes linear ubiquitination of NEMO, RIPK1 and other adaptors, whereas cIAP1 and cIAP2 mainly mediate K63-linked ubiquitination. The ubiquitin scaffold generated in Complex I is a pivotal checkpoint for induction of NF-κB, apoptosis, and necroptosis. This is mostly achieved through recruitment and activation of TAK1 and the IKK complex. IKKα/β within the IKK complex phosphorylates IκBα, leading to its K48-linked ubiquitination and proteasomal degradation. In addition, IKKα/β can directly phosphorylate RIPK1 to inhibit its death-inducing function [8].

Phosphorylation of IκBα exposes the nuclear localization signal on NF-κB, resulting in its translocation to the nucleus. NF-κB is the most widely known to drive expression of inflammatory mediators. In addition, NF-κB also stimulates expression of pro-survival genes such as c-FLIP, an inactive homolog of Caspase-8 that lacks protease activity. Although the long isoform of c-FLIP (c-FLIP$_L$) forms a heterodimer with Caspase-8 to promote its activity [9], it can also inhibit Caspase-8 and apoptosis when overexpressed. It is therefore noteworthy that overexpression of c-FLIP$_L$ has been reported in many tumors [10–12]. In addition to c-FLIP$_L$, the E3 ligases cIAP1 and cIAP2, which ubiquitinate RIPK1 to sterically interfere with binding to other death-inducing signal adaptors, are also well-known targets of NF-κB. The induction of pro-survival factors explains why inhibition of NF-κB is required to achieve optimal cell death in response to TNF (Fig. 13.1).

The membrane-associated Complex I is short-lived and is quickly internalized into the cytosol within the first hour. Internalization of Complex I leads to dissociation of TNFR1 and recruitment of FADD and Caspase-8 to the complex. This cytosolic complex, termed Complex II (Fig. 13.1) [7], is the key signaling node for cell death. The mechanism that regulates the transition of Complex I to Complex II is still under investigation, but is believed to involve de-ubiquitination of RIPK1 in Complex I [13]. This process critically requires the de-ubiquitinating enzyme cylindromatosis (CYLD), which also regulates RIPK1 ubiquitination in Complex II [14]. In addition, IKKα/β directly phosphorylates RIPK1 to impede downstream Complex II assembly [15]. Interestingly, a similar complex called the ripoptosome is assembled in response to chemotherapeutic agents independent of receptor stimulation [16,17]. Hence, Complex II or the ripoptosome can be assembled in response to multiple stimuli.

Complex II is normally an apoptosis-inducing complex (Complex IIa). When Caspase-8 is inhibited, such as that during certain viral infections [18], RIPK1 recruits RIPK3 via the RIP homotypic interaction motif (RHIM) to form the necrosome or Complex IIb (Fig. 13.1). The RHIM is a protein interaction motif marked by a highly conserved tetrapeptide core and flanking β-strand dominant residues. The RHIMs of RIPK1 and RIPK3 form an amyloid-like complex that facilitates downstream necroptosis signaling [19]. The requirement for caspase inhibition for necroptosis is explained by the fact that Caspase-8 cleaves RIPK1 and RIPK3 at the boundary of the kinase domains [20,21]. Thus, cleavage of RIPK1 and RIPK3 separates the kinase domain from the RHIM and prevents kinase activation within Complex II. Although RIPK1 kinase activity is essential for RIPK3 phosphorylation and activation in response to TNF, it is noteworthy that TRIF and DAI/ZBP-1, two other RHIM-containing adaptors that promote necroptosis, are not kinases. Hence, it is likely that RIPK1 does not activate RIPK3 through direct phosphorylation. Rather, RIPK1 kinase activity may play a more important role in transitioning of Complex I to Complex II.

Once RIPK3 is phosphorylated, it recruits and phosphorylates its downstream effector mixed lineage kinase domain-like (MLKL). MLKL is a pseudokinase that has no enzymatic activity [22]. Upon phosphorylation by RIPK3, MLKL assembles into an oligomer and translocates to the plasma membrane and other cellular membranes [23–25] (Fig. 13.1). Recent studies reveal that MLKL oligomers form channels on membranes, suggesting that it may directly trigger the plasma membrane rupture that is characteristic of necroptosis [25–27]. Besides TNFR-1 and related death receptors, RIPK3 can also be activated by TRIF or DAI/ZBP-1 in response to TLR3/TLR4 stimulation or herpesvirus infection, respectively [28,29]. Although the upstream activating signals are different, the downstream mechanisms stimulating MLKL appear to be conserved for all the necroptosis stimuli.

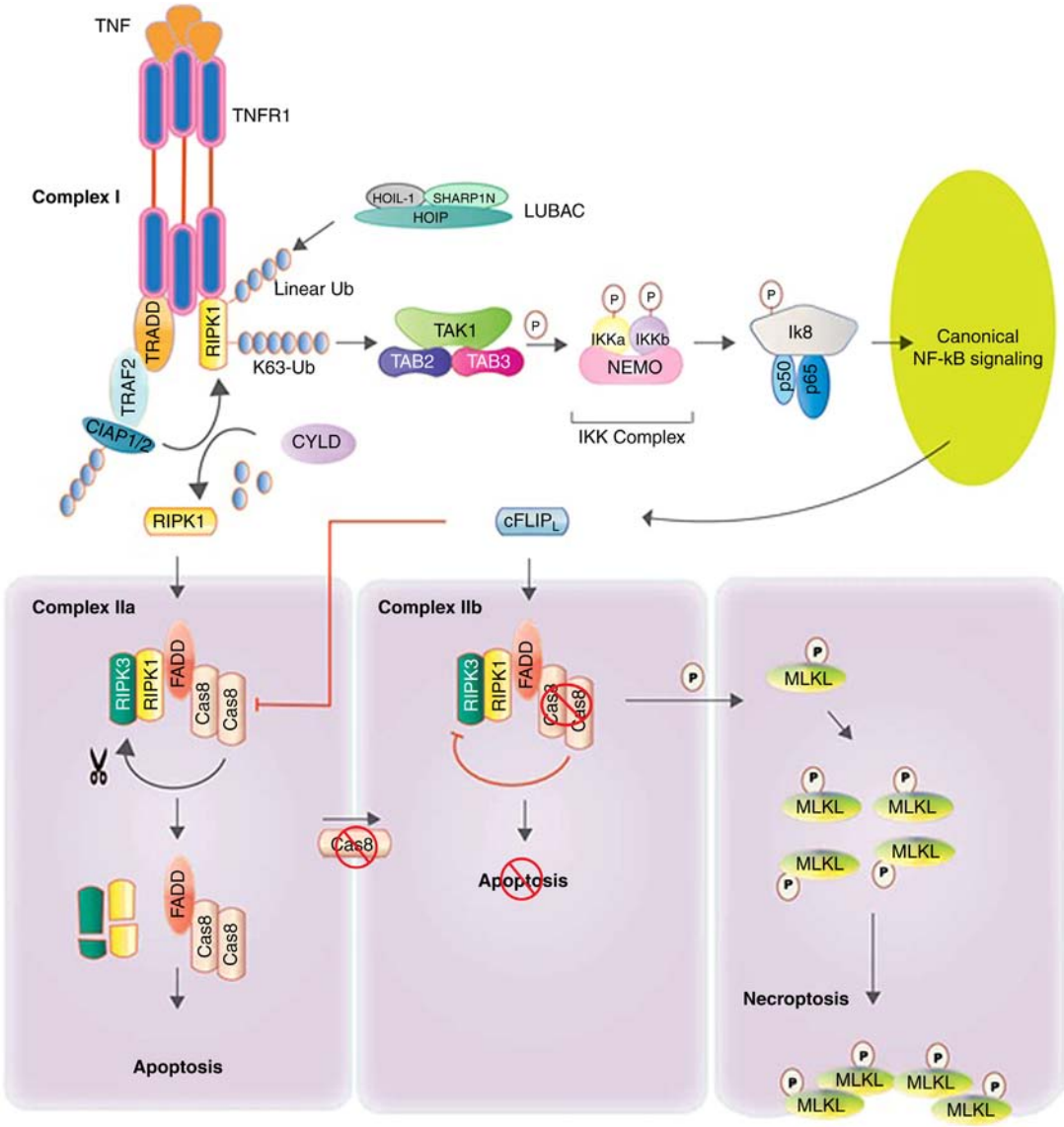

FIG. 13.1 Molecular mechanism of TNF-induced necroptosis. TNF stimulation leads to formation of a TNFR1-associated complex whose main function is to activate the latent transcription factor NF-κB. Poly-ubiquitination of adaptors such as RIPK1 in this complex promotes recruitment of the kinase TAK1 through the adaptors TAB2 and TAB3, which in turn activates the IKK complex, IκBα phosphorylation and degradation, and NF-κB dependent gene transcription. De-ubiquitination of RIPK1 by CYLD marks the transition of Complex I to cytosolic Complex II. Active Caspase-8 in Complex IIa is responsible for apoptosis induction and inhibition of necroptosis through cleavage of RIPK1 and RIPK3. NF-κB-dependent expression of survival factors such as $cFLIP_L$ inhibits apoptosis by suppressing Caspase-8 activity. When Caspase-8 is inactive, RIPK1 and RIPK3 interact through their RHIM domains, leading to Complex IIb assembly and MLKL phosphorylation. Phospho-MLKL undergoes oligomerization and translocates to the plasma membrane to trigger membrane rupture and necroptosis. Abbreviations: Casp8, Caspase-8; $cFLIP_L$,

13.3 NECROPTOSIS AND CANCER

13.3.1 Differential Expression of Necroptosis Regulators in Normal and Cancer Cells

Resistance to apoptosis is a major hallmark of cancers. This resistance is often acquired by reduced expression/function of pro-apoptotic molecules. Likewise, several reports showed that pro-necroptotic molecules are similarly downregulated in cancer cells. For instance, expression of RIPK3 and CYLD was found to be significantly reduced in various cancer tissues compared to adjacent normal tissues [30–33]. In addition to RIPK3 and CYLD, MLKL expression was also reduced in primary leukemia [34–39]. Similar loss of RIPK3 expression is found in many cancer cell lines commonly used in the laboratory [31,32]. Various mechanisms such as DNA hypermethylation or hypoxia have been attributed to cause downregulation of RIPK3 expression in cancer cells (Fig. 13.2) [31,32]. In addition, mutations in critical amino acids of pro-necroptotic molecules, such as D156N in the DLG motif of the kinase domain of RIPK1, V458M in the RHIM of RIPK3, and L291P in the pseudokinase domain of MLKL, have been found in cancer tissues [40]. In addition to these mutations, single-nucleotide polymorphism (SNP) analysis revealed strong correlation between SNPs in the *Ripk3* gene and non-Hodgkin lymphoma [6]. The reduced expression of MLKL and CYLD is associated with poor prognosis in pancreatic adenocarcinoma, cervical squamous cell carcinoma, melanoma, and leukemia patients [36,38,41,42]. Germline loss-of-function mutations of CYLD were linked to familial cylindromatosis, Brooke–Spiegler syndrome, and multiple familial trichoepithelioma, all of which are autosomal dominant genetic disorders marked by multiple skin tumors [43–48]. Consistent with the data from human patients, *Ripk3*$^{-/-}$ mice were more prone to develop inflammation-driven colorectal cancer [49,50]. These results strongly suggest that necroptosis limits tumor generation and progression.

Despite the large number of studies suggesting a tumor-suppressive role for necroptosis, several recent reports show that RIPK3 can also promote tumor growth. For example, necroptosis-dependent release of the chemokine CXCL1 appears to promote pancreatic ductal adenocarcinoma [51]. Two recent studies reported that RIPK3 expression in endothelial cells promotes extravasation of cancer cells and metastasis [52,53], although these reports differ on whether RIPK3 exerts this function through necroptosis. How can we reconcile these disparate reports on the role of RIPK3 and necroptosis in cancer? One possible explanation lies in the recent discovery that RIPK3 can promote inflammatory cytokine expression independent of cell death [54]. Thus, it is important to remember that these cell death-independent functions of RIPK3, as well as necroptosis, can both contribute to tumor progression and metastasis [52].

cellular FLICE-like inhibitory protein; cIAP, cellular inhibitor of apoptosis 1; CYLD, cylindromatosis; FADD, Fas-associated via death domain; IKK, inhibitor of κB kinase; LUBAC, linear ubiquitin chain assembly complex; MLKL, mixed lineage kinase domain-like; NEMO, NF-κB essential modulator; P, phosphorylation; TAB, TAK1-binding protein; TAK, TGFβ-activated kinase; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TRADD, TNF receptor-associated death domain; TRAF, TNF receptor-associated factor 2; Ub, ubiquitin.

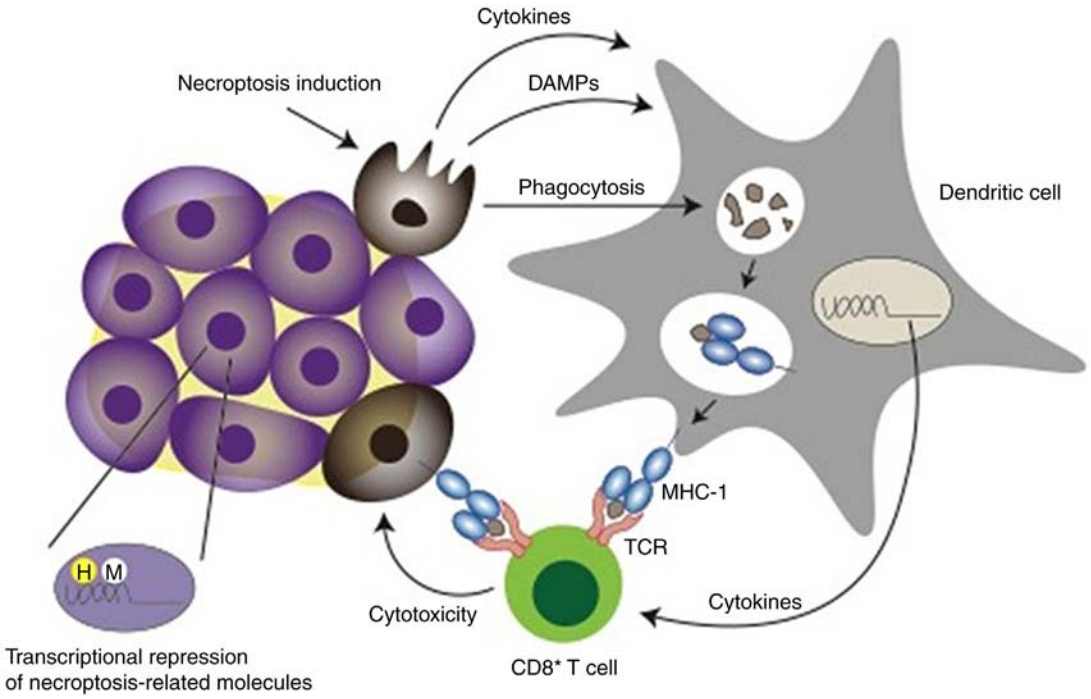

FIG. 13.2 **Schematic overview of necroptosis in cancer.** Necroptotic cells release immuno-stimulating molecules called DAMPs through the ruptured plasma membrane. In addition, necroptotic cells can actively secrete cytokines through NF-κB-dependent transcription. Both these factors promote functional maturation of DCs. The remnants of necroptotic cells are engulfed by DCs and processed in the endosomes. Tumor-associated peptides are loaded on MHC class I (MHC-I) complex and recognized by T-cell receptors (TCRs) expressed on naïve $CD8^+$ T-cells. This cross-presentation generates effector $CD8^+$ T-cells with cytotoxic function against cancer cells. On the other hand, cancer cells at the center of the tumor tissues that is under hypoxic stress (H, yellow circle) can lead to transcriptional silencing of RIPK3. In addition, RIPK3 expression can also be suppressed by DNA methylation (M, white circle). These competing events ultimately determine whether the developing tumor survives or is eliminated by immune surveillance mechanisms. Abbreviation: DAMPs, damage-associated molecular patterns.

13.3.2 Necroptosis and Anticancer Drug

Necroptosis is mainly induced by cell-surface receptors such as TNFR-1. Certain conventional anticancer agents have been reported to stimulate this canonical necroptotic pathway by stimulating autocrine TNF production. For example, cisplatin induces cancer cell necroptosis through TNF-dependent and -independent mechanisms [55,56]. Autocrine TNF also plays a key role in necroptosis of colon carcinoma in response to 5-FU and pan-caspase inhibitor [57]. Neoalbaconol, a compound extracted from fungus, also induced necroptosis through autocrine TNF production [58].

Although many anticancer agents cause necroptosis through autocrine TNF production, there are also examples in which tumoricidal compounds can trigger necroptosis independent of TNF. For instance, the Bcl-2 inhibitor obatoclax and the novel chalcone derivative chalcone-24 potently induced necroptotic cancer cell death [59–63]. The kinase inhibitors

sorafenib and staurosporine induced necroptosis in lymphoma and prostate cancer cells [64–66]. Interestingly, the DNA-damaging agents 5-FU, etoposide, and camptothecin induced MLKL-dependent, but RIPK3-independent necroptosis in colon cancer cells that lack caspase 3 [67]. The combination of simvastatin and metformin induced necroptosis in metastatic castration-resistant prostate cancer cells [68]. Although TNF is not involved in these cases, other mechanisms such as autophagy, reactive oxygen species production, and degradation of inhibitors of apoptosis protein have also been implicated. How the necroptosis machinery interacts with these processes at the molecular level, however, is not clear. In this light, it is noteworthy that many of these studies relied on the RIPK1 kinase inhibitor necrostatin-1 to determine whether cell death was caused by necroptosis [62–64,66,69,70]. Although RIPK1 kinase activity is crucial for TNF-induced necroptosis, it is also required for certain types of apoptosis [16,17]. Moreover, off-target effects have been reported on necrostatin-1 [71,72]. Hence, necrostatin-1 and other RIPK1 kinase inhibitors are not the best criteria to determine if necroptosis is the causative cell death modality. In fact, RNAi-mediated knockdown of RIPK3 expression may also be insufficient to determine whether necroptosis is involved, since RIPK3 can promote apoptosis in a kinase-independent manner in certain situations [15,73–77]. These caveats highlight the importance of using multiple criteria and approach to interrogate the role of necroptosis in cancer cell death.

13.3.3 Necroptosis in Antitumor Immunity

The immune system not only protects the body from infectious, nonself agents, but also limits abnormal proliferation of cancer cells that can be viewed as noninfectious "self". Immunotherapy against cancer has been shown to be effective and emerged as a promising alternative to current standard chemotherapy [78]. $CD8^+$ cytotoxic T-cells play a major role in the immune surveillance and elimination of cancer cells. Activation of tumor-specific $CD8^+$ T-cells requires tumor antigen cross-presentation by professional antigen-presenting cells [79]. This can be achieved through phagocytosis of necrotic tumor cells by dendritic cells (DCs) or macrophages [80]. Indeed, this notion is corroborated by recent reports that necroptotic tumor cells taken up by DCs can efficiently cross-prime cytotoxic $CD8^+$ T-cell response against tumor grafts [81,82]. Interestingly, RIPK1-dependent NF-κB activation and gene induction appears to further enhance efficient cross-priming of tumor-specific $CD8^+$ T-cells (Fig. 13.2) [81]. In addition to immunization with necroptotic tumor cells, TLR3 stimulation could directly induce IL-1α secretion and IL-1α-induced necroptosis of cervical cancer cells. In this case, the dying cancer cells stimulated DC-mediated IL-12 production, suggesting an indirect adjuvant effect of the necroptotic cells [83]. These results are in agreement with the widely accepted notion that DAMPs released from necroptotic cells stimulate innate inflammation, which in turn promotes a robust adaptive immune response against cancer cells. Based on these results, immunization with necroptotic cells appears to be a promising strategy for therapeutic cancer vaccine.

13.4 CONCLUDING REMARKS

Intensive studies in the last decade have established a critical role for the phosphorylation-driven RIPK3-MLKL pathway in cell death and inflammation. In contrast, our knowledge on the role of necroptosis in cancer development, progression, and metastasis is still evolving.

Since inflammation is a double-edged sword that can promote or inhibit tumor growth [84], the effect of necroptosis on carcinogenesis is likely going to be context dependent. Having said that, given the reduction of essential necroptosis regulators RIPK3 and MLKL in many cancers, we can postulate that necroptosis has a general tumor-suppressive function. It is noteworthy that while many cancer cell lines are normally refractory to necroptosis, the combination of agents that primes cells to necroptosis and other standard chemotherapeutic agents can often break this resistance to necroptosis [85]. Hence, both experimental results and observations in human patients give credence to the idea that one can harness the power of necroptosis in anticancer therapy. Besides elimination of the tumor itself, necroptosis offers the additional advantage of stimulating tumor-specific cytotoxic T-cell responses. Hence, it will be interesting to determine whether necroptosis-sensitizing agents can also enhance the efficacy of immune checkpoint inhibitors in cancer therapy.

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