Relevance of necroptosis in cancer

Najoua Lalaoui, Gabriela Brumatti

Cite this article as: Najoua Lalaoui, Gabriela Brumatti, Relevance of necroptosis in cancer, *Immunology and Cell Biology* accepted article preview 6 December 2016; doi: 10.1038/icb.2016.120.

Received 31 October 2016; revised 1 December 2016; accepted 1 December 2016;
Accepted article preview online 6 December 2016

Relevance of Necroptosis in cancer

Najoua Lalaoui[1,2] and Gabriela Brumatti[1,2]

[1]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia

[2]Department of Medical Biology, University of Melbourne, Parkville, Victoria 3050, Australia

ABSTRACT

Resistance to caspase-dependent apoptosis is often responsible for treatment failures in cancer. Finding novel therapeutic strategies that can activate alternative cell death programs appears to be appealing. Necroptosis is a form of programmed necrosis that occurs under caspase deficient conditions. This alternative form of cell death has recently emerged as a potential anti-cancer therapy that could overcome apoptosis resistance. A growing understanding of the molecular events triggering necroptosis helped to examine its implication in cancer development and to define new therapeutic strategies. Genetic and proteomic analysis suggest that necroptosis is deregulated in many cancers. Various preclinical and clinical compounds induced necroptosis and demonstrated significant therapeutic efficacy. Moreover, accumulating evidence has shown that necroptosis promotes anti-cancer immune response. A better knowledge of the cascade of events regulating necroptosis is expected to assess the feasibility of its therapeutic exploitation for cancer therapy.

INTRODUCTION

Evading cell death is indisputably a hallmark of cancer to ensure cancer cells survive under stress conditions[1]. The programmed cell death apoptosis is often deregulated in cancer. In the last three decades, enormous research efforts have made great strides in decrypting the molecular mechanisms that govern apoptosis. Those discoveries led to the development of anti-cancer agents that reactivate apoptosis to kill cancer cells. However, therapeutic interventions aiming to induce apoptosis often face resistance arising from activation of survival pathways. Thus, the finding of more thoughtful combinations therapies that simultaneously target alternative cell death and survival pathways is one of the main focuses in cancer research.

While the role of apoptosis in cancer has been largely characterised, the relevance of alternative cell death pathways such as necroptosis has been far less studied. Necroptosis is a relatively newly discovered programmed form of necrosis[2, 3]. The term 'necroptosis' arise from its ability to share apoptotic and necrotic features. Necrosis is considered as an accidental death resulting from an over-whelming cytotoxic insult, and does not require specific molecular events in order for it to occur. In contrast, necroptosis is highly regulated and shares molecular events with apoptosis. Like in necrosis, the ultimate stage of a necroptotic process is the swelling and rupture of the cell membrane, releasing Damage-Associated Molecular Patterns (DAMPs), which can elicit immune responses[4].

The potential immunogenicity of necroptosis and its ability to kill cancer cells are two attractive characteristics for this type of cell death to be seen as a new therapeutic approach. In addition, triggering necroptosis could be used when drugs failed to induce apoptosis. Recently, a number of studies have provided new insights in the molecular regulation of necroptosis. This has helped defining and designing necroptotic stimuli that can be potentially use for cancer therapy. However, there is still a debate on whether this type of therapy is tolerable, feasible or just conceptual. Given the rising significance of necroptosis in cancer, a better understanding of its implication in cancer development and maintenance is a prerequisite for the design of appropriate drugs. It is therefore a timely and important question to review necroptosis and its relevance in cancer. In this review, we will discuss the role of necroptosis in tumour development and progression. We will also describe and comment on the importance of this pathway in cancer immune surveillance and therapy.

NECROPTOSIS PATHWAY IN BRIEF

A detailed view of the molecular cascade triggering necroptosis in different scenarios can be found in other reviews in this issue. We will therefore succinctly cover the main molecular events leading to necroptosis.

Necroptosis can be activated by the engagement of Tumor Necrosis Factor (TNF) Receptor superfamily, T-Cell Receptors, Pattern Recognition Receptors, Interferons Receptors, genotoxic or oxidative stresses and various anti-cancer drugs[5]. In contrast to apoptosis, necroptosis requires inactivation of caspase-8, which leads to the activation of serine/threonine Receptor Interacting Proteins kinases RIPK1 and RIPK3.

One of the best-characterised signalling cascades leading to necroptosis is the one engaging the TNF/TNFR1 signalling pathway. In a physiological situation TNF activates the transcription of survival and inflammatory genes. However, in some circumstances deregulation of TNF signaling or high dose of TNF causing systemic inflammatory response syndrome lead to TNF-mediated cell death[6]. The binding of TNF to TNFR1 induces the formation of a membrane bound complex (complex I) where cellular Inhibitor of APoptosis proteins (cIAP1&2) ubiquitylate RIPK1 (Figure 1). Ubiquitylation of RIPK1 facilitates the recruitment kinases that activate the NF-κB and MAPK signaling pathways. This leads to the transcription of downstream genes such as the caspase-8 inhibitor cFLIP[7, 8]. In parallel, in complex I deubiquitinase enzymes such as CYLD remove ubiquitins from RIPK1 to limit a sustain activation of NF-κB[9].

Ubiquitylated RIPK1 also prevents formation and activation of RIPK1-dependent apoptotic and necroptotic complexes. Indeed, perturbations of RIPK1 ubiquitylation, by the absence of cIAP1&2

for instance, promote the formation of the cytosolic complex II so-called "Ripoptosome", containing RIPK1, FADD and caspase-8, and reduce cFLIP expression leading to caspase-8 activation and apoptosis[10, 11] (Figure 1). In complex II activated caspase-8 cleaves and inactivates RIPK1 and RIPK3 to block necroptosis. In some cell types or conditions, activation of caspase-8 is compromised and RIP kinases are no longer cleaved. Subsequently, a series of auto- and cross-phosphorylations between RIPK1 and RIPK3 result in the formation of the necrosome, where MLKL is phosphorylated by RIPK3, which stimulates its oligomerization and translocation to the plasma membrane to trigger necroptosis[12-14] (Figure 1).

NECROPTOSIS AND TUMORIGENESIS AND CANCER PROGRESSION

During the course of tumorigenesis accumulations of genetic and epigenetic alterations allow cancer cells to evade cell death, acquire proliferative advantage, induce angiogenesis and invade the body[1]. Although the study of the role of necroptosis in cancer is still in its prelude, there is accumulating evidence demonstrating that necroptosis is deregulated in cancer.

Mutations

According to the COSMIC database, somatic mutations in *RIPK1*, *RIPK3*, and *MLKL* genes have been observed in human cancers. One of these mutations V458M, resides within the tetra-peptide core of the RHIM domain of RIPK3 and may provoke the disruption of RHIM-mediated protein interaction and signaling[15]. Moreover, several missense mutations in the kinase domain of RIPK1 were found in different types of cancers and might alter its signaling function. The reported MLKL mutations F398I did not affect MLKL necroptotic function, while L291P mutation may represent loss of function mutants[13] (Table 1).

In addition, Single-Nucleotide Polymorphisms (SNP) in the *RIPK1* and *RIPK3* genes were detected in CML and non-Hodgkin lymphoma patients respectively[16, 17] (Table 1). RIPK1, RIPK3 and MLKL functions are regulated by sequential phosphorylations and ubiquitylations events as well as homotypic interactions *via* their Death Domains and RHIM domains[18]. A better knowledge of the phosphosites and the ubiquitin sites would therefore help on identifying whether the COSMIC mutations or SNP found in RIPK1 and RIPK3 affect their functions.

Expression in cancer patients

A pleiotropic range of cancer cell lines lack RIPK3 expression[19, 20-22]. Consistently, RIPK3 down-regulation has been found in human samples of several types of cancer (Table 1). RIPK3 transcripts were found to be reduced in AML patient samples, whereas *RIPK1* expression did not differ

significantly when compared to healthy donors[23]. Interestingly, AML patients with low expression of *RIPK3* presented high level of methylations near the transcription start site of *RIPK3*[19]. In both studies, the sample sizes were too small to draw meaningful conclusions on whether RIPK3 expression was correlated with any AML subtypes. However, recently a group has interrogated 2 large datasets of *de novo* primary AML patient samples and found a significant reduction in both *RIPK3* and *MLKL* expression in several AML subtypes including AMLs with FLT3 mutations and AML1/ETO9a translocations[24] (Table 1). Consistent with this, loss of *Ripk3* or *Mlkl* accelerated the leukemogenesis in mouse model of FLT3-ITD and AML1/ETO9a. In contrast *RIPK3* and *MLKL* expressions in AML patients carrying MLL translocations were comparable to healthy donors and genetic deletions of *Ripk3* or *Mlkl* did not affect the leukemia progression of mouse model of MLL-ENL[24, 25]. The examination of the expression profile of human CLL samples revealed that *RIPK3* and *CYLD* were frequently down-regulated, while no difference in *RIPK1* expression were found compared with healthy controls[26] (Table 1). CYLD removes ubiquitins from RIPK1 and increases its ability to form apoptotic and necroptotic cell death complexes[9, 27]. Consistent with this, CLL samples were refractory to necroptotic stimuli[26].

Suppression of *RIPK3* expression was also documented in several solid cancers. Like in AML patients, in primary breast cancer samples RIPK3 loss correlated with methylation of the genomic region near *RIPK3* gene's transcription start site suggesting that a methylation-mediated mechanism regulates RIPK3 expression during breast cancer development[19] (Table 1). Several reports found that *RIPK3* is down-regulated in human colon and colorectal cancers compare to adjacent normal tissues[22, 28, 29]. Accordingly, loss of *Ripk3* increased colon tumorigenesis induced by carcinogenes in mice. The acceleration of tumorigenesis in *Ripk3*-deficient mice was more likely due to an excessive inflammation[28]. Therefore, the role of RIPK3 in dampening inflammation during colorectal tumorigenesis contrasted with its role in others inflammatory scenarios, where loss of *Ripk3* has been described to limit inflammation[30]. It has been suggested that hypoxia could account for RIPK3 silencing in colon cancers[22].

Collectively, these studies suggest that necroptosis may have a tumor suppressor role in cancer. However, shutting down the necroptosis pathway does not seem to be a general mechanism for all types of cancer cells to survive and progress. In some cancers, the expression of necroptotic players was found to be elevated. For instance, RIPK1 was found upregulated in gliobastoma and lung cancer[31, 32] (Table 1). Furthermore, analysis of TCGA database showed that there is an enrichment for *RIPK3* expression in serous ovarian cancer[33]. Interestingly, RIPK1, RIPK3 and MLKL were highly expressed in pancreatic cancer samples[34, 35] (Table 1). High *RIPK3* levels correlated with high *CXCL1* expression in human pancreatic cancer samples, a potent chemo-attractant of myeloid

cells. Accordingly, genetic deletion of *Ripk3* or inhibition of RIPK1 protected against pancreatic oncogenesis in mice, reduced CXCL1 expression and decreased the infiltration of tumour-associated macrophages *in vivo*[34]. In contrast to its role in colon cancer, necroptosis seemed to drive a tumour immuno-suppressive environment facilitating the progression of pancreatic cancer.

Prognosis

It is clear now that deregulation of necroptotic regulators occurs in cancer and accumulating evidence has in fact associated level of expression and patient survival. For instance, low RIPK3 levels correlated with poor outcome for colorectal and breast patients[19, 22, 28, 29] (Table 1). This indicates that RIPK3 expression may be negatively selected during development or progression of breast and colorectal cancers. Likewise, reduced expression of MLKL was significantly associated with decreased overall survival of gastric, ovarian, cervix, colon and pancreatic cancers[35-39] (Table 1). Thus, MLKL expression could potentially serve as a prognostic biomarker for those cancers.

However, it is noteworthy that although low MLKL expression correlated with poor prognosis for colon cancer patients, high level of phospho-MLKL was reported to be associated with worse prognosis for colon and esophageal cancer patients[28, 40]. The questions to be asked are why and how cancer cells would keep a high level of phopho-MLKL to progress. One would predict that those cells would die through necroptosis. Two hypotheses can be formulated amongst many others. One is that a subset of cancer cells would activate necroptosis to manipulate the immune system. The other reason could be that phospho-MLKL has another function beside its role in executing necroptosis.

Metastasis

The role of necroptosis in metastasis is poorly characterised. To disseminate tumour cells need to evade the anti-tumour attacks. A recent study uncovered the involvement of RIPK3 in regulating NKT cell responses[41]. As NKT cells participate on immune response against metastasis, expression of RIPK3 in the hematopeitic system may limit cancer invasion. Consistently, genetic deletion of *Ripk3* compromised NKT cell activation and resulted in a defect of anti-metastatic response *in vivo*. The authors also found that mitochondrial phosphatase PGAM5 played a role in NKT cell activity and this required DPR1[41]. PGAM5 has been proposed to function downstream of MLKL to promote DRP1 dimerization responsible for the mitochondria fission during necroptosis[42]. However, the implication of the mitochondria in necroptosis has been recently questioned by several reports, which exposed the dispensability of both proteins in executing necroptosis[13, 43-46]. The role of PGMA5-DRP1 in anti-metastatic response is therefore potentially independent of the necroptosis

During the metastastic process, extravasation of tumour cells refers to their exit from the blood vessels to the secondary site. Recently Strilic and colleagues demonstrated that co-cultures of cancer cells and endothelial cells resulted in necroptotic endothelial cell death[47]. Similarly, injection of metastatic tumour cells induced necroptosis of lung epithelial cells in mice. Consistently, inhibition of the necroptotic pathway reduced tumour cell migration and metastatic burden *in vivo*. A siRNA screen revealed that DR6 (or TNF receptor superfamily member 21) promoted endothelial cell death and tumour cells transmigration through its cognate ligand Amyloid Precursor Protein (APP)[47]. The authors found that APP/DR6 induced epithelial cell death with necrotic features. The mechanism by which APP/DR6 induce necrotic/necroptotic epithelial cells death has not been fully explored in this study. Nevertheless, it is interesting to note that DR6 has a death domain that might be able to recruit other death domain containing proteins such as RIPK1 to activate necroptosis.

Although the work of Strilic and colleagues suggest that necroptosis can, in some situations, promote cancer cell metastasis, a body of evidence has endorsed evasion of necroptosis as hallmark of cancer. Caspase-8 expression is suppressed in many cancers[48]. This is probably a natural evolution for cancer cells to suppress necroptosis. It would be informative to determine the correlations between the expression of caspase-8 and the necroptotic players. Intriguingly, amongst the necroptotic regulators RIPK3 seems the most deregulated in cancers. The absence of *Ripk1* in mice is detrimental while loss of *Ripk3* does not have any overt effect[44, 49-53]. One could think that suppression of RIPK1 could potentially be fatal for cancer cells while suppression of RIPK3 could protect cancer cells against any necroptotic stimuli. In addition, in contrast to MLKL, RIPK3 regulates cytokine production[54-56]. Therefore, tumour cells might silence RIPK3 to block necroptosis and manipulate the anti-tumoral immune attacks. Although, mouse models of cancer have corroborated the low level of RIPK3 observed in human cancer, the studies cited above did not explore the role of RIPK3 in the different cellular compartments (e.g. cancer *vs* immune *vs* stromal cells). Given the role of necroptosis in the immune system, tissue specific deletion of RIPK3 would clarify the role of necroptosis in tumour surveillance.

NECROPTOSIS AND CANCER IMMUNE SURVEILLANCE

The immune system can specifically eliminates pre-cancerous and cancerous cells based on their tumour-specific antigens expression. Tumour surveillance is mainly mediated by Dendritic Cells (DC), cytotoxic $CD8^+$ T cells, tumoricidal macrophages and Natural Killers (NK). Tumour antigens are presented by DC to $CD8^+$ T cells, leading to their activation and expansion in response to

antigen recognition, a process known as cross-presentation or cross-priming. Unlike T cells, recognition of tumour cells by NK cells is not governed by tumour-antigens specificity. NK cells express several receptors that recognise and attack cancer cells presenting aberrant expression of MHC class 1 molecules and stress markers[57]. NKT cells are subsets of NK that also participate in anti-tumoral responses[58]. While RIPK3 was found dispensable for B and T cell receptors signalling, it appeared to regulate the functions of NKT[59, 60]. RIPK3 dependent activation of NKT cells induced anti-tumor and anti-metastatsic responses, which was independent of RIPK1[60]. These findings mirrored the role of RIPK3 in DC activation during inflammation and tissue repair [61].

In order to grow and invade the body cancer cells subvert anti-tumoral immune responses. Induction of immunogenic cell death is an attractive approach to re-activate or enhance anti-tumour immune responses. Given that necroptosis induces the release of DAMPs, which initiate adaptive immunity, it became obvious to investigate its role anti-cancer immune responses. A number of studies have shown that inhibition of caspases, in particular inhibition of caspase-8, switched cell death induced by some cytotoxic agents to necroptosis. For instance, Poly I:C and zVAD can induce necroptosis in some cancer cells causing the release of DAMPs, which activate immune effectors to eliminate cancer[62, 63] (Figure 2). Similarly, combining caspase inhibitors with radiotherapy or chemotherapy reduced the tumor growth due to recruitment of $CD8^+$ T cells and DC and less T-reg cells[64] (Figure 2).

Recently more targeted strategies have been used to evaluate side-by-side apoptotic and necroptosis immunogenicities. Two independent groups have designed inducible and 'dimerizable' caspase-8 and RIPK3 constructs to obtain 'pure' apoptotic and necroptotic cells. Immunisation with pure necroptotic cells induced $CD8^+$ T cell cross-priming and provided a significant anti-tumour immune response[65, 66] (Figure 2). Necroptotic cells stimulated production of multiple immune effectors leading to a greater recruitment of immune cells as compared to apoptotic cells. Although necrosis releases DAMPs and is believed to be highly immunogenic, in both studies accidental or secondary necrotic cells were poor inducers of $CD8^+$ T response *in vivo*, suggesting that programmed necrosis is more immunogenic than an unprogrammed necrosis[65, 66]. Consistently, Yatim and colleagues found that RIPK1 was required for necroptosis immunogenicity as necroptotic cells lacking RIPK3 RHIM domain had a reduced immunogenicity (Figure 2). This suggested that late stages of necroptosis (e.g. MLKL activation, death and DAMPs release) are not solely responsible for the necroptosis immunogenicity. Indeed, both studies showed that necroptotic dying cells activated NF-κB to drive the expression of cytokines[65, 66]. Importantly, RIPK1 was not only required for immunogenicity induced by necroptosis but also induced by Poly I:C-induced apoptosis[66]. These

findings support several reports highlighting the cell-death-independent function of RIPK1 and RIPK3 as inducers of cytokines[54-56]. The direct role of necroptotic DAMPs has not been fully explored in those studies, therefore DAMPs may not be, as predicted, the main drivers of necroptosis immunogenicity but rather amplifiers. In contrast to RIPK1 and RIPK3, MLKL seems to be dispensable for cytokines production[54, 56]. Thus, studying the role of MLKL in necroptosis immunogenicity may offer a clarification on the involvement of the necroptosis-associated cytokines expression *vs* the necroptosis-mediated cell death during an anti-tumour response.

According to the current concept of immunogenic cell death, necrosis is believed to be more immunogenic than apoptosis[67]. However those recent findings have demonstrated that a programmed necrosis that couple gene expression and death might be the best arsenal to turn on the immune system to fight cancer. The immunisation of patients with necroptotic cells might take sometime before becoming a clinical practice to treat cancer. However, inducing necroptosis in cancer cells may have the same outcome on anti-cancer immune response.

NECROPTOSIS FOR CANCER THERAPY?

Targeting necroptosis is an emerging and attractive therapeutic strategy allowing to bypass acquired apoptotic resistance and potentially switch on anti-tumour responses. A plethora of cancer cell lines can undergo necroptosis. The strategies to induce necroptosis in cancer are various and include classic necroptosis inducers, chemotherapeutic agents or natural compounds.

Necroptosis induced by Autophagy

There is still debate about whether autophagic cell death is caused by autophagy itself or induced or associated with another type of cell death[5]. Several studies have linked autophagy activation and necroptosis execution using preclinical and clinical compounds. BMI-1 has an essential role in regulating the proliferative activity of leukaemic stem cells and inhibiting its activity could be a potential therapeutic intervention in the clinic[68]. The BMI-1 inhibitor PTC-209 induced autophagy and RIPK3 upregulation leading to necroptosis of ovarian cancer cells[69]. Moreover, autophagy induced by chalcone resulted in c-IAP1/2 degradation and formation of the Ripoptosome that contributed to activate necroptosis to kill cancer cells[70]. On the other hand, the autophagic cell death triggered by the tyrosine kinase inhibitor sorafenib led to the accumulation of autophasomes where p62 and RIPK1 co-localised. Inhibition of RIK1 kinase blocked sorafenib cell death suggesting that the autophagic cell death in this context was triggered by necroptosis[71].

Similarly, the clinical Bcl-2 inhibitor obatoclax induced accumulation of autophagosomes and promoted the interaction of ATG5, a component of autophagosomal membranes, with RIPK1 and RIPK3[72]. Futhermore, when combined to dexamethasone obatoclax activated autophagy and killed

ALL cells in a RIPK1 and CYLD dependent manner[73]. Collectively, those studies provide evidence of an existing cross-talk between the autophagic and the necroptotic machineries. The ubiquitin-binding protein p62, present in the autophagosomes may act as the adaptor to recruit necroptotic players as it has been shown to interact with RIPK1 and induce caspase-8 aggregation in response to TNFR super family members[74, 75].

Necroptosis induced by natural compounds

Many natural compounds derived from plants or microbes have demonstrated potential anti-cancer properties. Several reports from the same group have suggested that a component of a Chinese herbal medicine named shikonin killed cancer cells through the necroptosis pathway[76-78]. Similarly, another group has reported that a small compound isolated from the fungus, Albatrellus confluens, neoalbaconol can trigger necroptosis in cancer cell lines[79, 80]. The precise molecular targets in the necroptotic signalling engaged by both compounds remain unknown. Mitochondrial production of ROS has been suggested to act as second messenger in the signaling pathway leading to necroptosis[80, 81]. However, the blockade of RIPK1 decreased the killing induced by neoalbaconol but failed to block ROS productions, suggesting that it was an independent molecular event to necroptosis[80]. The direct contribution of necroptosis by shikonin and neoalbaconol should be interrogated as it was observed only at high doses. It is therefore tempting to speculate that activation of necroptosis was due to an overwhelming cytotoxic insult provoked by high dose of both compounds.

Necroptosis induced by chemotherapy

Anti-cancer chemotherapeutic agents trigger not only apoptosis but also other modes of cell death. The recent analysis of the contribution of necroptosis in the chemo-sensibility led to divergent conclusions. For instance, expression or re-expression of RIPK3 was required to sensitise a range of cancer cell lines to DNA-damaging agents[19]. However, this was only true in a limited number of cancer cell lines as RIPK3 was dispensable for chemo-sensitisation of colon and breast cancers[22]. The molecular mechanism by which chemotherapy activates necroptosis is still unclear. It is worthily to note that certain chemotherapeutic agents induced degradation of cIAP1/2 leading to the formation of the Ripoptosome[11]. Therefore, one could consider that in some cancer cell lines where caspase-8 function is impaired due to mutations or gene hypermethylation, cIAP1/2 degradation induced by chemotherapy would lead to necroptosis. Consistently, inhibition of caspases (presumably caspase-8) primed cancer cells to necroptosis induced by 5-FU[82]. Another way to drive cancer cells to undergo chemotherapy-mediated necroptosis is by antagonising IAPs with smac-mimetics. Once more, this was only possible when caspases were inhibited[25, 83]. On the other hand,

since RIPK3 is often silenced through methylation of its promoter, demethylation agents such as 5-Aza-2′-deoxycytidine (5-Aza) has been used to restore RIPK3 expression and to sensitise cancer cells to necroptosis induced by smac-mimetics or to cell death induced by DNA damaging agents[19, 84]. Furthermore, the combination IAP and caspase inhibitors sensitized AML cells to other epigenetic modifiers such as HDAC inhibitors[85].

Those studies suggest that the toxicity of chemotherapeutic agents may be partially dependent on necroptosis. However, in several reports this conclusion was mainly drawn from the use of RIPK1 inhibitor. As RIPK1 has other functions besides executing necroptosis, the legitimacy of these studies requires further examination. In fact, the idea of a chemotherapy-induced necroptosis should be viewed cautiously as in most scenarios chemical inhibition of IAP and caspase-8 was required. As such those classical necroptotic inducers (e.g. smac-mimetic, caspase inhibitor) might be the appropriate alternative to fully exploit the power of necroptosis in anti-cancer therapy.

Clinical classical necroptotic inducers

Specific induction of necroptosis requires the coordination of RIP Kinases activation and caspase-8 inhibition. The conjugation of ubiquitin chains to RIPK1 by cIAPs represses its death function. Depletion of cIAP1/2 by smac-mimetics unleashes RIPK1 from its scaffolding survival function and induces its recruitment to the Ripoptosome. Within this complex the necroptotic activities of RIP kinases are repressed by cleavage mediated by caspase-8. Genetic deletion or inhibition of caspase-8 leads to the formation of the necrosome in which uncleaved RIPK1 and RIPK3 are phosphorylated, which in turn phosphorylates MLKL (reviewed in this issue). Therefore, targeting simultaneously IAPs and caspase-8 unequivocally leads to necroptosis. However, the formation of RIPK1 containing complexes (e.g. Ripoptosome and necrosome) often requires the binding of TNF super family to their receptors. Interestingly treatment with smac-mimetic induces production of TNF and leads to the formation of the Ripoptosome and smac-mimetic sensitive cells die through apoptosis[10, 11, 86-88]. In contrast, smac-mimetic resistant cancer cells lack the ability to produce TNF. Consistent with this, addition of TNF, TRAIL or Fas or specific induction of TNF sensitise resistant cancer cell to smac-mimetic[89-91].

Along those lines, combination of smac-mimetic and TNF induced necroptosis in leukemic cells that lacked FADD and caspase-8[92]. This suggest that smac-mimetic drugs can provide an alternative outcome in cancer cells that have silenced FADD or caspase-8 or overexpressed $cFLIP_L$, only if the necroptotic regulators function appropriately. It also provided the rational to combine smac-mimetic with caspase inhibitors to specifically induce necroptosis in cancer cells that have intact caspase-8 function. Accordingly, combination of smac-mimetic and caspase inhibitors sensitised pancreatic cancer cell lines and ovarian and AML patients samples[25, 93, 94]. Notably, chemical caspase

inhibition increased smac-mimetic-induced TNF and induced necroptosis in a TNFR1 dependent manner[25, 93, 94]. The secretion of TNF (and presumably other cytokines) induced by smac-mimetic/caspase inhibitor treatment could therefore potentially induce a anti-tumour response as it has been shown in mice immunised with necrototic cells[65, 66]. Importantly, this combined treatment overcame smac-mimetic-induced apoptosis resistance and was effective in chemo-resistant AML and ovarian patients samples[25, 93]. This suggests that acquired resistance to chemotherapy might not affect the necroptosis pathway raising the possibility that targeting necroptosis could be used as a second line treatment. Interestingly, in some ALL patient samples smac-mimetic induced necroptosis without the requirement of chemical caspase inhibition, implying that those patients have low caspase-8 activation[95].

Genetic deletions of IAP genes have helped to predict smac-mimetics' tolerability in vivo[56, 96, 97]. For instance, smac-mimetics that target cIAP1/2 preferentially to XIAP such as birinapant are well tolerated in human. The safety of caspase-8 inhibition *in vivo* could possibly be a concern as genetic deletion of caspase-8 is also embryonic lethally because of overwhelming activation of necroptosis[98, 99]. However, the clinical caspase inhibitor emricasan has been tested in the clinic for the treatment of liver diseases and when combined to the smac-mimetic birinapant, it was tolerable and provided a significant therapeutic efficacy in AML[25]. It has been proposed that the caspase-8/cFLIP$_L$ heterodimer is important for inhibiting necroptosis[98, 100]. Accordingly, emricasan has offered a greater activation of necroptosis compared to other caspase inhibitors such as zVAD or QVD. This was due to its higher ability to inhibit caspase-8/cFLIP$_L$ heterodimer[25]. Altogether those studies suggest that specifically targeting necroptosis might be a safe approach to treat cancer patients especially the ones who relapsed after chemotherapy intervention. The half-life of emricasan is less than 50 minutes in the plasma, thus the development of more specific and stable caspase-8/cFLIP$_L$ heterodimer inhibitor should be the focus to give greater outcomes.

CONCLUDING REMARKS

The deregulation of the necroptosis pathway observed in several cancers suggests its implication in cancer progression. Given the effect of necroptosis on the immune system, some cancer cells might have repressed necroptosis to escape immune attacks. The intriguing question is when does necroptosis occur during cancer development to drive tumour cells to silence it. Targeting necroptosis seems to be a plausible therapeutic intervention to boost the immune system. However, cancers that have suppressed necroptosis might be less likely responsive to such therapeutic strategy. The use of clinical compounds that specifically induce necroptosis and their tolerability in animal models exhibit great promise for translation. Yet, the clinical feasibility still needs to be wisely

assessed. In fact, necroptotic immunogenicity could act as a double-edged sword, since repetitive induction of necroptosis can possibly lead to the development of chronic inflammatory diseases. Moreover, the release of pro-inflammatory molecules can recruit tumor-promoting immune cells capable of fostering angiogenesis, cancer cell proliferation, and invasiveness. A clarification of the role of the inflammatory molecules released during necroptosis might be critical to understand the opposite functions of the immune system in cancer immune surveillance and tumour promotion.

The requirement of caspase inhibition for an effective necroptosis activation represents a paradigm shift in cancer therapy as most current therapeutic strategies have aimed to activate caspases for decades. Hence, inhibiting caspases could be detrimental for cancer patients that lack necroptosis regulators. Specific biomarkers of response should be rigorously identified. Moreover, a deeper understanding of the genetic and epigenetic context of necroptotic regulators will help the development and administration of appropriate therapeutics.

The remarkable clinical success of cancer immunotherapies utilising checkpoint blockade has generated considerable excitement. However, immunologically inert tumours fail to respond to those therapies. The ability of necroptosis to recruit and activate immune cells at the tumour site may therefore increase checkpoint blockade efficacy. Alternatively, combining checkpoint blockade with vaccination with necroptotic cells may provide better outcomes.

In conclusion, accumulating evidence suggests that necroptosis plays a role in cancer development. Recent studies have defined various therapeutic strategies to explore its efficacy. Targeting necroptosis for treatment of cancer presents several advantages over current strategies. However, a greater understanding of this pathway is essential to assess the clinical achievability.

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144:** 646-674.

2. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S *et al.* Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nature immunology* 2000; **1:** 489-495.

3. Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W *et al.* Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *The Journal of experimental medicine* 1998; **187:** 1477-1485.

4. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* 2013; **38:** 209-223.

5. Lalaoui N, Lindqvist LM, Sandow JJ, Ekert PG. The molecular relationships between apoptosis, autophagy and necroptosis. *Seminars in cell & developmental biology* 2015; **39:** 63-69.

6. Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T *et al.* RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. *Immunity* 2011; **35:** 908-918.

7. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF-kappaB signals induce the expression of c-FLIP. *Molecular and cellular biology* 2001; **21:** 5299-5305.

8. Bertrand MJM, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J *et al.* cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Molecular cell* 2008; **30:** 689-700.

9. Wright A, Reiley WW, Chang M, Jin W, Lee AJ, Zhang M *et al.* Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD. *Dev Cell* 2007; **12**: 705-716.

10. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M *et al.* cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell* 2011; **43:** 449-463.

11. Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F *et al.* The Ripoptosome, a Signaling Platform that Assembles in Response to Genotoxic Stress and Loss of IAPs. *Mol Cell* 2011, **43:** 1-18.

12. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M *et al.* Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 2009; **137:** 1112-1123.

13. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S *et al.* The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity* 2013; **39:** 443-453.

14. Sun L, Wang H, Wang Z, He S, Chen S, Liao D *et al.* Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 2012; **148:** 213-227.

15. Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS *et al.* The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell* 2012; **150:** 339-350.

16. Bruzzoni-Giovanelli H, González JR, Sigaux F, Villoutreix BO, Cayuela JM, Guilhot J *et al.* Genetic polymorphisms associated with increased risk of developing chronic myelogenous leukemia. *Oncotarget* 2015; **6:** 36269-36277.

17. Cerhan JR, Ansell SM, Fredericksen ZS, Kay NE, Liebow M, Call TG *et al.* Genetic variation in 1253 immune and inflammation genes and risk of non-Hodgkin lymphoma. *Blood* 2007; **110**: 4455-4463.

18. Murphy JM, Silke J. Ars Moriendi; the art of dying well - new insights into the molecular pathways of necroptotic cell death. *EMBO reports* 2014; **15:** 155-164.

19. Koo G-B, Morgan MJ, Lee D-G, Kim W-J, Yoon J-H, Koo JS *et al.* Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. *Cell Res* 2015; **25**: 707-725.

20. Fukasawa M, Kimura M, Morita S, Matsubara K, Yamanaka S, Endo C *et al.* Microarray analysis of promoter methylation in lung cancers. *J Hum Genet* 2006; **51**: 368-374.

21. Geserick P, Wang J, Schilling R, Horn S, Harris PA, Bertin J *et al.* Absence of RIPK3 predicts necroptosis resistance in malignant melanoma. *Cell Death Dis* 2015; **6**: 1884-1812.

22. Moriwaki K, Bertin J, Gough PJ, Orlowski GM, Chan FK. Differential roles of RIPK1 and RIPK3 in TNF-induced necroptosis and chemotherapeutic agent-induced cell death. *Cell Death Dis* 2015; **6**: 1636-1611.

23. Nugues A-L, El Bouazzati H, tuin DHe, Berthon C, Loyens A, Bertrand E *et al.* RIP3 is downregulated in human myeloid leukemia cells and modulates apoptosis and caspase-mediated p65/RelA cleavage. *Cell Death Dis* 2014; **5**: 1384-1312.

24. Höckendorf U, Yabal M, Herold T, Munkhbaatar E, Rott S, Jilg S *et al.* RIPK3 Restricts Myeloid Leukemogenesis by Promoting Cell Death and Differentiation of Leukemia Initiating Cells. *Cancer Cell* 2016; **30**: 75-91.

25. Brumatti G, Ma C, Lalaoui N, Nguyen NY, Navarro M, Tanzer MC *et al.* The caspase-8 inhibitor emricasan combines with the SMAC mimetic birinapant to induce necroptosis and treat acute myeloid leukemia. *Sci Trans Med* 2016; **8:** 339-369.

26. Liu P, Xu B, Shen W, Zhu H, Wu W, Fu Y *et al.* Dysregulation of TNFalpha-induced necroptotic signaling in chronic lymphocytic leukemia: suppression of CYLD gene by LEF1. *Leukemia* 2012; **26:** 1293-1300.

27. Moquin DM, McQuade T, Chan FK. CYLD deubiquitinates RIP1 in the TNFalpha-induced necrosome to facilitate kinase activation and programmed necrosis. *PloS One* 2013; **8:** e76841.

28. Bozec D, Iuga AC, Roda G, Dahan S, Yeretssian G. Critical function of the necroptosis adaptor RIPK3 in protecting from intestinal tumorigenesis. *Oncotarget* 2016; **7**: 46384-46400.

29. Feng X, Song Q, Yu A, Tang H, Peng Z, Wang X. Receptor-interacting protein kinase 3 is a predictor of survival and plays a tumor suppressive role in colorectal cancer. *Neoplasma* 2015; **62:** 592-601.

30. Silke J, Rickard JA, Gerlic M. The diverse role of RIP kinases in necroptosis and inflammation. *Nature immunology* 2015; **16**: 689-697.

31. Park S, Hatanpaa KJ, Xie Y, Mickey BE, Madden CJ, Raisanen JM *et al.* The Receptor Interacting Protein 1 Inhibits p53 Induction through NF- B Activation and Confers a Worse Prognosis in Glioblastoma. *Cancer Res* 2009; **69**: 2809-2816.

32. Wang Q, Chen W, Xu X, Li B, He W, Padilla MT *et al.* RIP1 potentiates BPDE-induced transformation in human bronchial epithelial cells through catalase-mediated suppression of excessive reactive oxygen species. *Carcinogenesis* 2013; **34**: 2119-2128.

33. McCabe KE, Bacos K, Lu D, Delaney JR, Axelrod J, Potter MD *et al.* Triggering necroptosis in cisplatin and IAP antagonist-resistant ovarian carcinoma. *Cell Death Dis* 2014*;* **5:** 1496-1498.

34. Seifert L, Werba G, Tiwari S, Ly NNG, Alothman S, Alqunaibit D *et al.* The necrosome promotes pancreatic oncogenesis via CXCL1 and Mincle-induced immune suppression. *Nature* 2016; **532**: 245-249.

35. Colbert LE, Fisher SB, Hardy CW, Hall WA, Saka B, Shelton JW *et al.* Pronecrotic mixed lineage kinase domain-like protein expression is a prognostic biomarker in patients with early-stage resected pancreatic adenocarcinoma. *Cancer* 2013: **119:** 3148-3155.

36. Ertao Z, Jianhui C, Kang W, Zhijun Y, Hui W, Chuangqi C *et al.* Prognostic value of mixed lineage kinase domain-like protein expression in the survival of patients with gastric cancer. *Tumor Biology* 2016; **37**: 1-7.

37. He L, Peng K, Liu Y, Xiong J, Zhu FF. Low expression of mixed lineage kinase domain-like protein is associated with poor prognosis in ovarian cancer patients. *Onco Targets Ther* 2013; **6:** 1539-1543.

38. Li X, Guo J, Ding AP, Qi WW, Zhang PH, Lv J *et al.* Association of Mixed Lineage Kinase Domain-Like Protein Expression With Prognosis in Patients With Colon Cancer. *Technol Cancer Research Treat* 2016; 1-7.

39. Ruan J, Mei L, Zhu Q, Shi G, Wang H. Mixed lineage kinase domain-like protein is a prognostic biomarker for cervical squamous cell cancer. *Int J Clinl Expl Pathol* 2015; **8:** 15035-15038.

40. Liu X, Zhou M, Mei L, Ruan J, Hu Q, Peng J *et al.* Key roles of necroptotic factors in promoting tumor growth. *Oncotarget* 2016; **7**: 22219-22233.

41. Kang YJ, Bang BR, Han KH, Hong L, Shim EJ, Ma J *et al.* Regulation of NKT cell-mediated immune responses to tumours and liver inflammation by mitochondrial PGAM5-Drp1 signalling. *Nature Comm* 2015; **6:** 8371.

42. Wang Z, Jiang H, Chen S, Du F, Wang X. The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell* 2012; **148:** 228-243.

43. Moriwaki K, Farias Luz N, Balaji S, De Rosa MJ, O'Donnell CL, Gough PJ *et al.* The Mitochondrial Phosphatase PGAM5 Is Dispensable for Necroptosis but Promotes Inflammasome Activation in Macrophages. *J Immunol* 2016; **196:** 407-415.

44. Remijsen Q, Goossens V, Grootjans S, Van den Haute C, Vanlangenakker N, Dondelinger Y *et al.* Depletion of RIPK3 or MLKL blocks TNF-driven necroptosis and switches towards a delayed RIPK1 kinase-dependent apoptosis. *Cell Death Dis* 2014; **5:** e1004.

45. Tait SW, Oberst A, Quarato G, Milasta S, Haller M, Wang R *et al.* Widespread mitochondrial depletion via mitophagy does not compromise necroptosis. *Cell Rep* 2013; **5:** 878-885.

46. Moujalled DM, Cook WD, Murphy JM, Vaux DL. Necroptosis induced by RIPK3 requires MLKL but not Drp1. *Cell Death Dis* 2014; **5:** e1086.

47. Strilic B, Yang L, Albarrán-Juárez J, Wachsmuth L, Han K, Müller UC *et al.* Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. *Nature* 2016: **536**: 215-218.

48. Olsson M, Zhivotovsky B. Caspases and cancer. *Cell Death Diff* 2011; **18:** 1441-1449.

49. Dannappel M, Vlantis K, Kumari S, Polykratis A, Kim C, Wachsmuth L *et al.* RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. *Nature* 2014; **513:** 90-94.

50. Kaiser WJ, Daley-Bauer LP, Thapa RJ, Mandal P, Berger SB, Huang C *et al.* RIP1 suppresses innate immune necrotic as well as apoptotic cell death during mammalian parturition. *PNAS* 2014; **111:** 7753-7758.

51. Rickard JA, O'Donnell JA, Evans JM, Lalaoui N, Poh AR, Rogers T *et al.* RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell* 2014; **157:** 1175-1188.

52. Roderick JE, Hermance N, Zelic M, Simmons MJ, Polykratis A, Pasparakis M *et al.* Hematopoietic RIPK1 deficiency results in bone marrow failure caused by apoptosis and RIPK3-mediated necroptosis. *PNAS* 2014; **111:** 14436-14441.

53. Dillon CP, Weinlich R, Rodriguez DA, Cripps JG, Quarato G, Gurung P *et al.* RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. *Cell* 2014; **157:** 1189-1202.

54. Najjar M, Saleh D, Zelic M, Nogusa S, Shah S, Tai A *et al.* RIPK1 and RIPK3 Kinases Promote Cell-Death-Independent Inflammation by Toll-like Receptor 4. *Immunity* 2016; **45**: 46-59.

55. Alvarez-Diaz S, Dillon CP, Lalaoui N, Tanzer MC, Rodriguez DA, Lin A *et al.* The Pseudokinase MLKL and the Kinase RIPK3 Have Distinct Roles in Autoimmune Disease Caused by Loss of Death-Receptor-Induced Apoptosis. *Immunity* 2016; **45:** 513-526.

56. Wong WW, Vince JE, Lalaoui N, Lawlor KE, Chau D, Bankovacki A *et al.* cIAPs and XIAP regulate myelopoiesis through cytokine production in a RIPK1 and RIPK3 dependent manner. *Blood* 2014; **123**: 2562-2572.

57. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986; **319:** 675-678.

58. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nature reviews. Immunology* 2012; **12:** 239-252.

59. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *MCB* 2004; **24:** 1464-1469.

60. Kang YJ, Bang B-R, Han KH, Hong L, Shim E-J, Ma J *et al.* Regulation of NKT cell-mediated immune responses to tumours and liver inflammation by mitochondrial PGAM5-Drp1 signalling. *Nature Comm* 2015; **6**: 1-15.

61. Moriwaki K, Balaji S, McQuade T, Malhotra N, Kang J, Chan FK. The necroptosis adaptor RIPK3 promotes injury-induced cytokine expression and tissue repair. *Immunity* 2014; **41:** 567-578.

62. Takemura R, Takaki H, Okada S, Shime H, Akazawa T, Oshiumi H *et al.* PolyI:C-Induced, TLR3/RIP3-Dependent Necroptosis Backs Up Immune Effector-Mediated Tumor Elimination In Vivo. *Cancer Immunol Res*, 2015; **3**: 902-914.

63. Schmidt SV, Seibert S, Walch-Rückheim B, Vicinus B, Kamionka E-M, Pahne-Zeppenfeld J *et al.* RIPK3 expression in cervical cancer cells is required for PolyIC-induced necroptosis, IL-1α release, and efficient paracrine dendritic cell activation. *Oncotarget* 2015; **6**: 8635-8647.

64. Werthmoller N, Frey B, Wunderlich R, Fietkau R, Gaipl US. Modulation of radiochemoimmunotherapy-induced B16 melanoma cell death by the pan-caspase inhibitor zVAD-fmk induces anti-tumor immunity in a HMGB1-, nucleotide- and T-cell-dependent manner. *Cell Death Dis* 2015; **6:** e1761.

65. Aaes TL, Kaczmarek A, Delvaeye T, De Craene B, De Koker S, Heyndrickx L *et al.* Vaccination with Necroptotic Cancer Cells Induces Efficient Anti-tumor Immunity. *Cell Rep* 2016; **15**: 274-287.

66. Yatim N, Jusforgues-Saklani H, Orozco S, Schulz O, Barreira da Silva R, Reis e Sousa C *et al.* RIPK1 and NF- B signaling in dying cells determines cross-priming of CD8+ T cells. *Science* 2015; **350** : 328-334.

67. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *An Rev Immunol* 2013; **31:** 51-72.

68. Wang MC, Li CL, Cui J, Jiao M, Wu T, Jing LI *et al.* BMI-1, a promising therapeutic target for human cancer. *Oncology letters* 2015; **10:** 583-588.

69. Dey A, Mustafi SB, Saha S, Dwivedi SKD, Mukherjee P, Bhattacharya R *et al.* Inhibition of BMI1 induces autophagy-mediated necroptosis. *Autophagy* 2016; **12**: 659-670.

70. He W, Wang Q, Srinivasan B, Xu J, Padilla MT, Li Z *et al.* A JNK-mediated autophagy pathway that triggers c-IAP degradation and necroptosis for anticancer chemotherapy. *Oncogene* 2014; **33:** 3004-3013.

71. Kharaziha P, Chioureas D, Baltatzis G, Fonseca P, Rodriguez P, Gogvadze V *et al.* Sorafenib-induced defective autophagy promotes cell death by necroptosis. *Oncotarget*, 2015; **6**: 37066-37082.

72. Basit F, Cristofanon S, Fulda S. Obatoclax (GX15-070) triggers necroptosis by promoting the assembly of the necrosome on autophagosomal membranes. *Cell Death Diff* 2013; **20**: 1161-1173.

73. Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK *et al.* Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. *J Clin Invest* 2010; **120:** 1310-1323.

74. Sanz L, Sanchez P, Lallena MJ, Diaz-Meco MT, Moscat J. The interaction of p62 with RIP links the atypical PKCs to NF-kappaB activation. *EMBO J* 1999; **18:** 3044-3053.

75. Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR *et al.* Cullin3-Based Polyubiquitination and p62-Dependent Aggregation of Caspase-8 Mediate Extrinsic Apoptosis Signaling. *Cell* 2009; **137**: 721-735.

76. Xuan Y, Hu X. Naturally-occurring shikonin analogues--a class of necroptotic inducers that circumvent cancer drug resistance. *Cancer Lett* 2009; **274:** 233-242.

77. Han W, Xie J, Li L, Liu Z, Hu X. Necrostatin-1 reverts shikonin-induced necroptosis to apoptosis. *Apoptosis* 2009; **14**: 674-686.

78. Han W, Li L, Qiu S, Lu Q, Pan Q, Gu Y *et al.* Shikonin circumvents cancer drug resistance by induction of a necroptotic death. *Mol Cancer Ther* 2007; **6:** 1641-1649.

79. Yu X, Deng Q, Li W, Xiao L, Luo X, Liu X *et al.* Neoalbaconol induces cell death through necroptosis by regulating RIPK-dependent autocrine TNFalpha and ROS production. *Oncotarget* 2015; **6:** 1995-2008.

80. Deng Q, Yu X, Xiao L, Hu Z, Luo X, Tao Y *et al.* Neoalbaconol induces energy depletion and multiple cell death in cancer cells by targeting PDK1-PI3-K/Akt signaling pathway. *Cell Death Dis* 2013; **4:** e804.

81. Schenk B, Fulda S. Reactive oxygen species regulate Smac mimetic/TNFalpha-induced necroptotic signaling and cell death. *Oncogene* 2015; **34:** 5796-5806.

82. Metzig MO, Fuchs D, Tagscherer KE, ne H-JGo, Schirmacher P, Roth W. Inhibition of caspases primes colon cancer cells for 5- fluorouracil-induced TNF-α-dependent necroptosis driven by RIP1 kinase and NF-κB. *Oncogene* 2015; **35**: 3399-3409.

83. Chromik J, Safferthal C, Serve H, Fulda S. Smac mimetic primes apoptosis-resistant acute myeloid leukaemia cells for cytarabine-induced cell death by triggering necroptosis. *Cancer Lett* 2014; **344:** 101-109.

84. Steinhart L, Belz K, Fulda S. Smac mimetic and demethylating agents synergistically trigger cell death in acute myeloid leukemia cells and overcome apoptosis resistance by inducing necroptosis. *Cell Death Dis* 2013; **4:** e802.

85. Steinwascher S, Nugues A-L, Schoeneberger H, Fulda S. Identification of a novel synergistic induction of cell death by Smac mimetic and HDAC inhibitors in acute myeloid leukemia cells. *Cancer Lett* 2015; **366**: 32-43.

86. Vince JE, Wong WW-L, Khan N, Feltham R, Chau D, Ahmed AU *et al.* IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007; **131:** 682-693.

87. Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J *et al.* Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell* 2007; **12:** 445-456.

88. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P *et al.* IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007; **131:** 669-681.

89. Lalaoui N, Hanggi K, Brumatti G, Chau D, Nguyen NY, Vasilikos L *et al.* Targeting p38 or MK2 Enhances the Anti-Leukemic Activity of Smac-Mimetics. *Cancer Cell* 2016; **29:** 145-158.

90. Geserick P, Hupe M, Moulin M, Wong WW, Feoktistova M, Kellert B *et al.* Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. *J Cell Biol* 2009; **187:** 1037-1054.

91. Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science* 2004; **305:** 1471-1474.

92. Laukens B, Jennewein C, Schenk B, Vanlangenakker N, Schier A, Cristofanon S *et al.* Smac Mimetic Bypasses Apoptosis Resistance in FADD- or Caspase-8-Deficient Cells by Priming for Tumor Necrosis Factor α-Induced Necroptosis. *Neoplasia* 2011; **13:** 971-929.

93. McCabe KE, Bacos K, Lu D, Delaney JR, Axelrod J, Potter MD *et al.* Triggering necroptosis in cisplatin and IAP antagonist-resistant ovarian carcinoma. *Cell Death Dis* 2014; **5:** e1496.

94. Hannes S, Abhari BA, Fulda S. Smac mimetic triggers necroptosis in pancreatic carcinoma cells when caspase activation is blocked. *Cancer Lett* 2016; **380:** 31-38.

95. Mccomb S, Aguadé-Gorgorió J, Harder L, Marovca B, Cario G, Eckert C *et al.* Activation of concurrent apoptosis and necroptosis by SMAC mimetics for the treatment of refractory and relapsed ALL. *Scien Trans Med* 2016; **8**: 339-370.

96. Moulin M, Anderton H, Voss AK, Thomas T, Wong WW-L, Bankovacki A *et al.* IAPs limit activation of RIP kinases by TNF receptor 1 during development. *EMBO J* 2012; **31**: 1679-1691.

97. Condon SM, Mitsuuchi Y, Deng Y, LaPorte MG, Rippin SR, Haimowitz T *et al.* Birinapant, a smac-mimetic with improved tolerability for the treatment of solid tumors and hematological malignancies. *J Med Chem* 2014; **57:** 3666-3677.

98. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C *et al.* Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* 2011; **471:** 363-367.

99. Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R *et al.* RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 2011; **471:** 368-372.

100. Pop C, Oberst A, Drag M, Van Raam BJ, Riedl SJ, Green DR *et al.* FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. *The Biochem J* 2011; **433:** 447-457.

Table 1: Deregulation of necroptosis signalling in cancers and the impact on disease prognosis

Gene	Deregulation		Tumor	Prognosis
	Nature	Outcome		
RIPK1				
	SNP	*nd*	CML[16], non-Hodgkin lymphoma[17]	High risk
	nd	High protein level	Glioblastoma[31], lung and pancreatic [32]cancers	Poor prognosis in Glioblastoma[31]
RIPK3				
	COSMIC mutation V458M	Potential disruption of RHIM interactions	Melanoma	*nd*
	SNP	*nd*	CML[16], non-Hodgkin lymphoma[17]	High risk
	nd	Low RNA level	Breast [19], colon and colorectal[22, 28, 29] cancers, AML[23, 24], CLL[26]	Poor outcome in breast[19], colon and colorectal [22, 28, 29]
		High RNA level	Ovarian cancer [33]	*nd*
	Methylation	Low protein level	AML[19], breast cancer[19], CLL[26]	Poor prognosis in breast cancer[19]
		High protein level	Pancreatic cancer[34]	*nd*
MLKL				
	COSMIC mutations F398I L291P	Non-functional Loss-of-function	Gastric cancer	*nd*
	nd	Low RNA level	AML[23]	*nd*
	nd	Low protein level	Pancreatic [35], cervix[36], gastric[36], ovarian[37], colon[38] cancers	Low overall survival in pancreatic [35], cervix[36], gastric[36], ovarian[37], colon[38] cancers
		High protein level	Pancreatic cancer[34, 35]	High overall survival[35]
	Phosphorylation	High	Colon[28], Esophageal[40] cancers	Worse prognosis in Colon[28], Esophageal[40] cancers
CYLD				
	nd	Low protein level	CLL[26]	*nd*

nd : non determined