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Review

Programmed necrosis and its role in management of breast cancer

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ABSTRACT

Breast cancer is one of the major causes of cancer related deaths in women worldwide. A major factor responsible for treatment failure in breast cancer is the development of resistance to commonly used chemotherapeutic drugs leading to disease relapse. Several studies have shown dysregulation of molecular machinery of apoptosis, the major programmed cell death pathway in breast malignancies. Thus, there is an unmet need to search for an alternative cell death pathway which can work when apoptosis is compromised. Necroptosis or programmed necrosis is a relatively recently described entity which has attracted attention in this context. Classically, even in physiological conditions necroptosis is found to act if apoptosis is not functional due to some reason. Recently, more and more studies are being conducted in different malignancies to explore the possibility and utility of inducing cell death by necroptosis. The present review describes the key molecular players involved in necroptotic pathway and their status in breast cancer. In addition, the research done to utilize this pathway for treatment of breast cancer has also been highlighted.

1. Introduction

Breast cancer represents a set of highly heterogeneous diseases mainly classified on the basis of estrogen (ER) & progesterone receptor (PR) and HER2 status [1,2]. Triple negative breast cancers (TNBCs), accounting for 15-20% of breast malignancies are phenotypically defined by the lack of expression of hormone receptors and HER2. Consequently, these are characterized by lack of a targeted therapy [3]. Several studies on breast cancer show that there is dysregulation of apoptotic program leading to escape from cell death. Apoptosis resistance mainly arises due to up regulation of anti-apoptotic factors like inhibitors of apoptosis proteins (IAPs), overexpression of Bcl2 and or downregulation of proapoptotic factors like FAS, FASL and caspase8 in cancer cells. The above coupled to up regulation of pro- survival pathways like AKT/mTOR and Nuclear factor κB (NF-κB) is believed to result in unbridled proliferation of cancer cells. The other major mode of cell demise that is autophagic cell death is also believed to be dysregulated in cancers [4]. The defunct conventional cell death pathways in malignancies like breast cancer thus warrant the search for alternative pathways to kill the malignant cells. Necroptosis is a relatively novel mode of cell death which is being intensively investigated for its potential as a therapeutic target in different cancers which are resistant to apoptosis. So far, the work done on the role of necroptosis in breast cancer is still in its nascent stages. In this review article we intend to provide insight into mechanisms and key molecules involved in necroptosis, status of necroptotic pathways in breast cancer and discuss how this mode of cell death can be harnessed for treatment of breast cancer.

2. Resistance to drug therapy in breast cancer: causes and magnitude of the problem

Breast cancer is an important cause of morbidity and mortality amongst females. Besides surgery, administration of chemo radiation, hormonal, and immunotherapy constitute the major modalities of treatment for breast cancer. Chemotherapeutic agents are used primarily as adjuvant or neoadjuvant based approaches. However, despite advances in therapeutic management, nearly 30% of patients who are in early stage of cancer develop recurrent disease or show tumor progression after a variable period of time [5]. Development of metastasis seriously compromises the possibility of a complete cure. Resistance to therapy, de novo or acquired is believed to be the root cause responsible for above. Whereas, the former includes the patients who do not respond to targeted therapies from the beginning itself, the later, includes those responding to targeted therapies at the outset but becoming refractory later in the course of the disease [6]. Genetic and epigenetic factors leading to emergence of resistant clones characterized by altered transport carriers, metabolizing pathways and intracellular targets of

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drugs are proposed to be some of the mechanisms behind tolerance to systemic therapies in the cancer cells [5,7,8].

Dysregulation of cell death pathways has been increasingly reported as the strategy utilized by cancer cells to evade drug therapy. Apoptosis has been believed to be the commonest mode of cell death involved in chemotherapy mediated killing of cancer cells [9].

3. Resistance to conventional programmed cell death modes: scheming to evade drug therapy

Triggering of apoptotic or autophagic cell death is proposed to be the major mechanism involved in therapy induced killing of tumor cells. Most conventional chemotherapeutic agents, for example, taxols, anthracyclines, platins and etoposide are known to affect mitochondrial dynamics and function resulting in triggering of apoptotic machinery. Chemotherapeutic drugs are believed to induce changes in mitochondrial membrane permeability, alter mitochondrial bioenergetics, release lipid messengers like ceramides, cause alkalinization of matrix, increase generation of reactive oxygen species (ROS) and induce activation of proapoptotic proteins of the extrinsic and intrinsic apoptotic pathway [10–12]. Mitochondria are signaled to release proapoptotic factors into the cytoplasm in response to activation of checkpoint proteins like p53 [13]. Drugs like paclitaxel have been shown to induce accumulation of factors like Bim resulting in apoptosis in epithelial tumors [14]. Defects in the apoptotic pathways are therefore believed to promote progression of tumor and resistance to therapy.

Breast cancer cells have been observed to evade the apoptotic program in variety of ways. These may include alterations in signaling pathways like PI3K/Akt pathway, increase in FAK phosphorylation, enhanced expression of the IAPs like X-linked IAP (XIAP), cellular IAPs (cIAPs) and livin or suppression of the proapoptotic factors like caspases 3 and 9 and Poly ADP-ribose polymerase 1 (PARP-1) [15]. Alterations of tumor suppressor gene PTEN which negatively regulates PI3K pathway have also been reported [15–19]. NF-κB has been observed to be constitutively activated in both ER+ & ER- cell lines as well as primary cancers. NF-κB not only plays a crucial role in cell proliferation but also in cell survival & synthesis of several anti-apoptotic proteins. Alterations in regulators of intrinsic apoptotic pathway like Bcl2, Bcl-xL, Mcl-1 and Bax have also been reported in malignant cells. Wu et al., in their study showed decreased expression of caspase8 in breast cancer cells caused by CpG site promoter methylation [4]. Paradoxically, in some studies, caspase 3 overexpression has been correlated with poor patient survival [20]. Further, TNF-related apoptosis-inducing ligand receptor (TRAIL R) 1 and 2 mutations have also been linked to metastasis in breast cancer [21]. Survivin, a key member of IAP family has also been observed to be over expressed particularly in ER- breast cancers [22] (Table 1).

Autophagy is primarily a self-eating process that helps in recycling of the damaged organelles thus enabling the cell to survive in presence of any external or internal stress. However, under certain set of circumstances autophagy may cause cell death. Some studies have suggested that excessive levels of autophagy can result in death of apoptosis deficient cells [23,24]. However, perturbations of autophagy related proteins (Beclin-1) and failure of autophagic cell death have been identified as one of the mechanisms in tumorigenesis [25]. Harvey et al., in their study showed that the enhanced expression of protein Brk prevents autophagic cell death in breast cancer [26]. Nevertheless, due to paucity of markers for autophagic cell death, it has been difficult to discern the exact role of this mode of programmed cell death (PCD) either with or without chemotherapy in breast and other malignancies (Table 1) [27].

Table 1
Status of apoptosis regulators in ER- & ER + breast cancer.

S.No	Molecule	Function	Expression In ER-	Expression in ER+	References
1)	Caspase8	Apoptosis	Less/ down regulated		[4]
2)	Survivin	IAP	More	Less	[22]
3)	Caspase-3	Apoptosis	Less	More	[20]
4)	LC3A, LC3B and beclin-1	Autophagy	More	Less	[27]

4. Necroptosis: an alternative cell death pathway in breast cancer

Programmed necrosis, unlike necrosis, is planned and genetically programmed mode of cell death which can be of several types like pyroptosis, ferroptosis, NETosis etc. Necroptosis, a type of programmed necrosis is a relatively recently identified cell death pathway with roles in cellular physiology and development especially when apoptosis is rendered non-functional due to one or the other reason. The necroptosis pathway shares some of its molecules with the apoptosis [28].

Necroptosis, classically described as caspase independent cell death was first observed in L929 cells and Fas mediated killing of T-cells by Peter Vandenabeele's group (1997) and J Tschopp *et al.,* (2000) respectively [29–33]. The term Necroptosis was first coined by Yuan and colleagues in 2005 who also used Receptor Interacting Kinase (RIPK) 1 inhibitor necrostatin -1 to inhibit it [34–36]. The key molecular networks regulating it were identified by Hitomi et al., (2008) [37]. This was followed by decoding of the role of RIPK in necroptosis and discovery of the agents which could modulate necroptosis. The executioners of necroptosis like Mixed Lineage Kinase Domain like Pseudo kinase (MLKL), Dynamin-related protein 1 (Drp1) and Phosphoglycerate mutase family member 5 (PGAM5) were discovered subsequently.

Morphologically, necroptosis is characterized by cellular and organelle swelling, early plasma membrane damage and permeabilisation and an inflammatory reaction owing to cell lysis resulting in spillage of intracellular contents (Table 2) [38–43].

5. Necroptosis: the molecular doo hickey!

Necroptosis may be triggered via several pathways and a wide variety of stimuli like TNF-α, interferons, toll-like receptors (TLRs), viruses and various physical and chemical stressors. The key molecules involved in necroptosis can be categorized as:

5.1. Molecules constituting ripoptosome complex

"Ripoptosome" comprising of core molecules like RIPK1, FADD, and caspase8 is the key platform involved in necroptosis. Genotoxic stress induced depletion of molecules like XIAP, cIAP1 and 2 or treatment with Smac-mimetics are believed to promote the assembly of ripoptosome. Further, assembly of ripoptosome also requires kinase activity of RIPK1 which can result in assembly of either Complex IIa or Complex IIb resulting in caspase8 mediated apoptosis or caspase-independent necroptosis respectively. Contrarily, the molecules like FLIP, XIAP and cIAP1 & 2 negatively regulate the ripoptosome formation. The latter two are believed to target components of ripoptosome complex for degradation by ubiquitylation [44].

5.2. The RIP kinases

RIPK1 and 3 belong to the family of serine threonine kinases which contain an N-terminal Kinase domain (KD) which is signature domain of the family. RIPK1 also comprises of a C-terminal death domain (DD) and a connecting intermediate domain (ID) harboring a RIP homotypic interaction motif (RHIM). RIPK3 has a unique C-terminal sequence containing RHIM but lacks the ID [2].

RIPK1, a 671(76 KDa) amino acid long protein plays a crucial role in signal transduction by interacting with proteins like TNF receptor associated factor (TRAF-2) and TRADD (TNF receptor associated death

Table 2
Timeline depicting important discoveries in the field of necroptosis.

Year	Discovery	References
1995	Caspase independent cell death was discovered requiring participation of death domains containing serine/Threonine kinase RIPK1	[38]
1998	Cell death occurred when L929 cells were treated with TNF-α in presence of pan-caspase inhibitors	[33]
2000	Jurket cell lines deficient in DISC components found to be sensitive to TNF-α-induced caspase independent cell death	[30]
2004	SiRNA mediated knockdown of caspase8 leads to caspase independent cell death regulated by DISC	[39]
2005	Development of Necrostatin inhibitor of RIPK1 &TNF-α induced necroptosis in FADD deficient jurkat cells. Term "Necroptosis" coined	[36]
2008	Catalytic activity of RIPK-1 found to be vital for Necroptosis	[40]
2009	Necroptosis may serve as backup pathway to ensure timely demise of lymphocytes following their clonal expansion	[41]
	RIP kinase family member RIPK3 also required for TNF-α induced necroptosis. Complexes with RIPK1 by cross phosphorylation	
2010	RIPK1/RIPK3 containing "Necrosome" promotes downstream signals promoting induction of necroptosis	[42]
2012	Necrosome leads to phosphorylation of MLKL necessary for necroptosis	[43]
	Role of PGAM5, Drp1, ROS, Ca ionophores etc as downstream executioners of necroptosis identified	

domain) [45]. RIPK1 is essential for the TNF-mediated activation of NF-κB [46]. The DD in RIPK1 is homologous to the DD of various proteins such as FAS, TNFR1, and TRAILR 1 & 2. RIPK1 binds to the DD of these death receptors and further activates the signaling cascade. Thus RIPK1 is called a DD kinase [47]. RIPK1 is also involved in caspase activation and recruitment. If RIPK1 expression is increased then it leads to cell death whereas its deficiency makes the cells resistant to caspase-independent cell death [48–50]. However, RIPK1 has also been believed to prevent inappropriate activation of both necroptosis (RIPK3-dependent) and apoptosis (caspase8-dependent) [51–57].

RIPK3, a 518 (57KDa) amino acid long protein is another key signaling molecule of the necroptotic pathway [2]. RIPK3 is recruited to the TNF receptor 1 (TNFR1) complex in a RIPK1 dependent manner [58]. In addition to RIPK1, RIPK3 also interacts with TNFR1 and TRAF2. Interaction with the former occurs via RHIM domain thus activating itself and forming RIPK1-RIPK3 necroptosis-inducing complex [59]. The RIPK1-RIPK3 dimer interacts with MLKL and PGAM5 leading to the phosphorylation of PGAM5 and enhancement in its phosphatase activity. RIPK3 has been shown to bind and increase the activity of three metabolic enzymes (Glutamate ammonia ligase, Glutamate dehydrogenase 1 and Glycogen phosphorylase) associated with oxidative phosphorylation and increased ROS production [60–62]. Once necroptosis is activated, RIPK3 is also believed to inhibit NF- κB activation.

5.3. MLKL

This protein kinase superfamily member is a pseudokinase which is activated by phosphorylation by RIPK3 at positions T357 and S358 [63,64]. On induction of necroptosis it interacts with PGAM5, RIPK1 & 3 and helps in appropriate targeting of the later two to PGAM5. On phosphorylation, MLKL forms a homotrimer and moves towards plasma membrane thus facilitating calcium influx and plasma membrane damage [65–67]. Inhibition of MLKL by using MLKL inhibitors or its knockdown has been found to lead to inhibition of TNFα-induced necroptosis. Necrosulfonamide, an inhibitor of necroptosis acts by targeting MLKL [68].

5.4. Downstream executioners of necroptosis

Wang et al in 2012 reported the role of mitochondrial membrane protein PGAM5 in induction of necroptosis via its interaction with RIPK1-RIPK3-MLKL complex and subsequent dephosphorylation of DRP1 to cause mitochondrial fission and fragmentation [43]. Studies by Moriwaki *et al.* and He et al. also showed that the PGAM5/DRP1 axis is dispensable for TNF-α induced necroptosis in many cells and conditions [69,70]. Lu et al., in their work however showed that PGAM5 may actually protect the cells from necroptosis by promoting mitophagy [71]. Similarly, DRP1 mediated mitochondrial fission is recognized as an obligatory step in execution of necroptosis in some studies but others have suggested that it may not be necessary for necroptosis [72].

Alterations in mitochondrial metabolism and overproduction of ROS resulting in macromolecular damage may further contribute to the execution of necroptosis [73]. Activation of calcium-regulated enzymes like calpain facilitated by release of calcium due to mitochondrial damage also may result in permeabilization of the lysosomal membrane and release of their acidic contents leading to the destruction of cellular components [74]. During the execution phase of necroptosis other enzymes like sphingomyelinases may become activated there by resulting in further permeabilization of lysosomal membrane and consequently damaging the cell membrane [75,76]. Iron storage compartment may be altered in response to stress kinase c-Jun N-terminal kinase (JNK) pathway activation by RIPK1-RIPK3 thereby further contributing to necroptosis [76,77]. In contrast to apoptosis which is largely non-immunogenic, necroptotic cell death is characterized by immunogenic and inflammatory responses owing to early cell membrane rupture resulting in release of cellular contents as danger associated molecular patterns (DAMPs) [78].

5.5. Regulators of necroptosis

Apart from key molecules involved in necroptotic pathways many other proteins involved in TNF signaling pathway may also positively or negatively influence necroptosis.

Positive Regulators:-

1) **CYLD (cylindromatosis):**- It is a deubiquitinating enzyme responsible for removal of linear ubiquitin chains from RIPK1 & TRAFs. It increases TNF-α induced death signaling by promoting necrosome formation [79,80].
2) **Heat shock protein (HSP) 90:**- The Cdc37- HSP90 co-chaperone system which regulates protein kinases is believed to enhance protein stability of kinases involved in necroptotic pathway like RIPK1, RIPK3 and MLKL and hence promotes necroptosis. HSP-90 has also been shown to be involved in transport of necrosome from cytoplasm to cell membrane [81–83].
3) **Gβγ-Src signaling**:-Transmembrane G (guanine nucleotide binding) -proteins, Gβ and Gγ coupled to activation of downstream target Src kinase are believed to act as initiators of an alternative necroptosis pathway [84]. Further, Gβγ signaling has been found to be crucial for oligomerization of MLKL and its translocation to membranes [85].

Negative regulators:-

1) **Caspase8:**-Caspase8 has been demonstrated to negatively regulate necroptosis and its deficiency has been found to be associated with uncontrolled necroptotic activity leading to embryonic death [86]. Caspase8 is believed to cleave inducers of necroptosis like RIPK1 [87], RIPK3 [88], and CYLD [79] thus inhibiting necroptosis [89].
2) **cIAP proteins:**- The cIAPs which are E3 ubiquitin ligases cause ubiquitylation of RIPK1 resulting in triggering of TNF-NF-κB pro-

Table 3
Molecules regulating Necroptosis

Molecule	Function	References
Positive regulators		
1) **CYLD**	Deubiquitinating enzyme, removes linear ubiquitin chains from RIPK-1 & TRAFs	[79,80]
1) **HSP-90**	Regulator of protein kinases, Interact with RIPKs and enhances their stability by protecting them from proteasomal degradation.	[81,82,83]
1) **MLKL**	Plays a critical role in TNFα induced necroptosis via its phosphorylation by RIPK3.Phosphorylated MLKL causes loss of membrane integrity and cell death	[63,64] [67,68],
1) **Gβγ-Src signaling**	Alternative pathway that operates in parallel with TNF-induced necroptosis. Perturbation of Gβγ signaling disrupted MLKL oligomerization and translocation to membranes	[84,85]
Negative regulators		
1) **Caspase8**	RIPK-1 & RIPK-3 cleavage	[86,87] [88,89]
1) **IAP proteins**	RIPK1 ubiquitylation and prosurvival TNF-induced NF-κB activation	[90]
1) **TRAF2**	Antinecroptotic effect via cIAP1/2 recruitment to TNFR complexes & ubiquitylation of RIPK1and by limiting MLKL association with RIPK3	[91,92,93]
1) **Ppm1b**	Phosphatase dephosphorylating mouse RIPK3 Deficiency results in modest elevation in cell death	[94]
1) **A20**	Deubiquitinating enzyme, restricts TNF-mediated NF-κB signaling and inflammation. Deubiquitinates RIPK1 and causes its proteasomal degradation. Prevents RIPK1-RIPK3 complex formation.	[95]

survival pathway. XIAP acts as a direct inhibitor of apoptotic caspases. Therefore, loss of above proteins can confer sensitivity to TNFα mediated necroptosis if accompanied by caspase inhibition. In tune with the above, other ubiquitin ligases like Makorin RING finger protein-1 can also repress necroptosis via ubiquitination of RIPK1 [90].

3) **TRAF2:-** Recruitment of cIAPs to TNFR complex is brought about by binding to TRAF2 via its cIAP interaction motif [91]. Thus the loss of TRAF2 may expose the cells to necroptotic cell death [92,93]. Also, TRAF2 has been reported to exert its effect by directly limiting the interaction of MLKL with RIPK3 [93].
4) **Ppm1b:-** Ppm1b,a phosphatase acting as a de-phosphorylator of mouse RIPK3 has been found to reduce RIPK3 catalytic activity and hence necroptosis [94].
5) **A20:-** A20 is a deubiquitinating enzyme which removes K63 linked ubiquitin chains from RIPK1 thus limiting TNFα induced NFκB signaling. Also, it has been found to inhibit the formation of RIPK1-RIPK3 complex via inhibition of RIPK3 ubiquitination [95]. (Fig. 2, Table 3)

6. Pathways leading to necroptosis

Various pathways may trigger necroptotic cell death depending upon the stimulus. Out of these TNF-α mediated necroptosis has been studied most extensively.

6.1. TNF-α mediated necroptosis

It is induced when TNF-α binds with the surface receptor TNFR1 leading to its oligomerization followed by recruitment of other proteins like TRADD, RIPK1, cIAPs, TRAF2 and TRAF5. The above form a multimeric complex called complex I on cell membrane. Polyubiquitination of RIPK1 by cIAPs results in recruitment of TGFβ–activated kinase 1 and IκB kinase thereby leading to the activation of the NF-κB signaling pathway. Also there may be activation of cellular FLICE-like inhibitory protein (c-FLIP), thus preventing DISC formation and cell survival [96,97]. Alternatively, TNFR may be internalized following binding of the ligand leading to posttranslational modification of CYLD which causes deubiquitination of RIPK1. This forms a complex called cytoplasmic cell death complex or complex II that includes RIPK1, TRADD, FAS-associated death domain protein (FADD) and caspase8. If caspase8 is active it leads to apoptosis [76,96,97] however, if it is deleted or mutated, phosphorylation and activation of RIPKs is triggered to initiate necroptosis pathway [76,89,98–100]. RIPK1 then interacts with RIPK3 and forms a protein complex in the cytoplasm known as necrosome which serves as a scaffold for necroptosis.

6.2. PARP-1 mediated necroptosis

Necroptosis may also occur via PARP-1 pathway [76,101]. In TNFα induced necroptosis ROS production leads to DNA damage [43,102]. This in turn activates PARP-1, a nuclear enzyme which causes depletion of ATP and NAD thus helping in the execution of necroptosis [103]. Also PARP-1 can activate pro-necroptotic RIPK1 that in turn activates JNK1 which induces necroptosis through mitochondrial permeability transition [104,105]. The above events induce massive fragmentation of DNA characteristic of necroptosis by activation of Bax and breakdown of apoptosis inducing factor (AIF) from mitochondria [106].

6.3. Necroptosis through TLRs

Cellular damage, stress and infection are sensed by different receptors including TLRs. TLR activation pathway is identified as another important mode of necroptosis in the cell. TLR3 activation has been shown to result in recruitment of cytosolic protein called TRIF (Toll/IL-1 receptor domain-containing adaptor protein inducing interferon) which is involved in activation of NF-κB signaling and induction of type I IFN release [107]. TLR4 also recruits TRIF [108]. TRIF has a RHIM-domain which interacts with RIP kinases. Further agonists of TLR3 like polyinosine-polycytidylic acid [poly (I:C)] and TLR4 like lipopolysaccharide (LPS) can result in RIPK1 independent but RIPK3 and MLKL dependent TRIF mediated necroptosis. However, studies have shown that necrostatin mediated inhibition of RIPK1 activity prevents the programmed necrosis induced by combination of above two agonists with zVAD-fmk. It is possible that inhibition of kinase activity converts it to inhibitor of TLR3 or 4 induced activation of RIPK3 [109,110].

6.4. DNA-dependent activator of IFN regulatory factors (DAI) mediated necroptosis

This protein known as Z-DNA binding protein also contains a RHIM domain. This induces NF-κB activation as well as RIPK3 mediated necroptosis in presence of viral double stranded DNA [111].

6.5. FAS/TRAIL mediated necroptosis

The apoptosis inducer TRAIL has also been found to induce necroptosis in some studies. In malignant cell lines acidic pH in extracellular environment has been found to switch TRAIL-induced apoptosis to necroptosis [112]. TRAIL induced necroptosis also involves RIPK1, RIPK3 and PARP-1 activation. Binding of FAS-L or TRAIL to their respective receptors on cells leads to assembly of membrane associated DISC aided by FADD. The later recruits caspase8 and leads to the apoptosis; however, when cIAPs and caspase8 are inhibited it leads

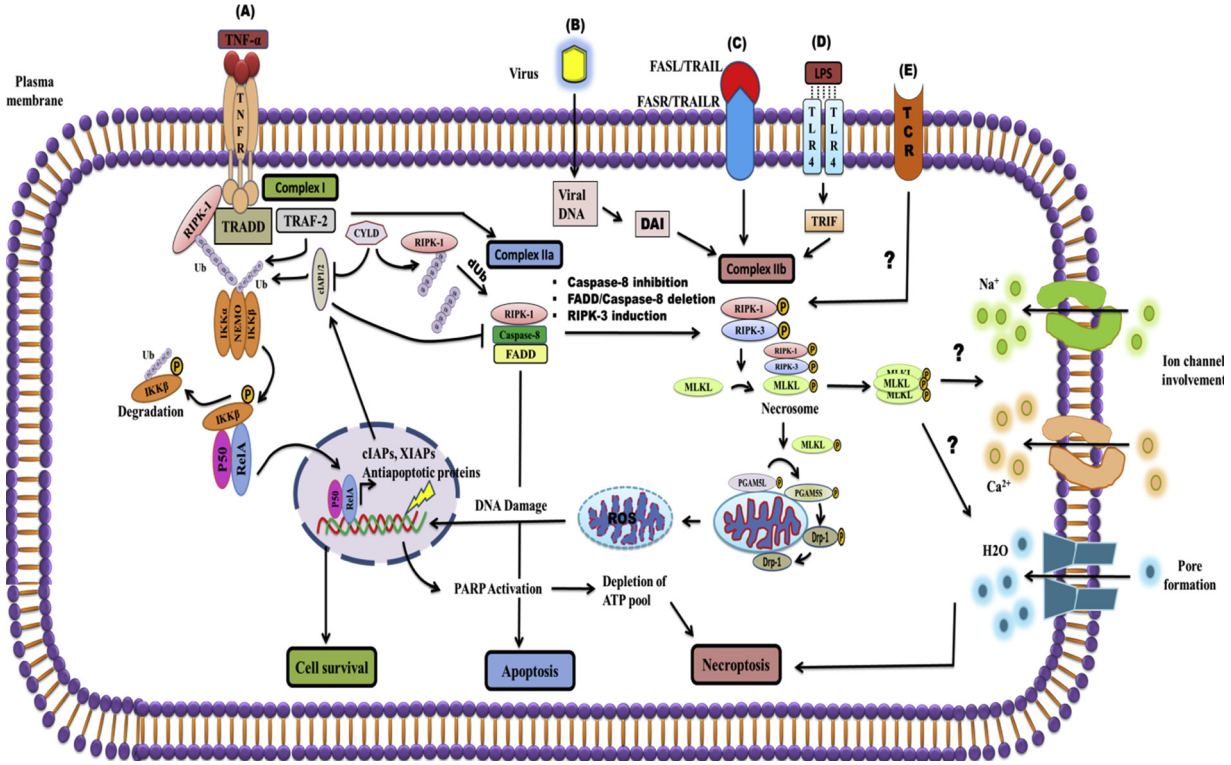

Fig. 1. Figure showing Necroptosis triggered by various stimuli including TNF-α (A) TNF-α interacts with TNFR to trigger cell survival (Complex I) by activating NF-κB pathway, apoptosis (Complex II a) by activating Caspase8 or necroptosis (Complex II b) by activating RIPK-1&3. Also shown are (B) DAI dependent (C) FAS/TRAIL dependent (D) TLR mediated (E) TCR dependent pathways leading to necroptosis.

to the recruitment of RIPK1 to trigger necroptosis [68]. (Fig. 1)

7. Role of necroptosis in physiological conditions

Several studies indicate involvement of necroptosis in several normal developmental processes especially in the absence of apoptosis. Mice with defective apoptotic machinery (Apaf-1^{-}/bax^{-}/bak^{-}/caspase mutants) were found to develop into normal adults indicating maintenance of tissue homeostasis by cell death pathway other than apoptosis [113,114]. Further, necroptosis has also been implicated both in innate and acquired immune responses however, the mechanism by which it contributes to the above are poorly understood. Ripoptosome assembly has been found to result from genotoxic stress as well as during antigenic stimulation of T-cells lacking caspase8 or FADD [103].

8. Role of necroptosis in pathological conditions

Although mainly studied in context of malignancies programmed necrosis has been implicated in variety of pathological conditions like viral and bacterial infections, inflammatory disorders, ischemia–reperfusion injury and neurodegeneration [78]. Mocarski *et al.,* in their work on virus induced necroptosis opined that the later may be considered as a safety mechanism to protect unrestricted proliferation of cells due to viral infections that tend to inhibit apoptosis to evade host immunity [115,116]. In case of pathogen attack (bacteria, viruses, yeast, and fungi) necroptosis has been found to be triggered via TLR3 & 4 [117].

9. Necroptosis and cancer

Cancer cells may undergo genetic and epigenetic remodeling to evade immunity or chemotherapy-induced apoptosis; however, it is hypothesized that this may render them susceptible to necroptosis. In recent years induction of necroptosis with or without chemotherapy is being explored as an alternate route to kill the malignant cells especially when the conventional cell death programs are deranged. Studies are being carried out in different types of cancers like breast, ovarian, colorectal cancers and pancreatic and brain tumours. Necroptosis has been found to be triggered by reduced procaspase8 and active RIPK1 & 3. In some cancers like breast cancer, caspase8 is down regulated making them resistant to apoptosis. Therefore, induction of necroptosis is being tried as a strategy to kill tumour cells [4]. In case of glioblastoma targeting necroptosis with edelfosine has been used as a novel approach to destroy apoptosis resistant tumour cells [118]. Induction of necroptosis has also been attempted in non-responsive tumours e.g. cisplatin and IAP antagonist resistant ovarian carcinoma [119]. Zhang *et al.* showed that TRAIL treatment can induce either apoptosis or necroptosis in pancreatic cancer cell lines under specific conditions [120]. In cervical cancer, induction of necroptosis was found to be associated with pro-inflammatory conditions and dendritic cells mediated IL-12 release indicating triggering of anti-tumor immune response [121]. Also, more recent studies have demonstrated the benefits of combining pro- necroptotic agents with immune checkpoint inhibitors to promote the anti- tumour immunity [122]. Although necroptosis has also been linked to reduced incidence of metastasis in cancer the literature regarding the same is scanty [123]. McComb et al. in their work induced necroptosis in the drug resistant and relapsed cases of acute lymphoblastic leukemia by using Smac- mimetic brinipant and found that there is delay in engraftment, decreased leukemia burden, and prolonged survival of xenografted mice [124]. Fu et al., in their work on osteosarcoma demonstrated that induction of necroptosis by using shikonin increased the survival rates as well as decreased the lung metastasis [125] (Table 4).

However, studies on different cancers show that like apoptosis, necroptosis may also dysregulated or disturbed in malignant cells and upregulation or downregulation of necroptotic pathway molecules have been found to be associated with decreased or increased survival in different cancers. In chronic lymphocytic leukemia defects in the key

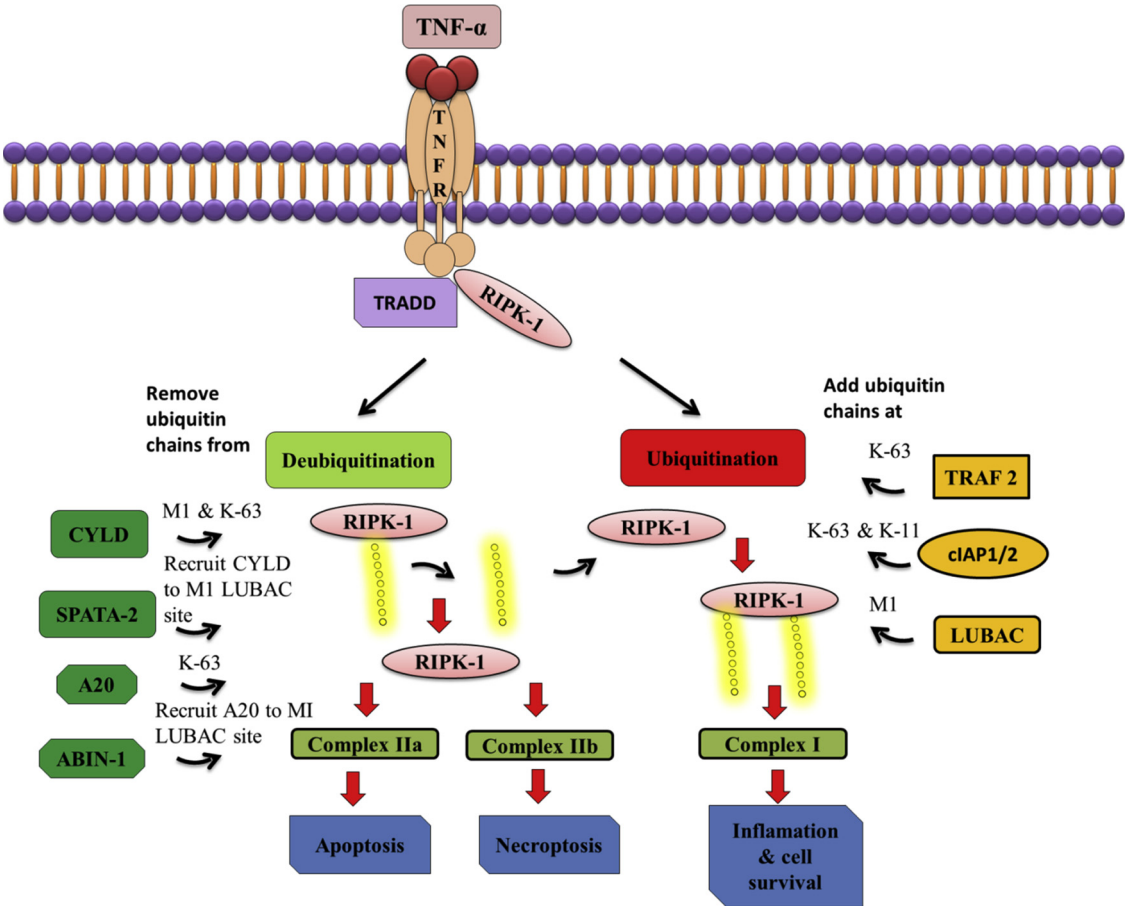

Fig. 2. Role of ubiquitination & deubiquitination of RIPK-1 in TNF-α mediated cell survival & cell death pathways. Also indicated are the molecules which regulate the ubiquitination- deubiquitination dynamics of RIPK1. K-63 = Lysine-63, M1 = methionine-1,LUBAC = Linear Ubiquitination Assembly Complex, SPATA-2 = Spermatogenesis-Associated protein 2, ABIN-1 = A20 Binding and Inhibitor of NF-κB.

signal transduction components of the necroptotic pathway like CYLD & RIPK3 have been observed [126]. Further, RIPK3 gene polymorphisms have been reported in patients with non-Hodgkin lymphoma [127]. RIPK3 was shown to be less expressed and having a tumour suppressor function in colorectal cancer by Feng *et al.* [88]. Further, the expression of RIPK3 was reported to be decreased in malignancies like Acute Myeloid Leukemia & colorectal cancer [2,128–130]. Similarly, the expression of MLKL was also found to be low in gastric and ovarian cancers in various studies [129,131,132]. In Head & neck squamous cell cancers a low RIPK1 expression was reported to be associated with enhanced tumorigenesis [133]. However, in malignancies like glioblastoma and lung cancer an increased expression of RIPK1 was shown to be associated with a poorer prognosis and progression of tumour [134,135]. Similarly, in pancreatic cancer the necroptosis pathway molecules (RIPK1, 3 and MLKL) were reported to be highly expressed. Also, their expression was found to be associated with the tumour progression due to the suppressive effect on immune microenvironment of the tumour [136]. A recent study by Hou *et al.* demonstrated the role of necroptosis in promoting metastasis in melanoma and lung carcinoma [137].

9.1. Status of necroptosis in breast cancer

A decreased expression of key molecules of necroptosis like RIPK3 has been shown in some breast cancer cell lines like MDA-MB-231, MCF-7 etc. [138]. Also, some studies have demonstrated that key

Table 4
Different agents used for the induction of necroptosis in various cancers.

Molecules	Cancer	Mode of action	References
Shikonin	Breast cancer	Naturally occurring naphthoquinone, induces necroptosis in drug and apoptosis resistant cancer cells overexpressing P-glycoproteins, MRP-1, BCRP, Bcl-2 or Bcl-XL	[146,152]
FTY720	Lung cancer	Ceramide mimetic targeting the I2PP2A/SET oncoprotein, resulting in the reactivation of PP2A and induction of necroptotic cell death	[153]
UCD38B	Glioblastoma	Derivative of 5′-benzylglycinyl-amiloride, triggering necroptosis via loss of mitochondrial membrane potential and causing translocation of AIF from mitochondria to cytosol and nucleus	[154]
UCD74A	Glioblastoma	Derivative of glycinyl-amiloride functions in the same way as UCD38B	[154]
BI2536	Prostate cancer	Inhibitor of mitotic kinase Plkl-1 causes necroptosis in androgen-resistant prostate cancer cells	[155]
Obatoclax	Acute lymphoblastic leukemia (ALL)	Inhibitor of antiapoptotic Bcl-2 proteins; induces autophagy-dependent-necroptosis in glucocorticoid resistant childhood ALL	[113]
BV6	Leukemia	Antagonist of XIAP, cIAP1 and cIAP2; sensitizes the apoptosis resistant leukemia cells to TNF-α induced necroptosis	[114]
Edelfosine	Glioblastoma	Triggers cell death in human glioblastoma cells with the characteristics of necrosis	[118]

Table 5
Agents causing necroptosis in breast cancer cells.

S.No	Molecule	Function	Type of cancer/cell line	References
1)	Quercetin	Induce apoptosis and necroptosis in MCF-7	Breast cancer cell line	[138]
2)	Shikonin	Circumvents cancer drug resistance by induction of a necroptotic death	Breast cancer cell line (MCF-7)	[146]
3)	Honokiol	Enhanced apoptosis additionally Necroptosis	Multidrug resistant breast cancers	[156]
4)	Smac- mimetic LCL161	Inhibits inhibitors of Apoptosis	Breast cancer	[148]
5)	Geldanamycin DHQ3	HSP-90 inhibitor and increased the amount of RIPK-1 & RIPK-3	TNBC Cell lines (MDA-MB-231)	[149]
6)	Nickel(II)-Dithiocarbamate Phenanthroline Complex	Inhibits mamosphere formation, Triggers MLKL phosphorhylation, oligomerization and Translocation	Breast cancer stem cells	[144]
7)	Goniothalamin	Triggers Necroptosis via TNF-α pathway	TNBC cell line (MDA-MB-231)	[150]

molecules of necroptosis like RIPK3 may be silenced in TNBC due to hypermethylation of its genomic sequence. Hypoxic environment prevalent within the tumors has also been shown to be responsible for down regulation of the expression of molecules like RIPK1, RIPK3 and MLKL in breast cancer [2,139]. Recent studies have suggested that glycemic conditions can decide the mode of PCD (either apoptosis or necroptosis) in cells. However, whether the same is applicable to malignant cells and tumor microenvironment remains to be explored! [140].

Stoll *et al.,* reported a decreased expression of MLKL & RIPK3 in breast cancer as compared to the normal tissue. Also, a correlation of pro-necrotic gene products like RIPK1, RIPK3, MLKL and PGAM5 was observed with immune-related metagenes in different types of cancers including breast cancer [141]. In contrast, some of the studies have also highlighted the role of necroptosis in the tumor promotion and metastasis. Liu et al, in their study on necroptotic gene knockout models demonstrated reduced tumorigenicity and enhanced sensitization of the tumour to radiotherapy [142]. Karsch et al. in their work in MDA-MB-231 cells also showed increased proliferation, angiogenesis and metastasis in xenograft models of breast cancer on exposure to necrotic malignant cell lysates [143].

9.2. Strategies for harnessing necroptosis as treatment modality in breast cancer

Several strategies are being explored to restore the necroptosis pathway in breast cancer cells. Hypomethylating agents or virus mediated expression of RIPK3 and anti-hypoxia drugs have been investigated [139]. Necroptosis induction in cancer stem cell population by metal based drugs is also being explored as a strategy to overcome resistance in breast cancer [144]. Apart from killing of cancer cells, the release of cytoplasmic contents as DAMPs in necroptotic pathway may result in cancer specific immune response and subsequent death of malignant cells. Stoll et al., in their work in mouse models of breast cancer showed that MLKL and RIP kinases facilitated local expression of genes involved in interferon α and γ thus promoting anti cancer immunity [141]. However, necroptosis related inflammation may have a pro tumorigenic effect [139]. Also, it is believed to carry the potential of inducing inflammatory diseases. Pro-necroptotic therapy in combination with chemotherapeutic drugs has been found to be useful in inducing cell death in tumors previously resistant to the above agents due to switching over from apoptosis to necroptosis. Koo et al., have shown that necroptotic pathway is involved in chemotherapy induced killing of cancer cells and modulating it may help to improve chemosensitivity in breast cancer [139].

Several agents have been observed to induce necroptotic cell death in breast cancer in different studies. Shahsavari et al., in their study on ER + T-47D cells showed successful induction of necroptosis by shikonin, a naturally occurring naphthoquinone [145]. Further, Han et al., in their work demonstrated the potency of shikonin in causing necroptosis in drug resistant breast cancer cells also [146]. Chen et al., demonstrated that depletion of amino acid cystine causes cell death by necroptosis and ferroptosis in TNBC cell lines [147]. In a study on MCF-7 cells, Khorsandi et al., reported quercetin induced killing by both apoptosis and necroptosis. Further, they also observed an increase in expression of molecules like RIPK1 and RIPK3 in response to the above drugs in MCF-7 cells [138]. Jin et al., in their study involving Smac-mimetic LCL161 showed its potential as a therapeutic agent for inducing multiple forms of PCD including necroptosis in breast cancer [148]. In a study by Zhang et al., non- benzoquinone analog of geldanamycin DHQ3 was found to induce necroptosis in TNBC cell lines by increasing the expression of RIPK1 and RIPK3 [149]. Khaw-On et al., in their study on MDA-MB-231 cells showed that chemical compound Goniothalamin caused death of malignant cells via induction of TNF-α mediated necroptosis [150]. PARP inhibitors are being investigated as augmenters of chemotoxicity in breast cancer cells. However, excess

PARP in tumor microenvironment may promote cell death by necroptosis in response to chemotherapy [151] (Table 5).

Thus, from the above review of literature, it is clear that resistance to cell death is one of important characteristic of cancer cells. This resistance may not only promote tumor progression but may also reduce the effectiveness of currently available chemotherapeutic agents. Apoptosis is the most commonly studied cell death pathway and various approaches have been designed to enhance apoptosis in cancer cells. Necroptosis is a relatively newly recognized cell death entity, and the details of the pathway are still being explored. Studies have shown that necroptosis induction in cancers including breast cancer may help to promote killing of tumor cells. However, many issues remain to be sorted out before it can be harnessed to destroy apoptosis resistant tumor cells. Specificity of necroptosis for killing of malignant cells, suppressing pro-tumor inflammatory component of necroptotic end products without suppressing their anti-tumor effects, up regulation of impaired necroptotic machinery molecules in tumors, combining necroptosis induction with hypoxia antagonists, understanding of factors which may help to promote necroptosis in tumor microenvironment etc remain some of the questions which require immediate attention.

Declaration of Competing Interest

The authors declare that there is no conflict of interest

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