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Omics approaches to better understand the molecular mechanism of necroptosis and their translational implications

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Abstract

Necroptosis is a type of programed cell death characterized by an inflammatory phenotype due to extensive membrane permeabilization and rupture. Initiation of necroptosis involves activation of tumor necrosis factor receptors by tumor necrosis factor alpha (TNFα) followed by coordinated activities of receptor-interacting protein kinases and mixed lineage kinase-like protein (MLKL). Subsequently, MLKL undergoes phosphorylation and translocates to the plasma membrane, leading to permeabilization. Such permeabilization results in the release of various cytokines and causes extensive inflammatory activity at the organismal level. This inflammatory activity is one of the major differences between apoptosis and necroptosis and links necroptosis to several human pathologies that exhibit inflammation, in addition to the ultimate cell death phenotype. Given the crosstalk between the activation of cell death pathway and inflammatory activity, approaches that provide insights on the regulation of transcripts, proteins and their processing at the global level have substantially improved our understanding of necroptosis and its involvement in different disease states. In this review, we highlight recent omic studies probing the transcriptome, proteome and lipidome which elucidate potential new mechanisms and signaling pathways during necroptosis and the necroptosis-associated inflammatory activity observed in various diseases. We specifically focus on studies investigating the transcriptome and intracellular and released proteome that contribute to inflammatory nature of necroptotic cells. We also highlight different lipids that have been implicated in necroptosis and lipidomic studies identifying lipid players in necroptosis. Finally, we review studies which suggest certain necroptosis-related genes as potential prognosis markers for different cancers and discuss their translational implications.

1. Introduction

Biological events involve a complex network of signaling cascades. Cell signaling translates a signal, either initiated internally or via an extracellular stimulus to achieve a biological response, resulting in different cell fates, including cell death and cell survival.[1] The multiple steps involved in any signaling event begin from the recognition and processing of signal by different targets such as receptors or enzymes followed by activation of other downstream partners to eventual conversion of the signal to biological response. Activation mechanisms range from post-translational protein modifications such as phosphorylation, ubiquitylation, lipidation to change in ion concentrations, protein-protein or protein/DNA interactions or translocation of proteins to different cellular compartments.[1, 2] Dissecting the contribution and function of these different molecules require the use of different approaches that are well-suited for high-throughput and their specific analysis in the presence of complex cellular matrix. Further, to obtain a better understanding of these interconnected events, integrative information of the changes occurring at multiple levels is required. Such information becomes essential in disease states where the basal cellular functioning is either modified or disrupted and many cellular functions/events are affected.

"Omic"-based approaches have emerged as a forefront player in understanding biological events. In this paper, we will review recent studies that utilize proteomics, transcriptomics and lipidomics to gain new insights on the molecular mechanism of a specific type of programmed cell death, programmed necrosis or necroptosis, and the implications of these findings from mechanistic and disease perspectives. We focus on proteomics and lipidomics studies to better understand signaling and different protein and lipid involvement in the context of necroptosis-associated inflammation and membrane dynamics. We also review recent transcriptomic studies which reveal several necroptosis-related genes involved in different cancers and other disease states.

2. Necroptosis is a complex process that requires protein, lipid and membrane modeling

2.1. *Necroptosis, a type of programmed cell death.* The different death pathways that cells follow involve a complex and tightly controlled regulation. Such regulation or programming is essential as cell death not only plays a role in cellular homeostasis and development but also in disease development.[3, 4] Apoptosis is one of the major and most well-studied form of programmed cell death. Apoptosis can be initiated by an intrinsic signal such as intracellular damage or via an extrinsic signal such as binding of ligands to different death ligands such as tumor necrosis factor

alpha (TNFα) to TNF receptors (TNFR, **Figure 1**).[5-7] Both pathways eventually involve activation of caspases which ultimately results in cellular shrinkage, blebbing of plasma membrane, nuclear condensation and fragmentation and release of apoptotic bodies. Apoptosis was thought to be the only type of programed cell death for a long time, with necrosis, on the other hand, was thought of as an accidental process involving cell swelling and rupture.[5] However, it was found that a different type of regulated cell death resembling the morphological changes of necrosis could be triggered by stimuli.[8] This type of programmed necrosis was termed as necroptosis[8, 9] and was characterized by necrosis-like morphological and cellular changes including disrupted plasma membrane, cell organelle swelling, ATP depletion and leakage of intracellular contents (**Figure 1**). *In vitro*, necroptosis differs from apoptosis in the rupture of plasma membrane early in the presence of death signal and release of intracellular contents and damage-associated molecular patterns (DAMPs). At the organismal level, this rupture and release of intracellular material results in an extensive inflammatory activity.[10]

2.2. *Mechanism of necroptosis.* Necroptosis is dependent on reduced caspase activity and activation of receptor interacting protein kinase 1 (RIPK1) and RIPK3.[11] Similar to the extrinsic pathway of apoptosis, necroptosis is often triggered by activation of the TNF receptor by the same family of cytokines.[12] The binding of TNFα to TNF receptor can lead to different cell fates dependent upon the various protein players involved and their levels. Cells can proceed toward a cell survival pathway due to ubiquitination of RIPK1, whereas deubiquitination of RIPK1 can result in programmed cell death (**Figure 1**). Such deubiquitination of RIPK1 is caused by deubiquitinases such as cylindromatosis (CYLD, a tumor suppressor protein[13]) or second mitochondria-derived activator caspase (SMAC), which regulates cellular inhibitor of apoptosis proteins 1/2 (cIAP1/2).[12] Deubiquitinated RIPK1 then assembles multiple adaptor proteins and promotes activation of procaspase-8 initiating apoptosis. When caspases exhibit reduced activity, however, necroptotic cell death pathway is activated.[14] RIPK1 binds to RIPK3 leading to its autophosphorylation and eventual interaction with mixed lineage kinase domain like pseudokinase (MLKL), a key protein in signaling necroptosis.[15] Binding of MLKL to RIPK3 activates MLKL via phosphorylation and causes its oligomerization and subsequent translocation to the plasma membrane where MLKL oligomers cause membrane disruption.[16-18] How MLKL causes membrane disruption is the subject of multiple studies and is not entirely understood. Previous studies suggested that MLKL translocation to the membrane disturbs ion homeostasis by causing influx of Na^{+} [17], Mg^{2+}, Ca^{2+} either by forming cation channels[19] or by affecting some other ion channel forming protein[20]. Further, MLKL has been shown to disrupt membranes by

binding to phosphatidylinositol phosphates (PIPs) at the plasma membrane in vitro, in reconstituted membranes[21] or directly interact with membranes via its N-terminal 4-helix bundle domain and potentially causing leakage.[22]

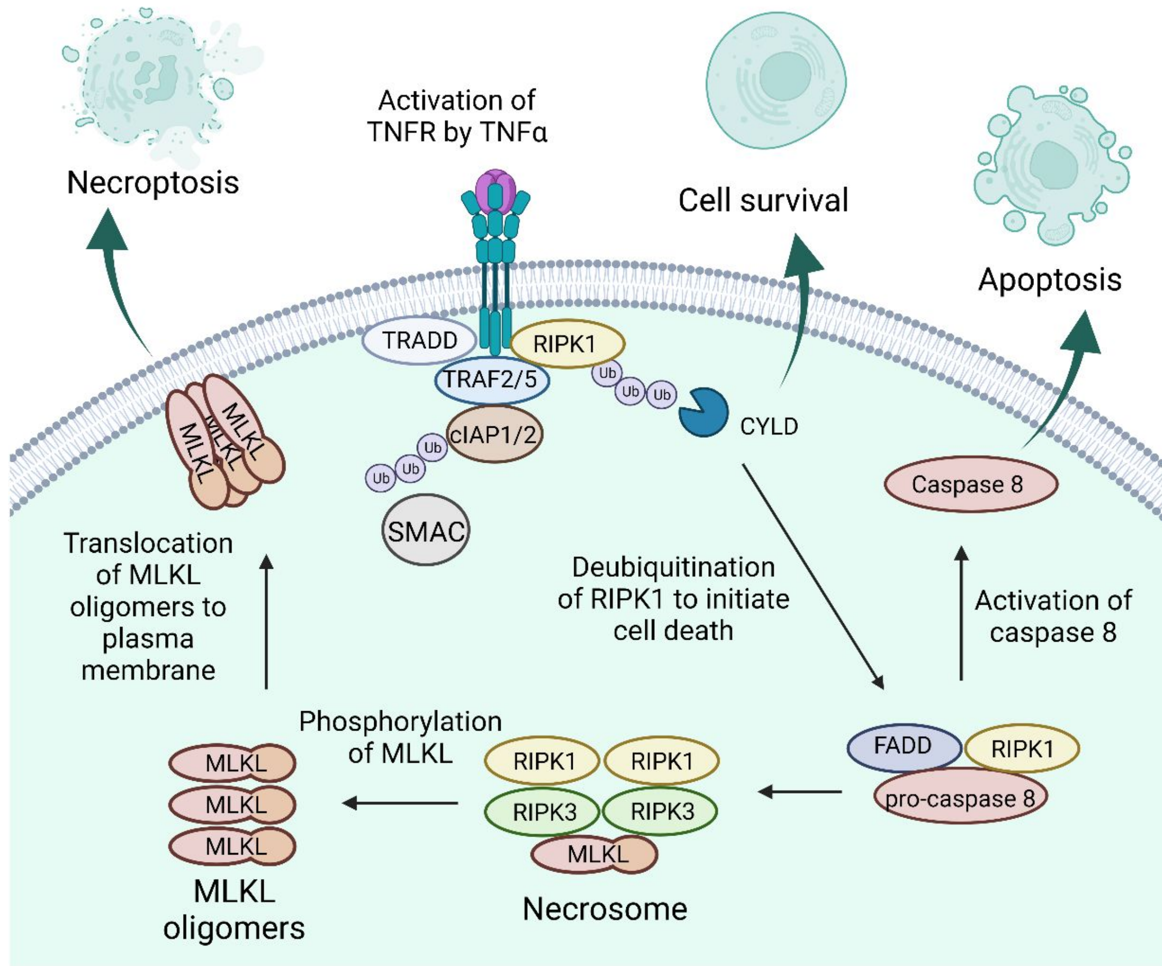

Figure 1: Different cell fates following initiation of TNFα signaling depend upon the ubiquitination of RIPK1 and activity of caspases. TNF receptor activation by binding to TNFα causes recruitment of several adaptor proteins such as receptor interacting protein kinase 1 (RIPK1), TNF receptor type-1 associated death domain (TRADD), TNF receptor associated factor 2 (TRAF2), TNF receptor associated factor 5 (TRAF5) and cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2). cIAPs can cause ubiquitination of RIPK1 and initiate a cell survival signal. Cylindromatosis (CYLD) or second mitochondria-derived activator caspase (SMAC) can deubiquitinate RIPK1 and initiate a cell death signal. RIPK1 can recruit Fas associated via death domain (FADD) and procaspase 8 to initiate apoptosis or form a necrosome with receptor interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain like pseudokinase (MLKL) in the absence of caspase 8 to initiate necroptosis. Phosphorylated MLKL oligomerizes and translocates to the plasma membrane leading to necroptosis-associated membrane permeabilization. Figure created with BioRender.com.

2.3. *Models to study necroptosis.* As we discussed above, binding of TNFα to TNFR can signal either necroptosis or apoptosis, indicative of a close interrelation between the two types of programmed cell death processes. This binding event signals different phenotypic outcomes depending on downstream effectors, their levels and activity. Hence, differentiating between apoptosis and necroptosis can become challenging due to the common activation pathways and players that regulate both these processes, especially in tissues and *in vivo* models with different genetic and biochemical backgrounds. Therefore, *in vitro* systems, where necroptosis can be induced in the absence of apoptotic activity, have been the primary source of information regarding the molecular markers of necroptosis and to understand the mechanism.[11, 16, 17] The elimination of apoptotic activity is commonly achieved by using pan-caspase inhibitors (e.g., Z-VAD-fmk) and cIAP inhibitors (e.g., BV6) *in vitro*.[23, 24] Markers specific to apoptosis (e.g. cleavage of poly ADP-ribosome polymerase) or necroptosis (e.g. MLKL phosphorylation) can then be used to distinguish between these processes. HT-29 (human colorectal adenocarcinoma) and U937 (human histiocytic lymphoma) have been the most common cell lines of choice to study necroptosis due to their increased RIPK1 and RIPK3 activity and ease of culturing due to their cancerous nature.[25] The findings from these *in vitro* systems have greatly helped to explain *in vivo* observations, making them useful models to study different aspects of necroptosis. However, the complex environment and potential crosstalk between different signaling pathways in *in vivo* systems including disease models or clinical samples make it essential to obtain findings that are translational to human health.

2.4. *Disease relevance of necroptosis.* Several pathological conditions have been attributed to the inflammatory response generated by the different cytokines and intracellular molecules released during necroptosis.[10] Cytokines released from necroptotic cells can promote inflammation.[10] Bacterial infections can involve necroptosis in response to the pathogenic cytokines released by the bacteria.[26, 27] *In vivo* studies in mice and patients have been used to provide evidence of RIPK-MLKL-mediated necroptosis and caspase involvement in other pathological conditions. For instance, elevated expression of RIPK1 in neurodegenerative conditions like Alzheimer's disease[28], increased activation of RIPK1/3 and MLKL as well as impaired activation of caspase-8 in multiple sclerosis [29] and increased expression of RIPK3 and phosphorylated MLKL (pMLKL) in non-alcoholic fatty liver disease[30] show a link between necroptosis and different types of inflammatory diseases. RIPK1-dependent necroptosis contributes to renal ischemia and reperfusion injury[31] and inflammatory conditions like Crohn's disease was shown to have increased levels of RIPK3.[32] In fact, RIPK1 inhibitor Necrostatin-1

(Nec-1) has shown amelioration of necroptosis in various cell lines and animal models of different inflammatory conditions and neurodegenerative diseases.[33]

Viral infections like influenza has been found to rigger necroptosis.[34] Recently, necroptosis has also been implicated in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) along with other types of cell death in lung cells, initiating an inflammatory response.[35] While necroptosis led to enhanced immune response which can help control viral infection, the subsequent inflammatory nature of necroptosis such as the excessive release of inflammatory cytokines causes SARS-CoV-2-related complications.[36, 37] Following such findings, use of necroptosis inhibitors in coronavirus infections is being studied with certain necroptosis inhibitors currently under clinical trials.[38]

In parallel to inflammatory diseases, necroptosis has been implicated in different cancers, exhibiting context-dependent anti- and pro-tumor effects.[39, 40] Expression levels of RIPK1/3 have been associated with a poor prognosis of some cancers and inhibited progression of some types of cancers.[40, 41] For instance, RIPK1 and RIPK3 expression was found to be increased in pancreatic ductal adenocarcinoma and deleting RIPK1/RIPK3 protected against oncogenesis in mice by immune suppression. However, this *in vivo* observation contradicted with the *in vitro* observation in the same study where deleting RIPK3 enhanced pancreatic cancer cell proliferation.[40] Similarly, RIPK3 was found to control proliferation of tumor cells and inflammation associated with colorectal cancer in mice models by regulating the expression of tumorigenic and inflammatory genes.[41]

While previous studies have improved our understanding of necroptosis and provided critical information regarding the different signaling molecules and the mechanism involved, there are still gaps in our knowledge, primarily involving intra- and extracellular membrane trafficking that mediates inflammatory phenotype of necroptosis and how these impact different disease states. The field of 'omics' has recently emerged capable of analyzing large-scale transcriptome, proteome and lipidome data sets[42], advancing the understanding of multitude of fields not limited to human disease. In the past two decades, omics studies have taken the stage for the elucidation of mechanism of necroptosis. These studies include extensive proteomic studies to understand transcripts and their proteoforms that are relevant for necroptotic signaling and their role in inflammation and studies focusing on the lipidome to better understand extensive membrane remodeling that occurs during necroptosis.

3. Transcriptomics and proteomics of necroptotic cells and their secreted extracellular components.

3.1. *Studies on necroptotic signaling via phosphoproteomics.* The major protein regulators of necroptotic signaling have been well-established. However, necroptosis being a dynamic process involving membrane permeabilization, release of intracellular content and associated inflammation, recent studies providing a global overview of proteins involved in different steps of necroptosis have enabled a better understanding of the necroptotic signaling. Since necroptosis involves kinases such as RIPK1 and RIPK3 that can activate proteins via phosphorylation, including MLKL, the executioner protein of necroptosis, understanding the phosphoproteome of necroptotic cells is important to investigate different players in this process.[43] Zu and co-workers conducted a comprehensive analysis of the phosphoproteome to elucidate the different signaling pathways downstream of RIPK1 kinase activation and how they affect inflammation in necroptosis, specifically via phosphorylation.[43] Quantitative proteomics analysis was conducted in TNFα-induced necroptotic Jurkat cells (human T lymphocyte) and around 7000 phosphorylated proteins were quantified. Among the proteomics hits that showed differential phosphorylation during necroptosis, Tripartite-motif containing 28 (TRIM28) phosphorylation showed a time-dependent increase and was inhibited by Necrostatin-1s (Nec-1s), an inhibitor of RIPK1 **(Figure 2)**, suggesting that TRIM28 phosphorylation occurs downstream of RIPK1 activation. TRIM28 is a multi-domain protein and involved in cell proliferation.[44] Depending on its phosphorylation state, TRIM28 can promote or inhibit tumor progression.[45] Zu et al. showed that Ser473 in TRIM28 undergoes phosphorylation in late necroptosis downstream of RIPK1 activation and MLKL oligomerization, but not during apoptosis. Since phosphorylated TRIM28 acts as a transcription factor, they carried out transcriptomics to investigate the effect of TRIM28 phosphorylation on gene expression in necroptosis. Of the number of genes that showed differential expression, several ones involved in inflammatory signaling were upregulated in necroptosis, indicating the role of phosphorylated TRIM28 in promoting transcription of inflammatory genes. They also showed that TRIM28 phosphorylation is mediated by p38 MAPK and Transforming growth factor-β (TGF-β) activated kinase 1 (TAK1) downstream of RIPK1 activation in late necroptosis.[43] These studies established the involvement of p38 MAPK in necroptotic signaling, primarily in the activation of RIPK1.

3.2. *Transcriptomics to understand the associated inflammatory phenotype of necroptosis.* Zhu et al. conducted gene expression analysis in necroptotic HT29 cells using RNA sequencing, where they compared the transcriptome of necroptotic cells to that of cells stimulated by TNFα

alone.[46] Given the inflammatory nature of necroptosis, expression of proinflammatory cytokines like C-C motif chemokine ligand 20 (*ccl20*), *TNFα*, CXC motif chemokine ligand 8 (*cxcl8*) and macrophage colony stimulating factor-1 (*csf1*) were upregulated during necroptosis (**Figure 2**). A more pronounced increase in the expression of these genes was observed in late necroptosis as compared to earlier time points and to stimulation with TNFα alone, showing the progressive nature of the changes in gene expression. This phenomenon was confirmed in different cell lines [i.e. mouse embryonic fibroblasts, Jurkat, HT22 (mouse hippocampal neuronal cells) and L929 (mouse connective tissue cells)] with different stimuli to induce necroptosis [i.e. TNF-related apoptosis-inducing ligand (TRAIL), SM-164, a SMAC mimetic[47] and zVAD-fmk], indicating the increased expression of inflammatory cytokines as a hallmark of necroptosis. Two possible mechanisms were found to be responsible for the increased expression of inflammatory cytokines in late necroptosis: Nuclear factor kappa B (NF-κB) and p38 MAPK pathway (**Figure 2**). The activation of NF-κB pathway was dependent on scaffolding function of RIPK1. p65, a component of NF-κB critical for transcription, showed increased translocation to the nucleus and IκBα, inhibitor of NF-κB,[48] showed enhanced degradation in necroptotic cells as compared to control group in a RIPK1-RIPK3-MLKL dependent manner. This study also showed that the RIPK1/RIPK3/MLKL complex was necessary for the activation of p38 pathway, although the mechanisms are yet to be identified.[46] In parallel, this study provided important insights on the mechanisms of increased cytokine production and suggested that enhanced inflammatory cytokine production is mainly triggered by RIPK1/RIPK3/MLKL activity, and less likely to be triggered externally by DAMPs.[46] Overall, these findings improved our understanding of the global changes in gene expression and new mechanistic insights on how enhanced expression of inflammatory cytokines are regulated during necroptosis.

3.3. *Proteomics to understand the secretory phenotype associated with inflammatory activity in necroptosis.* Necroptosis involves extensive membrane remodeling both mediating the rupture of the plasma membrane and the repair of damaged membrane regions. pMLKL accumulating at the plasma membrane leads to membrane damage which accelerates necroptosis in neighboring cells which have also been stimulated with a TNFα-mediated necroptotic signal.[49] Studies also link MLKL to events related to membrane remodeling like exocytosis, membrane shedding and the release of intracellular contents in necroptosis (**Figure 2**).[50-52] One study showed that MLKL facilitates endocytic trafficking and vesicle generation and its release from the cells after incorporation in extracellular vesicles in necroptosis (**Figure 2**).[52] These findings highlighting the role of protein trafficking, membrane remodeling and vesicular processes occurring in necroptosis

and establish proteome-wide investigations in the released or extracellular content in necroptosis as critical for a comprehensive understanding of the proteins involved in these dynamic membrane changes.

Two recent studies investigated the proteome of extracellular vesicles released by necroptotic cells (**Figure 2**). One study focused on the proteome of extracellular vesicles in necroptosis.[53] They investigated the role of vesicular trafficking in necroptosis and explored the idea of taking advantage of extracellular vesicles as anticancer therapeutics. They found that extracellular vesicles released from necroptotic cells carry several proteins, such as Rab family proteins, MLKL and TNF, involved in vesicular trafficking and inflammation. Another study analyzed the secreted proteins form necroptotic and apoptotic cells, providing a comprehensive account of common and different proteins released in apoptosis and necroptosis. Several receptor proteins such as mannose receptor C type 2 (MRC2), cell surface protein CD44, sortilin 1 (SORT1) were common to both apoptotic and necroptotic cells, while nucleosomal proteins like histones were released in apoptosis but not necroptosis. They also found that cytokines and lysosomal proteins were released into the extracellular matrix only in necroptosis, highlighting protein components associated with inflammatory phenotype of this process.[54]

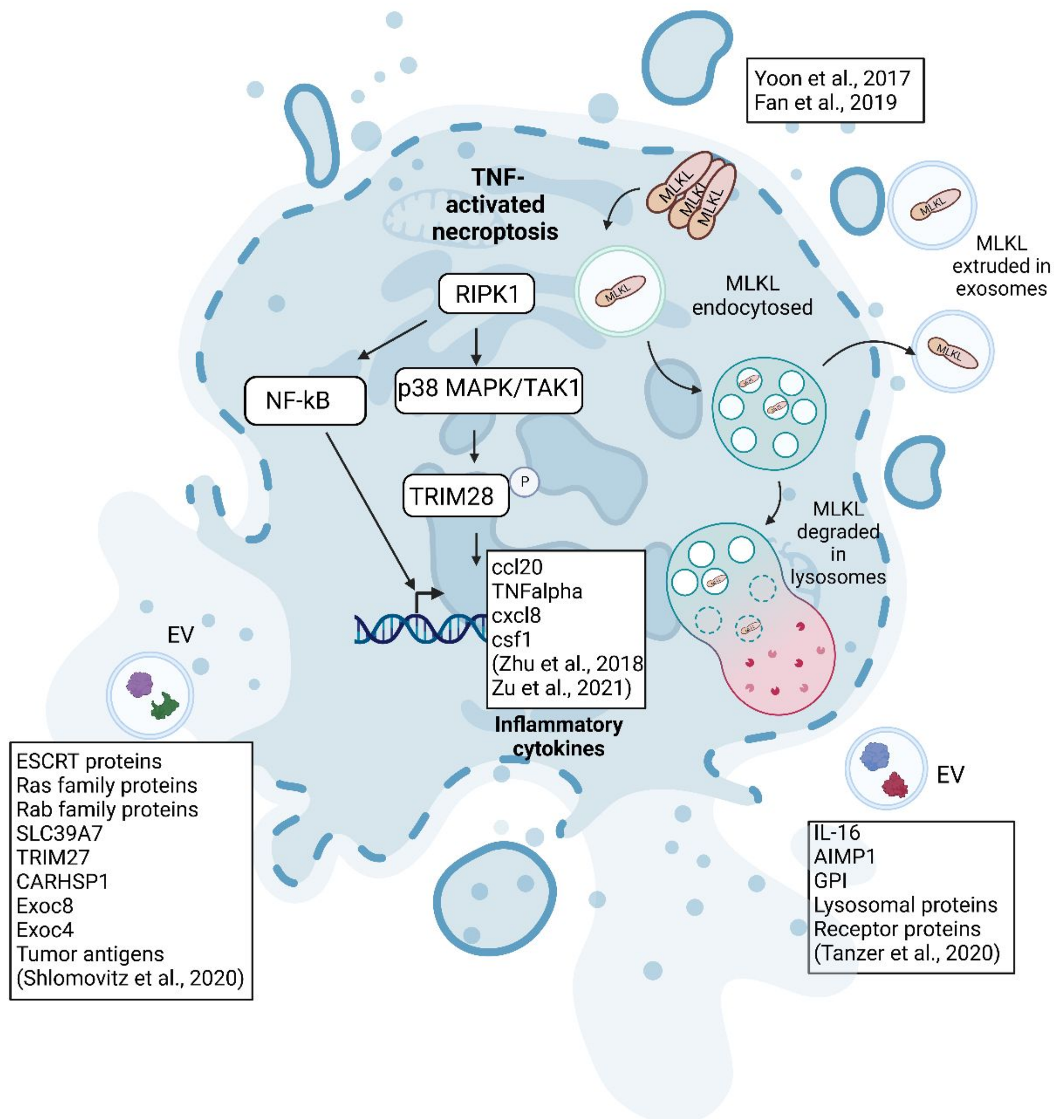

Figure 2: Necroptosis results in increased expression of inflammatory cytokines. TRIM28 initiates transcription of inflammatory cytokines downstream of p38 MAPK/TAK1 following RIPK1 activation. Similarly, NF-κB pathway activation downstream of RIPK1 activation increases expression of inflammatory cytokines. The secretory phenotype in necroptosis shows the extensive membrane remodeling occurring. Supernatants and extracellular vesicles enriched in proteins involved in vesicular trafficking, membrane dynamics and inflammation are observed. Figure created with BioRender.com.

Shlomovitz et al. characterized the proteome of extracellular vesicles of necroptotic U937 cells to obtain a global overview of the secretory phenotype during necroptosis.[53] The approximately 3000 identified proteins were then compared with existing human exosome

proteome databases to validate the proteomic results based on the presence of proteins like endosomal sorting complexes required for transport (ESCRT) accessory proteins including Alix, flotillins, Ras family proteins which are commonly EV-associated. Bioinformatics analysis of proteins unique to necroptotic extracellular vesicles showed an enrichment of various inflammatory signaling pathways like the Toll-like receptor signaling pathway, type 1 interferon production regulation, antigen processing and presenting as well as proteins involved in vesicle formation and endosomal trafficking such as ESCRTIII family members strengthening the relationship between vesicles released during necroptosis and their potential role in exacerbating inflammation. Supporting the role of extracellular vesicles in promoting inflammation, necroptotic extracellular vesicles were also found to be phagocytosed by macrophages inducing an increased secretion of inflammatory cytokines. Further analysis of proteins revealed the upregulation of Rab family proteins, which are essential in exocytosis of pMLKL-rich regions form the plasma membrane.[52] Other upregulated cargo proteins revealed included SLC39A7, a zinc carrier protein, TRIM27 and calcium-regulated heat-stable protein 1 (CARHSP1) which are involved in necroptotic signaling[53, 55-57], and exocyst complex component 8 and 4 (Exoc8 and Exoc4), components of exocyst, an eight-subunit complex that tethers vesicles to the plasma membrane and mediates vesicle-membrane fusion.[58] This study also suggested that extracellular vesicles derived from necroptotic cells could be potential carriers for tumor antigens and could be used as anticancer therapy due to activation of the immune cells leading to an anti-tumorigenic effect.[53]

The secretory phenotype of necroptosis is distinct from apoptosis.[59,60] Tanzer et al. conducted proteomics on the supernatants and extracellular vesicles-enriched fractions obtained from control and necroptotic U937 cells and human primary macrophages to investigate the proteome of TNFα-induced apoptosis and necroptosis to provide a comparative analysis of the secreted protein during these two programmed cell death processes.[54] They identified >2000 proteins in the supernatants from necroptotic cells showing the most variation in cytosolic and ER- and mitochondrial protein levels over different time points. Supernatant from necroptotic cells had decreased levels of TNF-induced cytokines including conventional cytokines like granulin (GRN), CSF1 and pro-platelet basic protein (PPBP), interleukin-8 (IL8) and C-C motif chemokine ligand 2 (CCL2). This reduction in the release of conventionally released cytokines during necroptosis might also be due to decreased transcription of TNF-induced targets. Some of the cytokines which are released when cell membrane integrity is compromised, the non-conventionally released cytokines, such as interleukin-16 (IL16) and aminoacyl tRNA synthase complex-interacting multifunctional protein (AIMP1), increased in necroptotic supernatants (**Figure 2**). This corroborates not only the phenotype of membrane permeabilization but also the

subsequent release of inflammatory cytokines associated with necroptosis. Interestingly, several receptors were released to similar extents in necroptotic and apoptotic cells, which is mediated by ADAM metalloproteinase family, instead of being exocytosed (**Figure 2**). This finding provides a new insight on the signaling mediated intra- and intercellularly due to protein shedding during necroptosis. In this context, the release of receptor proteins via ectodomain shedding corroborates with previous results showing MLKL-mediated activation of these metalloproteases and promoting membrane disruption and inflammation in surrounding cells.[61] Key differences in extracellular vesicles was the enrichment of nucleosome component proteins in apoptotic supernatants, and mature lysosomal component proteins in necroptotic supernatants (**Figure 2**).[54] The increased release of luminal lysosomal proteins in supernatant from necroptotic cells was attributed to lysosomal exocytosis, which might serve as a mechanism for membrane repair.[62] Overall, this study provides invaluable data on intra- and extracellular membrane trafficking and insights on the involvement of multiple proteins which affect signaling and extent of inflammation in necroptosis.

4. Lipidomics to study lipid involvement and membrane modeling in necroptosis

Since necroptosis ultimately results in permeabilization of the membrane, membrane modeling is a core process in necroptosis. As we describe earlier, MLKL has been associated with membrane shedding, wherein, during necroptosis, MLKL associated with the plasma membrane results in shedding of membrane in the form of ‘bubbles’[51]. This process is mediated by some components of the ESCRT-III machinery and acts as a last resort to rescue cells from necroptotic death.[51] Such membrane dynamics occurring in necroptosis suggest involvement of different lipids, major components of cell membranes, working in parallel with key protein components. Inspired from these, several groups, including ours, have studied the involvement of different lipid classes in membrane-related transformations during necroptosis (**Figure 3**).

4.1. *The involvement of phospholipids in necroptosis.* The N-terminal domain of MLKL consists of a 4-helix bundle which is linked to the C-terminal pseudokinase domain via a linker or a brace region.[15] The 4-helix bundle of MLKL is essential for the recruitment of MLKL to the plasma membrane and essential to induce necroptosis.[21] This 4-helix bundle was found to bind to phosphatidylinositol phosphate species, namely, phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol (4,5)-bisphosphate (PIP2, **Figure 3**) and phosphatidylinositol (3,4,5)-triphosphate (PIP3) as demonstrated in liposomes and cause leakage in these systems.[16, 21] This interaction between the 4-helix bundle and the PIP species is through electrostatic interactions

between the negatively charged lipids and positively charged amino acids, mainly multiple lysine and arginine residues, in the helical region. Quarato et al. show initial low affinity binding of between the basic residues of one helix of the 4-helix bundle with the PIPs, followed by a 'rolling over' mechanism of the brace region leading to increased interactions between the second helix and PIPs.[63] Thus, this interaction is important for MLKL recruitment to the plasma membrane in necroptosis.

In addition to PIPs, dysregulated phosphatidylcholine (PC) synthesis has been implicated in necroptosis in certain tissues (**Figure 3**). Kennelly et al. showed impaired PC synthesis due to deletion of phosphocholine cytidyltransferase α, a rate limiting enzyme in PC biosynthesis, caused necroptosis-mediated colitis in mice. These mice had depleted levels of PCs in the epithelial cells of the colon along with increased intestinal permeability and altered structures. This model was used to mimic gastrointestinal inflammatory conditions which also have lowered PC levels. The altered membrane composition brought about by PC imbalance caused activation of the unfolded protein response and ER stress, causing necroptosis.[64]

4.2. *Ceramides, well-known pro-apoptotic lipids, are regulated in necroptosis.* Ceramides (**Figure 3**), a member of sphingolipids, are well-established pro-apoptotic lipids. The levels of ceramides increase via *de novo* biosynthesis during apoptosis.[65] Different mechanisms are suggested for the apoptotic activity of ceramides including ceramides forming channels in mitochondrial membranes,[66] a direct permeabilization of mitochondrial membranes by interacting with pro-apoptotic proteins like Bax potentially via forming ceramide channels stabilized by Bax[67] and binding of ceramides to voltage anion dependent channels to regulate shuttling of pro-apoptotic proteins[68] among others. Recent studies on necroptosis investigated the role of ceramides in this process and related diseased conditions and proposed different mechanisms. Activated *de novo* biosynthesis of ceramides[69] and breakdown of sphingomyelin species[70] both have been implicated in the accumulation of ceramides observed in necroptosis. Another study shows reactive oxygen species (ROS)-mediated accumulation in ceramides seen in necroptosis.[71] Other studies suggested generation of ROS via the mitochondrial electron transport chain in TNFα- mediated necroptosis. This involved a ceramide signaling pathway where decreasing ceramides by inhibiting sphingomyelin breakdown reduced ROS-associated toxicity. Increased ceramide levels were also shown to affect mitochondrial ROS generation by signaling ceramide-activated protein kinase (**Figure 3**).[72, 73] Ceramide was also found to cause necroptosis in *in vitro* placental cells and preeclamptic tissue.[74] Similarly, ceramide nanoliposomes were found to induce MLKL activation and oligomerization to induce necroptosis in ovarian cancer cell lines

(Figure 3), thus serving as a therapeutic tool. However, the exact mechanism by which ceramides target MLKL is yet unknown.[75]

Figure 3: Lipid involvement in necroptosis. Lipid classes involved in necroptosis include lipids present extensively at the plasma membrane and lipids located subcellularly, contributing to membrane modeling and signaling associated with necroptosis. Figure created with BioRender.com.

4.3. *The involvement of cholesterol in necroptosis.* Cholesterol is a major component of eukaryotic membranes (**Figure 3**).[76] Cholesterol modulate membrane rigidity and interaction with different proteins which have roles in cholesterol trafficking, lipid homeostasis and signaling.[77] Cholesterol is trafficked within different organelles; transported from endoplasmic reticulum to the plasma membrane, Golgi, mitochondria and lysosomes to maintain homeostasis.[77] Cholesterol also serves as a precursor for steroidal hormones and bile acids which have roles in signaling as well as other physiological processes.[78] With cholesterol playing an important role in cellular lipid homeostasis and membrane dynamics, involvement of this lipid in necroptosis is not surprising. Induction of necroptosis-like cell death in neuronal cells using L-Norephedrine, an alkaloid with

dopamine receptor and adrenergic agonist properties, caused an upregulation of gene expression of low-density lipoprotein receptor (LDLR) and Niemann-Pick disease Type C 2 (NPC2) and sterol regulatory element binding protein-2 (SREBP-2). LDLR and NPC2 are involved in regulating cholesterol transport and SREBP-2 is the master regulator of both cholesterol uptake and biosynthesis. The subsequent accumulation of cholesterol was found to be concentrated in lysosomes indicative of impaired trafficking from lysosomes and other vesicles to plasma membrane (**Figure 3**). This accumulation of cholesterol at the lysosomes was accompanied by induction of necroptosis. They observed that necroptosis was the mode of cell death induced by L-Norephedrine based on the presence of pRIP3 and the sensitivity of cell death towards Nec-1s, an inhibitor of RIPK1. However, the role of pMLKL in L-Norephedrine-induce cell death remains unknown.[79] Another study found 24(S)-hydroxycholesterol to induce necroptosis. 24(S)-hydroxycholesterol treatment resulted in the formation of lipid droplets which consisted of esterified 24(S)-hydroxycholesterol (**Figure 3**). This esterification was mediated by Acyl-CoA:cholesterol acyltransferase 1 (ACAT1) and thought to contribute to initiating necroptosis.[80] Although these studies have provided important links between cholesterol and necroptosis, more studies are needed to understand the molecular mechanism of how cholesterol mediates necroptosis as an important component of cellular membranes and a signaling molecules that controls lipid homeostasis.

4.4. *Mitochondrial lipids in necroptosis.* Similar to mitochondrial involvement in apoptosis as described in section 4.2, necroptosis also involves mitochondrial function and related proteins. A mitochondrial protein phosphatase, PGAM5, has been identified to be involved in not only TNFα-induced necroptosis but also in necroptosis induced by reactive oxygen species or calcium overload. After binding to the necrosome and activation, PGAM5 contributes to mitochondrial fragmentation, leading to necroptosis.[81] Mitochondrial lipids also affect necroptosis. Cardiolipins are a class of phospholipids predominantly found in the inner membrane of the mitochondria. Any dysfunction in cardiolipin metabolism can adversely affect mitochondrial function and cause cell death.[82, 83] The N-terminal bundle of MLKL binds to cardiolipins *in vitro*, in liposomes that mimic the cardiolipin composition of the mitochondrial membranes, causing leakage.[16, 21] A similar interaction between MLKL and cardiolipins might potentially be at play in necroptotic cells and remains to be shown (**Figure 3**).

4.5. *The role of fatty acids in necroptosis.* Our group took an untargeted LC-MS-based approach to investigate lipid changes in colon cancer and lymphoblast cell line models of

necroptosis.[69] Untargeted lipidomics showed saturated very long chain fatty acids (fatty acids with acyl chains > 20 carbons[84]), (**Figure 3**) constituted the most profound accumulations in the cellular lipidome during necroptosis. Follow up studies suggested that the accumulation of very long chain fatty acids contribute to membrane permeability during necroptosis.[85] Interestingly, we recently showed that saturated very long chain fatty acids can be incorporated into proteins including MLKL and pMLKL mediated by zDHHC family of S-acyltransferases. We further showed that this acylation was functionally important as the acylation increases the membrane binding of these proteins, exacerbating necroptotic activity.[86] Overall, the results from our studies identified a new lipid-mediated mechanisms that contributes to membrane permeabilization during necroptosis.

4.6. *Other studies on lipid involvement in necroptosis.* Other lipid classes have also been linked to necroptotic activity. One study showed the effect of fenofibrate, a lipid reducing drug, to potentiate necroptosis by increased pMLKL and pRIPK3 levels on a hepatoma cell line, suggesting the anti-tumorigenic role of necroptosis.[87] Another study showed the effect of eicosanoids, a class of bioactive lipids, on necroptosis.[88] Lipidomics conducted on mice model of influenza and *Staphylococcus aureus* infection showed an accumulation of CYP450 eicosanoids which activate peroxisome proliferator-activated receptor-alpha (PPARα), a transcription factor that is involved in fatty acid metabolism and promotes transcription of proinflammatory genes, suggesting that an inflammatory type of cell death such as necroptosis could be observed during an infection **(Figure 3)**.[88]

5. Implications and opportunities for diagnostics and therapeutics targeting necroptosis

Different research groups have undertaken omics-based approaches to study how necroptosis affects the genome, transcriptome, proteome, metabolome as well as the lipidome in different diseases. These studies used *in vitro* samples, animal models, clinical samples of actual diseased conditions and various tools to analyze the omics data and provide valuable information in terms of different disease markers and prognosis, therapy approaches.

As previously described in section 2.4, necroptosis can exhibit pro- or anti-tumorigenic effects, depending on tissue and cell types and the expression levels of proteins involved in necroptotic signaling. Some studies suggest the role of necroptosis and necroptosis-related proteins to suppress tumor growth[25, 41] as well as activation of an immunogenic response in necroptosis due to RIPK1 and NF-κB signaling.[39] On the other hand, Liu et al. reported contradictory effects where proteins involved in necroptosis promoted tumor growth, possibly by

NF-κB-mediated pro-tumor growth cytokines. In fact, increased pMLKL levels were associated with poor survival in some cancers.[89] Therefore, discovery of necroptosis-related genes and potential biomarkers of necroptosis play an important role in shedding light on its involvement in different diseases, especially malignancies. A recent study conducted by Yang et al. analyzed the transcriptome and clinical data of hepatocellular carcinoma patients obtained from The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC), and carried out gene ontology and pathway analysis, multiple algorithms to identify prognostic molecules, risk prognosis models and survival analysis based on differential gene enrichment.[90] Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the transcriptome showed many differentially expressed genes enriched in necroptosis-related signaling pathways. These included TNF receptor-associated factor 2 (TRAF2), PGAM5, autophagy related 16 like 1 (ATG16L1), caspase recruitment domain family member 9 (CARD9), phosphate cytidylyltransferase 1A (PCYT1A), toll-like receptor 2 (TLR2) and poly(ADP-ribose) polymerase (PARP2). These genes were determined as potential prognosis markers in hepatocellular carcinoma. Other studies also suggested the involvement of these genes in necroptosis,[81, 91-96] strengthening the connection between necroptosis and hepatocellular carcinoma.

Pancreatic cancer is characterized by a rapid disease progression and high mortality. Combined with a late diagnosis and limited treatment options, it is imperative to establish certain prognosis markers for the disease for a better therapeutic outcome. A recent study analyzed the expression of necroptosis-related genes in pancreatic cancer.[97] This study screened different necroptosis-related genes in the transcriptome data of pancreatic cancer patients obtained from TCGA and proposed five necroptosis-related genes as markers for pancreatic cancer using regression analysis and that can be used to divide patients into a low risk and high-risk category. These genes are glutamate dehydrogenase 1 (GLUD1), spermatogenesis associated 1 (SPATA1), H2A clustered histone 8 (H2AC8), glycogen phosphorylase L (PYGL) and TNF superfamily member 10 (TNFSF10). GLUD1 and PYGL have been shown to be activated downstream of RIPK3 in necroptosis.[98] Other groups have also used similar transcriptomics and bioinformatic approaches to establish a necroptosis-related prognostic model in different types of cancers, such as cervical cancer[99], renal carcinoma[100], pancreatic adenocarcinoma[101] and other cancers where regulation of necroptosis-related genes affects tumorigenesis[102].

In addition to cancer, a transcriptomic and metabolic profiling study showed the involvement of necroptosis in chronic obstructive pulmonary disease (COPD).[103] This study investigated the role of melatonin in COPD as it has been found to have an ameliorative effect on

COPD-associated lung inflammation and decrease in the levels of inflammatory cytokines. They utilized mouse models to investigate the transcriptome and metabolome profiles using gas chromatography-mass spectrometry and pathway analysis. Melatonin treatment reduced expression of necroptosis-related genes such as TNF, allograft inflammatory factor 1 (AIF1) among others. Their findings show involvement of RIPK1/RIPK3/MLKL associated-necroptosis in the disease progression via amino acid metabolism and that melatonin can potentially prevent necroptosis evidenced by decreased levels of RIPK1/3 and MLKL protein levels in mouse lung lysates. Since increased RIPK3 expression has been reported in COPD patients, this study provides a potential mechanism of necroptosis inhibition to combat COPD with further in-depth investigations underway.[103]

6. Conclusions

Necroptosis is a highly dynamic cellular process that involves extensive remodeling of the transcript, protein and lipid landscape. The initial studies to understand the molecular mechanism of this process established the role of RIPKs and MLKL following TNFα induction and eventual progression of necroptosis. Recently, omics has helped improve our understanding of necroptosis on a global scale. Transcriptomics and proteomics have helped elucidate the regulatory and inflammatory aspect of necroptosis via gene regulation and involvement of proteins such as the NF-κB pathway, p38 MAPK family and TRIM28. The secreted proteome in necroptosis provides information about the membrane dynamics and the inflammation-associated signaling. Furthering the knowledge of necroptotic membrane dynamics, lipidomics have shown the involvement of several classes of lipids such as sterols, ceramides and fatty acids in necroptosis. Omics have also greatly benefited *in vivo* studies to identify prognostic markers for multiple diseases involving necroptosis. Encouraged by all these recent advances in our fundamental understanding of necroptotic activity and its implications in diseases, many new questions emerge: What are the fundamental mechanisms that govern the regulation of the transcriptome and proteome? What are some of the organelle-specific protein and membrane remodeling that contribute to the activated vesicular trafficking during this process? And, how can we take advantage of these regulations and alterations to modulate necroptotic activity for therapeutic interventions targeting cancers and inflammatory pathologies? We believe that the integration of these omics approaches to obtain a system-levels understanding followed by mechanistic investigations will pave the way for new discoveries to better understand and target necroptosis.

Conflict of Interest

Authors declare no conflict of interest.

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