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Necroptotic cell death consequences and disease relevance

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Arguably one of the most surprising revelations in the field of cell death research was the discovery that cellular necrosis, a lytic and inherently messy cell death with far-reaching consequences for human physiology, can be genetically encoded. There is no single necrotic pathway either, as compelling evidence exists for distinct necrotic modalities such as pyroptosis, necroptosis and ferroptosis. The recent momentum of molecular, structural and disease-relevant findings has opened the door to targeting necrotic machinery to prevent collateral tissue damage and inflammatory diseases. In this Review, we evaluate the case for targeting the necrotic cell death pathway called necroptosis. We examine the organs and cell types where the human necroptotic machinery is expressed, identifying a lymphocytic ZBP1, RIPK1, RIPK3 and MLKL signature, review knowledge into the immunogenic consequences of necroptotic signaling and highlight building evidence that necroptosis is engaged in humans and can be triggered by ischemic injuries. Finally, we note several limitations of mouse studies due to fundamental differences with the human necroptotic apparatus and critically appraise the evidence for necroptosis being a disease-driving factor that, if successfully targeted, could be of clinical benefit.

'Death is not the opposite of life, but a part of it' (Haruki Murakami)

Organs with self-renewing capacities exist in a homeostatic equilibrium of birth and death, with billions of their cells dying each day and billions being born. This process ensures the removal of aged, damaged, malignant and infected cells. In other words, without genetically encoded cell death, we would not be alive. Tissues that are in constant turnover include the skin, intestine and bone marrow, and these also happen to involve major cell types, such as barrier and immune cells, that are continuously being exposed to potential damage and are the primary responders to microbial and environmental cues. The constant regeneration of these tissues is largely enabled by apoptotic cell death. Here, the apoptotic cysteine-aspartate proteases, termed caspases, are activated by intrinsic or extrinsic signals. Caspase cleavage of diverse protein substrates leads to both protein activation and inactivation, orchestrating an orderly cellular demolition and clearance. This highly coordinated molecular process is the biochemical equivalent of a Tchaikovsky concerto; a dramatic masterpiece that is complex and requires exquisite timing. Indeed, without the finely tuned regulation of apoptotic caspase activity that shuts down necrotic pathways[1–4], a compromised cell undergoes membrane lysis, spilling immunogenic intracellular content that, collectively, can drive lethal immune responses[5].

A case in point that speaks to the importance of cell death is the loss of the death ligand FasL, the Fas receptor (TNFRSF6) or downstream apoptotic caspase-8, which causes autoimmune lymphoproliferative syndrome (ALPS) and an increased susceptibility to lymphoma[6]. This example also serves to illustrate how the evasion of programmed cell death represents a hallmark of cancer, and this malignancy has been the public poster child for spurring the development of targeted pro-cell death drugs, such as BH3 and Smac mimetics, for therapeutic benefit[7]. On the other hand, excess cell death can contribute to diverse conditions such as infections, tumor lysis syndrome, type 2 diabetes and neurodegenerative diseases (for example, Parkinson's disease and Alzheimer's disease). Several inflammatory disorders arising from

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in-born errors in genes required to inhibit programmed cell death highlight its ability to drive damaging inflammation and aptly demonstrate why the cell death machinery needs to be tightly kept in check[8].

Despite the tremendous progress in deciphering and targeting apoptotic signaling with rationally designed small molecules, it is cellular necrosis that has long been associated with tissue damage and potent inflammatory responses. The molecular and functional distinction between these cell death modalities is of fundamental importance, as the mechanism of programmed cell death triggered can dictate whether one lives or dies, with necrotic cell death capable of triggering cytokine storms and organ failure[5,9], whereas apoptosis signaling can harbor remarkable anti-inflammatory and, in relevant organs, tissue-regenerative capacities[10,11]. It is thus imperative to understand the drivers and signaling pathways that lead to necrotic cell death and consequent release of cytokines and damage-associated molecular patterns (DAMPs) that act as potent inflammatory mediators.

Cellular necrosis can represent a passive process caused by overwhelming chemical or physical stressors and a loss of survival factors. This is the case for the necrotic modality referred to as ferroptosis, which is not actively executed by dedicated death signaling machinery but occurs owing to a failure of antioxidant defense systems and iron-dependent accumulation of lipid peroxides. On the other hand, the necrotic cell death pathways of necroptosis and pyroptosis are actively triggered by host and pathogen molecules to engage distinct energy-dependent cell death signaling components and terminal effector proteins. In this review article, we focus on recent developments in necroptotic cell death. We examine evidence for its disease relevance in humans, including tissue and cell-type expression patterns of the core necroptotic machinery, and highlight the often-overlooked dearth of knowledge into which necroptotic factors are responsible for driving pathological inflammation. Readers are referred to excellent review articles on the alternate necrotic cell death programs gasdermin-driven pyroptosis[12] and iron-mediated ferroptosis[13], which also cause cell lysis and are implicated in distinct, but also sometimes the same, conditions.

Necroptotic triggering and signaling

The molecular unraveling of the programmed necrotic cell death pathway now known as necroptosis began in the late twentieth century, when the death ligands TNF and FasL were observed to induce cellular necrosis that could not be blocked by caspase inhibitors[14,15]. Subsequently, death receptor-induced necroptosis was shown to require receptor-interacting serine/threonine protein kinase-1 (RIPK1)[16], which is inhibited by necrostatin-1 (ref. 17), and RIPK3 (refs. 18–20). The target of RIPK3 kinase activity, and apparent terminal membrane-rupturing necroptotic effector, mixed lineage kinase domain-like pseudokinase (MLKL) was then discovered in 2012 (ref. 21), and MLKL-deficient mice and the structure of MLKL were reported in 2013 (ref. 22). Since then, an intensive focus of the field has been to define the involvement of necroptosis signaling in disease[5]. Because RIPK1 and RIPK3 have many important non-necroptotic signaling functions, including cytokine production, activation and apoptosis induction[23–29], the role of necroptosis is best determined through analyzing MLKL activity and the impact of *Mlkl* deletion.

Besides TNF superfamily death ligands (for example, TNF, FasL and TRAIL), other widely accepted necroptotic triggers include pathogen molecule-sensing Toll-like receptor 3 (TLR3) and TLR4 and Z-nucleic acid binding protein-1 (ZBP1; also known as DAI or DLM1; Fig. 1). Like TNF receptor 1 (TNFR1), these sensor proteins signal to RIPK1, resulting in its autophosphorylation and engagement of RIPK3 via conserved RIP homotypic interaction motifs (RHIMs). However, RIPK1 is not universally required for necroptosis as mouse, but not human[30], ZBP1 activates RIPK3 in the absence of RIPK1, which otherwise inhibits ZBP1 via heterotypic RHIM interactions[31,32]. In some contexts, TLR3 and TLR4 can also engage RIPK3 independent of RIPK1 via the RHIM-containing adaptor protein TRIF to cause necroptosis[28,33], and this may be dictated by target expression levels and hence availability[34].

The formation of TRIF and/or RIPK1 and RIPK3 complexes, often termed necrosomes, results in RIPK3 phosphorylation of MLKL protein within its pseudokinase domain[21]. This event triggers a conformational change in MLKL, formation of MLKL tetramers and, via a yet to be clearly defined Golgi–microtubule and actin trafficking pathway, redistribution to the plasma membrane[35–38]. Here, MLKL interacts with membranes via a patch of positively charged residues in its four-helical bundle (4HB) domain binding to negatively charged phosphatidylinositol phosphates[39,40]. Once a threshold of plasma membrane-localized MLKL accumulates, it causes membrane damage and cell lysis, resulting in the release of DAMPs into the extracellular milieu. Whether nonstochastic trafficking events also dictate MLKL targeting of intracellular organelles, which can promote cell death, free radical generation and extracellular vesicle release, has yet to be examined (reviewed in Wang et al.[41]) (Fig. 1). It is pertinent that unlike the necrotic pathways of pyroptosis and ferroptosis, efficient necroptotic-mediated plasma membrane rupture is not reliant on NINJ1 (ref. 42), and both the mouse and human 4HB domains of MLKL alone suffice to rapidly permeabilize liposomal membranes[38,39,43].

Although apoptosis is required for normal embryonic development, necroptosis is dispensable but may represent a mechanism by which defective embryogenesis can be aborted. For example, in mice, necroptotic signaling requires caspase-8 proteolytic activity to be disabled. In the absence of caspase-8, TNFR1, ZBP1 and the TLR adaptor TRIF contribute to RIPK3 triggering of MLKL to cause lethality during embryogenesis, and the targeted deletion of *Casp8* and resulting necroptotic pathway activation is fatal to many cell types[2,27,44,45]. The caspase-8 substrates RIPK1, RIPK3, CYLD and cFLIP have all been suggested to be important for caspase-8-mediated inhibition of necroptosis. However, caspase cleavage mutant RIPK3, cFLIP and CYLD mice are viable, showing that their processing by caspase-8 is not essential for preventing necroptosis signaling during development[46]. However, cells derived from caspase cleavage mutant cFLIP mice are more susceptible to necroptotic killing[47], as are caspase cleavage mutant RIPK1 cells, with RIPK1 cleavage mutant mice also exhibiting heightened caspase-8-driven apoptosis and embryonic lethality[46,48]. Although caspase cleavage mutant RIPK3 mice and cells do not display altered necroptotic signaling[49,50], macrophages isolated from these animals have exaggerated RIPK3-driven NLRP3 inflammasome activation of interleukin-1β (IL-1β)[49]. Similarly, in graft-versus-host disease, RIPK3-mediated transcriptional responses mean that its deletion confers greater disease protection than MLKL loss[51]. Collectively, these and other findings highlight how RIPK3 can signal inflammatory, yet necroptotic-independent, outcomes[23,28,52,53].

Expression patterns of the necroptotic machinery

Evidence demonstrates that the necroptotic machinery is tightly regulated by epigenetic mechanisms and cytokine networks, particularly type I and type II interferons (IFNα/IFNβ and IFNγ, respectively), which dynamically modulate its activation threshold[54,55]. For example, in mice, IFNγ induces the expression of two MLKL isoforms, one of which can bind MLKL and limit its interaction with RIPK3 via an eight-amino-acid insertion in the pseudokinase domain to reduce necroptotic sensitivity[56]. Similarly, cytosolic DNA–cGAS–STING signaling can drive ZBP1 and MLKL expression to enable necroptosis in caspase-8-deficient epidermis[57]. Understanding the tissue and cell-type expression of necroptotic components is therefore key for understanding how and where necroptotic signaling is regulated and able to be activated.

A recent study described low to undetectable expression of necroptosis pathway members such as ZBP1, RIPK1, RIPK3 and MLKL in many tissues at steady state[58]. Their expression in mice was largely restricted to fast cycling barrier tissue such as intestinal epithelial cells and immune cells, which are the cells first exposed to pathogens

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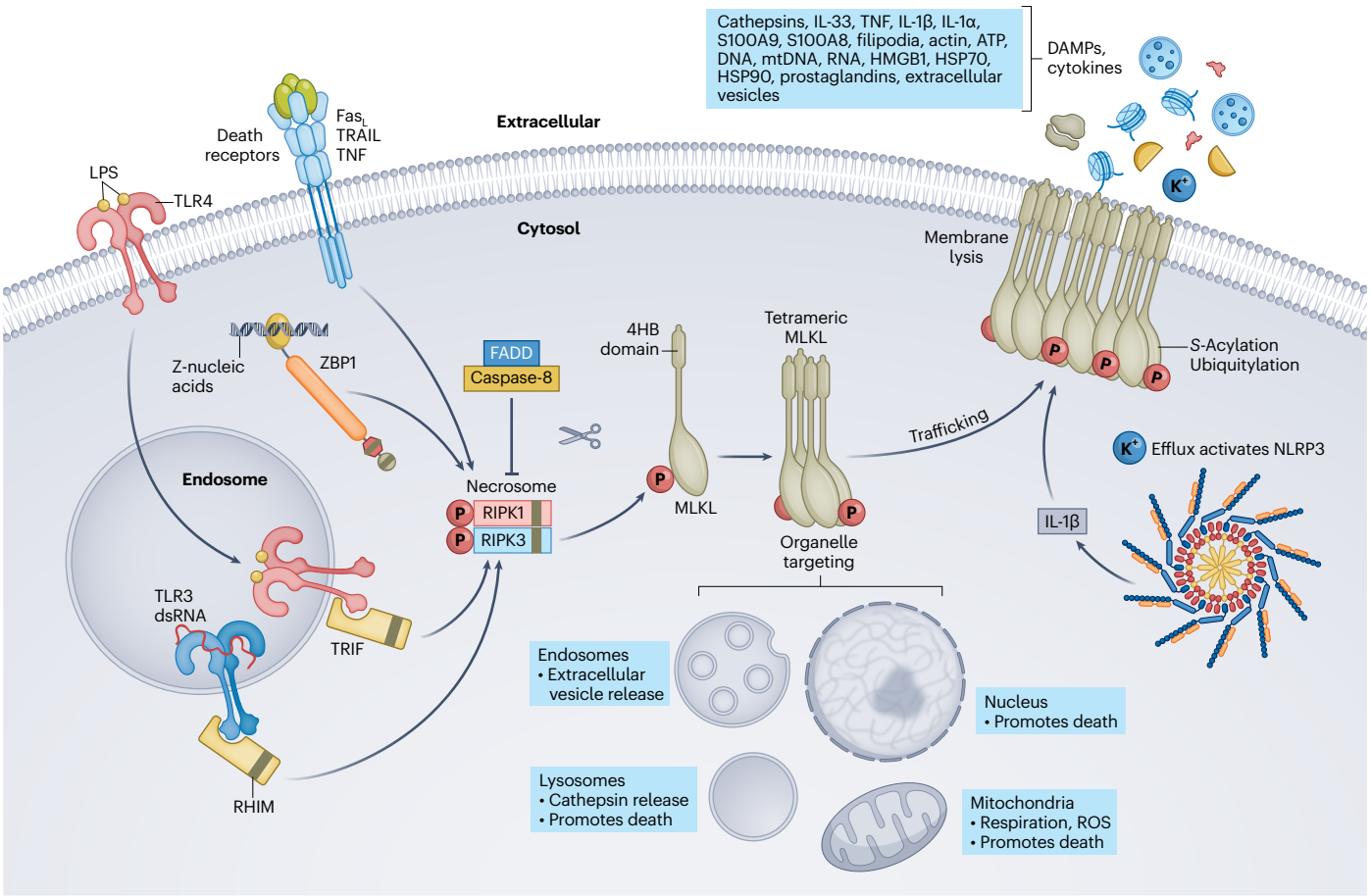

Fig. 1 | Overview of necroptosis triggers and signaling pathway. A RIPK1- and RIPK3-containing necrosome is formed downstream of death receptor, TLR and ZBP1 signaling and requires reduced caspase-8 activity. RIPK3 phosphorylation of MLKL induces a conformational change, resulting in MLKL tetramer formation and trafficking to the plasma membrane. Here, positively charged residues within the 4HB domain of MLKL enable it to embed into the membrane, which is also facilitated via PTMs, such as ubiquitylation and *S*-acylation. Once a threshold of MLKL oligomers is reached, the plasma membrane is lysed, and DAMPs are released into the extracellular milieu. In myeloid cells undergoing necroptosis, MLKL-driven potassium ion efflux activates the NLRP3 inflammasome to generate bioactive IL-1β, which escapes via MLKL-damaged membranes and does not require pyroptotic pore-forming gasdermins. MLKL targeting of intracellular organelles has also been suggested to promote cell death, with lysosomal and nuclear targeting preceding the detection of MLKL at the plasma membrane; dsRNA, double-stranded RNA; LPS, lipopolysaccharide; mtDNA, mitochondrial DNA; ROS, reactive oxygen species.

and impacted by inflammation. However, human tissue necroptotic machinery expression patterns may differ from other animals. Therefore, using one of the latest label-free proteomic datasets, we analyzed levels of the four main necroptotic components at steady state across various human tissues and compared protein levels to mRNA[59]. This dataset revealed that necroptotic protein (Fig. 2a) and RNA (Fig. 2b) expression correlated well, with broader coverage observed at the protein level. Unlike other necroptotic players, RIPK1 was detected in all tissues and displayed the highest abundance in most, consistent with important additional roles in regulating death and pattern recognition receptor responses beyond necroptosis, including cell survival, gene transcription and apoptosis. ZBP1, which is IFN inducible, displayed the lowest expression across most tissues, whereas all necroptotic pathway members were best represented in the gastrointestinal tract, tonsil and lung. Unexpectedly, RIPK3 was absent in the liver, despite reports describing necroptosis in this organ[5]. This highlights a key limitation of bulk tissue analysis, as expression in less common cell populations, such as lymphocytes or Kupffer cells in the liver, may be missed. To address this issue, we examined human necroptotic transcript abundance at single-cell resolution using CZ CELLxGENE Discover[60] (Fig. 2c). This analysis further confirmed *RIPK1* as the most widely expressed necroptotic gene and identified *MLKL* as the most heterogeneously expressed at steady state. Myeloid cells, endothelial cells and lymphocytes were the main cell types within most organs containing *RIPK1*, *RIPK3* and *MLKL* transcripts. While comparative expression of IFN-inducible ZBP1 was low, lymphocytes were the exception as these cells in the liver, lung, kidney, small intestine and spleen all showed robust ZBP1 expression, in addition to RIPK1, RIPK3 and MLKL (Fig. 2c). Notably, this necroptotic signature and high steady-state ZBP1 levels were displayed in several lymphocyte subsets, including natural killer cells, innate lymphoid cells, plasma cells and a variety of T cells (Fig. 2d). Although early work, mainly via caspase-8 deletion or inhibition, has shown that necroptosis can occur in mouse lymphocytes such as T cells, most subsequent studies shifted focus toward how dying cells present antigens and stimulate T cells[61]. Our findings provide a strong rationale to investigate how necroptosis signaling might be engaged in relevant human lymphocyte subsets without the artificial manipulation of caspase activity.

Increased expression of MLKL has been reported in conditions such as inflammatory bowel disease (IBD)[58,62,63], Alzheimer's disease[64], idiopathic pulmonary fibrosis[65], chronic obstructive pulmonary disease[66] and graft-versus-host disease[67], indicating a potential role for necroptosis in diverse pathologies. The mechanisms driving the disease-associated upregulation of MLKL requires further study, although ZBP1, RIPK1, RIPK3 and MLKL protein levels can be modulated via epigenetic, transcriptional and post-translational

mechanisms. For example, *RIPK3* and *ZBP1* are frequently silenced by promoter methylation in cancer to prevent necroptosis, and *RIPK3* is also silenced in hepatocytes[68,69]. By contrast, the epigenetic silencing of *RIPK1* and *MLKL* remains unclear and requires further investigation. Transcriptional regulation of necroptotic pathway members has been reported in response to IFNs in healthy and pathogenic conditions such as IBD[54,55,58,62], whereas RIPK1 and RIPK3 are induced by *Escherichia coli* and *Salmonella typhimurium*, and MLKL is upregulated in response to *Mycobacterium tuberculosis* infection[70].

Although increased expression of necroptotic components indicates a potential role in the relevant tissue cells, it does not indicate necroptosis pathway activation. As such, to study the triggering of necroptosis, a major focus has been identifying the activating post-translational modifications (PTMs) of its main players, including via phosphorylation, ubiquitylation, ribosylation (most likely poly(ADP)-ribosylation (PARylation)), acetylation, GlcNAcylation, proteolytic cleavage, acylation, acetylhypusination and disulfide bonding[71]. The most critical PTMs that activate necroptosis are phosphorylation in the activation loop of human RIPK1 Ser166 (ref. 72), human RIPK3 Thr224/Ser227 (refs. 21,73) and human MLKL Thr357/Ser358 (refs. 21,40). A range of PTMs on different sites of these proteins have been identified, and many of them tune their activation or inhibition, such as activating ubiquitylation at Lys219 of MLKL[74] and MLKL *S*-acylation via the zDHHC21 palmitoyl transferase, which enables efficient membrane insertion[75]. Nine further ubiquitylation sites and 11 phosphorylation sites on MLKL have been reported and many more for RIPK1 and RIPK3 (refs. 71,74,76,77). Functions of ribosylation and likely also PARylation of RIPK3, acetylation of RIPK1 and other PTMs remain to be defined[78] but underscore the importance of fine-tuning necroptosis. Given its crucial role in pathogen recognition, and the ability of pathogens to exploit different PTMs to manipulate the necroptotic pathway for their benefit, it may be advantageous for the host to have additional PTMs in place to rapidly counteract pathogen manipulation. Importantly, most enzymes responsible for adding and removing PTMs have not been identified, and the way diverse PTMs interplay with each other to regulate necroptotic component activation, oligomerization and translocation still needs addressing.

Consequences of necroptotic cell death

Although there is a wealth of research on the regulation of RIPK1, RIPK3 and MLKL, fewer studies have focused on the physiological consequences of necroptotic versus apoptotic or pyroptotic cell death, particularly in terms of their overlapping and unique impacts. This knowledge gap limits confident attribution of cell-death-mode-specific cargo release and downstream effects. Consequently, rigorous head-to-head studies directly comparing necroptotic killing to other cell death modalities in the same cell types will be essential for properly defining specific and generalizable cell death-induced immunity. However, as discussed below, emerging evidence points toward both similarities and differences into the factors released during different modes of cell death that can impact inflammatory responses.

One might predict a difference between the molecules released by necroptotic plasma membrane rupture and those released during apoptotic plasma membrane blebbing. Indeed, a head-to-head systems-level analysis comparing the proteins released by necroptosis in a monocytic cell line to those released by apoptosis over time revealed distinct processes activated at later stages of cell death[79]. In this in vitro setting, apoptotic cells released nucleosome components and cytokines in response to TNF-induced apoptotic cell death, whereas necroptotic cells primarily released lysosomal components and fewer cytokines. A separate study likewise detected lysosomal components in the supernatant of pyroptotic cells, most likely reflecting lysosomal exocytosis triggered by plasma membrane permeabilization[80]. The reduced cytokine release under necroptotic conditions may occur due to the faster kinetics of TNF-induced necroptosis observed in vitro, while the increased production of cytokines by apoptotic cells cannot be directly extrapolated to in vivo conditions, where dying cells are rapidly cleared. The engineering of inducible apoptotic, pyroptotic and necroptotic systems in vivo would help define the overlapping and distinct physiological consequences to these different types of cell death.

Apoptotic cells have been long known to release a range of signals, such as ATP, sphingosine-1-phosphate, C-X-C motif chemokines and other metabolites, that primarily attract phagocytic cells for clearance[81]. Although these factors are typically described in the context of apoptosis, lytic cells can release them indiscriminately[82]. Conversely, immunogenic molecules commonly associated with necroptosis (Fig. 1), such as heat shock protein 70, high mobility group box 1, IL-1α, uric acid and mitochondrial content, can be released by both pyroptotic and apoptotic cells[83–86], the latter of which can undergo secondary necrosis if not efficiently removed.

Recently, cell-intrinsic MLKL-driven triggering of the NLRP3 inflammasome and consequent IL-1β activation and release has emerged as a necroptotic disease determinant[87,88] (Fig. 1). This MLKL–NLRP3 signaling axis has been reported to contribute to arthritis in mice lacking the deubiquitylating enzyme A20 (ref. 89) and limit acute myeloid leukemia progression[90] and has also been linked to infectious diseases[91–94]. Findings have pointed toward IL-33 and TNF as being two additional important necroptotic cell death-associated inflammatory factors in vivo[95–97]. Both pyroptosis and necroptosis have also independently been associated with the production and release of molecules involved in tissue regeneration and wound healing[98–100], while necroptotic and pyroptotic, but not apoptotic, cell corpses are adorned with actin-containing filopodia[101]. These filopodia were shown to stimulate Clec9A on surrounding dendritic cells, leading to enhanced antigen presentation. Together, these studies underscore several overlapping consequences of necroptotic and pyroptotic killing.

This convergence raises a critical question: how functionally distinct are the extracellular environments or supernatants produced by these lytic cell death modalities in vivo? Although substantial overlap is expected due to shared similarities in plasma membrane rupture, pyroptosis uniquely favors robust IL-1β and IL-18 activation and signaling, as these potent inflammatory cytokines are predominantly

Fig. 2 | Protein and transcript levels of necroptotic players in human tissue. **a**, Wang et al.[59] analyzed the proteome of 29 healthy human tissues using a label-free approach in data-dependent acquisition mode; $\log_2$-transformed intensities of ZBP1, RIPK1, RIPK3 and MLKL are displayed in a heat map with high expression levels shown in yellow and low expression levels shown in dark blue. Blank entries indicate no protein detected. **b**, The corresponding transcript levels across the same 29 tissues were measured using RNA sequencing. High transcript expression of *ZBP1*, *RIPK1*, *RIPK3* and *MLKL* is also shown in yellow, low expression is shown in dark blue, and blank entries indicate no transcript detected. Protein and RNA expression levels showed good correlation, with RIPK1 being the highest expressed necroptotic member throughout the body. **c**, The expression of *ZBP1*, *RIPK1*, *RIPK3* and *MLKL* in select human tissue from CZ CELLxGENE[60]. Dot size indicates the percentage of cells with evidence of gene expression, and color indicates the mean expression after normalization (natural log of counts per 10,000 + 1). Cell types were subset to the 20 most common within each tissue, using intermediate-level cell-type annotations only (excluding overly broad and overly specific categories). Blank entries indicate that data were not available on CELLxGENE. **d**, As lymphocytes expressed ZBP1 in a higher proportion of cells than other cell types, gene expression within lymphocytic populations using CELLxGENE was examined further. For each tissue, only the 20 most common cell types are shown. Because cell-type annotations are hierarchical, individual cells may be represented in more than one cell type within the figure. NK cell, natural killer cell.

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activated by the pyroptotic caspases. The physiological outcome will also depend on context, including the type and number of dead cells, the concentration of released molecules, the makeup of cell corpses and the antigens present. Therefore, although studies have identified several important factors released during necroptotic cell death, research has yet to establish a necroptosis-specific extracellular signature. Addressing this gap is a compelling challenge, particularly because capturing cells in the act of dying in vivo remains notoriously difficult owing to the transient nature of cell death, which often occurs in a spatially and temporally restricted manner. Defining such a signature, if it exists, would represent a major advance, enabling the identification of human diseases driven by overactive necroptosis signaling.

Clearance of necroptotic cells

There is growing interest in how the origin and type of dead cells influence the reprogramming of engulfing cells, particularly macrophages. Studies have shown that macrophages engulf necroptotic cells, although engulfment efficiencies can vary. Some reports indicate enhanced engulfment of necroptotic cells compared to apoptotic cells, while others suggest the opposite[102,103]. So far, very few studies have directly compared the impact of different cell identities and types on the programming of engulfing cells. Early research found that macrophages engulfing necrotic neutrophils displayed greater proliferation and higher TNF and antigen presentation receptor expression than when engulfing apoptotic neutrophils[104]. Whether this difference reflects altered efferocytosis efficiency has not been examined. Liebold et al.[105] reported that macrophage engulfment of apoptotic neutrophils, in combination with IL-4, triggers a robust wound healing response that facilitates the resolution of *Schistosoma mansoni* infections. Notably, this effect was specific to apoptotic neutrophils, as similar responses were not observed with apoptotic thymocytes or hepatocytes. Additionally, the upregulation of tissue remodeling-associated genes was absent when necrotic neutrophils were engulfed. Whether this phenomenon is driven by the activation of different phosphatidylserine receptors engaged upon the sensing of different cell types or cells undergoing different cell death modalities, the presence of differentially processed cargo within the dead cells or proteins released by necrotic cells that prime macrophages before engulfment remains to be determined. A comprehensive analysis comparing the effects of engulfing apoptotic versus necrotic cells on macrophage programming could help clarify why necrotic neutrophil engulfment fails to trigger a tissue remodeling signature. This has notable implications for mesenchymal stem cell (MSC) therapy, used in conditions such as IBD. Current studies suggest that the clearance of apoptotic MSCs supports wound healing and anti-inflammatory functions[10,105]. However, whether the clearance of necroptotic MSCs can achieve similar outcomes needs to be defined. A recent study highlighted that Gas6 in extracellular vesicles released by dying MSCs has a critical role in wound healing[106]. Given that necroptotic cells are also known to release extracellular vesicles, it will be interesting to examine whether these vesicles contain Gas6. Further in vivo studies that induce specific cell death pathways across different disease models and therapeutic contexts are needed to elucidate the role of necroptosis in disease progression and tissue regeneration.

Evidence for necroptotic signaling in disease states

Evidence suggests that the necroptotic cell death pathway evolved to counteract pathogens that inhibit apoptosis to avoid immune detection and enable intracellular replication[107]. This evolutionary arms race, and the selection for RIPK3 and MLKL in primates, has led to pathogens targeting components of the necroptotic pathway, including MLKL itself[108–110]. In some cases, pathogens copy or co-opt necroptosis with detrimental consequences. For example, a nonstructural protein of the severe fever with thrombocytopenia syndrome virus can engage necroptosis to drive lethal pathology[111], while the norovirus NS3 protein mimics the MLKL 4HB killer domain to induce the death of infected cells and facilitate viral egress[112]. However, because necroptosis invariably requires apoptotic failure, it might be viewed as being of secondary importance compared to the primary pathogen-driven apoptotic and pyroptotic cell death pathways. Indeed, mice deficient in necroptotic cell death generally show limited phenotypes compared to those lacking the pyroptotic or apoptotic machinery, although the deletion of *MLKL* has been documented to confer protection against, or increase susceptibility to, a range of both inflammatory and infectious diseases (Fig. 3).

Necroptotic pathway dysregulation in humans

In humans, the upregulation of Ser358-phosphorylated MLKL, which marks its activation, has been observed in various conditions such as cutaneous graft-versus-host disease[67], chronic obstructive pulmonary disease[66], idiopathic pulmonary fibrosis[65], liver fibrosis[113], psoriasis[114], IBD[58,62,63,115] and metabolic dysfunction-associated steatohepatitis[116]. Although this alone does not indicate whether necroptosis signaling in these scenarios acts in a damaging capacity, provides disease protection or is simply a bystander event, it suggests that the inhibition of necroptosis by caspase-8 is overcome in several important disease states.

Individuals with mutations in key members of cell death signaling, directly linked to the disease, provide stronger evidence for the involvement of these pathways. Several autoactivating mutations in inflammasome sensor proteins that cause pyroptosis have been identified[12]. However, as these conditions often respond well to anti-IL-1 therapies, in this context, the pyroptotic gasdermin pores may simply act to facilitate IL-1 release, with cell lysis playing a secondary role. There is little evidence of similar activating mutations that drive aberrant necroptosis signaling. For example, the MLKL-activating polymorphism Ser-132Pro in humans does not cause a clearly distinct phenotype despite increased necroptotic function when expressed in human cells[117]. Similarly, the regulation of necroptosis and apoptosis by caspase-8 in mice is not conserved in humans. First, unlike the embryonic lethality of caspase cleavage mutant RIPK1 mice, RIPK1 caspase cleavage mutant humans develop normally but suffer from an autoinflammatory condition termed cleavage-resistant RIPK1-induced autoinflammatory (CRIA) syndrome[48,118]. CRIA syndrome increases cellular sensitivity to apoptosis and necroptosis signaling, but exactly why RIPK1 kinase activity increases IL-6 in these individuals, who clinically respond to IL-6 inhibition, and if this IL-6-driven pathology is related to apoptotic and/or necroptotic cell death, remains unclear[48,118]. Second, unlike mice lacking caspase-8, human caspase-8 mutations resulting in a near complete loss of caspase-8 protein also do not impact development[119], suggesting redundancy with caspase-10, a human-specific paralog, in suppressing necroptotic pathway activation[55]. Instead, individuals with mutant caspase-8 develop immunodeficiencies and very early onset IBD[119,120], but whether these manifestations result from the triggering of necroptosis requires further investigation. However, the fact that increased levels of intestinal necroptotic markers, including phosphorylated MLKL, have been detected in individuals with IBD across several different studies[58,62,63,115] is indicative of consistent necroptotic pathway upregulation and signaling in this condition.

The diversity of RIPK1 and RIPK3 functions makes it difficult to ascertain if loss-of-function variants in RIPK1 (IBD, infections, immunodeficiency, arthritis and developmental delay)[121–123] and RIPK3 (herpes simplex encephalitis)[124] is primarily driven by resistance to necroptotic cell death or through defective RIPK1- or RIPK3-mediated gene transcription and apoptosis. However, MLKL does not appear to moonlight in a diverse number of other signaling scenarios, and MLKL loss-of-function variants have been linked to neurodegenerative disease[125,126] and maturity-onset diabetes[127], although these disease associations require further experimental validation. Nevertheless, the former phenotype could result from prolonged survival

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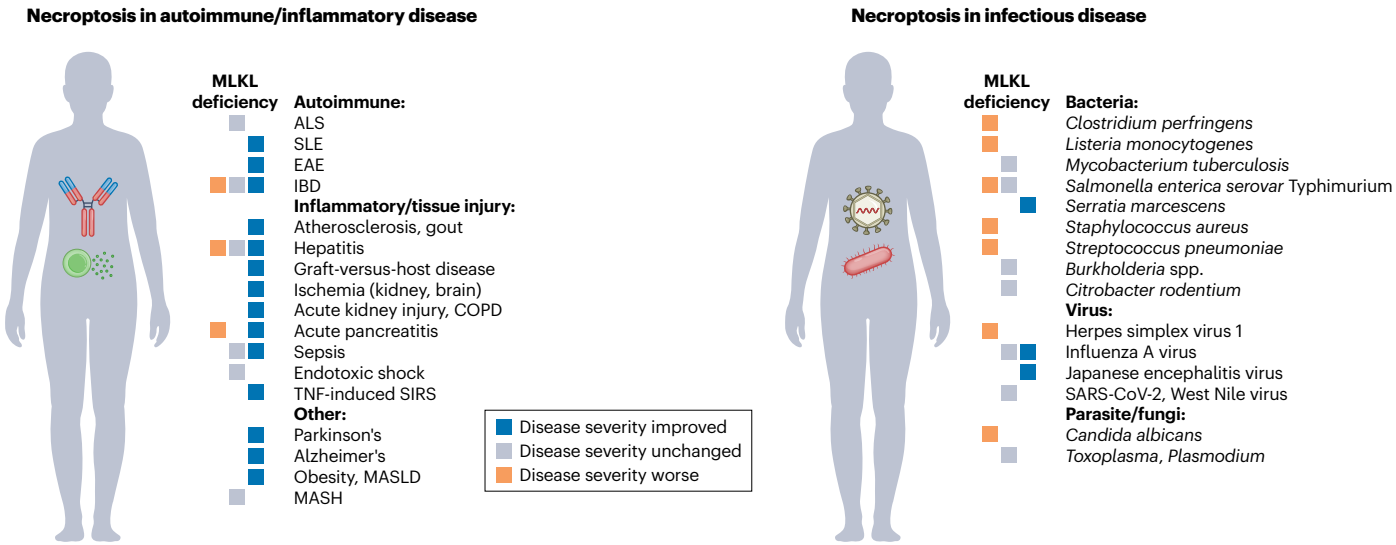

Fig. 3 | The role of MLKL in autoimmune, inflammatory and infectious diseases. Severity of autoimmune, inflammatory and infectious diseases assessed in wild-type and MLKL-deficient mice. Blue squares indicate improved disease outcomes in the absence of MLKL, gray squares show no change, and red squares reflect worsened disease severity. ALS, amyotrophic lateral sclerosis; COPD, chronic obstructive pulmonary disease; EAE, experimental autoimmune encephalomyelitis; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatoic liver disease; SIRS, systemic inflammatory response syndrome; SLE, systemic lupus erythematosus.

of disease-driving cells or one of the rare reported non-necroptotic roles for MLKL in nerve regeneration[128]. An increased frequency of *MLKL* polymorphisms linked to a gain-of-function phenotype has been implicated in chronic recurrent multifocal osteomyelitis, with relevant MLKL-activating mutant mice displaying perinatal lethal inflammation[129]. Other conditions, such as progeroid syndrome (premature aging) resulting from loss-of-function mutations in *ZMPSTE24*, may also cause excess activation of MLKL, with the increased inflammation, tissue damage and survival of ZMPSTE24-deficient mice largely prevented by MLKL co-deletion[130]. Interestingly, in line with the idea that MLKL has a role in aging, MLKL disruption alone confers some protection from sterile age-related increases in inflammation[131]. Similarly, intestinal deletion of the histone methyltransferase *Setdb1* in mice allows for endogenous retroviral activation of ZBP1 to drive apoptosis and, at the same time, MLKL activation, contributing to terminal ileitis and colitis that is partly rescued by *Mlkl* deletion[115]. Although it was also reported that intestinal SETDB1 expression was decreased in the mucosa of individuals with IBD, this finding has been questioned, although rare *SETDB1* missense variants predicted to impact its function have been identified[132].

Ischemia-driven necroptosis activation

Additional animal models to establish a role for necroptosis in disease pathogenesis have been examined, with a consistent role for necroptosis driving pathology in ischemia-driven injuries. For example, the loss of MLKL protects from kidney[133,134], hepatic[135,136] and gut ischemia-reperfusion injury[137] and also ischemic stroke[138]. Studies have also reported that pharmacological targeting of RIPK1 or RIPK3 can limit ischemia-induced damage[139–142].

Recently, ischemia was shown to drive endothelial cell necroptosis, leading to complement activation causing red blood cell lysis and microvascular obstruction, which was reduced following endothelial cell *Mlkl* deletion[137]. Endothelial necroptotic markers associated with red blood cell hemolysis were also observed in ischemic tissue from individuals with COVID-19, as well as in human tissues following acute myocardial infarction or ischemia-reperfusion injury[137]. Interestingly, the NLRP3 inflammasome drives complement-induced red blood cell lysis[143], highlighting how cell death signals can potentially spread from one cell type to another and involve distinct cell death modalities.

Whether there is a common trigger of necroptotic signaling following ischemic insult remains to be determined, although hypoxia and reactive oxygen species generation can result from tissue ischemia and contribute to necroptotic pathway activity[20,144,145]. However, despite the building evidence that ischemia is a physiological necroptotic trigger, the protection afforded from the loss of MLKL is often partial, with evidence indicating that in some cases multiple cell death pathways can be engaged under ischemic conditions in addition to necroptosis, such as ferroptosis[146], apoptosis[134], pyroptosis[147] and mitochondrial permeability transition pore-induced necrosis[148]. Regardless, endothelial cells appear particularly prone to MLKL-dependent killing. Endothelial *Casp8* deletion drives fatal intestinal necroptotic killing in response to microbial sensing[149,150], whereas in wild-type animals, TNF-driven systemic inflammatory response syndrome also causes lethality via the activation of endothelial cell necroptosis[151]. Cerebral endothelial cell necroptosis has also been implicated in blood–brain barrier damage and Alzheimer's disease pathology[152]. It will be of interest to explore the contribution of endothelial cell necroptosis to other MLKL-associated conditions.

Mouse model limitations

Despite sequence, structural, PTM and signaling differences between the human and mouse necroptotic machinery (Table 1), the fundamentals of necroptotic signaling (RIPK1 to RIPK3 to MLKL to death) are conserved. An exception, however, is the recent discovery that human ZBP1 requires RIPK1 to execute necroptosis[30], whereas mouse RIPK1 inhibits ZBP1 binding to RIPK3 and hence blocks necroptosis[31,32]. This poses issues for the testing of RIPK1 inhibitors in mouse disease models where ZBP1-driven necroptosis may have a role, such as ADAR1-associated autoinflammatory disease and IBD. Mouse studies are also often limited by the inherent constraints of the models, which do not accurately replicate the multifactorial traits of human diseases and are sometimes difficult to reproduce across different laboratories. For example, despite necroptotic death of endothelial cells and red blood cell hemolysis being detected in human tissue from individuals with COVID-19, in mouse COVID-19 models, red blood cell hemolysis in relevant organs is mild compared to humans[137], and *Mlkl* deletion has no impact on disease progression[153]. Different laboratories have also reported that MLKL loss either protects from severe influenza infection

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Table 1 | Human and mouse differences in necroptosis

Necroptotic component	Human	Mouse	References
Caspase-8 deficiency	Caspase-8 loss results in immunodeficiency similar to ALPS. Includes impaired immune cell activation and recurrent infections and/or life-threatening end organ lymphocytic infiltration. Can also trigger IBD.	Unlike humans, *Casp8* deletion causes RIPK3- and MLKL-driven embryonic lethality.	2,27,44,119,120,168
RIPK1 deficiency	Loss of RIPK1 can result in immunodeficiency, arthritis or IBD and may also cause intellectual disability.	Unlike humans, *Ripk1* deletion results in lethal caspase-8, ZBP1, RIPK3 and MLKL signaling.	97,121–123,169,170
RIPK1 caspase cleavage mutant	Predisposes cells to necroptosis and apoptosis. Causes autoinflammatory disease (CRIA syndrome), with individuals responding to IL-6 inhibition.	Also sensitizes cells to necroptosis and apoptosis, but, unlike RIPK1 cleavage mutant humans, caspase cleavage RIPK1 mutant mice are embryonically lethal.	46,48,118
Caspase-10	Functional in humans and may combine with caspase-8 to limit necroptosis. Inactivating mutations linked to immune dysregulation/ALPS, although requires further research.	Absent in mice.	55,171
MLKL activation loop	RIPK3 target site Thr357/Ser358 phosphomimetic mutants fail to activate MLKL and cause human cell death.	In mouse cells, RIPK3 target site Ser345 phosphomimetic mutation triggers MLKL-dependent killing in the absence of upstream signals.	21,22,172,173
MLKL pseudokinase domain	Stable presignaling binding to RIPK3. Adopts a closed active kinase-like fold with intact R-spine: ATP binding stabilizes MLKL in a closed, autoinhibited conformation.	Transient signaling-induced RIPK3 interactions. Displays an open pseudokinase fold lacking the regulatory spine; oligomerization more readily triggered by activation loop modifications.	21,22,174–176
RIPK3 and MLKL cross-species complementation	Human MLKL cannot complement mouse cells. Human RIPK3 fails to activate mouse MLKL due to divergence in activation loop and interface sequences.	Mouse RIPK3 and MLKL cannot complement human counterparts.	177,178
ZBP1 regulation by RIPK1	ZBP1-induced necroptosis requires RIPK1 to stabilize ZBP1–RIPK3 amyloid-like complexes.	RIPK1 inhibits ZBP1. When activated, ZBP1 binds directly to RIPK3 via RHIM–RHIM interactions and activates necroptosis independent of RIPK1.	30–32
ZBP1 isoforms	Complex splicing with at least seven predicted isoforms.	Two main isoforms, ZBP1 full-length and short, the latter lacking RHIMs and acting as an endogenous ZBP1 full-length inhibitor.	179; see also UniProt Q9H171

or has no impact[154]. Similarly, despite MLKL mutations being associated with neurodegeneration[125,126] and inactive mutant MLKL-G316D being linked to one diabetic family[127], MLKL loss in mice does not cause obvious cognitive defects or diabetes. By contrast, MLKL loss conferred protection in a mouse model of Alzheimer's disease[155] and improved metabolic dysfunction-associated fatty liver disease, including protection from high-fat-diet-induced weight gain and insulin resistance[156]. MLKL deficiency has also been reported to reduce carbon tetrachloride- and bile duct ligation-induced liver injury and fibrosis[113], or MLKL activation contribute to liver cancer[157], findings that, when taken together, suggest a broader role of necroptosis in diverse liver pathologies. These selected examples collectively highlight the need to validate mouse findings in humans using carefully curated markers of necroptotic pathway activation before proceeding to costly clinical trials.

Targeting necroptosis and future challenges

The key intracellular proteins in the necroptosis pathway (ZBP1, RIPK1, RIPK3 and MLKL) each represent distinct pharmacological targets with unique challenges and opportunities for therapeutic intervention. These targets all harbor non-necroptotic functions that must be carefully evaluated for their impact in relevant conditions, as well as the long-term consequences that their disruption might have on altered immunity and susceptibility to infection. In some cases, the inhibition of necroptotic-independent functions, such as induction of apoptosis and gene transcription, may provide added benefit in inflammatory diseases compared to the removal of downstream MLKL[134].

So far, several potent allosteric RIPK1 inhibitors have progressed to phase 1 and 2 clinical trials for conditions such as ulcerative colitis, cutaneous lupus erythematosus, neurodegenerative conditions and psoriasis (reviewed in Yao et al.[158]). Although often well-tolerated and safe, some trials have been discontinued owing to a lack of efficacy, such as in ulcerative colitis[159] and rheumatoid arthritis[160], or long-term nonclinical toxicology[161]. The results of other trials, including for conditions such as acute graft-versus-host disease and cardiac surgery-associated kidney damage, have yet to be published. Targeting RIPK3 is problematic as the inhibition of its kinase activity can trigger on-target RIPK1-mediated apoptosis. However, a new class of RIPK3 inhibitors have recently been developed that maintain RIPK3 in an inactive conformation and do not trigger apoptotic toxicity; although human-based studies have yet to be conducted[162]. Several MLKL inhibitors have been generated that covalently bind Cys86 of MLKL[163], the first developed and most widely used being necrosulfonamide[21]. These cysteine-reactive compounds may display off-target effects, as demonstrated for necrosulfonamide[164], making them unlikely candidates for progressing past preclinical studies. Lipid nanoparticle delivery of intrabody mRNAs that inhibit MLKL represent a small-molecule alternative therapeutic modality[165], while *MLKL* mRNA or RIPK1 PROTAC treatment to drive immunogenic cell death are also being explored as pronecroptotic anticancer strategies[166,167].

As new and better necroptotic inhibitors progress through preclinical studies, their deployment in relevant clinical trials will require a better understanding of necroptotic pathway activation in the human setting via the use of three-dimensional organoid systems, development of accurate necroptotic molecular signatures and analysis of clinical tissue. Nevertheless, emerging evidence in human-based studies points to damaging MLKL activation in physiological scenarios

such as IBD and ischemic conditions. In such contexts, where MLKL is not genetically triggered by *FADD* or *CASP8* deletion, determining how necroptotic signaling overcomes its inhibition by caspase-8 or a cell's propensity for survival may point to additional scenarios that necroptosis is activated in. The plasticity of cell death signaling could present another challenge for necroptosis inhibitors, as genetic studies in cell lines and mice indicate that optimal disease inhibition often requires targeting multiple cell death pathways. Thus, although accumulating evidence shows that targeting necroptosis holds significant therapeutic promise, careful consideration of disease context, cell death signaling cross-talk, drug specificity and immunomodulatory consequences will be essential for successful clinical translation.

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Author contributions

J.E.V. and M.C.T. conceived and wrote specific sections of the original manuscript draft. N.M.D. performed the human CELLxGENE analysis of necroptotic pathway expression and contributed to the writing of the relevant text. All authors reviewed and edited the final manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors have no competing interests to declare.

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