Review

NECROPTOSIS IN INTESTINAL INFLAMMATION AND CANCER: NEW CONCEPTS AND THERAPEUTIC PERSPECTIVES

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Abstract: Necroptosis is a caspases-independent form of programmed cell death exhibiting intermediate features between necrosis and apoptosis. Albeit some physiological roles during embryonic development, tissue homeostasis and innate immune response are documented, necroptosis is mainly considered a pro-inflammatory cell death. Key actors of necroptosis are the receptor-interacting-protein-kinases, RIPK1 and RIPK3, and their target, the mixed-lineage-kinase-domain-like protein, MLKL. The intestinal epithelium has one of the highest rates of cellular turnover in a process that is tightly regulated. Altered necroptosis at the intestinal epithelium leads to uncontrolled microbial translocation and deleterious inflammation. Indeed, necroptosis has been associated to chronic inflammatory diseases and cancer. Drugs that inhibit necroptosis could, therefore, be used therapeutically for the treatment of these diseases, and researches to develop such inhibitors are already underway. In this Review, we outline pathways for necroptosis and its role in chronic inflammation and cancer. We also discuss current and developing therapies that target necroptosis machinery.

Keywords: programmed cell death; inflammation; cancer; intestinal diseases; inhibitors.

1. Introduction

Cell death is crucial during the development and maintenance of tissue homeostasis in multicellular organisms. Until recently, apoptosis has been considered the only molecularly controlled type of cell death, as opposed to necrosis that was classically described as nonregulated accidental cell death. Several types of regulated necrotic cell death have been recently identified, which share common morphological features, including increase of the cellular volume, swelling of organelles, and disruption of the plasma membrane but are activated in response to different triggers and are executed by distinct biochemical pathway [1] Necroptosis is a form of regulated necrotic cell death induced by multiple extracellular and intracellular stimuli, including tumor necrosis factor (TNF) family members, Fas ligands, interferon, and oxidative stress [2-4].

The receptor-interacting protein kinase 1 (RIPK1) and 3 (RIPK3), as well as their target, the mixed lineage kinase domain-like protein (MLKL), have been shown to be essential for signal transduction pathways in necroptosis [5-7]. Necroptosis has been implicated in mediating multiple human diseases including ischemic reperfusion injury, inflammatory, neurodegenerative, infectious, autoimmune diseases and cancer [8-18]. More Recently, necroptosis has also been involved in mediating organ rejection in both cardiac and renal allografts [8,12,19-22]

Despite the pathological connotation that have typically characterized necroptosis at the beginning of its identification, emerging evidence points to its crucial role in physiological phenomena such as development, immunology and differentiation. For example, mutant mice with kinase-dead RIPK1 or RIPK3 and MLKL deficiency show no detrimental phenotype in regard to development and adult homeostasis [11]. Furthermore, several authors reported that necroptosis is able to induce the innate immune response to viral infections, when virus inhibits host apoptotic machinery and cells are

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committed to alternative cell death to limit viral replication or generate an immune response [23-27]. Necroptosis is also implicated in the regulation of antigen-activated T-cell proliferation and survival [28,29].

In this review, we discuss the latest insights into the role of necroptosis in the development of chronic inflammation and cancer of the gastrointestinal (GI) tract as well as novel therapeutic alternatives targeting the necroptosis machinery.

2. Necroptosis molecular pathway

Necroptosis is a cellular response to environmental stress that can be caused by chemical and mechanical injury, inflammation, or infection. Traditionally, this process is known to be activated by different triggers, including members of the tumor necrosis factor receptor (TNFR) superfamily, Toll-like-receptor 3 and 4 (TLR3/4), T cell receptors (TCRs), viral DNA and interferon receptor (IFNR) (**Figure 1**).

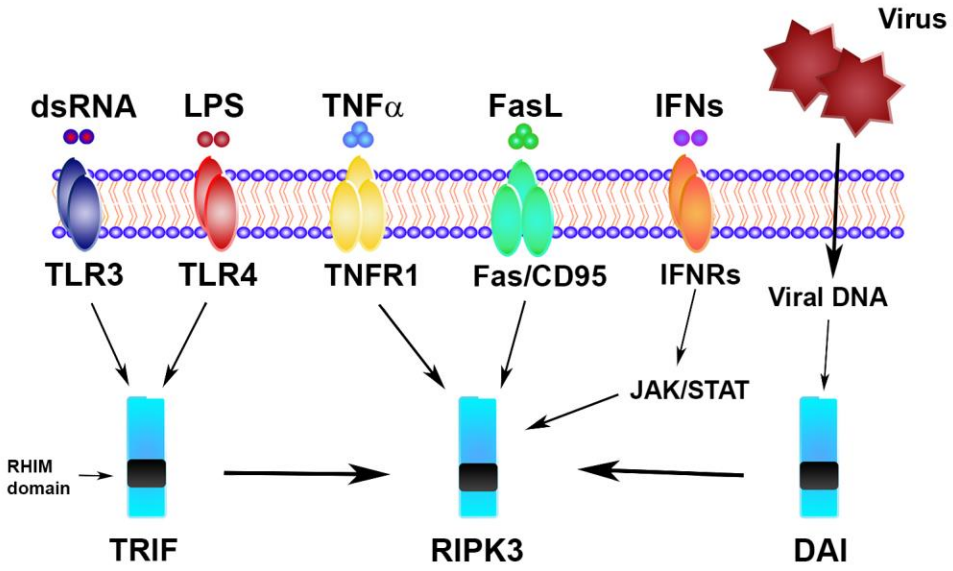

Figure 1. The necroptosis marker RIPK3 is activated in response to different triggers. TLR, Toll-like receptor; LPS, Lipopolysaccharide; TNFα, tumor necrosis factor alpha; TNFR1, tumor necrosis factor receptor; FasL, Fas ligand; IFN, interferon; IFNR, interferon receptor; TRIF, Toll/interleukin-1 receptor (TIR) domain-containing interferon-β; DAI, DNA-dependent activator of IFN regulatory factors; RHIM, RIP-homotypic-interaction-motif domain; RIPK3, receptor-interacting protein kinase 3.

More recently, several evidences showed that necroptosis can be also induced by DNA damage, environmental stresses, such as hypoxia and glucose level, and several chemotherapeutic agents **[30-34]**. Among the various triggers, the TNFα/TNFR pathway is considered a prototype and has been the most intensively investigated **[8,9,35-38]**. The principal steps of the TNFα/TNFR signaling are described below. Binding of TNF to TNFR1 induces a conformational change in TNFR1 trimers, leading to the recruitment of a multiprotein platform named complex I, that includes RIPK1, TRADD (TNFR-associated death domain), cIAP1 (cellular inhibitor of apoptosis protein 1), cIAP2 and TRAF (TNFR-associated factor)2. The polyubiquitination of RIPK1 by cIAP1/2 causes the

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recruitment of complexes IKK (IKKα,IKKβ and NEMO) and TAK1 (TAK1, TAB1 and TAB2) leading to the activation of the transcription factor NF-kB and cell survival. Deubiquitination of RIPK1 by deubiquitinase cylindromatosis (CYLD) or A20 ubiquitin-editing complex marks the transition from complex I to complex II and inhibits NF-κB activation [**39**]. TRADD and RIPK1 dissociate from TNFR1, recruiting FADD and pro-caspase-8 and leading to the formation of complex IIa, that results in the activation of caspase-8 cleavage followed by apoptosis [40].

The enrollment of RIPK3 causes the formation of complex IIb. Several evidences support a role of cellular FLICE-inhibitory protein (cFLIP) as a critical switch to control cell survival and death in various tissues. cFLIP protein is evolutionarily conserved and expressed as functionally different isoforms, named long FLIP(L) and short FLIP(S) splice forms. In vitro studies have shown that cFLIP(L) blocks both apoptosis and necroptosis, while cFLIP(S) blocks apoptosis but promotes necroptosis [41,42]. Thus, the presence of cFLIP(L) at complex IIb inhibits caspase-8, blocking apoptosis, and prevents the interaction between RIPK1 and RIPK3 impeding necroptosis as well [43]. Differently, the presence of cFLIP(S), that binds to FADD with higher affinity compared to procaspase-8, prevents the recruitment of caspase-8 and apoptosis [43]. However, in case of high levels of RIPK3 or caspase-8 inactivation, due to pharmaceutical or genetic intervention, the necrosome is formed: RIPK1 and RIPK3 interact and trans/autophosphorylate developing RIPK1/3 amyloid structures, then, RIPK3 phosphorylates its substrate MLKL that oligomerizes and translocates to the plasma membrane to execute necroptosis [44-48].

The interaction between RIPK1 and RIPK3 occurs through their common RIP-homotypic-interaction-motif (RHIM) domain. However, other RHIM-containing proteins, such as Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-ß (TRIF) and DNA-dependent activator of interferon-regulatory factor (DAI, also known as ZBP1), may interact and activate RIPK3, regardless of RIPK1, leading to necroptosis [**35**]. In addition to MLKL, other effectors of necroptosis, downstream RIPK3, have been identified, such as the mitochondrial protein phosphatase (PGAM5) mediating the mitochondrial fragmentation, [49] and Ca2+/calmodulin-dependent protein kinase (CAMK)-II regulating the ion channels activation by phosphorylation with following influx of extracellular ions [45] (**Figure 2**).

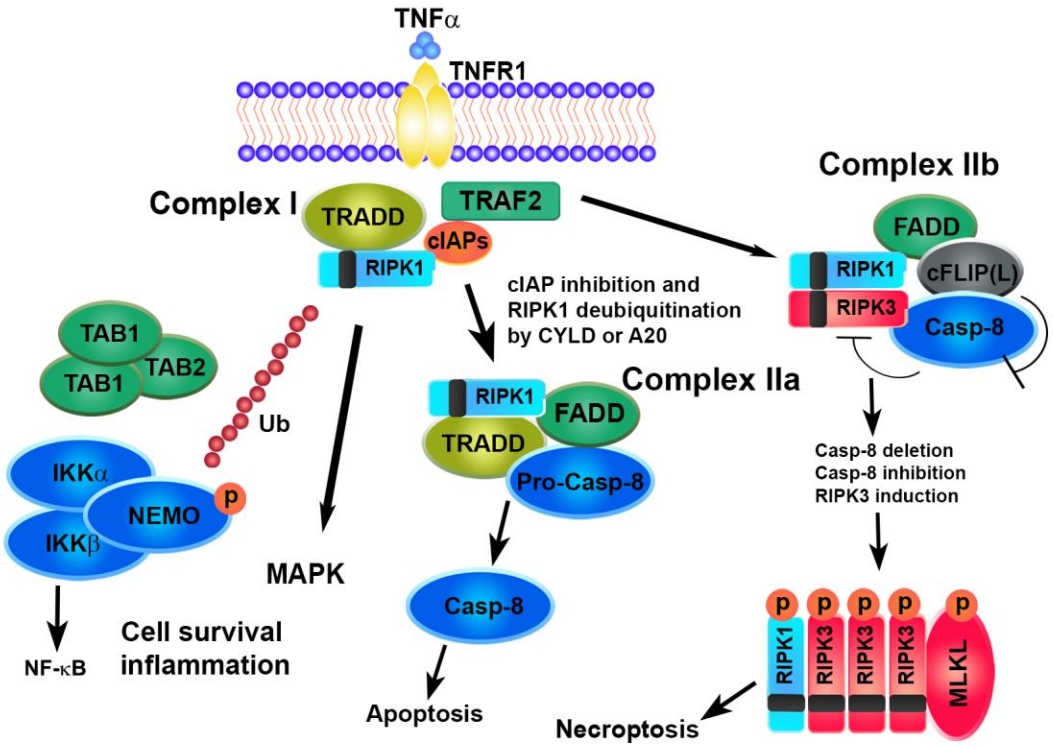

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Figure 2. Survival or cell death pathways activated by TNFα/TNFR1. TNFα, tumor necrosis factor alpha; TNFR1, tumor necrosis factor receptor; TRADD, TNFR-associated death domain; TRAF2, TNFR-associated factor, cIAPs, cellular inhibitors of apoptosis protein, RIPK, receptor-interacting protein kinase, TAK1, transforming growth factor-activated kinase 1; TAB, TAK1-binding protein; IKK, inhibitor of NF-kB kinase; NEMO, NF-kB essential modulator; FADD, Fas-associated protein with death domain; CYLD, cylindromatosis lysine 63 deubiquitinase; cFLIP, cellular FLICE-like inhibitory protein; MLKL, mixed lineage kinase domain like protein.

Causing inflammation, necroptosis is finely controlled by several negative regulators. For example, TBK1 and IKKε prevent TNF-induced cell death by RIPK1 phosphorylation [50, 51]. Moreover, absent, transient and sustained levels of TAK1-mediated RIPK1 phosphorylation may represent distinct states in TNFR1 signaling complex to dictate the activation of alternative cell death mechanisms [52]. Carboxyl terminus of Hsp70-interacting protein (CHIP, also known as STUB1) is a bona fide negative regulator of the RIPK1-RIPK3 necrosome formation leading to desensitization of TNF-mediated necroptosis [53]. A20 (also known as TNFAIP3, TNF-induced protein 3) and ABIN-1 (also known as TNIP1, TNFAIP3-interacting protein 1), candidate susceptibility genes for several autoimmune or inflammatory diseases, synergistically restrict death by inhibiting TNF-induced caspase-8 activation and RIPK1 kinase activity blocking both apoptosis and necroptosis [54].

Finally, pharmaceutical inhibition of RIPK3 shows a curative effect in mice deficient in SET-domain-bifurcate-1 (SETDB1), a histone methyltransferase that mediates the trimethylation of histone H3 at lysine 9, suggesting that targeting necroptosis of intestinal stem cells may represent an approach for the treatment of severe inflammatory bowel disease (IBD) [55].

3. Interplay between necroptosis, apoptosis and autophagy

The interplay between necroptosis and other important cellular processes, such as apoptosis, autophagy or pyroptosis, and the identification of their converging points are crucial for developing novel therapeutic approaches in inflammatory diseases and cancer. Apoptosis occurs during the development as a homeostatic mechanism but also as a defense tool in immune reactions or in the presence of damaged cells [56].

In most situations, apoptosis is the default cell death modality, whereas necroptosis intervenes when key apoptotic mediators are blocked by pharmacological inhibition or genetic ablation or after a cellular stress, such as energy ATP depletion or in certain viral infections [57,58].

Caspase-8 represents the molecular switch to control the balance between apoptosis and necroptosis [59,60]. Mice with intestinal epithelial cells (IEC)-specific deletion of caspase-8 or its adapter FADD develop colitis and ileitis with loss of Paneth cells and these effects are rescued by RIPK3 deficiency, underlying the importance of necroptosis in driving the pathology in both the small and large intestine [61,62].

Moreover, in mice with IECs specific deletion of caspase-8, the stimulation of TLRs after *Salmonella Typhimurium* infections causes a RIPK3-dependent epithelial necroptosis with more severe mortality and tissue damage [63,64]. Mice with IEC-specific FADD or caspase-8 deficiency develop colitis dependent on MLKL-mediated epithelial cell necroptosis. Besides, caspase-8 and gasdermin-D (GSDMD), the effector of pyroptosis, are both required for the development of MLKL-independent ileitis in mice with epithelial FADD deficiency [65].

 doi:10.20944/preprints202009.0084.v1

Transgenic (Tg) mice wherein CFLARs, the gene encoding cFLIP, was integrated onto the X chromosome, died perinatally due to severe ileitis. Deletion of RIPK3 or MLKL prevented both necroptosis and apoptosis, and rescued lethality of CFLARs Tg mice [66].

A crosstalk between necroptosis and autophagy, an essential cellular process for the maintenance of cellular and tissue homeostasis, has also been demonstrated, albeit molecular mechanism remains poorly defined [67,68]. Autophagy regulates the degradation of cytoplasmic proteins and organelles within lysosomes, provides nutrients and energy under various stresses, including starvation, cellular and tissue remodeling, and cell death [69]. Recently, it has been shown that autophagy machinery can control the mechanism of programmed cell death, switching between apoptosis and necroptosis, by serving as a scaffold rather than by degrading cellular material [70].

Moreover, the autophagy gene ATG16L1 is essential in the intestinal epithelium for preventing loss of Paneth cells and a variant of ATG16L1 is associated with poor survival in allogeneic hematopoietic stem cell transplant recipients and Crohn's disease, one of the two forms of IBD. Intestinal organoids lacking ATG16L1 reproduced this loss in Paneth cells and displayed TNFα-mediated necroptosis indicating that, in contrast to tumor cells in which autophagy promotes caspase-independent cell death, ATG16L1 maintains the intestinal barrier by inhibiting necroptosis in the epithelium [71]. It has also been shown a role for ATG16L1 in coordinating the outcome of IL-22 signaling in the intestinal epithelium leading to transient ER stress and subsequent activation of type I interferon (IFN-I) signaling that amplifies epithelial TNF production and contributes to necroptotic cell death [72]. Besides, a critical function has recently emerged for mammalian target of rapamycin (mTOR), an evolutionarily-conserved protein kinase controlling the balance between cell growth and autophagy, in the regulation of RIPK3 expression and necroptosis in the gut epithelium. Epithelial mTOR hyperactivation, associated with the Western diet, dysbiosis, or ablation of its repressor TSC1, has been shown to inhibit RIPK3 degradation in autophagolysosome and promote necroptosis of epithelial cells, barrier disruption and chronic inflammation. Hyperactive mTOR and aberrant necroptosis has been found to be correlated in humans IBD [73].

Furthermore, the immunity-related GTPase family M protein (IRGM), that plays a role in innate immunity by regulating autophagy in response to several intracellular pathogens, has been found to regulate necroptosis and release of damage-associated molecular patterns (DAMPs) inducing gastrointestinal inflammation. Interestingly, IRGM has been identified in genome wide association studies as a genetic risk factor in CD. IRGM overexpression by phosphorylated eukaryotic translation initiation factor 2 (pEIF2A) plays a major role as a checkpoint allowing host cell survival by autophagy or host cell elimination by necroptosis [74].

Similarly, necroptosis may promote or repress autophagy, although mechanisms are still unclear [75]. For example, necroptotic stimulation has been shown to reduce autophagic activity, as evidenced by enlarged puncta of the autophagic substrate Sequestosome 1 (SQSTM1/p62) and its increased colocalization with microtubule-associated protein 1A/1B-light chain 3 (LC3), attenuating autophagic flux before the lysosome fusion step [76]. Activation of necroptosis in mouse dermal fibroblasts and HT-29 human colorectal cancer cells results in accumulation of the autophagic marker, lipidated LC3B in an MLKL-dependent manner. Unexpectedly, the necroptosis-induced increase in lipidated LC3B was due to inhibition of autophagic flux, not the activation of autophagy [77].

4. Necroptosis and inflammation

Molecular mechanisms of cell death have regulatory roles in inflammation and molecular changes associated with different forms of cell death may affect the course of inflammation in different ways [78]. In the past few decades, emerging knowledge on cell death and inflammation has enriched our

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molecular understanding of the signaling pathways that mediate various programs of cell death and multiple types of inflammatory responses.

Necroptosis is typically considered a highly pro-inflammatory mode of cell death, due to release of DAMPs, which can promote inflammation and activate an immune response, either alone (in the context of sterile injury) or in combination with pathogen-associated molecular patterns (PAMPs) [79-81]. Necroptosis promotes the activation of macrophages and dendritic cells that increase the levels of pro-inflammatory cytokines, including interleukin (IL)-1 superfamily, thus triggering acute and chronic inflammatory diseases [5,35,36,79,80].

Recent studies have revealed unexpected complexity in the regulation of cell death programs by RIPK1 and RIPK3 with the possibility that necroptosis is one mechanism by which these kinases promote inflammation [81]. For example, RIPK3 seems to control a separate, necrosis-independent pathway of inflammation by regulating NF-κB activation, dendritic cell (DC) biology, innate inflammatory-cytokine expression, and injury-induced tissue repair [82]. Several studies have documented that RIPK3 mediates the formation of NATCH, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome and following activation of caspase-1 and -11, through the interaction with different molecules, including caspase-8 and LPS [83-86]. It is unclear whether MLKL is involved in this process [87]. However, a recent paper reported that MLKL promotes intestinal epithelial barrier function by enhancing inflammasome activation [88].

In contrast with the traditional view of a pro-inflammatory mode of necroptosis, some authors suggest that it might have anti-inflammatory effects in certain settings, through curbing excessive TNF- or TLR-induced inflammatory cytokine production [36, 37, 89-91].

5. Necroptosis and intestinal diseases

5.1. Inflammatory Bowel Disease (IBD)

IBD is a chronic, debilitating intestinal disease with various clinical presentations whose main forms are ulcerative colitis (UC) and Crohn's disease (CD). The cause of IBD is still unknown, although it is believed to be a combination of multiple environmental factors along with the genetic inheritance patterns, all leading to an excessive and abnormal immune response against commensal gut flora [92].

The first demonstration of the involvement of necroptosis in the pathogenesis of IBD derives from studies reporting that genetic ablation of either Casp8 or Fadd in IECs is sufficient to induce necroptosis and spontaneous ileitis and/or colitis [61,62]. Later, RIPK3 overactivation has been reported in inflamed tissues of pediatric and adult IBD patients [61,93].

Recently, our group showed that RIPK3-driven necroptosis seriously affects intestinal inflammation by increasing pMLKL, activating different cytokines and alarmins, and altering epithelial permeability. The inhibition of necroptosis caused a significant decrease of all these effects [94]. Accordingly, the use of the RIPK1 inhibitor Necrostatin-1 (Nec-1) reduced the intestinal inflammation and colitis-associated tumor growth in mice with dextran sulfate sodium([DSS)-induced colitis [95], while, MLKL deficiency inhibited the colitis by preventing inflammatory cytokines production and MAPK signaling activation [96]. MLKL$^{-/-}$ mice were highly susceptible to colitis and colitis-associated tumorigenesis, which was associated with massive leukocyte infiltration and increased inflammatory responses, suggesting a protective role of the necroptosis effector [89]. The necroptosis inhibitor necrosulfonamide (NSA) repressed necroptosis in intestinal epithelial cells in vitro suggesting a potential beneficial effect against IBD [97]

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More recently, the expression of a secreted form of interferon lambda (IFN λ) in mice resulted in loss of Paneth cells and increased cell death, via STAT1 and MLKL, controlled by caspase-8, in intestinal tissues **[98]**. Zhang and Coll. **[99]** showed that inhibition of HtrA2 by the serine protease UCF-101 alleviated DSS-induced colitis by preventing necroptosis of intestinal epithelial cells. Furthermore, an unexpected connection between necroptosis and members of the disintegrin and metalloproteinase (ADAM) protease family has been reported [100]. Hence, the disintegrin metalloprotease ADAM17, with a critical role in intestinal inflammation and regeneration in mice, as illustrated by the dramatically increased susceptibility of ADAM17 hypomorphic ($ADAM17^{ex/ex}$) mice to dextran sulfate sodium (DSS)-induced colitis, was shown to be involved in necroptosis regulation. Indeed, murine embryonic fibroblasts derived from $ADAM17^{ex/ex}$ mice were protected from tumor necrosis factor (TNF)-induced necroptosis and failed to show phosphorylation of MLKL and RIPK3 after induction of necroptosis by TNF, revealing a novel, undescribed role of the protease ADAM17 in necroptosis [101].

Interestingly, the circadian rhythm disruption (CRD) is suggested as a risk factor for IBD. Genetic ablation of circadian clock function or environmental CRD in mice increased susceptibility to severe intestinal inflammation and epithelial dysregulation, accompanied by excessive necroptotic cell death and a reduced number of secretory epithelial cells, thus suggesting that CRD increases intestinal necroptosis, rendering the gut epithelium more susceptible to inflammatory processes [102].

5.2. *Necrotizing enterocolitis (NEC)*

Necrotizing enterocolitis (NEC) is a rare but devastating gastrointestinal disease that predominately affects preterm neonates [103]. The pathogenesis of NEC is incompletely understood and appears to be multifactorial. From a mechanistic viewpoint, it has been shown that bacterial activation of the innate immune receptor Toll-like receptor 4 (TLR4) in the intestinal epithelium leads to barrier injury and the inflammatory microenvironment and is required for the development of NEC. Indeed, the expression of TLR4 in the intestinal epithelium is higher in the premature vs full-term intestine and mice lacking TLR4 in the intestinal epithelium are protected from NEC [104].

Recently, it was reported that necroptosis is activated in the intestinal epithelium upon TLR4 signaling and is required for NEC development [105]. Imbalance of IEC cell death, resulting in increased intestinal permeability and barrier dysfunction, leads to several acute and chronic intestinal diseases, including NEC [106].

Recently, a study showed that miR-141-3p protects intestinal epithelial cells from LPS damage by suppressing RIPK1-mediated inflammation and necroptosis, providing an alternative perspective to explore the pathogenesis of NEC [107].

5.3 *Bacterial intestinal infections*

Infections with bacterial pathogens often results in the initiation of programmed cell death, including necroptosis, as part of the host innate immune defense, or as a bacterial virulence strategy [108].

The sexually transmitted pathogen *Chlamydia trachomatis* is able to replicate and survive in human intestinal epithelial cells and to induce apoptosis and necroptosis [109].

Moreover, *Salmonella* outer protein B (SopB) has a role in modulating necroptosis to facilitate bacteria escape epithelial cell and spread to systemic sites through a *Salmonella*-induced colitis model [110], while, caspase-8 knockout mice infected with *Salmonella typhimurium* showed more severe mucosal injury and intestinal epithelial cell death as compared to wild-type mice [63].

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Listeria monocytogenes, a bacterial foodborne pathogen, efficiently spread and caused systemic infection in RIPK3-deficient mice while almost no dissemination was observed in wild-type mice. Intriguingly, MLKL was shown to directly bind to *Listeria* and inhibits their replication in the cytosol. Suggesting a novel functional role of the RIPK3-MLKL pathway in nonimmune cell-derived host defense against *Listeria* invasion [111]. *Clostridium perfringens* uses its large arsenal of protein toxins to produce histotoxic, neurologic and intestinal infections in humans and animals, resulting in the activation of intracellular pathways with a variety of effects, commonly including cell death, such as apoptosis, necrosis and/or necroptosis [112]. MLKL$^{-/-}$ mice were more susceptible to *Salmonella* infection compared with wild-type counterparts, with higher mortality rates, increased body weight loss, exacerbated intestinal inflammation, more bacterial colonization, and severe epithelial barrier disruption [88].

6. Inhibitors of necroptosis

Accumulating data, particularly in mouse models, suggest that targeting cell death has therapeutic potential in chronic inflammatory diseases [113]. Several inhibitors of RIPK1, RIPK3 and MLKL have been developed showing promise towards the treatment of inflammatory intestinal disorders [114]. Necrostatins are tryptophan-based compounds that inhibit the kinase activity of RIPK1 [115]. Necrostatin-1 [Nec-1; methyl-thiohydantoin-tryptophan (MTH-Trp)] as well as its improved version Nec-1s, was found to selectively target the kinase activity of RIPK1 [116,95], suggesting a protective role in IBD [61,94,95]. Nec-1 administration inhibited the up-regulation of RIPK1 and RIPK3 and enhanced the expression of caspase-8 in DSS-induced colitis and, in addition, the anti-inflammatory effect was confirmed by in vitro analyses [95].

Moreover, GSK2982772, the first oral, monoselective, small-molecule inhibitor of RIPK1 developed to treat chronic inflammatory diseases, is currently in phase 2a clinical studies for psoriasis, rheumatoid arthritis, and ulcerative colitis [117,118]. Blockade of RIPK3 kinase activity has been considered for therapeutic intervention in patients with inflammatory disorders, as well. Three selective small molecule compounds, GSK'840, GSK'843, and GSK'872, are shown to inhibit RIPK3 kinase-dependent necroptosis, although their therapeutic value is undermined by a surprising, concentration-dependent induction of apoptosis [119,120].

Dabrafenib, belonging to B-Raf (V600E) inhibitors that are an important anticancer drug class for metastatic melanoma therapy, showed a potent inhibition on RIPK3, which was achieved by its ATP-competitive binding to the enzyme [121]. The anti-cancer pan-RAF inhibitor LY3009120 is a necroptosis inhibitor and may serve as a potential therapeutic drug for colitis, indeed, it significantly alleviated (DSS)-induced colitis as indicated by prevention of body weight loss, colon shortening, and decreased mortality [122]. Recently, a novel class of RIPK1/RIPK3 dual inhibitors, which completely block necroptosis in both human and murine cells, have been identified [123]. For example, Ponatinib and Pazopanib inhibited necroptotic cell death induced by various cell death receptor ligands in human cells, while not protecting from apoptosis. Ponatinib inhibited both RIPK1 and RIPK3, while Pazopanib preferentially targeted RIPK1 [124].

The novel MLKL inhibitors provide powerful tools to study the biological function of MLKL and demonstrate that MLKL should be viewed as a druggable target. Yan and coworkers reported the development of novel MLKL inhibitors with single nanomolar potency (compound 15 is also named as TC13172) showing that the inhibitors covalently bind to Cysteine-86 (Cys-86) of MLKL [125]. Moreover, necrosulfonamide (NSA), blocking MLKL, inhibits necroptosis in intestinal epithelial cells in vitro conferring a potential protective effect against IBD [97]. Numerous microRNAs (miRNAs), endogenous non-coding RNAs that target mRNAs for cleavage or translational repression, regulate programmed cell death including necroptosis. MiR-155 has been reported to prevent necroptosis in human cardiomyocyte progenitor cells by directly targeting RIPK1 [126]. CLYD, the key deubiquitinating enzyme in the apoptosis/necroptosis pathway, was directly

targeted by miR-181b-1 and miR-19, resulting in the hyperactivation of the NF-κB signaling pathway and in a high inflammatory status in cancer cells; these events contribute to tumor progression [127,128]. Numerous compounds originated from the natural sources display promising anti-necroptotic potentials. Hesperetin, a flavonoid from citrus fruits shows protective effects on DSS-induced colitis by inactivating RIPK3/MLKL necroptosis signaling [129]. Furthermore, flaxseed oil, a rich source of α-linolenic acid (ALA), which is the precursor of the long-chain n-3 polyunsaturated fatty acids (PUFAs), attenuates intestinal injury induced by LPS by regulating necroptosis and TLR4/NOD signaling in a piglet model. [130]. Recently, celastrol, a triterpene from the root bark of the Chinese medicinal plant Tripterygium wilfordii, was shown to inhibit necroptosis in DSS-mice model [131].

7. Necroptosis and GI cancer

Recent research attributes to necroptosis both a tumor suppressive and tumor promoting activity depending on the type, stage and localization of the tumor. The tumor suppressive activity of necroptosis has been shown in many types of cancer in which the expression of RIPK3 [132-133] or MLKL [135] was silenced by methylation [18,34,135] or altered by polymorphisms in coding genes [132,136], suggesting that cancer cells evading apoptosis, may also escape from necroptosis [13,18]. Hence, RIPK3 expression is downregulated in many cancer cell lines [18,137] as well as in several human cancers, including breast cancer [18], colorectal cancer (CRC) [17], acute myeloid leukemia (AML) [138] and melanoma [137]. Therefore, induction of necroptosis may also represent an alternative strategy for killing cancer cells [139-140].

The tumor-suppressing activity of RIPK3 has been documented in CRC. Overexpression of RIPK3 significantly suppressed the proliferation of colorectal cancer cells in vitro suggesting that it may play a suppressive role in the colorectal carcinogenesis. Accordingly, low RIPK3 expression has been associated with poor clinical outcomes in human CRC, while high RIPK3 expression reduced migration and invasion of cancer cells [17]. Moreover, RIPK3 knockout mice have increased risk to develop colitis-associated CRC producing increased amount of pro-inflammatory or tumor-promoting factors, such as the transcription factor STAT3 [90]. Although the signaling events that induce different forms of programmed cell death are well defined, the subsequent immune responses to dying cells in the context of cancer remain relatively unexplored. It has been proposed a role for RIPK1/RIPK3 activation as a beneficial proximal target in the initiation of tumor immunity, thus, maximizing the immunogenicity of dying cells within the tumor microenvironment through specific activation of necroptosis may represent a beneficial treatment approach [141].

Remarkably, the release of cell content, including DAMPs and cytokines/chemokines, characterizing necroptosis, underlies the immunogenic nature of necroptotic cancer cells and their ability to induce efficient anti-tumor immunity [142]. As well, decreased RIPK3 in CRC boosted tumorigenesis via accumulation and immunosuppressive activity of myeloid-derived suppressor cells (MDSC). Indeed, decreased RIPK3 in MDSC and colorectal cancer cells elicited NF-κB-transcribed COX-2, which catalyzed the synthesis of prostaglandin E_2 (PGE_2). PGE_2 exacerbated the immunosuppressive activity of MDSCs and accelerated tumor growth [143].

Differently, some authors suggest that inflammation underlying necroptosis may promote tumor development by providing genomic instability, angiogenesis, cell proliferation, and accelerating metastasis [144]. Higher phosphorylation level of MLKL has been correlated with a poorer prognosis and shorter survival in human patients with colon and esophageal cancer and the use of MLKL inhibitor, NSA, was shown to significantly delay the tumor growth, highlighting the role of necroptosis in tumor promotion [145].

Targeting non-apoptotic forms of cell death including necroptosis, may have therapeutic benefits in apoptosis-defective cancer cells. Necroptotic cell death has been identified as an important effector

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mechanism of 5-fluorouracil (5-FU)-mediated anti-tumoral activity [146]. Moreover, RIPK3, which is highly expressed in mouse models of CRC and in a subset of human CRC cell lines, was shown to be the deciding factor of cancer cell susceptibility to second mitochondria-derived activator of caspase (SMAC) mimetic-induced necroptosis [147]. The 3-Bromopyruvate (3BP) can induce colon cancer cell death by necroptosis and apoptosis at the same time and concentration in the SW480 and HT29 cell lines [148]. More recently, resibufogenin, a member of bufadienolide family, was proven to suppresses the growth and metastasis of CRC by inducing necroptosis in vivo through upregulation of RIPK3 and phosphorylation of MLKL at Ser358 [149]. In a recent paper, Van Hoeche describes a generic antitumor therapy based on the intratumor delivery of mRNA that codes for the necroptosis executioner MLKL showing that this intervention stalls primary tumor growth and protects against distal and disseminated tumor formation in syngenic mouse melanoma and colon carcinoma models [150]. However, the role of necroptosis in cancer is complex and further precise characterization of necroptosis, will be critical for the validation of the significance of necroptosis in different types of cancer.

In the following table the principal alterations of necroptotic genes in *in vivo* and *ex vivo* models and their effects are reported. **Table 1**.

Gene alteration	Model	Effects	Ref.
In vivo:			
FADD$^{\Delta IEC}$ /RIP3-/-or MLKL-/-Casp8$^{\Delta IEC}$ /RIP3-/- or MLKL-/-		**Prevention of colitis, cell death and inflammation**	**65**
Setdb1$^{\Delta IEC}$/Ripk3-/- or Mlkl-/-		**Inhibition of stem cell death**	**55**
MLKL$^{\Delta IEC}$	**High protein diet and DSS-induced colitis.**	**Rescue of IEC death and intestinal inflammation**	**73**
IEC Casp8 $^{C362S/C362S}$ Mlkl−/−		**Severe intestinal inflammation**	**60**
Ripk3−/− or Mlkl−/− Casp8$^{\Delta IEC}$	**IFNλ injection**	**Rescue of INFλ–induced necroptosis in Paneth cells**	**98**
cFLIPs Tg mice RIPK3-/- or MLKL-/- or RIPK1$^{DN/DN}$		**Partially rescue of lethality, epithelial cell death and villous destruction**	**66**
Mlkl-/-	**AOM i.p. injection + DSS-induced colitis**	**Susceptibility to colitis and colitis-associated tumorigenesis**	**89**
Mlkl-/-	**DSS-induced colitis**	**Inhibition of intestinal inflammation independent from microbiota**	**96**
Mlkl-/-	**DSS-induced colitis**	**Prevention of body weight loss and mortality**	**99**
Ripk3−/− or Mlkl−/−	**NEC**	**Reduced IEC death and mucosal inflammation**	**105**
Casp8$^{\Delta IEC}$Ripk3−/− or Mlkl−/−	***Salmonella*-induced gastroenteritis**	**Rescue of IEC death, body weight loss and mucosal destruction**	**63**
Ripk3-/-	**AOX+ DSS-induced CAC**	**Promotion of colorectal carcinogenesis and infiltration of myeloid-derived suppressor cells**	**143**

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Mlkl-/-	*Salmonella*-induced colitis	Rescue of barrier integrity	88
Casp8$^{\Delta tumor}$	SMAC mimetic administration	Increased cancer susceptibility in SMAC mimetic induced necroptosis	147
ATG16L1$^{\Delta IEC}$	MNV + DSS-induced colitis + GSK547	Rescue of clinical score	71
Casp8$^{\Delta IEC}$Ripk3-/-	LPS or poly (I:C) i.p. injection	Rescue of IEC death and destruction of crypt-villus architecture	64
Ripk3-/-	Induced CRC	Higher susceptibility to CRC	90
Ripk3-/-_	DSS-induced colitis+LPS	Higher susceptibility to colitis	81
Chip-/-/Ripk3-/-		Reduction of cell death induced by Chip deletion in the small intestine	53
Ripk3-/-	DSS-induced colitis	RIPK3 protective against colitis	82
Casp8$^{\Delta IEC}$	Spontaneous ileitis	Increased cell death of Paneth cells	61
Fadd$^{\Delta IEC}$	Spontaneous colitis	IEC cell death, loss of Paneth cells, enteritis and severe colitis.	62
Ex vivo:			
IEC TSC1$^{\Delta IEC}$ RIP3+/- organoid	Z-VAD-FMK + poly I:C/TNFα/IFNβ	Cell death attenuation	73
MLKL$^{\Delta IEC}$ organoid	IL-22 + BafA	Reduced cell death	72
ATG16L1$^{\Delta IEC}$ organoid	Z-VAD-FMK + Nec-1	Inhibition of cell death	71
Casp8$^{\Delta IEC}$ organoid	Poly I:C + Nec-1	Inhibition of cell death	64
Casp8$^{\Delta IEC}$ organoid	TNFα + Nec-1	Inhibition of cell death	61

Table 1. In vivo and ex vivo studies reporting necroptotic genes alterations. IEC, intestinal epihelial cells; DSS, dextran sulfate sodium, IFN, interferon; AOX, azoxymethane; i.p., intraperitoneal; NEC, necrotizing enterocolitis; CAC, colitis-associated cancer; SMAC, second mitochondria-derived activator of caspase; Nec-1, necrostatin-1; GSK547 (RIPK1 inhibitor); MNV, murine norovirus; LPS, lipopolysaccharide; poly (I:C), polynosine-polycytidylic acid (I:C); CRC, colorectal cancer; NSA, necrosulfonamide; zVAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone (pan-caspase inhibitor); IL, interleukin; BafA, bafalomycin A.

8. Conclusions

Necroptosis is now recognized as a major form of regulated cell death. It is mediated by RIPK1/RIPK3 activation, followed by MLKL phosphorylation and membrane disruption. Necroptosis is becoming increasingly attractive as a field of study given its implications in the

pathogenesis of inflammatory diseases and cancer, including those of the GI tract. Targeting RIPK3 and RIPK1 may help to overcome therapeutic hurdles in the treatment of these complex disorders. Although the necroptosis field has developed rapidly in recent years, it also faces many challenges. For examples, how cells select survival, apoptosis, necrosis and necroptosis according to different physiological and pathological conditions, the regulation of RIPK family activity and its substrates or molecular mechanisms of MLKL. As well, it is necessary to unveil the molecular mechanism through which necroptosis process regulates the phenotype of immune cells in the gut.

The further investigation of the molecular mechanism of necroptosis, we will be more aware of cell death and develop new ideas for exploration of the pathogenesis of various human diseases.

Author Contributions: literature search, A.N. and E.C.; writing review and editing, A.N., E.C. and L.S.; supervision, S.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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