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Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium

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ABSTRACT

Intestinal epithelial cells (IEC) are organised as a single cell layer which covers the intestine. Their primary task is to absorb nutrients present in the intestinal lumen. However, IEC also play an important role in the immune defence of our body by building a barrier that separates the bowel wall from potentially hazardous bacteria present in the gut lumen. The life cycle of IEC is determined by the time span in which cells migrate from their place of origin at the crypt base to the villus tip, from where they are shed into the lumen. Cell death in the intestinal epithelium has to be tightly regulated and irregularities might cause pathologies. Excessive cell death has been associated with chronic inflammation as seen in patients with Crohn's disease and ulcerative colitis. While until recently apoptosis was discussed as being essential for epithelial turnover and tissue homeostasis in the intestinal epithelium, recent data using gene deficient mice have challenged this concept. Moreover, an apoptosis-independent mode of programmed cell death, termed necroptosis, has been identified and described in the intestinal epithelium. The following article reviews previous studies on cell death regulation in IEC and a potential role of necroptosis for gut homeostasis.

THE LIVE CYCLE OF IEC

Although the skin is the most obvious surface of the human body, the gut with an area of about 300 m^2 represents the largest boundary against the external environment, characterised by dietary components and the bacterial flora in the gut lumen. To allow for efficient nutrient absorption, the small intestine is folded to form a number of tubular invaginations, denoted crypts, and finger-like villus structures.[1] Dehydration of water takes place in the large intestine.[2] Since villi would hamper the passage of stool through the colon, the colonic epithelium has a smooth surface with crypt structures but lacking villi.[3] The intestinal barrier is established by a single layer of intestinal epithelial cells (IEC) which separate the intestinal lumen from the lamina propria with its resident mucosal immune cells. Opposed to its barrier function, the intestinal epithelium has to be permeable to allow efficient absorption of nutrients and water. In order to control this semi-permeability, the intestinal epithelium is organised in an intricate manner. In addition to the complexity established by the 3D structure of the gut surface, additional complexity is given by the fact that the intestinal epithelium does not represent a homogeneous cell population.[4] It is composed of different cell types, each with highly specialised functions. In general, the epithelial cell lineage consists of epithelial stem cells and proliferating progenitor cells located within the crypt region as well as terminally differentiated cells along the crypt or crypt–villus axis, respectively.[5 6] Differentiated epithelial cells can be further divided into two main groups, the absorptive and secretory lineages. The absorptive lineage is represented by enterocytes, the most numerous cell type in the intestinal epithelium.[5 6] Enterocytes provide the physical barrier of the intestinal epithelium by forming close contacts via tight junctions. Since their main function is the efficient absorption and transport of nutrients from the luminal side into the blood stream, enterocytes have further enlarged their luminal surface with microvilli.[7] Epithelial cells of the secretory lineage, the Paneth cells, goblet cells and enteroendocrine cells develop from the same epithelial progenitor cells but differ from enterocytes both in morphology and function.[5] Paneth cells express antimicrobial peptides like α-defensins, lyzosozym or phospholipase A and thereby contribute to host defence against a broad spectrum of bacteria, fungi and some viruses.[8] Within Paneth cells, antimicrobial peptides are stored in cytoplasmatic granules from which they can be released by exocytosis into the gut lumen.[8–10] Paneth cells are not evenly distributed in the gut epithelium. They are restricted to the crypt in the small intestine, where they are located at the crypt base together with intestinal stem cells.[11] In contrast to Paneth cells, goblet cells can be found along the crypt–villus axis of the small intestine and in the crypts of the large bowel.[11] Goblet cells provide a protective function against physical and chemical injury by the secretion of high molecular weight glycoproteins called mucins.[12] These mucins are composed of a polymeric protein backbone structure, linked to numerous hygroscopic and hydrophilic oligosaccharide side-chains that contribute to the formation of a gel-like matrix, covering the intestinal epithelium. Antimicrobial peptides together with a thick mucus gel film provide innate immune defence by hampering access to and survival of bacteria directly adjacent to the epithelium.[13] Finally, enteroendocrine cells belong to the

enteric endocrine system and coordinate gut function by secretion of specific gut hormones.[11 14 15] They can be found along the crypt–villus axis especially in the upper part of the small intestine.[16]

A striking feature of the intestinal epithelium is its enormous self-renewing capacity.[17] It is completely replaced by newly generated cells within only 4–5 days[5] (figure 1). Undifferentiated stem cells proliferate within the crypt region of the small and the large intestines, then undergo up to six rounds of cell division forming a pool of transit-amplifying cells, which are already partially differentiated.[18] Intestinal epithelial progenitors then stop cell division and differentiation into the different epithelial lineages proceeds while the cells migrate upwards along the crypt–villus axis presumably by the constant pressure exerted by newly generated cells demanding space within the crypt. After 3–4 days, terminally differentiated cells reach the tip of the villi from where they are shed by a yet poorly understood mechanism.[5 15] In contrast to all other differentiated epithelial cell types, Paneth cells escape the upward migration by an unknown mechanism and settle down at the crypt base.[11] Moreover, compared with enterocytes, goblet and enteroendocrine cells, whose lifespan is generally determined by the speed of migration from the crypt to the villus tip, Paneth cells can survive for more than 3 weeks.[19] The mechanisms controlling the live cycle of Paneth cells and of proliferating cells within the crypt is incompletely understood. Given the complex structure of the intestinal epithelium, proliferation, differentiation and cell death have to be tightly controlled. Excessive cell death might result in barrier defects and as a consequence thereof uncontrolled access of bacteria into the gut wall.[20] On the contrary, resistance to cell death is believed to be a driving force of tumour development in the gut.[21]

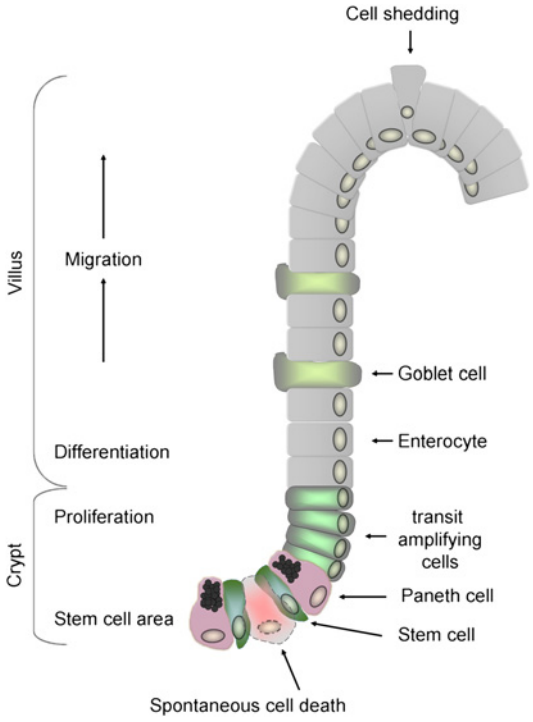

Figure 1 The intestinal epithelial cell layer. Stem cells reside next to Paneth cells at the base of small intestinal crypts. The crypt–villus border harbours stem cell progeny, the transit-amplifying cells that undergo four to five rounds of cell division before they stop proliferation and differentiate into the mature epithelial lineages. Ultimately, aged epithelial cell are shed into the lumen at the villus tip. Spontaneous cell death can be observed within in the crypt region, where cells presumably lack the capacity to undergo cell shedding.

APOPTOSIS AND NECROSIS OF IEC IN HEALTH AND DISEASE

The structural integrity of the gut and an efficient intestinal barrier are only maintained if the rates of epithelial cell proliferation and cell death are tightly regulated. Although, under steady state conditions, cell death is rarely observed along the length of the villus, two hotspots of epithelial cell death were described, the villus tip and the crypt region.[22] Aged epithelial cells, after travelling from the crypt base to the villus tip in the small intestine, or to the surface epithelial cuff in the colon, are thought to die from anoikis, a special form of programmed cell death which is induced in anchorage-dependent cells after detachment from their matrix.[23–26] Although the precise mechanisms controlling this process of shedding associated cell death are still poorly understood, a number of studies implicate that this process is actively regulated and involves caspase-3.[27 28] In fact, caspase-3 activation has been described to occur together with morphological changes associated with cell shedding.[28] This theory has also been supported by recent experimental studies showing that excessive cell shedding induced by TNF administration could be inhibited if caspase activation is blocked.[28] On the contrary, mice deficient for caspase-3 were described to show no morphological differences in the development of the gastrointestinal tract,[23 29–31] suggesting that caspase-3 might be dispensable for tissue homeostasis in the gut. Similarly, mice deficient for caspase-8 or Fas associated protein with death domain (FADD) in the intestinal epithelium showed no changes in the general structure of the gut.[32 33] The fact that mice deficient for central molecules of apoptosis show little if any structural changes implies that apoptosis might not be required for epithelial turnover in the gut at least in the steady state. These findings are in agreement with the hypothesis that epithelial cell shedding might be a rather passive process induced by the spatial constraints of densely packed epithelial cells at the villus tip and shedding associated cell death might be a consequence rather than a cause of shedding.

As mentioned above, spontaneous cell death of IEC has also been described within the crypt region.[34] In the small intestine, sporadical crypt epithelial cell death has been described in the stem cell region but only rarely in other parts of the crypt.[35] Conversely, in the colonic epithelium, only

very few cell death events were reported directly within the stem cell area, whereas cell death was described in the upper part of the crypt.[35] Compared with the shedding associated cell death at the villus tip, even less is known about the regulation and physiology of cell death in the crypt region. The finding that stem cells within the crypts of the colon was more resistant to spontaneous cell death than those in the small intestine, however, implicates differences in the regulation of cell death between the two bowel parts.[17 35 36] Indeed, recent data suggested that the expression of the antiapoptotic gene bcl-2 in epithelial progenitors of colonic crypts might protect these cells from spontaneous apoptosis.[37] This hypothesis was supported by studies in which genetic ablation of bcl-2 was demonstrated to increase the amount of cell death events within the stem cell region of the large intestine whereas the level in the small intestine remained unaffected.[36] Differences in cell death regulation might provide an explanation for the increased susceptibility of the colon for neoplastic transformation.

Although apoptosis seems to be dispensable for the structural integrity of the gut, there is little doubt that dysregulated or excessive apoptosis can lead to severe gut pathology. In line with this hypothesis, several studies have demonstrated either spontaneous gut inflammation or increased susceptibility towards induced gut inflammation in mice with phenotypes of elevated apoptosis in the intestinal epithelium. For example, mice deficient in the gene NEMO (nuclear factor kappa B (NFκB) essential modulator) specifically in IEC are characterised by chronic colitis developing shortly after birth.[38] It was demonstrated that NEMO deficiency leads to excessive tumour necrosis factor (TNF)-dependent apoptosis within the epithelium, followed by a barrier breakdown and the translocation of bacteria into the bowel wall driving inflammation. Blocking TNF or TLR signalling in these mice prevented the development of colitis providing a strong link among NFκB activity, epithelial apoptosis, bacterial translocation and intestinal inflammation.[38] Similarly, deletion of other members of the NFκB pathway, RELA, TAK1 or both IKK1 and IKK2, resulted in an increased susceptibility to colitis, underlining the importance of NFκB for epithelial cell survival and immune homeostasis in the gut.[39–41] In another study, it was shown that spontaneous enteritis can originate from deficient expression of the transcription factor XBP1 in IEC.[42] The authors moreover described an association of XBP1 with both Crohn's disease (CD) and ulcerative colitis, indicating that dysregulated XBP1 levels might actually occur in inflammatory bowel disease (IBD) patients.[43] On the molecular level, the authors reported that XBP1 deletion induces endoplasmatic reticulum stress resulting in increased sensitivity of IEC to cell death and spontaneous apoptosis of differentiated Paneth and goblet cells. While this finding suggested defects in intestinal barrier function of XBP1 deficient mice, the authors suggested that the absence of Paneth cells was not by itself causative to the spontaneous enteritis,[43] but rather a contributing factor supporting intestinal inflammation. In support of this view, another study reported that Paneth cell deficiency by experimental manipulation did not result in detectible effects in host–microbial interactions or in intestinal inflammation.[44]

Another important regulator of epithelial cell death is Stat3. Conditional knockout mice with an IEC-specific deletion of Stat3 show defects in epithelial restitution and are highly susceptible to DSS-induced colitis.[45 46] Analysis of cell death in these mice demonstrated an increased sensitivity of IEC towards apoptosis upon treatment with DSS. Taken together, there is now compelling evidence from various experimental mouse models that excessive cell death in the intestinal epithelium is sufficient to induce intestinal inflammation. It is therefore tempting to speculate that a dysregulation of cell death pathways in IEC is involved in the pathogenesis of IBD in humans.

In line with this hypothesis, a large number of apoptotic bodies were found in colonic biopsies routinely taken from patients suffering from ulcerative colitis.[47 48] Additionally, several studies demonstrated increased apoptosis in patients with CD and also in patients infected with human pathogens like Salmonella, *Escherichia coli* and *Helicobacter pylori*.[22 49–52] Although, these studies implicated a role for excessive cell death in IBD pathogenesis, it remains unclear whether increased IEC death is a secondary event caused by the inflammatory environment or whether it could be a causative event as in the genetic mouse models described above. In favour of the first hypothesis, comparative studies in CD of non-inflamed and inflamed intestinal epithelium could demonstrate an increased number of apoptotic enterocytes in inflamed tissue compared with healthy tissue from the same patient.[49] However, no difference of the cell death rate was observed in uninvolved areas and normal intestine suggesting that the increased incidence of cell death in CD patients is triggered by the inflammatory reaction and therefore might be a secondary or contributing event.

Although most studies have linked apoptotic cell death to the pathogenesis of gastrointestinal disease, other forms of cell death like necrosis might influence intestinal inflammation.[34 53–55] Unfortunately, the physiological and pathophysiological role of necrosis in the gut is largely unknown. Until very recently, necrosis was considered a passive effect, secondary to cellular trauma like plasma membrane injury observed in intestinal ischaemia and infarction, as seen in patients with necrotising enterocolitis.[56] In general, necrosis can be observed under physiological stress, inflammation and infection and is caused by external environmental changes such as the occurrence of toxins, hypoxia, cytolysins and significant changes in temperature.[57–59] Necrosis might play an important pathogenic role in infectious gastrointestinal diseases caused by pathogens since several cytotoxic bacteria can kill host cells by

inducing necrosis.[34 60] Epithelial necrosis has also been observed in patients suffering from CD. In 1983, Dourmashkin *et al* described for the first time the detection of necrosis in the gut of CD patients.[61] This was shown in an electron and light microscopic study of rectal biopsy specimens taken from patients with CD, ulcerative colitis or controls. The authors reported necrotic epithelial cells within the colon of patients with CD. Moreover, they demonstrated that this form of cell death was also present in areas without acute inflammation supporting the notion that increased levels of necrotic cell death might be a primary mechanism for the development of inflammation rather than a secondary effect of CD. In 1999, a study by Barkla and Gibson presented evidence pointing to a physiological role of necrosis in the human large intestine.[53] The authors used biopsies of the colon and analysed them histologically for different cell death modes. They observed that shedding cells located at the surface of the epithelium undergo apoptosis, as described in the literature, whereas epithelial cells located within the crypts demonstrated necrotic features. Moreover, the authors reported necrotic cell death in patients without active IBD, an unexpected finding demonstrating that necrosis of epithelial cells occurs more frequently than previously expected. However, whether necrosis of IEC represents a physiological form of cell death in the colon or is associated with intestinal inflammation remained elusive. In a more recent study, necrosis of Paneth cells in the terminal ileum has been linked to the pathogenesis of IBD.[32] This study demonstrated crypts in the inflamed terminal ileum of CD patients with dying cells showing features of necrosis, including mitochondrial swelling and extensive vacuole formation. In contrast, classical features of apoptosis like cell blebbing and nuclear fragmentation were rarely observed in the lower part of the crypt. Necrotic cell death of Paneth cells at the crypt base in IBD patients might explain decreased production of antimicrobial peptides and other Paneth cell defects described in patients with IBD.[62]

Despite compelling evidence that in addition to apoptosis necrosis is involved in the physiology and pathophysiology of the gut, little data are available on how necrosis of IEC might stimulate gut inflammation.[34 53–55] Similar to apoptosis, necrotic cell death might compromise the epithelial barrier and allow infiltration of bacteria from the lumen into the bowel wall. Necrotic cells might also sustain inflammation by releasing endogenous proteins that cause tissue damage in the environment leading to the recruitment of granulocytes and other immune cells.[58]

NECROPTOSIS: WHEN APOPTOSIS MEETS NECROSIS

In addition to necrosis and apoptosis, a new mode of cell death has recently been described, termed necroptosis.[63–65] Necroptosis shares with necrosis the fact that dying cells show the morphological features of necrosis but not of apoptosis.[23 66] On the other hand, necroptosis differs from necrosis as a passive mode of cell death as it shares with apoptosis the fact that it is highly regulated by an intracellular protein platform.[59]

The discovery of necroptosis goes back to studies on the physiological relevance of apoptosis in vivo using mice in which relevant genes had been deleted by homologous recombination. It was found that germline deletion of either caspase-8 or FADD, two molecules essential for the extrinsic apoptosis pathway, was associated with early embryonic lethality.[67 68] The phenotype of these mice demonstrated that, despite their important role in inducing apoptosis, caspase-8 and FADD might in fact have pro-survival functions in certain tissues and under certain conditions.[69–76] This finding represented an unresolved mystery since deletion of pro-apoptotic molecules had been expected to promote survival rather than death. Interestingly, mice with a deletion of caspase-3, the most important downstream effector of caspase-8 and FADD, showed a decreased apoptosis frequency in the brain but did not show embryonic lethality as observed in mice lacking caspase-8 or FADD.[77–80] Similarly, mice deficient for cell surface receptors, important for the induction of apoptosis, including the TNF receptor (TNFR), did not result in a phenotype comparable with FADD or caspase-8 deficient mice.[81] The finding that FADD and caspase-8 were essential for embryonic development but molecules up or downstream were not indicated a death receptor- and caspase-3-independent function of caspase-8 and FADD during embryogenesis.

The concept of regulated apoptosis and unregulated necrosis was challenged when it was discovered that TNF stimulation can trigger different ways of cell death.[57 69 82–86] One type of cell death was associated with the typical morphological features of apoptosis characterised by apoptotic body formation, pseudopode retraction, chromatin condensation, pyknosis and blebbing of intact plasma membranes. However, under certain experimental conditions and in certain cell lines, TNF stimulation could also result in cell death with signs associated with necrosis, including organelle swelling, extensive vacuole formation and intact condensed nuclei.[82 87 88] Thus, despite the paradigm that necrosis occurs in a rather passive and unregulated manner, these studies demonstrated that necrosis could actually be induced by TNFR signalling in certain cells. It is important to note that death receptor-induced necrotic cell death had only been observed when apoptosis was inhibited by blocking of caspase activity, therefore questioning the physiological relevance of this form of cell death.[84 87 89] In 2003, Chan and colleagues demonstrated on the molecular level that the kinase receptor-interacting protein 1 (RIP1) was critically involved in this apoptosis-independent TNF-induced cell death and for the first time connected the terms 'necrosis' and 'programmed' to programmed necrosis.[69] Programmed necrosis was then later denoted necroptosis.[63]

Support for a physiological relevance of necroptosis in vivo came from studies demonstrating that the severe embryonic phenotype of caspase-8 and FADD deficient mice was directly mediated by RIP1 and by another cellular kinase, RIP3.[72 90] The authors had generated mice lacking caspase-8 or FADD together with RIP1 or RIP3, respectively. In contrast to the lethal phenotype of caspase-8 or FADD deficient mice, caspase-8/RIP3 or FADD/RIP1 double mutant mice were born without exhibiting any gross phenotype. Using in vivo stimulation experiments, these studies demonstrated that cells from these mice were resistant to death receptor-induced cell death. In summary, these in vivo studies for the first time demonstrated that the supposed pro-survival function of FADD and caspase-8 is to control and regulate RIP mediated cell death during embryonic development.

The discovery that necrosis is not necessarily a passive process but can be induced under certain conditions involving an intracellular signalling pathway raised the question which molecular mechanisms regulate necroptosis versus apoptosis. Interestingly, it turns out that there is a high degree of overlap in the molecules that regulate apoptosis and necroptosis.[59 65 91–95] Under physiological conditions, TNFR ligation results in the assembly of one of the following complexes: the TNFR complex I which mediates survival functions and involves the molecules TRADD (TNFR type 1 associated death domain protein), TRAF2/5 (TNFR associated factor 2/5), cIAP1/2 (cellular inhibitor of apoptosis 1/2) and the polyubiquitinated RIP1.[96] The formation of this complex represents the apical stimulus for the canonical NFκB activation pathway and therefore promotes cell survival[97] (figure 2). In contrast, dependent on the cell type, cell activation state as well as environmental influences TNFα binding can induce formation of an alternative TNFR complex, the TNFR complex II, better known as the death inducing signalling complex (DISC).[59 65] TNFR complex II promotes apoptosis and is composed of the molecules FADD, TRADD and caspase-8[98] (figure 2). Recent data now suggest that the TNFR complex II triggers apoptosis and induces and regulates necroptosis upon recruitment of RIP1 and RIP3.[59 86 99–102] According to this model, RIP1 is initially deubiquitinated in an enzymatic step involving the deubiquitinase Cyld by a yet to be identified stimulus.[59 103 104] RIP1 can then translocate to the TNFR complex II and bind through the RIP homotype interaction motif, RIP3.[96 105] Studies have demonstrated that under steady state conditions, caspase-8 controls RIP1 and RIP3 activity by proteolytic cleavage, thereby blocking necroptosis.[86 106 107] In addition, Cyld itself is a substrate of caspase-8. Following TNFα stimulation, caspase-8 cleaves Cyld,[108] thereby preventing the deubiquitination of RIP1, resulting in the association of ubiquitinated RIP1 with the prosurvival complex.[108] However, when caspase-8 is inactivated by gene deletion or by pharmacological methods, complex II can no longer prevent the activation of RIP1 and RIP3 resulting in autophosphorylation of the latter kinases and finally to induction of necroptotic cell death by a yet to be defined mechanism (figure 3).[65] Expression of RIP1 and RIP3 is considered to be essential for the execution of necroptosis and RIP3 has been shown to determine the sensitivity of cells towards necroptosis.[59 99]

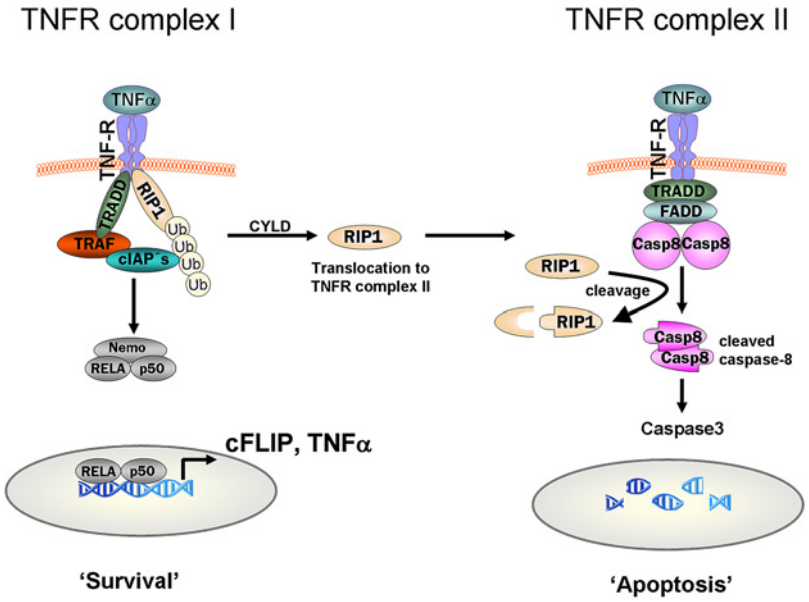

Figure 2 Model of TNFR mediated NFκB activation and extrinsic apoptosis. TNFα binding results in the assembly of the TNFR complex I, including TRADD, TRAF, cIAP and polyubiquitinylated RIP1. Assembly of this multiprotein complex represents the apical stimulus for the canonical NFκB pathway. Deubiquitination of RIP1 through CYLD contributes to the release of this kinase from complex I and translocation to the TNFR complex II, resulting in the assembly of the DISC containing TRADD, FADD, the deubiquitinated RIP1 and a homodimer of caspase-8 (casp8). Under apoptotic conditions, casp8 is activated which leads to the release of a homodimeric catalytic domain into the cytoplasm and results in the initiation of the classical caspase cascade. cFLIP, cellular FLICE-inhibitory protein; cIAP, cellular inhibitor of apoptosis; DISC, death inducing signalling complex; FADD, Fas associated protein with death domain; NFκB, nuclear factor kappa B; RIP, receptor-interacting protein; TNF, tumour necrosis factor; TNFR, TNF receptor; TRADD, TNFR type 1 associated death domain protein; TRAF, TNFR associated factor; CYLD, Cylindromatosis.

Collectively, there is now substantial evidence that activation as well as inactivation of caspase-8 can both lead to alternative modes of cell death, thus raising the question of how caspase-8 can inhibit necroptosis without at the same time inducing apoptosis. A possible explanation comes from recent studies on the cellular FLICE-inhibitory protein (cFLIP), an inhibitor of caspase-8. Similar to caspase-8, cFLIP contains two death effector domains, allowing it to bind to the DISC.[109–112] Moreover, cFLIP carries a caspase-like domain that is very similar to that of caspase-8, but lacks some active site residues, rendering it catalytically inactive.[113] cFLIP shares structural features with caspase-8, and can build a heterodimer with caspase-8 at the DISC.[114 115] cFLIP has been demonstrated to inhibit caspase-8 mediated apoptosis in a number of studies.[109 113–116] However, conflicting studies had also shown that cFLIP under certain conditions can actually induce cell death.[93 114 117 118] Recent data suggest that

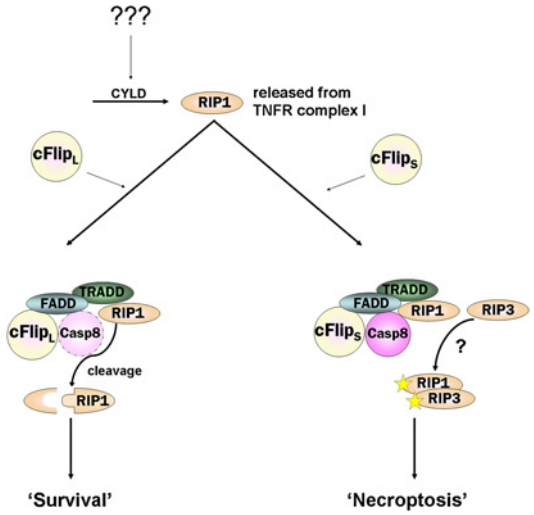

Figure 3 Necroptosis. cFLIP can act as a dominant negative regulator of caspase-8 (casp8). Heterodimers of cFLIP and casp8 are important mediators of survival and cell death. The impaired catalytic activity of the casp8/the long isoform of cFLIP ($cFLIP_L$) heterodimer is insufficient to induce apoptosis but still sufficient to prevent RIP3-dependent necroptosis by cleaving RIP1 and RIP3. In contrast, the short isoform of cFLIP ($cFLIP_S$) blocks casp8 activity in a way that prevents apoptosis induction but promotes necroptosis by suppressing RIP1 inactivation. As a consequence, RIP3 is recruited to RIP1, resulting in the phosphorylation of both RIP kinases. cFLIP, cellular FLICE-inhibitory protein; FADD, Fas associated protein with death domain; RIP, receptor-interacting protein; TNFR, tumour necrosis factor receptor; TRADD, TNFR type 1 associated death domain protein; CYLD, Cylindromatosis.

although cFLIP inhibits caspase-8 activation, the heterodimer of both still possesses some catalytic activity.[114 117 118] In this context, cFLIP allows the initial partial processing of caspase-8 into its 43kD proform but inhibits the final cleavage and release of the active enzyme into the cytoplasm and thereby blocks apoptosis.[109 119] Of note, it has recently been demonstrated that the partially impaired proteolytic activity of caspase-8 is sufficient to prevent RIP-dependent necroptosis by cleaving RIP1.[93 107 120] Moreover, it has been shown that under physiological conditions the caspase-8/cFLIP heterodimer assembles with a higher preference and exhibits an increased stability and therefore represents the preferred form.[118] Consequently, cFLIP might have a key role as an inhibitor of apoptosis as well as necroptosis. This is supported by studies demonstrating early embryonic lethality in mice with genetic ablation of cFLIP.[120]

Interestingly, cFLIP can be expressed at two different isoforms.[115] In addition to this long isoform ($cFLIP_L$), a shorter isoform exists ($cFLIP_S$) that contains only the two death effector domains but not the pseudocaspase domain present in the long form.[115] It has been shown that $cFLIP_S$ unlike $cFLIP_L$ additionally prevents the initial cleavage step of procaspase-8 and therefore cannot contribute to the inactivation of RIP1.[95 116] RIP1 and RIP3 become phosphorylated and can promote necroptosis.[95] Thus, while $cFLIP_L$ inhibits both apoptosis and necroptosis, $cFLIP_S$ might even trigger necroptosis. The role of c-FLIP in the regulation of necroptosis in vivo and especially in the gut has yet to be investigated. However, it has been shown that both isoforms of cFLIP are upregulated in biopsies taken from patients with CD or ulcerative colitis when compared with control patients.[121] The authors of this study reported that in normal gut cFLIP is rapidly degraded by the proteasome pathway, whereas in inflamed gut of CD and ulcerative colitis patients, a reduced degradation of $cFLIP_L$ and $cFLIP_S$ was observed.[121] Therefore, it is tempting to speculate that upregulation of $cFLIP_S$ in human IEC might inhibit caspase-8 activation, resulting in the activation of necroptosis. However, further studies have to focus on the expression of the different isoforms in the gut of IBD patients.

A CASE FOR NECROPTOSIS IN IBD?

Although there is now compelling evidence that necroptosis occurs in vivo, little is known on whether this cell death pathway is involved in pathophysiological processes. Two recent studies however have demonstrated that necroptosis of IEC can lead to intestinal inflammation with features, similar to IBD in humans.[32 33] By generating conditional knockout mice the authors demonstrated that deletion of FADD or caspase-8 specifically in IEC results in spontaneous intestinal inflammation associated with immune cell infiltration and enhanced cytokine levels. An interesting feature of caspase-8 as well as FADD deficient mice was a complete absence of Paneth cells and decreased expression of antimicrobial peptides in these mice. The authors could further demonstrate that Paneth cells do develop in the absence of caspase-8 but undergo necroptosis in vivo. Similar to embryonic lethality described above in caspase-8 knockout mice, Paneth cell necroptosis was dependent on RIP1 and RIP3, suggesting that RIP kinases have important regulatory functions in the gut. The afore-mentioned studies also indicate that Paneth cells might be specifically sensitive to this mode of cell death. The underlying cause of this sensitivity is yet to be defined. Low levels of caspase-8 expression or activity in Paneth cells can be hypothesised. Alternatively, constitutive expression or activation of RIP1 and RIP3 in Paneth cells might provide an explanation for this observation. Indeed, one study demonstrated a high level expression of RIP3 in Paneth cells both in humans and in mice.[32] In summary, recent studies have demonstrated that a deregulation of the expression of molecules involved in apoptosis and necroptosis can lead to excessive epithelial cell death and intestinal inflammation. Although this has not been formally proven, enhanced necroptosis of Paneth cells is likely to cause barrier defects and invasion of bacteria into the bowel wall.

It has been hypothesised that abnormalities of Paneth cells might contribute to the development of CD.[10 122–124] Indeed, biopsies taken from the terminal ileum of patients suffering from CD demonstrate dying cells at the crypt base, with signs of necrosis.[32] Comparable with the murine situation, the necroptosis mediator RIP3 was constitutively located in human Paneth cells,

implicating a potential role for necroptosis in the pathological changes observed in the small intestine of CD patients. The presence of RIP3 in Paneth cells as well as the fact that CD patients display necrosis at the crypt base suggests a model in which Paneth cells undergo necroptotic cell death.

Interestingly, although FADD and caspase-8 are functionally dependent on each other, the phenotypes of the conditional IEC-specific knockout mice slightly differed.[32 33] While mice lacking FADD developed both colitis and ileitis,[33] caspase-8 deficient mice showed ileitis but no colitis.[32] Different housing conditions and composition of the microbiota between the animal facilities might explain this observation. However, this finding might also hint at additional caspase-8-independent functions of FADD in controlling epithelial cell death and inflammation in the colon. The colonic inflammation of FADD deficient animals could be rescued by an additional deletion of Cyld (a deubiquitinase that regulates necroptosis downstream of TNF), MyD88 or by treating these animals with antibiotics, suggesting that colitis development is induced by bacteria.[33] However, this was not the case for the enteritis and the Paneth cell depletion observed in FADD deficient mice that occurred independent from the presence of the commensal flora.[33] Further studies will be needed to understand these different observations.

Necroptosis, like apoptosis, can be induced by TNFα and other death receptor ligands.[101] TNFα is a cytokine produced by macrophages, Th17 cells and also by IEC themselves.[125–128] The latter mentioned cytokine has been shown to influence and regulate inflammatory processes and it affects the integrity of the IEC barrier by inducing cell death in IEC.[129 130] Moreover, TNFα is considered as an important contributor to the pathogenesis of intestinal inflammation and patients suffering from CD or ulcerative colitis show increased levels of TNFα.[131–136] Additionally, genetic studies identified a chromosomal region that encompasses the TNFα gene as an IBD susceptibility locus.[137–139] The pathogenic function of TNFα in the inflammatory processes of CD patients is further underlined by the therapeutic effects of anti-TNF treatment using biological drugs.[140–143] The capacity of the cytokine to regulate survival and death of epithelial cells might provide an explanation for the connection between TNF signalling in the gut and the development of IBD. Although necroptosis was discovered upon treatment of cells with TNF, the role of TNF in necroptosis regulation in the intestinal epithelium is currently under debate. TNF has been shown to induce more severe damage in the gut of conditional caspase-8 deficient animals than in wild-type mice, suggesting that TNF is a strong inducer of necroptosis in the intestinal epithelium.[32] Although TNF deletion could strongly ameliorate the colitis phenotype associated with necroptosis in conditional FADD deficient mice,[33] Paneth cell death and enteritis were not affected, suggesting that another inducer might be responsible for Paneth cells necroptosis under steady state conditions. Thus, although TNF triggers necroptosis in the intestinal epithelium, other stimuli could provide additional stimuli and these might differ between the small and large bowel. In line with this conclusion, it has been shown that mice deficient for RIP3 are protected against TNFR-induced cell death but in a tissue-specific manner.[31] In this study, the authors analysed the contribution of necroptosis in the systemic inflammatory response syndrome and demonstrated that in contrast to wild-type mice that died after TNFα administration, RIP3 deficient mice were completely protected and survived. Surprisingly, although the liver of RIP3 deficient mice was protected against cell death and associated tissue damage, RIP3 deficiency did not influence tissue damage in the gut. In contrast, deletion of caspase-3 had no impact on the lethal course of experimental systemic inflammatory response syndrome but protected against gut damage indicating that TNF might induce necroptosis in the liver of wild-type mice while apoptosis induction dominated in the gut.

Collectively, accumulating evidence demonstrates that necroptosis is an alternative mode of cell death in the gut and that dysregulated necroptosis can drive intestinal inflammation. The finding that an inflammatory mediator like TNFα is a strong inducer of necroptosis supports the view that a dysregulated immune response and epithelial cell death might constitute a vicious cycle that leads to the perpetuation of intestinal inflammation as seen in patients with IBD. Currently, to our knowledge, no study has demonstrated an involvement of necroptosis-related genes as IBD-linked genes in genetic studies. However, many of these proteins are regulated at the post-transcriptional and post-translational level. More studies will be needed to verify whether necroptosis is involved in the physiology of the gut and in the pathogenesis of intestinal inflammation in humans.

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