Published in final edited form as:

Immunol Rev. 2017 November ; 280(1): 57–73. doi:10.1111/imr.12602.

Inflammatory cell death in intestinal pathologies

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Summary

The intestinal tract is a site of intense immune cell activity that is poised to mount an effective response against a pathogen and yet maintain tolerance towards commensal bacteria and innocuous dietary antigens. The role of cell death in gut pathologies is particularly important as the intestinal epithelium undergoes self-renewal every four to seven days through a continuous process of cell death and cell division. Cell death is also required for removal of infected, damaged and cancerous cells. Certain forms of cell death trigger inflammation through release of damage associated molecular patterns (DAMPs). Further, molecules involved in cell-death decisions also moonlight as critical nodes in immune signaling. The manner of cell death is, therefore, highly instructive of the immunological consequences that ensue. Perturbations in cell death pathways can impact the regulation of the immune system with deleterious consequences. In this review, we discuss the various forms of cell death with a special emphasis on lytic cell death pathways of pyroptosis and necroptosis and their implications in inflammation and cancer in the gut. Understanding the implications of distinct cell death pathways will help in the development of therapeutic interventions in intestinal pathologies.

Keywords

pyroptosis; necroptosis; cell death; colitis; colorectal cancer

Intestinal tract- an immunological tight rope

The intestinal tract presents an environment that experiences multitude of antigenic stimuli from microbial, dietary and environmental products. A single layered intestinal epithelium separates the underlying immune system from these potent triggers to maintain an equanimous environment. The immune system must apprehend and respond to pathogenic stimuli while maintaining tolerance against non-pathogenic antigens. This equilibrium between inflammatory and tolerogenic immune response needs to be perfectly calibrated and unregulated tipping of this balance can lead to deleterious consequences, like inflammation and cancer. The host employs several mechanisms to restrain unwarranted immune activation and yet mount an appropriate immune response when required. First, a sparsely colonized 50–100 μ m mucus layer termed as the “demilitarized zone” physically separates

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The authors have no competing financial interests.

microbiota and the epithelium. Second, the immune system that resides within the intestinal tract has through multiple mechanisms acquired a tolerogenic phenotype towards non-pathogenic stimuli. Third, the host is armed with several anti-microbial mechanisms ranging from secretory antibodies (IgA) to anti-microbial peptides and phagocytosis to neutralize a pathogen.

Many lines of evidence suggest a causal relation between inflammation and colorectal cancer (CRC). Firstly, patients with Inflammatory bowel disease (IBD) are at a significantly higher risk for development of CRC (1, 2). Secondly, multiple anti-inflammatory drugs such as anti-TNF antibodies and non-steroidal anti-inflammatory drugs (NSAIDs) reduce the incidence of CRC in human patients (3, 4). Thirdly, immune cells frequently infiltrate the tumor tissue and surrounding microenvironment. Immune cells can modulate tumorigenesis by secreting factors such as pro-inflammatory cytokines, matrix metalloproteases (MMPs), growth factors, and reactive oxygen species (ROS) that can promote DNA damage, aberrant cellular proliferation, angiogenesis, and metastasis (Figure 1) (5). Coordinated processes of cellular proliferation, migration, and angiogenesis are also involved in wound healing and injury repair, and tumors are therefore considered as “wounds that do not heal” (6).

The innate immune system forms a critical line of defense that needs to respond to a diverse range of stimuli, mount a rapid and dynamic response to combat an insult, and promote tissue repair once the deleterious agent has been eliminated. The innate immune cells distinguish between states of “alarm” and “resolve” through an array of germline encoded pattern recognition receptors (PRRs) that recognize conserved molecular patterns. The ligands for PRRs fall into two categories- pathogen associated molecular patterns (PAMPs) that represent microbial triggers and danger associated molecular patterns (DAMPs) that include host components released during cellular stress or damage. The PRRs are widely distributed within a cell to provide immune surveillance against extracellular, endocytic and cytosolic stimuli. The membrane bound Toll Like Receptors (TLRs) recognize extracellular or endosomal ligands and induce transcriptional upregulation of inflammatory genes like cytokines and chemokines. Cytosolic sensors that include nucleotide-binding oligomerization domain and leucine-rich repeat containing receptors (NLRs), the AIM2-like receptor (ALRs) and retinoic acid inducible 1 (RIG-I)-like receptors (RLRs) amongst others provide immune surveillance within the cytosol. Some NLRs and ALRs assemble into multimeric complexes called inflammasomes that activate the cysteine protease, caspase-1. Activation of caspase-1 through the inflammasome complex provides innate cells two functional outputs - Gasdermin-D (GSDMD) mediated inflammatory cell death termed as pyroptosis, and cleavage and release of pro-inflammatory cytokines, IL-1 β and IL-18. Pyroptosis also leads to release of alarmins such as IL-1 α and HMGB1 (7). The inflammasome activation, therefore, leads to removal of infected and/or damaged cells and production of inflammatory mediators that together combat the infectious or sterile insult.

Cell death pathways

Cell death has been observed as a pathological feature within inflamed tissue and was largely considered to be a consequence of inflammation. Recent studies have however identified cell death as an instigator and modulator of inflammation. Not surprisingly, cell

death is a complex, well-regulated process with huge implications under both homeostatic and pathophysiological conditions. While earlier literature considered apoptosis as an “immunologically silent” and coordinated event, necrosis was considered as incidental cell death with no defined molecular mechanisms. Recent studies have however clarified this long-held misconception and multiple forms of regulated necrotic cell death pathways such as necroptosis, ferroptosis, and NETosis have been defined (8). Necroptosis and pyroptosis involve the formation of pores in the cell membrane (9) that leads to loss of osmotic potential, cytoplasmic swelling, and the release of intracellular contents. The accompanying release of DAMPs provides a pro-inflammatory characteristic to these forms of cell death (Figure 2).

Apoptosis

Apoptosis is considered an immunologically silent form of programmed cell death that can be triggered by cell-intrinsic factors such as DNA or mitochondrial damage, and extrinsic factors such as engagement of death-receptors. Intrinsic apoptosis utilizes caspase-9 as the initiator caspase, while the extrinsic pathway uses caspase-8. Apoptosis ultimately involves activation of executioner caspases, caspase-3, and caspase-7 that cleave the cellular contents and, neutralize their inflammatory potential until scavenging cells can phagocytose the dying cell. Unsurprisingly, aberrations in the apoptotic pathways can lead to increased inflammation due to the breakdown of the epithelial barrier, or cancer due to non-removal of neoplastic cells (reviewed in (10, 11)). Apoptosis is also involved in the killing of cancer cells by chemotherapeutic drugs and radiotherapy (reviewed in (12)).

Pyroptosis

Pyroptosis is induced in response to caspase-1 activation within the inflammasome complex and caspase-11 (caspase-4/5 in humans) activation following intracellular LPS recognition. Caspase-1, and caspase-4/5/11 induce pyroptosis through cleavage and oligomerization of GSDMD (13–16). Caspase-1/4/5/11 can, therefore, be considered as initiator caspases, while GSDMD acts as the executioner molecule. GSDMD has a caspase cleavage site that releases its active N-terminus subunit, allowing it to undergo oligomerization, insert into the cellular membrane and form pores (10–14nM in size) (13–16). The formation of pores leads to loss of osmotic potential, cytoplasmic swelling, and release of intracellular contents. Pyroptotic cell death can be inhibited by glycine that restricts loss of osmotic potential and consequent death (9, 17). Expression of N-terminus domain of GSDMD is sufficient to induce pyroptosis in mammalian cells (13–16) and death in bacterial cells (15, 16). GSDMD induced pyroptosis further allows release of active forms of cytokines, IL-1 β and IL-18, and alarmins such as IL-1 α (13, 14).

GSDMD belongs to a family of proteins that share 45% sequence homology, and the active N-terminus domain is conserved amongst most of its members. The human genome encodes for GSDMA, GSDMB, GSDMC, GSDMD, GSDME (DFNA5) and DFNB59, and all but DFNB59 have the N-terminus domain that can potentially induce cell death in mammalian cells (13, 15). The expression of the GSDM family members is specifically localized to the epithelium of skin and gastrointestinal (GI) tract (18). Further, GSDMD and GSDMC are differentially expressed along the proximal-distal axis within the GI tract with highest

expression observed in the duodenum (18). Within esophageal and gastric cancer cells, the expression pattern suggests that *GSDMA*, *GSDMC*, and *GSDMD* could be potential tumor suppressors while *GSDMB*, which was overexpressed, could be an oncogene (19). Through a similar strategy of expression and promoter (methylation pattern) analysis in colorectal cancer (CRC) cell lines, *GSDME* was identified as a tumor suppressor and molecular marker for colon cancer (20, 21). The exact consequence of the expression pattern or the role of these homologues in cell death and GI pathologies has not been defined yet. Any deductions regarding the role of pyroptosis in gut pathologies are extrapolated from the role of inflammasome components. Pyroptosis is required for inflammasome mediated cell death and release of IL-1 β and IL-18 *in vitro* (13, 14), it remains to be seen whether similar dependence on pyroptosis or GSDMD (or another GSDM molecule) exists *in vivo* during intestinal pathologies. Further, whether inflammasome mediated cell death and cytokine release have distinct immunological consequences remains unknown. Identification of the pyroptosis executioner during *in vivo* pathologies will allow the investigators to delineate the effects of cell death versus cytokine release following inflammasome activation.

A recent study demonstrated that GSDME-expressing cells undergo pyroptosis in response to chemotherapy through caspase-3 mediated activation of GSDME (22). This switch from apoptotic to pyroptotic cell death has inflammatory implications as *Gsdme*^{-/-} mice were protected from chemotherapy (cisplatin) induced tissue damage and weight loss (22). This suggests that the GSDM molecules may be downregulated by cancer cells to evade cell death and that promoting their expression could overcome resistance to chemotherapeutic drugs.

Necroptosis

Receptor interacting protein kinase 1 (RIPK1) acts as a molecular switch between apoptosis and necroptosis forms of cell death. This process has been well described with respect to tumor necrosis factor receptor 1 (TNFR1) mediated signaling (23, 24). In response to trimeric TNF ligand binding, RIPK1 and TNFR1 associated death domain protein (TRADD) are recruited to TNFR1 (25, 26). TRADD recruits the E3 ubiquitin ligases, cellular inhibitors of apoptosis 1 and 2 (cIAP1/2) using TNF receptor associated factor 2 (TRAF2) as the scaffold (25, 26). RIPK1 ubiquitination by cIAP mediates activation of NF κ B activation through Tumor growth factor β -activated kinase (TAK1) and IKK α / β axis, to promote cell survival. However, inhibition or degradation of cIAPs leads to de-ubiquitination of RIPK1 by deubiquitinases, cylindromatosis (CYLD) and A20 and switch in its function from cell survival to cell death (27, 28). While TNF by itself does not induce cell death, inhibition of transcription or translation promotes cell death, suggesting that NF κ B mediated gene transcription (and translation) is required to actively ward off cell death (29, 30). Similarly, treatment with second mitochondrial derived activator of caspase (SMAC)-mimetic drugs that rapidly degrade cIAPs also sensitizes cells to TNF mediated cell death (29, 30). Following cell death trigger, RIPK1 binds to FADD and recruits procaspase-8 to form complex II. Once caspase-8 is activated, it induces apoptosis and simultaneously cleaves RIPK1 to halt the function of this kinase (29, 30). However, if caspase-8 activity is inhibited, RIPK1 transitions to induce a distinct form of cell death termed as necroptosis (31–34). During necroptosis, RIPK1 interacts with another kinase, receptor interacting

protein kinase 3 (RIPK3) through RIP homotypic interaction motif (RHIM) mediated interaction, resulting in the activation and auto-phosphorylation of RIPK3 (31–34). RIPK3 binds to and phosphorylates a pseudokinase, Mixed lineage kinase domain- like protein (MLKL), which acts as the executioner of necroptosis. In response to RIPK3 mediated phosphorylation, MLKL undergoes oligomerization and membrane translocation to induce membrane depolarization and cell death (32). While the above-described process is canonical for TNF induced cell death, multiple other stimuli including LPS or dsRNA that signal through TRIF (a RHIM containing protein) can directly engage RIPK3 to induce necroptosis (Figure 3) (35). Most studies that define a biological role for necroptosis are derived from the genetic loss of pro-survival or apoptotic molecules, suggesting that necroptosis is stringently inhibited during homeostatic conditions.

Cell death has multiple forms and functions. In response to sterile/infectious insults, cell death can be a marker for cell damage, involved in removal of damaged or neoplastic cells, induce or be a consequence of inflammation. Despite distinct triggers, initiators and executioners, certain aspects of morphology are shared between the above discussed forms of cell death. Pyroptosis and necroptosis proceed through formation of pores on the cell membrane, making loss of cell membrane integrity a characteristic of both (9). Apoptotic features of caspase- 3 and –7 cleavage (36, 37), and DNA fragmentation (9, 36, 38) are also observed during pyroptosis. Therefore, while cell death can be assessed through multiple markers, including Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining or caspase cleavage, it is hard to identify the form of cell death occurring *in vivo* based on these markers alone. Information regarding the nature or function of various forms of cell death can be gleaned from critical dependence on molecules involved in each pathway (Table 1).

Inflammasomes in Intestinal pathologies: Implications for pyroptosis

The family of cytosolic receptors that engage in inflammasome platforms includes NLRs, ALRs and Pyrin. The NLR family is characterized by N-terminus pyrin domain (PYD) or caspase activation and recruitment domain (CARD) and C-terminus leucine repeat rich (LRR) motif. This family includes extensively characterized inflammasomes such as NLRP3, NLRC4, NLRP1 and other putative inflammasome forming proteins such as NLRP6, NLRP12, and NLRP9b (39). Other molecules such as NLRP6 (40), and NLRP12 (41, 42) also have signaling functions independent of the inflammasome assembly. The ALR family is named after its conventional member, Absent in melanoma 2 (AIM2) which consists of N-terminus PYD and C-terminus hematopoietic interferon inducible nuclear proteins with 200 amino acid repeat (HIN200) domain. Within this family, AIM2 is distinct in terms of lacking a nuclear localization domain and forming a well-defined inflammasome. Other ALR members interact with stimulator of interferon genes (STING) or ASC to regulate interferon and inflammasome responses (43). Pyrin belongs to tripartite motif (TRIM) family of proteins, comprises of N-terminus PYD in addition to B-box, coiled coil and B30.2 domains.

All inflammasome complexes employ homotypic PYD-PYD or CARD-CARD interactions to assemble into multimeric complexes within which caspase-1 can undergo autocatalytic

activation to enforce downstream functional outputs of cell death and cytokine maturation. Mechanisms of inflammasome activation and regulation have been covered elsewhere (7) and in this review, we focus on the consequence of inflammasome activation and inflammatory cell death in intestinal pathologies.

Inflammasome activation is triggered in response to a wide range of endogenous and exogenous ligands. Some of these triggers are direct ligands such as dsDNA for AIM2 (44–47) and flagellin and type III secretion systems (T3SS) for NLRC4 (48–53). Other inflammasomes are activated in response to cellular perturbations such as Rho-inactivation by toxins for pyrin activation (54), NLRP1b cleavage by *B. anthracis* lethal factor (55), and multiple triggers such as K⁺ efflux, ROS production, and lysosomal disruption for NLRP3 activation (56–67). Several of these ligands and triggers can be presented in the intestinal microenvironment by infectious and sterile insults. Inflammasome activation is, therefore, a critical process during a pathological response. Expression analysis in human colorectal cancer samples has identified distinct patterns for several of these innate sensors. The expression of *NLRP3*, *NLRP1*, *NLRC4*, *AIM2*, and *NLRC3* was significantly reduced in CRC samples (68). Further, the expression level of AIM2 and *NLRC3* negatively correlated with cancer progression (68). It is, therefore, not surprising that multiple inflammasomes have been implicated in several intestinal pathologies as discussed below.

NLRP3

Triggers for activation of NLRP3 inflammasome include a wide array of microbial products such as microbial cell wall components- lipopolysaccharide (LPS) and muramyl dipeptide (MDP), nucleic acids, pore forming toxins and damage associated molecular patterns (DAMPs) such as ATP and uric acid crystals (63, 69–71). As the NLRP3 inflammasome assembles in response to a vast range of DAMPs, it is likely that it senses a common cellular distress signal induced by these molecules, instead of a direct interaction with all the ligands. NLRP3 activation is further regulated through mechanisms of transcriptional control and post-translation modifications (Figure 4, reviewed in (7)).

Mice lacking NLRP3, or common inflammasome components, ASC or caspase-1 have increased susceptibility to experimental colitis induced by oral administration of dextran sodium sulfate (DSS) or rectal administration of hapten 2,4,6-trinitrobenzenesulfonic acid (TNBS) (72–74). NLRP3 inflammasome activation protects mice from DSS-induced colitis by promoting epithelial barrier integrity, and NLRP3 activation in both hematopoietic and non-hematopoietic cells contributes to protection from DSS-induced damage (72, 74, 75). The loss of epithelial barrier integrity in *Nlrp3*^{−/−} is not associated with increased cell death as assessed by TUNEL staining (72). Consistent with colitis being a pre-disposing factor for colon cancer, NLRP3, ASC and caspase-1-deficient mice have increased tumor burden when subjected to the AOM-DSS model of colitis-associated colon cancer (CAC) which involves induction of DNA damage by azoxymethane (AOM), followed by repeated rounds of DSS administration to induce chronic inflammation (74, 76). Mice deficient in IL-18 signaling (*Il18*^{−/−} and *Il18r*^{−/−}) mirror this increased susceptibility to polyp development, while *Il1r*^{−/−} mice are like WT mice in susceptibility to CAC (76, 77). Further, the exacerbated colitis and epithelial hyperplasia phenotype in NLRP3-deficient mice is rescued by exogenous IL-18

supplementation (74, 76), highlighting the protective role of IL-18 in inflammasome deficient settings. NLRP3 also contributes to control of metastatic growth of colon cancer in the liver by promoting IL-18 mediated tumoricidal activity of NK cells (78). NLRP3 activation is also protective in colitis induced by another hapten, oxazolone, in a manner dependent on the production of both IL-1 β and IL-18 production (79).

In contrast to these studies, other studies have reported protection from DSS or TNBS-induced colitis or AOM-DSS induced CAC in mice deficient in the NLRP3 inflammasome components or IL-18 signaling (80–84). The discrepancies in the phenotypes observed by different groups could be due to differences in microbial composition in different animal facilities, precise genetic backgrounds of the knockout mice, and experimental protocols. *Nlrp3*^{-/-} and *Il18*^{-/-} mice have been suggested to harbor microbial composition distinct from WT mice, and cohousing the knockout mice with WT mice normalizes the colitis phenotype (73, 85). Colitis severity in these mice is also ameliorated by antibiotic treatment, suggesting a critical role of the microbiota composition in modulating immune responses during experimental colitis and colon cancer. Use of littermate controls, defined breeding and housing strategies and a thorough restriction of experimental variables is, therefore, required to determine the precise roles of any molecule in gut-microbial homeostasis, colitis, and colon cancer.

Commensal microbes can also harness inflammasome to modulate the immune tone in the gut. For example, colonization with protozoa *Tritrichomonas musculus* promotes inflammasome activation and IL-18 production in the colon (86). Increased IL-18 response promotes Th1 and Th17 differentiation to provide protection against enteric *Salmonella* infection but exacerbates T cell driven colitis and sporadic tumorigenesis (86). NLRP3, caspase-1 and IL-1 β axis also promotes enteropathy induced by non-steroidal anti-inflammatory drugs (NSAIDs) (87) and tumor growth after chemotherapy (88). Excessive inflammasome activation and IL-1 and IL-18 signaling has been shown to promote colitis (89–91), and biochemical or genetic ablation of NLRP3 and IL-1 β protects mice deficient in autophagy (92) or anti-inflammatory IL-10 signaling (93) from experimental colitis. Therefore, inflammasome activation must be precisely regulated to restrain colitis and colon cancer (Figure 5).

Caspase-11 and non-canonical NLRP3 inflammasome activation

Caspase-11 (caspase-4/5 in humans) is a binding partner and promoter of caspase-1 activation (94). The functional significance of caspase-11 was masked for a long time, since the earlier strain of caspase-1 deficient mice (generated on 129 background) also lacked caspase-11 due to high genetic linkage between the two genes (95). Caspase-11 was eventually identified as a mediator of the NLRP3 inflammasome activation by cytosolic LPS (95, 96). The CARD domain of caspase-11 binds directly to the lipid A component of LPS that is penta- or hexa-acylated (97). In naïve macrophages, caspase-11 expression and subsequent NLRP3 inflammasome activation is upregulated through TRIF-mediated signaling (96, 98). Consistent with its role as a cytosolic LPS sensor and NLRP3 inflammasome inducer, loss of caspase-11 is protective from LPS-induced septic shock (95).

Caspase-11 is essential for NLRP3 inflammasome activation and IL-1 β and IL-18 maturation in macrophages infected with enteric pathogens *E. coli* and *C. rodentium* (96, 99, 100). Caspase-4 is similarly required for IL-18 secretion from human myeloid and epithelial cells infected with *E. coli* or *Salmonella* (101, 102). Further, caspase-4 is required for epithelial cell expulsion and restriction of bacterial niches in response to enteric infections (101, 102). Inflammasome activation is protective from *C. rodentium*-induced colitis as mice deficient in caspase-11, caspase-1, NLRP3, IL-1 β or IL-18 have increased colonization upon infection with *Citrobacter*, concomitant with increased intestinal pathology (100). In line with these studies, macrophages deficient in nuclear-binding oligomerization domain-containing protein 2 (NOD2) or RIP2, that have increased ROS production and caspase-11 expression upon *C. rodentium* infection, also demonstrate increased NLRP3 inflammasome activation and IL-1 β and IL-18 secretion (103). Increased inflammasome activation protects the NOD2- and RIP2-deficient mice from *C. rodentium*-induced colitis (103).

Caspase-11 is also protective from DSS-induced colitis. Two studies suggested that caspase-11 deficient mice are defective in IL-18 maturation during DSS administration (104, 105). Like NLRP3, caspase-11 also exerts its protective influence in both hematopoietic and non-hematopoietic compartments, and exogenous IL-18 supplementation ameliorates disease severity (104, 105). However, caspase-11 deficiency did not affect AOM-DSS induced chronic colitis or associated tumorigenesis (104). Furthermore, another study showed that IL-18 maturation is controlled by caspase-1 but not caspase-11 (106). Instead, caspase-11 influences gut microbial ecology, which is characterized by decreased *Prevotella* and increased *Bacteroidetes* levels in *Caspase11*^{-/-} mice. However, despite having a normalized microbiota profile when co-housed with WT mice, caspase-11 deficient mice are still more susceptible to DSS-induced colitis (106). Therefore, further studies are needed to clarify the mechanisms by which caspase-11 protects from DSS-induced colitis.

NAIP-NLRC4

NLRC4 inflammasome is activated through sensing of flagellin and T3SS by NAIPs (NLR family apoptosis inhibitory protein) (48–53). The role of NLRC4 in CRC is controversial with independent studies demonstrating no role (3) or a protective role (107) in colorectal tumorigenesis. Loss of NAIPs1–6, specifically in epithelial cells leads to increased tumor burden that is associated with lower anti-apoptotic and higher proliferative response following AOM exposure (108). While no difference in inflammasome response is detected in the absence of NAIPs, tumor tissue isolated from NAIP1–6 deficient mice exhibits increased expression of pro-inflammatory cytokines, matrix metalloproteases (MMPs) and genes associated with tumor growth and invasion (108). In contrast to increased tumorigenesis, mice deficient in NAIP1–6 are protected from acute DSS-induced colitis (108). NLRC4 was also shown to play a protective role in DSS-induced colitis but not in response to spontaneous colitis induced by IL-10R blockade (109). Based on all these studies, it is hard to reconcile the exact role of the NAIP-NLRC4 inflammasome in colitis and CAC. Studies that simultaneously compare *Naip1*⁻⁶^{-/-} and *Nlrc4*^{-/-} mice with their respective littermate controls to equilibrate microbiota and genetic background variations would help determine the precise role of this inflammasome in intestinal disorders.

The importance of NAIP-NLRC4 axis in sensing flagella and T3SS components is underlined by its role in infectious enteritis during oral infection with multiple bacteria including *S. typhimurium* and *C. rodentium*. Loss of NLRC4 results in increased mortality in response to flagellated (but not aflagellated) *Salmonella* infection (110, 111). During oral infection, NLRC4 activation within the epithelium is sufficient to expel infected cells and restrict bacterial colonization (110, 111). This resistance to bacterial colonization is independent of IL-1 and IL-18 cytokines, implicating cell death as the mechanism that controls bacterial invasion (111). Another study, however, demonstrated that increased mortality in *Nlrc4*^{-/-} can be phenocopied by *Il1r*^{-/-} mice, with IL-1R signaling being protective in orogastric *Salmonella* infection through neutrophil recruitment (112). Systemic recognition of *Salmonella* or flagellin also induces hypothermia and diarrhea through eicosanoid production in response to NLRC4 activation (110, 113). Cytosolic delivery of flagellin (FlaTox) similarly triggers NLRC4 inflammasome activation and associated pathology (113). The pathology is associated with expulsion of epithelial cells through NLRC4, and redundant on caspase-1 and caspase-8 activation (110). Importantly, loss of Casp-1-GSDMD (in epithelial cells) axis prevents lytic death but not intestinal cell expulsion or intestinal pathology (110). Genetic loss of NLRC4 or ASC/Casp1/Casp11 protects against cell expulsion and inflammatory consequences (110). Therefore, while NLRC4 activation is required to restrict bacterial invasion, it also causes pathology through inflammatory cell death. NLRC4 also synergizes with NLRP3 to restrict bacterial burden and associated pathology during *C. rodentium* infection despite not being involved in inflammasome activation in macrophages (100). NLRC4 is required within the non-hematopoietic cells to restrict early colonization and innate response to *Citrobacter* infection (114). Mutations in NLRC4 are associated with enterocolitis and autoinflammation in humans (115–117) and incidence of severe neonatal enterocolitis was associated with a de novo mutation in the *NLRC4* gene (115). This mutation results in spontaneous ASC oligomerization, caspase-1 processing, cell death and release of inflammatory cytokines, IL-1 β and IL-18 (115). Regulation of NLRC4 activation is, therefore, critical during enteric infections and autoinflammatory disorders.

AIM2

AIM2 was identified as an IFN γ -inducible protein in a tumor suppressor screen and only later redefined as an inflammasome-forming ALR, specifically in response to dsDNA (44–47). Increased tumor burden is observed in the absence of AIM2 in multiple models of colon tumorigenesis including AOM-DSS (118, 119), and *Apc*^{min/+} mice (118). This tumor suppressor function of AIM2 is dominant in the non-hematopoietic cells. Unlike other inflammasome-forming sensors, the role of AIM2 in restraining CAC is independent of its role in inflammasome activation or production of inflammatory mediators (118, 119). Instead, it relies on the ability of AIM2 to control cellular proliferation by restraining Akt activation (118, 119). Further, increased cell proliferation in the colons of *Aim2*^{-/-} mice is also linked to decreased cell death as evidenced by lower cleavage of caspase-3 and caspase-7 (118, 119). Epithelial stem cells lacking AIM2 exhibit increased proliferation potential, both *in vivo* during AOM-DSS treatment and *ex vivo* during differentiation into epithelial organoids (119). Akt inhibition significantly reduces tumor burden in *Aim2*^{-/-} mice (118). Accelerated tumorigenesis, increased AKT activation and stem cell activity is

also observed in the absence of AIM2 in a small intestinal model of tumorigenesis induced in response to aberrant wingless-int (Wnt) signaling (119).

Other studies have however demonstrated that AIM2 functions as an inflammasome in the colon tissue to restrict inflammation during DSS-induced acute colitis (120). Increased colitis and CAC is associated with microbial dysbiosis observed in *Aim2*^{-/-} mice, and fecal transfer, treatment with antibiotics or co-housing with WT normalize the microbiota and equilibrate the colitis susceptibility in *Aim2*^{-/-} mice (120, 121). The authors also link lower inflammasome activation and IL-18 production in *Aim2*^{-/-} mice to increased IL-22BP and decreased anti-microbial peptides (AMP) production, predisposing *Aim2*^{-/-} mice to microbial dysbiosis and increased susceptibility to colitis (120, 121). Contrary to previous studies (118, 119), the authors did not observe any differences in epithelial cell death in the absence of AIM2 (120). While AIM2 is protective in CAC, its precise role in colitis varies depending on the genetic background and/or husbandry conditions. However, even under co-housed conditions (normalized microbiota), *Aim2*^{-/-} mice have increased tumor burden compared to the WT controls (119). Therefore, the synergy between microbiota and AIM2 mediated cell-proliferative signaling controls colitis and colon tumorigenesis.

NLRP1

The murine genome encodes for three paralogs (*Nlrp1a-c*) of the human *NLRP1* gene, of which NLRP1b forms an inflammasome platform in response to *B. anthracis* lethal toxin (55) and *Toxoplasma gondii* (122). Genetic deletion of *Nlrp1b* increases the susceptibility of mice to DSS-induced colitis and AOM-DSS induced colon tumorigenesis (123). Like AIM2, NLRP1b also functions in part by modulating the gut microbiota, such that co-housing transfers hyper susceptibility to WT mice and antibiotic treatment ameliorates disease in *Nlrp1b*^{-/-} mice (123). The hypersensitivity of *Nlrp1b*^{-/-} mice to DSS induced colitis is associated with reduced inflammasome output and supplementation with recombinant IL-18 and IL-1 β ameliorates the histological damage (123). Increased cell death, and impaired epithelial barrier function was observed in *Nlrp1b*^{-/-} mice following DSS-induced damage (123). NLRP1b also functions primarily within the non-hematopoietic compartment to restrict colon tumorigenesis (123). Genetic association studies have identified NLRP1 mutations linked to Crohn's disease that affect its auto-proteolytic cleavage and activity (124). Further studies into structural modifications and proteolytic activity of NLRP1 mutants would help delineate the molecular mechanisms involved in its activation and its functions in gut physiology.

NLRP6

NLRP6 is highly expressed in both small and large intestine of mice (125, 126) and in the small intestine of humans (127). *Nlrp6* expression is abundant in the intestinal epithelium, particularly within the enterocytes and goblet cells (125). Studies have characterized NLRP6 as a critical innate sensor in host defense primarily through its role in modulating NF κ B and MAPK activation, and type I interferon (IFN) production following bacterial and viral infections (40, 128). NLRP6 responds to viral RNA bound to DHX15, a RNA helicase, and interacts with mitochondrial adaptor protein, MAVS to induce production of type I IFNs and interferon-stimulated genes (ISGs). Mice deficient in NLRP6 are unable to restrict viral

loads in response to orogastric infection with encephalomyocarditis virus (EMCV) and murine norovirus (MNV) (128). However, the role of NLRP6 in regulating anti-viral immunity is restricted to the gut, as NLRP6 is dispensable for defense against systemic EMCV and MNV infections, highlighting the critical role of NLRP6 in enteric anti-viral responses (128).

Most studies implicating the role of NLRP6 as an inflammasome are derived from its role in mucosal responses. NLRP6 deficient mice are characterized as having a low level of spontaneous inflammation and epithelial hyperplasia that is exacerbated by induction of acute colitis by DSS administration (129, 130). This is a result of *Nlrp6*^{-/-} (and *Asc*^{-/-}) mice harboring a colitogenic microbiota as a function of impaired inflammasome activation. Further, *Nlrp6*^{-/-} and *Asc*^{-/-} mice can transfer colitis hyper-susceptibility to co-housed WT mice (129). Another study demonstrated that NLRP6 is expressed by the myofibroblasts within stem cell niches and is critically required for epithelial stem cell proliferation and migration, events necessary for reparative processes following mucosal damage induced by DSS (125). This function contributes to the susceptibility of *Nlrp6*^{-/-} mice to colorectal tumorigenesis (125). Another mechanism proposed for the anti-tumorigenic role for NLRP6 is restriction of CC-motif Chemokine Ligand-5 (CCL5)- IL-6 axis that promotes epithelial Signal Transducer and Activator of Transcription-3 (STAT-3) activation and proliferation (131). NLRP6 also functions in the inflammatory monocytes to protect from DSS induced colitis by promoting IL-18 production (132). Therefore, NLRP6 can function through multiple mechanisms to restrict colitis and CAC.

NLRP6 is also expressed in the goblet cells and is required for effective autophagy and associated mucus exocytosis (126). Consequently, *Nlrp6*^{-/-}, *Asc*^{-/-}, and *Caspase1/11*^{-/-} mice exhibit altered goblet cell function and lack a thick, continuous mucus layer (126). Abrogated mucus secretion into the lumen decreases the capacity of *Nlrp6*^{-/-} mice to clear enteric infections and resolve inflammation (126). This role of NLRP6 in the promotion of mucus barrier was confirmed in another study that demonstrated that NLRP6 activation in a specialized “sentinel goblet cell” is required to induce secretion of the mucin muc2, a major component of intestinal mucus layer (133). NLRP6 activation in this instance was triggered upon endocytosis of TLR ligands such as LPS and flagellin and mediated by reactive oxygen species (ROS) production (133). The authors, however, noted that NLRP6 and inflammasome deficient mice did not exhibit discontinuous inner mucus layer (133) like previously noted (126). In addition to microbial ligands, microbial metabolites have also been shown to modulate NLRP6 activation within the colonic microenvironment. Microbe-derived metabolite taurine was shown to positively engage NLRP6 activation, while histidine and spermine inhibit NLRP6 activation (134). Decreased IL-18 production due to abrogated NLRP6 activation leads to an altered AMP landscape that further promotes microbial dysbiosis and colitogenic response (134). Another study suggests that NLRP6 restricts abundance of mucin-degrading microbe, *Akkermansia muciniphila* through IL-18 production and thereby reduces spontaneous colitis in *Il10*^{-/-} mice (135). Surprisingly, the authors demonstrate that co-housing does not equilibrate *Akkermansia* colonization and colitis susceptibility. This study identified level of *Akkermansia* colonization as the critical determinant of colitis susceptibility in *Il10*^{-/-} mice and shows that the abundance of *Akkermansia* is actively regulated by NLRP6-IL-18 axis (135).

Contrary to above mentioned observation, a recent study did not find a role for NLRP6 in regulating the gut microbial landscape (in two geographically distinct facilities) or susceptibility to DSS-induced colitis (136). The authors found no differences in the fecal microbial landscape, or DSS-induced colitis in F2 *Nlrp6*^{-/-} and *Nlrp6*^{+/+} littermates even after the gut microbiota of these mice had the opportunity to evolve in the pre-weaning phase, and then for one year in separately housed setting (136). The observed changes in the microbial landscape were instead driven by cage and maternal co-variants (136). These studies underline the use of littermates as a prelude to studying the effect of a gene on microbial alterations and enteric immunity. The precise role and function of NLRP6, therefore, requires careful re-consideration.

NLRP12

NLRP12 was identified as a factor capable of modulating NF κ B activation and ASC speck formation (137). Although certain studies have suggested an inflammasome function for NLRP12 (138, 139), most studies have described an inhibitory role for NLRP12 in MAPK and non-canonical NF κ B activation (41, 42). The increased susceptibility to colitis and CAC in the absence of NLRP12 is linked to enhanced MAPK and NF- κ B signaling (41, 140). Expression of NLRP12 in both hematopoietic and non-hematopoietic cells is required to restrict inflammation, while it functions predominantly within the non-hematopoietic compartment to inhibit tumorigenesis (140). NLRP12 was shown also to interact with TRAF3 and NIK to restrain non-canonical NF κ B signaling (140). NLRP12 expression itself is downregulated during DSS induced inflammation through the TLR4-Blimp1 axis, perhaps to promote inflammatory response during colitis (141). A subsequent study showed that *Nlrp12*^{-/-} mice exhibit low-level of inflammation and decreased microbial diversity under homeostatic conditions, which predisposes them to DSS-induced colitis (142). NLRP12 functioned primarily in the hematopoietic compartment to restrain colitis and microbial dysbiosis (142). WT and *Nlrp12*^{-/-} mice raised under germ-free conditions have similar susceptibility to DSS-induced colitis, suggesting that the primary function of NLRP12 is to maintain a symbiotic gut microbial community (142). Antibodies against pro-inflammatory cytokines (IL-6 and TNF) and reconstitution with commensal *Lachnospiraceae* isolates could both correct colitis and microbial dysbiosis, highlighting the synergy between the immune system and the microbiota in determining susceptibility to colitis (142).

Contrary to its protective role in colitis and CAC, NLRP12 suppresses host immunity to enteric infections (42). The absence of NLRP12 leads to increased inflammatory signaling and anti-microbial activity in response to *Salmonella* infection, that promotes effective clearance (42). Therefore, NLRP12 mediated inhibition of inflammatory signaling can have both protective and pathogenic functions in enteric responses based on the inflammatory trigger.

NLRC3

Like NLRP12, NLR family CARD-containing 3 (NLRC3) has also been described as a non-inflammasome forming NLR that negatively regulates inflammatory and cell proliferative signaling (143–145). NLRC3-deficient mice have increased susceptibility to DSS-induced acute colitis, AOM-DSS induced colon cancer and sporadic intestinal tumorigenesis in the

Apc^{Min} background (145). Using cell-type specific NLRC3-deficient mice, authors demonstrate that NLRC3 functions primarily in the epithelial cells to protect from colon cancer. NLRC3 restrains aberrant epithelial cell proliferation by negatively-regulating the PI3K–mTOR pathway (145). NLRC3 also restricts the oncogenic potential through genes involved in cell cycle progression and stem cell proliferation during AOM-DSS challenge (146). *In vitro* studies suggest that NLRC3 reduces the activation of the PI3K p85 subunit by disrupting its association with the p110 α subunit (145). Further, reduced apoptotic cell death is observed in colons of *Nlr3^{-/-}* mice and could contribute to increased tumorigenesis (146). The implications of this negative regulation of the PI3K–mTOR pathway by NLRC3 in other intestinal or extra-intestinal disorders will be an interesting area of research.

NLRP9b

NLRP9b is a newly identified putative inflammasome sensor that mediates host defense against enteric rotavirus infection (39). Like NLRP6, NLRP9b is highly expressed in the epithelial cells of the small intestine and in a mechanism analogous to DHX15 and NLRP6 mediated ECMV and MNV detection, NLRP9b interacts with RNA-helicase DHX9 to induce anti-viral responses. Recognition of short dsRNA by DHX9 leads to recruitment of NLRP9b, which in-turn nucleates ASC to induce inflammasome formation, GSDMD activation, and IL-18 release (39). GSDMD-mediated pyroptosis induces death of the infected epithelial cells, thereby restricting viral replication (39). It remains to be seen if NLRP6 and NLRP9b have a concerted mechanism of activation against enteric viruses. Further, DHX9 has also been shown to interact with transcripts originating from Alu transposable elements (147). It is therefore unclear how RNA helicases and NLR complexes distinguish host RNA from viral RNA.

Caspase-8: Beyond apoptosis

Caspase-8 is a critical player in multiple distinct forms of cell death including apoptosis, pyroptosis, and necroptosis. Its activity must, therefore, be finely tuned to restrain unwarranted cell death. Caspase-8 (p55/53) has a prodomain that interacts with FADD and two catalytic subdomains that undergo sequential cleavage during activation. The linker between prodomain and catalytic subunits can be cleaved between Asp³⁷⁴ and Asp³⁸⁴ by caspase-8 itself to generate procaspase-8 (43/41) that is further processed into (p18)₂(p10)₂ heterodimer that induces apoptosis (148, 149). Processing of caspase-8 changes the range of downstream substrates and potency of its activity (150).

Within the intestinal epithelium, expression of caspase-8 increases in both the crypts and the villi between 5–15 weeks of age and it is constitutively present in its active form (p43) (151). Early enterocyte cell death, dependent on RIPK3 and morphologically characterized as necroptosis is observed with genetic loss of caspase-8 (152). Caspase-8 loss specifically within the intestinal epithelial cells (IEC)(Caspase-8^{IEC-KO}) also leads to spontaneous ileitis and increased susceptibility to DSS induced colitis in mice (153). Intestinal tissue from these mice have significantly reduced expression of antimicrobial peptides, and increased cell death-induced loss of paneth cells and goblet cells (153). Epithelial cell death in Caspase-8^{IEC-KO} mice is rescued by loss of TNF signaling or inhibition of necroptosis (153).

Increased cell death in paneth cells and increased expression of RIPK3 is also observed in the terminal ileum of caspase-8^{IEC-KO} mice and human patients with Crohn's disease highlighting the clinical relevance of necroptosis in IBD (153). Cellular FLICE-like inhibitory protein (cFlip) is a catalytically inactive homologue of caspase-8 that inhibits caspase-8 mediated apoptosis. IEC specific loss of cFlip leads to over activation of caspase-8 (and caspase-3), increased epithelial cell death and spontaneous inflammation within the intestinal tract (151). Unlike caspase-8^{IEC-KO}, c-Flip^{IEC-KO} exhibit increased epithelial cell death that is not mediated by RIPK3 or TNF signaling and could perhaps be due to increased apoptosis (not necroptosis) (151). Further, while c-Flip loss affects death in all intestinal cells, the loss of caspase-8 particularly induces death in paneth cells and goblet cells (153). Caspase-8 level and activity (both apoptosis and necroptosis) within the intestinal epithelium is, therefore, required to restrict cell death and inflammation during homeostasis.

Intestinal epithelial cells undergo shedding in response to microbial or viral infection to restrict "replication- niches" and induce an inflammatory response to control the infectious agent. Caspase-8 is critically involved in the process of epithelial shedding in response to TLR stimuli. Loss of caspase-8 specifically in the IEC leads to "switch" from apoptotic to necroptotic cell death, marked by epithelial cells that lack active caspase-3 but are TUNEL-positive (154). The necroptotic cell death is mediated by RIPK3 and leads to the loss of epithelial barrier integrity (154). While TLR4-induced necroptosis is dependent on TNFR1 expression on epithelial cells, TLR3-induced cell death is independent of TNFR1 or IFN λ (IL-28) signaling (154). TLR3 induced epithelial cell death is also independent of IL-1, IL-6, IL-15, BAX (bcl-2 associated X protein), MAVS (mitochondrial antiviral-signaling protein), PKR (ds-RNA activated protein kinase), IKK α/β (I κ kinase), IRF3 (Interferon regulatory factor 3), NK (natural killer) cells, dendritic cells and the adaptive immune system (155). Following dsRNA stimuli (through TLR3-TRIF axis), loss of caspase-8 triggers epithelial cell necroptosis to induce loss of epithelial barrier integrity, systemic microbial dissemination and consequent mortality (154, 155). Caspase-8 activation, therefore, needs to be regulated under homeostatic conditions and during inflammatory stimuli to restrict the epithelial damage.

In addition to being a determinant of cell death, caspase-8 has a role in modulating NF κ B and mitogen activated protein kinases (MAPK) activation downstream of TLRs or microbial stimuli (156, 157). Caspase-8 promotes both priming and activation of the NLRP3 inflammasome (158, 159). Consequently, FADD and caspase-8 are required for activation of NLRP3 inflammasome and clearance of enteric pathogen *Citrobacter rodentium* (158). Caspase-8 also acts in concert with caspase-1 to mediate pathogen clearance and causes intestinal damage in response to *Salmonella* infection (110). Caspase-8 can, therefore, modulate the inflammatory processes through its multifaceted roles in cell death, and inflammatory signaling.

Necroptosis in the gut

A significant overlap exists between signaling nodes that modulate inflammation, cell survival, and cell death. This suggests that an interplay between these biological processes functions to maintain homeostasis and prevent deleterious consequences. NF κ B signaling

and Receptor interacting protein kinase 1 (RIPK1) are examples of such signaling modules that regulate inflammatory signaling and fate of the cell.

The NF- κ B pathway controls transcription of multiple genes involved in inflammation and cell survival (160). Under resting conditions, NF- κ B dimers are held in an inactive state in the cytoplasm. NF- κ B activation follows two pathways with distinct regulators and biological outputs. Microbial or viral stimuli activate IKK α / β complex that phosphorylates I κ B, leading to its ubiquitination and proteasomal degradation. The liberated NF- κ B dimers translocate to the nucleus to initiate the transcription programming. The IKK complex in addition to the catalytic subunits, IKK α , and IKK β , also contains a regulatory subunit IKK γ (NEMO) that is required for NF- κ B activation (161). NF- κ B activation induces genes involved in immunity, blocking apoptosis and inducing proliferation, making it a critical node in promoting inflammation and cell survival.

The significance of NF κ B signaling in inhibiting cell death and maintaining intestinal homeostasis is highlighted by the occurrence of chronic colitis in mice deficient in NEMO specifically in the IECs (NEMO^{IEC-KO}) (162). NEMO^{IEC-KO} mice exhibit increased cell death in colonic epithelial cells and impaired AMP production that leads to loss of sterility within the mucosal layer. This triggers an inflammatory response and colitis through Myd88 and TNFR1 induced signaling (162). Protection from colitis is observed through genetic loss of RIPK3 alone (70% mice were protected), RIPK3 and FADD (100% mice protected), and loss of RIPK1 kinase activity, highlighting a critical role for cell death in instigating inflammation (163). NEMO^{IEC-KO} mice also exhibit paneth cell loss in the small intestine that is mediated by RIPK1 kinase activity and independent of TNFR1 signaling, NF κ B activation (RelA, RelB, and c-Rel deficient NEMO^{IEC-KO} mice exhibit paneth cell loss) and the RIPK3-FADD axis (163). Therefore, epithelial loss of NEMO triggers RIPK3 and FADD mediated cell death (apoptosis or necroptosis) and colitis, while paneth cell loss in small intestine occurs through RIPK1 kinase activity induced apoptosis. RIPK1 induced apoptosis also mediates paneth cell loss in RelA^{IEC-KO} (163). IEC-specific loss of RIPK1, itself, triggers caspase-8 mediated apoptotic cell death and intestinal inflammation (164). Therefore, a balance between NF- κ B and RIPK1 activation holds cell death induced intestinal inflammation at bay under basal conditions.

Loss of FADD in intestinal cells (FADD^{IEC-KO}) similarly triggers RIPK3 and CYLD dependent necroptosis, causing epithelial cell loss and spontaneous colitis (165). CYLD deficiency does not protect NEMO^{IEC-KO} mice suggesting that cell death in each case is mediated through distinct mechanisms (165). Even though RIPK3 promotes spontaneous colitis (NEMO^{IEC-KO} and FADD^{IEC-KO}), RIPK3-mediated signaling is protective in DSS induced colitis (166, 167). RIPK3 is required within the hematopoietic cells to induce reparative programming through IL-1 β /IL-23 induced IL-22 production (166). RIPK3 promotes inflammasome activation and IL-1 β release in cells of the innate immune system (168–170). Consistent with this innate cell intrinsic role of RIPK3, RHIM-domain mediated cytokine production within the dendritic cell compartment (CD11c) and not the kinase activity (necroptosis) is required for protection against DSS-induced damage (171). TNF induced toxicity is, however, mediated by epithelial RIPK3, as RIPK3^{IEC-KO} mice are resistant to systemic effects of TNF (172). Therefore, RIPK3 controls cell death and

production of inflammatory mediators (pro-repair or pro-damage) to regulate epithelial cell immunity.

Another example of the critical intersection of NF κ B signaling and cell death in gut physiology is observed in IEC-specific A20 knockout mice challenged with DSS (173). A20 is a ubiquitin editing enzyme that enacts a negative feedback control on NF- κ B signaling, including its activation downstream of RIPK1. Loss of A20 sensitizes the epithelial cells to TNF-induced apoptosis and leads to loss of barrier integrity and inflammatory response to commensal bacteria following DSS administration (173). Further, transgenic expression of A20 in IECs is protective against DSS-induced colitis, through restrained inflammation and cell death (174). A20 overexpression in IECs does not affect TNBS induced colitis (174) but exacerbates colitis in *Il10*^{-/-} mice (175), highlighting distinct roles NF κ B signaling in colitis induced by different triggers/cell types. While IEC specific A20 knockout mice (A20^{IEC-KO}) do not exhibit spontaneous colitis, further loss of A20 within the myeloid compartment (A20^{IEC-myel-KO}) leads to spontaneous colitis, increased cell death, and epithelial defects such as loss of paneth cells and goblet cells (176). These mice spontaneously produce a high level of inflammatory cytokines, exhibit increased epithelial apoptosis and develop benign adenomas in the colon upon aging to approximately 30 weeks. Innate immune cells mediate the pathology in these mice, as the absence of T and B lymphocytes (A20^{IEC-myel-KO} $\times$ *Rag*^{-/-} mice) does not inhibit the epithelial defects (176). Consistent with the pro-tumorigenic role of unrestrained NF- κ B activation highlighted in this study (albeit in a spontaneous genetic model) (176), enterocyte and myeloid cell-specific deletion of IKK β decreases tumor burden in the AOM-DSS model of colorectal cancer (177). Loss of IKK β in enterocytes increases apoptosis of carcinogen-exposed cells while myeloid specific IKK β loss reduces production of pro-tumorigenic and inflammatory mediators (177). CYLD deficiency similarly leads to increased NF- κ B activation, pro-inflammatory gene induction and increased susceptibility to colitis and CAC (178). Molecules of the necroptosis and NF- κ B pathway are therefore elegantly poised to control inflammation, cell survival, and cell death and thereby affect multiple physiological processes in the gut.

Conclusion

Over the decades, much progress has been made in our understanding of the mechanisms and consequences of various forms of cell death. The fact that many immortalized cell lines lack molecules involved in cell death pathways suggests that subverting these programs is an important step towards immortalization. The interplay between cell death, inflammation and damage can be harnessed to (a) trigger cell death in cancers recalcitrant to existing chemotherapeutics, (b) switch the form of cell death between “silent” and “inflammatory” to promote immune-surveillance, and (c) temper cell death in a way that it provides the benefit of an immune response to a pathogen, but does not cause excessive damage to the host tissue. Nowhere are these processes of continuous self-renewal and cell death more relevant in the mammalian system than the intestinal tract. Furthermore, some of these implications can be extended beyond the intestinal pathologies to liver, skin and blood disorders.

One confounding factor when studying intestinal disorders in animal models is the impact of gut microbiota. The gut microbiota can influence all aspects of gut physiology and disorders

and variations in the resident microflora in different mouse facilities lead to discrepancies in experimental outcomes. Use of appropriate controls such as littermates and rigorous strategies to delineate the “genetic” effect from “stochastic variations in microbiota” is essential to understand the true function of a molecule in shaping the gut microbial landscape and intestinal disorders. While we discussed aspects of synergy between gut microbiota and immune system in the context of gut pathologies, multiple studies have also uncovered a role for this synergy in regulating immunity and physiology at extra-intestinal sites. Finally, we recognize that the transition from mouse models to human patients would have to also consider the heterogeneity in genetic makeup, microbiome, dietary preferences, and medical history within the affected population. The intersection of cell death and the immune response is nonetheless an area of research that is exciting both from the standpoint of basic biology and therapeutic interventions.

Acknowledgments

Work from the lab is supported by grants from the National Institutes of Health (AI101935, AI124346, AR056296, and CA163507) and the American Lebanese Syrian Associated Charities (to T-D. K). We apologize to our colleagues whose work could not be cited due to space constraints. We would like to thank Drs. Ankit Malik and RK Malireddi for their helpful suggestions in writing and editing this manuscript.

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Figure 1. Pro-tumorigenic functions of inflammation

While controlled inflammation is required to both clear pathogenic insults and repair tissue damage, uncontrolled inflammation can lead to tumorigenesis through multiple mechanisms. Production of growth factors, anti-apoptotic and pro-survival factors signal to promote survival and proliferation of neoplastic cells. Inflammation also causes DNA damage through production of reactive oxygen species (ROS) and iNOS. Production of matrix metalloproteases (MMPs) and other proteases degrades extracellular matrix to promote migration and invasion of cancer cells. Furthermore, angiogenic factors promote the formation of blood vessels that provide oxygen and nutrients to the rapidly dividing cancerous cells.

Figure 2. Cell death and inflammation

Apoptosis is marked by membrane blebbing and surface exposure of signals such as phosphatidyl serine (PS), that allow phagocytes to recognize, engulf and clear apoptotic cells. During this process, damage associated molecular patterns (DAMPs) are inactivated and effectively cleared with the apoptotic bodies. Pyroptosis and necroptosis are inflammatory forms of cell death marked by loss of membrane integrity and release of intracellular contents (DAMPs) that signal via the membrane-bound and cytosolic receptors to trigger inflammation.

Figure 3. Necroptosis pathway of cell death

Necroptosis can be triggered by multiple stimuli, including TNF, LPS (through TRIF) and dsRNA and is mediated by the executioner molecule, mixed lineage kinase-like (MLKL). During necroptosis, receptor interacting protein kinase 1 (RIPK1) interacts with another kinase, receptor interacting protein kinase 3 (RIPK3) through RIP homotypic interaction motif (RHIM) mediated interaction, leading to activation and auto-phosphorylation of RIPK3. RIPK3 binds to and phosphorylates MLKL, p-MLKL undergoes oligomerization and membrane translocation to induce membrane depolarization and cell death. RIPK1 also induces a pro-survival signal through NFκB activation that is positively regulated by cellular

inhibitor of apoptotic protein (cIAP). De-ubiquitination by A20, and cylindromatosis (CYLD) switches the pro-survival signal to apoptotic cell death through Fas-associated protein with death domain (FADD)-Caspase-8 complex. Caspase-8 activity is inhibited by its enzymatically inactive homolog, cellular FLICE-like inhibitory protein (cFLIP). However, if RIPK1 pro-survival signal and caspase-8 activity are both compromised, RIPK1 activates RIPK3 to induce necroptosis.

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Figure 4. NLRP3 inflammasome activation

NLRP3 activation is a two-step process, involving priming and inflammasome assembly. The priming of NLRP3 inflammasome involves transcriptional upregulation of NLRP3 and its post-translational modifications. In response to cognate stimuli, primed NLRP3 interacts with the adaptor, ASC, which recruits caspase-1 to assemble a multimeric complex known as the NLRP3 inflammasome. Caspase-1 undergoes autocatalytic cleavage within the complex to yield its active subunit. Active caspase-1 cleaves pro-IL-1β and pro-IL-18 into their bioactive forms. The executioner molecule, Gasdermin D (GSDMD), mediates pyroptosis. GSDMD is cleaved by caspase-1 to yield its active N-terminus domain that oligomerizes and inserts into the cell membrane. Cell death is caused by loss of osmotic potential which leads to cell rupture and release of intracellular contents. This makes pyroptosis a potent pro-inflammatory mechanism of cell death.

**Epithelial repair,
AMP production,
Mucus production
Pathogen clearance**

**Inflammatory mediators,
Cell death and tissue damage
Microbial dissemination**

Figure 5. Consequences of inflammatory cell death in the gut

Pyroptosis and necroptosis are accompanied by release of DAMPs and bioactive inflammatory mediators, which have binary roles in gut pathology. Extensive cell death and inflammation leads to breakdown of the epithelial barrier and loss of goblet cells and paneth cells. However, certain inflammatory mediators also execute a reparative process by promoting pathogen expulsion, epithelial cell proliferation, antimicrobial peptide (AMP) and mucus production that help restore the integrity of the epithelial barrier. The activation of these inflammatory cell death pathways, therefore, needs to be tightly regulated to promote anti-pathogenic responses without causing excessive damage to the barrier.

Table 1

Cell death in intestinal pathologies.

I. Cell death in response to sterile/chemical/infectious insults				
Genotype	Model	Phenotype	Cell death assay	Reference
Pyroptosis				
<i>Nlrp3</i> ^{-/-}	DSS colitis	Susceptible	Similar TUNEL (+) cells, reduced cl-casp-1	(72, 179, 180)
		Protective	-	(181, 182)
	Oxazolone	Susceptible		(79)
	AOM-DSS	Increased tumor burden		(76, 179)
	<i>Citrobacter</i> infection	Susceptible	Increased bacterial invasion (lower cell death) *	(183)
<i>Casp11</i> ^{-/-}	DSS colitis	Susceptible	Increased/similar TUNEL (+) cells	(106, 184, 185)
	AOM-DSS	No difference		(185)
	<i>Citrobacter</i> infection	Susceptible	Increased bacterial invasion (lower cell death) *	(183)
WT (<i>T. musculus</i> colonization)	<i>Apc</i> ^{min/+} tumor	Susceptible	Increased TUNEL (+) cells	(86)
<i>Il33</i> ^{-/-}	DSS-colitis	Susceptible	Increased cell death (IL-1α release) *	(186)
<i>Naip1-6</i> ^{-/-}	DSS colitis	Protected		(108)
	AOM-DSS	Increased tumor burden	Decreased TUNEL (+) and cl-Casp3 (+) cells	(108)
<i>Nlr4</i> ^{-/-}	DSS colitis	Susceptible		(109)
	AOM-DSS	Increased tumor burden		(107)
		No difference		(179)
	<i>Salmonella</i> infection	Susceptible	Reduced expulsion of infected cells *	(109, 111, 112)
	<i>Citrobacter</i> infection	Susceptible	Increased bacterial invasion (lower cell death) *	(114, 183)
<i>Aim2</i> ^{-/-}	DSS colitis	No difference	Similar level of cl-casp-1	(118, 119)
		Susceptible	Reduced cl-casp1, similar TUNEL (+) cells	(120)
	AOM-DSS	Increased tumor burden	Decreased levels of cl-casp-3 and -7	(118, 119)
	<i>Salmonella</i> infection	Susceptible	Increased TUNEL (+) and cl-casp-3 (+)	(187)
<i>Nlrp1b</i> ^{-/-}	DSS	Susceptible	Increased TUNEL (+) cells	(123)
	AOM-DSS	Increased tumor burden		(123)
<i>Nlrp6</i> ^{-/-}	DSS colitis	Susceptible	Increased lesions in mucosa	(85, 125, 132, 188)
	AOM-DSS	Susceptible		(125, 130, 131)
	<i>Citrobacter</i> infection	Susceptible	Increased bacterial load and ulceration	(126)

I. Cell death in response to sterile/chemical/infectious insults				
Genotype	Model	Phenotype	Cell death assay	Reference
	Virus infection (EMCV and MNV)	Susceptible		(128)
<i>Nlrp12</i> ^{-/-}	DSS colitis	Susceptible	Similar TUNEL (+) cells and Bcl-XI expression	(41, 140, 141)
	AOM-DSS	Susceptible		(41, 140)
	<i>Salmonella</i> infection	Protected	Similar cell death in BMDMs	(42)
<i>Nlrp3</i> ^{-/-}	AOM-DSS	Susceptible	Decreased levels of cl-casp-3, -7 and -8, and similar cl-casp-1	(146, 189)
<i>Nlrp9b</i> ^{-/-} , <i>Casp-1/11</i> ^{-/-}	Rotavirus infection	Susceptible	Decreased TUNEL (+) cells and increased viral load	(39)
Necroptosis/Apoptosis				
<i>Casp8</i> ^{IEC-KO}	DSS	Susceptible		(153)
<i>Casp8</i> ^{-/-}	<i>Citrobacter</i> infection	Susceptible	Increased bacterial invasion (lower cell death) *	(158)
	<i>Salmonella</i> infection	Susceptible		(110)
<i>Ripk3</i> ^{-/-}	DSS	Susceptible	Similar TUNEL (+) cells, necroptosis independent	(166, 171)
<i>A20</i> ^{IEC-KO}	DSS	Susceptible	Increased TUNEL (+) cells	(173)
<i>A20</i> ^{IEC-KO}	TNF	Susceptible	Increased TUNEL (+) and caspase-active cells	(173)
<i>A20</i> ^{IEC-Tg}	DSS	Protected	Decreased TUNEL (+) cells	(174)
<i>A20</i> ^{IEC/myel-KO}	No stimuli	Spontaneous colitis and tumors on ageing	Increased TUNEL (+) and caspase-active cells	(176)
<i>IKKβ</i> ^{IEC-KO}	DSS	Susceptible	Increased TUNEL (+) cells	(177)
	AOM-DSS	Reduced tumor burden	Similar TUNEL (+) cells	(177)
<i>IKKβ</i> ^{myel-KO}	DSS colitis	Protected	Similar TUNEL (+) cells	(177)
	AOM-DSS	Reduced tumor burden	Similar TUNEL (+) cells	(177)
II. Genetic models of cell death pathways				
Model	Phenotype	Cell death status/ Mechanism	Cell death pathway	Reference
Caspase8 ^{IEC-KO} (spontaneous)	Goblet cell and paneth cell loss	TUNEL (+) but cl-Casp3 (-) cells/ Promoted by TNF and Inhibited by nec-1	Necroptosis	(153)
cFlip ^{IEC-KO} (spontaneous)	Epithelial cell death	TUNEL (+) and cl-casp-3 and -8 (+)/ Independent of TNF or RIPK3	Apoptosis	(151)
dsRNA (TLR3-TRIF axis)	Epithelial cell death	cl-casp-3 and -8 (+)/ Dependent on caspase-8 (within IECs)	Apoptosis	(155)
NEMO ^{IEC-KO}	Colitis	TUNEL (+) and cl-casp3 (+)/ TNF1, MyD88, RIPK3, RIPK1-kinase activity and microbiota dependent	Necroptosis/ Apoptosis	(162, 163)
NEMO ^{IEC-KO}	Enteritis (paneth cell loss)	cl-casp-3 (+)/ RIPK3+FADD, RIPK1-kinase activity dependent. Death domain and CYLD independent	Apoptosis	(163, 165)

II. Genetic models of cell death pathways				
Model	Phenotype	Cell death status/ Mechanism	Cell death pathway	Reference
RelA ^{IEC-KO}	Enteritis (paneth cell loss)	cl-casp-3 (+)/ RIPK1-kinase activity dependent	Apoptosis	(163)
RIPK1 ^{IEC-KO}	Intestinal inflammation	TUNEL (+) and cl-casp-3 (+)/ Caspase-8 dependent and RIPK3 independent	Apoptosis	(164)
FADD ^{IEC-KO}	Epithelial cell death	necrotic morphology (+) but cl- casp-3 (-)/ RIPK3, CYLD, MyD88 dependent	Necroptosis	(165)
FADD ^{IEC-KO}	Enteritis (paneth cell loss)	necrotic morphology (+) but cl- casp-3 (-)/ RIPK3 dependent	Necroptosis	(165)

* Denotes output possibly due to differences in cell death.

Abbreviations: DSS- dextran sodium sulfate; AOM- azoxymethane; WT- wild type; TUNEL- Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling; cl- cleaved; casp- caspase; IEC- Intestinal epithelial cells; KO- knockout; Tg- transgenic; myel- myeloid specific; TNF- tumor necrosis factor; RIPK- receptor interacting protein kinase; FADD- Fas-associated protein with death domain; CYLD- cylindromatosis.