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THE ROLE OF RIPK1 IN TNF-INDUCED CELL DEATH IN INTESTINAL EPITHELIAL CELLS

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per optar al títol de doctor per la Universitat de Barcelona

Treball realitzat sota la direcció del Dr. Michael Karin i la Dra. Monica Guma Uriel i tutelat per la Dra. Carme Caelles Franch en el laboratori de Gene Regulation and Signal Transduction de la University of California, San Diego.

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THESIS ABSTRACT

Inflammatory bowel diseases (IBD) are chronic inflammatory processes that affect the gastrointestinal tract. IBD is characterized by an intestinal inflammation and epithelial cell injury leading to a poor psychosocial wellbeing and increased the risk for cancer. IBD pathogenesis is multifactorial, involving genetic predisposition, epithelial barrier defects, dysregulated immune responses, and environmental factors. The current gold standard therapy is TNF blockade, a key proinflammatory cytokine. TNF activates the NF-κB pathway inducing an anti-apoptotic cell response and it plays an important role in the regulation of the intestinal homeostasis. However, in certain situations, TNF can also trigger cell death through different signaling cascades, a typical feature seen in IBD. This work shows how apoptotic IEC areas in IBD human samples co-localize with NF-κB activation and A20 upregulation. Furthermore, using animal models as well as in vitro studies, we show how NF-κB activation and A20 upregulation are required events to trigger TNF-dependent cell death in intestinal epithelial cells. We also show how other cytokines that are usually upregulated in IBD interact with TNF to induce cell death. Specifically, lymphotoxin β receptor activation synergizes with TNF to trigger apoptosis. We have proven that intestinal epithelial cells undergo apoptotsis downstream of TNFR in a RIPK1 dependent manner, and that kinase inhibition of RIPK1 prevents cell death. Chronic NF-κB induces apoptosis downstream of TNF in a ROS dependent manner and A20 requires its linear ubiquitin binding domain, zinc finger seven, to enhance the formation of complex IIb or ripoptosome after TNF stimulation. Overall these results help to further understand the pathogenesis of IBD and suggests RIPK1 as a possible target for new drugs to treat IBD.

RESUM DE LA TESI

Les malalties inflamatòries intestinals (MII) són patologies caracteritzades per una inflamació crònica del tracte gastrointestinal que indueix dany a l'epiteli intestinal incrementant la morbiditat del pacient i el risc de desenvolupar càncer. La patogènesis de les MII és multifactorial, i inclou susceptibilitat genètica, defectes en la barrera epitelial, una resposta immunitària descontrolada i factors ambientals. El tractament estàndard per les MII és el bloqueig del TNF, una citocina pro-inflamatòria. El TNF activa la via de senyalització del NF-κB induint una resposta anti-apoptòtica, al mateix temps que té una funció important en la regulació de l'homeòstasi intestinal. No obstant, en certs casos, el TNF es capaç d'induir mort cel·lular, una característica típica de les MII, a través de diferents vies de senyalització. Aquesta tesis mostra com les àrees apoptòtiques en l'epiteli de les MII correlacionen amb una activació de la via del NF-κB i un increment de A20. Usant models animals i experiments *in vitro*, demostrem com l'activació de NF-κB així com l'augment de A20 en les cèl·lules del epiteli intestinal, són events necessaris per induir la mort cel·lular depenent de TNF. També demostrem com altres citocines, que generalment estan augmentades en les MII, afavoreixen l'apoptosis secundaria a TNF. Específicament, l'activació de receptor de la limfotoxina beta incrementa la capacitat del TNF per induir mort cel·lular. Ensenyem com aquesta mort és secundaria a l'activitat quinasa de RIPK1 i com inhibint-la es pot prevenir la mort cel·lular. També mostrem com l'activació crònica de NF-κB indueix apoptosis secundaria al TNF degut a un increment de ROS mentre que A20 requereix el seu domini d'unió a cadenes d'ubiquitines lineals per afavorir la formació del complexe IIb o ripoptosoma posterior a l'estimulació per TNF. Així doncs, aquest treball aprofundeix més en el coneixement de la patogènesis de les MII i suggereix la inactivació de l'activitat quinasa de RIPK1 com una possible diana terapèutica.

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Chapter 1:
INTRODUCTION

1.1. INFLAMMATORY BOWEL DISEASE PATHOGENESIS

Inflammatory bowel disease (IBD) are inflammatory processes with a chronic relapsing course that are characterized pathologically by intestinal inflammation and epithelial injury and affect the different gastrointestinal (GI) linings. IBD are estimated to affect one in 200 people in developed countries with a rising incidence and prevalence in developing countries (Molodecky et al., 2012). Patients with IBD have a lifetime of debilitating physical symptoms that frequently lead to poor psychosocial wellbeing and a 18-fold increased risk of colorectal cancer when compared with the normal population (Gillen, Walmsley, Prior, Andrews, & Allan, 1994). Furthermore, IBD is a big financial burden with an estimate of 2.2 billion dollars per year in the USA alone (Everhart & Ruhl, 2009). It is, therefore, necessary to understand the physiopathology of the disease and find new approaches to treat IBD.

IBD include different inflammatory pathologies of the gastrointestinal track. The most prevalent ones are Crohn's Disease (CD) and Ulcerative Colitis (UC) (Table 1). CD can affect the whole gastrointestinal track, but it is commonly located in the distal ilium and colon. It is characterized by focal, asymmetric, and, sometimes, granulomatous inflammation that usually affects all layers of the intestine creating a wide variety of lesions from small to large and deep ulcers in multiple areas and swelling of the intestinal lining. It causes chronic or nocturnal diarrhea, abdominal pain, weight loss, fever and rectal bleeding with associated extraintestinal manifestations (EIM). Although the onset is usually subtle, CD can present in a fulminant manner at its onset or with the presence of toxic megacolon (Swan, Geoghegan, O'Donoghue, Hyland, & Sheahan, 1998). UC is limited to the colon and presents a diffuse mucosal inflammation. It is mainly localized in the rectum but can extend in a symmetrical, circumferential and diffuse pattern to other areas of the colon. The hallmark clinical symptom is bloody diarrhea.

	Crohn's Disease	Ulcerative Colitis
Incidence	3.1 to 20.2 cases per 100,000 individuals per year	2.2 to 19.2 cases per 100,000 individuals per year
Onset	Usually between 15 and 40 years	Usually between 15 and 40 years
Location	Gastrointestinal tract, frequently distal ileum and colon	Inflammation affects the colon only (distal colitis or proctitis (55%), left-sided colitis (25%) and pancolitis (20%))
Risk factors	Genotype and environment (smoking is a risk factor for aggressive forms)	Genotype and environment (smoking and appendectomy are protective factors)
Inflammation	Transmural	Mucosa and submucosa
Pathology	Discontinuous, patchy gut inflammation with skip lesions	Continuous areas of inflammation
Symptoms	Crampy abdominal pain, nausea, vomiting, diarrhea, blood and mucus in stool, weight loss, fever, fatigue	Crampy abdominal pain, bloody diarrhea, nausea, vomiting, anemia weight loss, fatigue
Complications	Fistulas, abscess, stenosis and colon cancer	Hemorrhage, toxic megacolon, rupture of the bowel and colon cancer

Table 1. Clinical Comparative chart between Crohn's Disease and Ulcerative Colitis.

The pathogenesis is multifactorial, involving genetic predisposition, epithelial barrier defects, dysregulated immune responses, and environmental factors. The gastrointestinal tract (in particular the terminal ileum and colon) contains a massive bacterial load that might initiate an acute inflammatory intestinal response if the mucosal barrier is breached and bacteria gain access to the lamina propria, as occurs in IBD. From the current understanding, the pathophysiological events in IBD involve a disturbance of the commensal microbiota, impairment of mucosal defense mechanisms, increased permeability of the gut's epithelial layer, and dysregulation of the innate and adaptive immune system, all of which eventually promote an aberrant immune response and subsequent tissue damage (Figure 1).

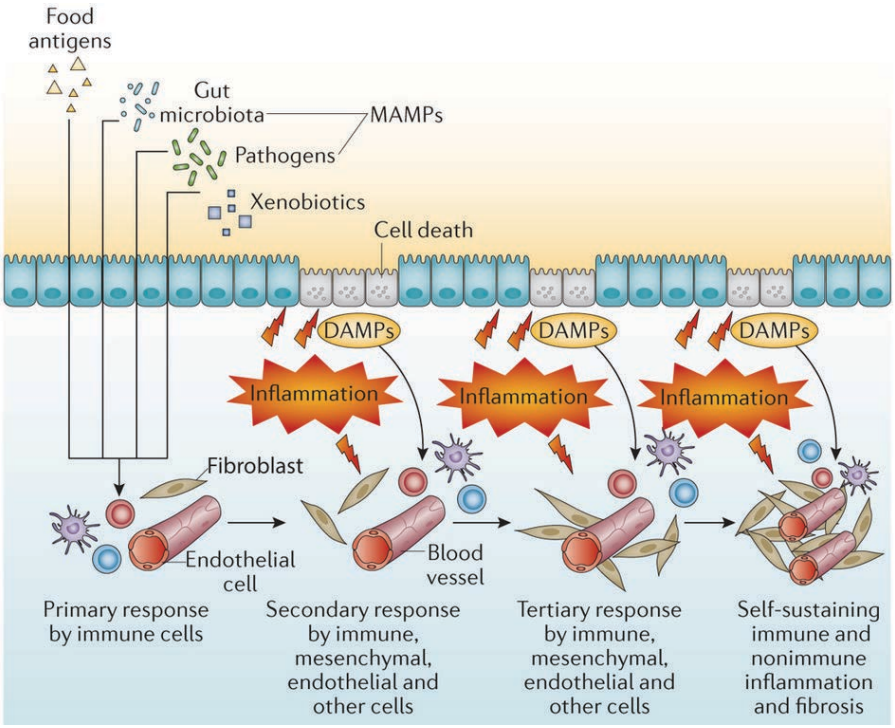

Figure 1. IBD chronification model. An initial translocation of food, gut microbiome or xenobiotics into the lamina propria can trigger an initial immune response. This primary inflammatory response induces proliferation of endothelial and mesenchymal cells, tissue damage and cell death resulting in the release of endogenous danger associated molecular patterns and further translocation of lumen antigens, which trigger a secondary inflammatory response mediated by immune and non-immune cells (endothelial, mesenchymal and other cells). This fuels the inflammatory process resulting in a self-sustaining cycle of chronic inflammation (de Souza & Fiocchi, 2015).

1.1.1. GENETIC PREDISPOSITION

Although family history is a risk factor for developing IBD, the concordance rate in monozygotic twins is only of 10–15% in UC, and 30–35% in CD, suggesting that non-genetic factors might play a bigger role (Spehlmann et al., 2008). Nonetheless, after the first genome-wide association study (GWAS) for Crohn's disease, undertaken in 2005 in Japan that identified the susceptibility locus of the tumor necrosis factor super family 15 gene (*TNFSF15*) (Yamazaki et al., 2005), several other studies have identified, in different ethnic

cohorts, 235 genetic markers in 200 susceptibility loci (Anderson et al., 2011; Franke et al., 2010; J. Z. Liu et al., 2015).

The first genetic risk variant identified for CD was the *NOD2* gene (Hugot et al., 2001; Ogura et al., 2001). Hugo et al. found three different polymorphisms in *NOD2* that comprise the frameshift mutation (L1007C), which causes a truncated protein transcript, and two non-synonymous polymorphisms (R702W and G908R). Carriage of one copy of any risk allele confers a modestly increased risk of developing CD (2 to 4-fold). However, having two copies or a combination thereof is associated with a 20- to 40-fold increased risk.

Another widely studied coding SNP, associated with autophagy, is located in the *ATG16L1* gene and was found to have a disease association with CD. This SNP is responsible for threonine to alanine substitution at amino acid 300 (T300A) that increases the odds ratio (OR) for CD to 1.62 in the Spanish population (Palomino-Morales et al., 2009).

Finally, another important gene related to autophagy is *IRGM* (immunity-related guanosine triphosphatase family M protein). It encodes a GTP-binding protein that induces autophagy and plays an important role in innate immunity against intracellular pathogens. Two flanking SNPs (rs13361189 and rs4958847) have been better associated with increased susceptibility to CD with an OR of 1.34 and 1.33, having the first SNP also confers a small association with UC (OR: 1.16) (Palomino-Morales et al., 2009).

Mice deficient in the above Crohn's-disease-associated genes, *Nod2* and *Atg16l1,* showed Paneth cell defects and susceptibility to intestinal inflammation (Balzola, Bernstein, Ho, & Lees, 2011; Cadwell et al., 2008). These results highlight the importance of the Paneth cell, a secretory cell that releases antimicrobial peptides and supports stem cells, biology such as the regulation of AMP production (*Nod2*) and granule exocytosis (*Atg16l1*), in the pathogenesis of the disease. Importantly, similar phenotypes have been observed in human disease, such that patients with Crohn's disease carrying the ATG16L1^{T300A} mutation show Paneth cell granule abnormalities (Cadwell et al., 2008).

SNPs in *TNFAIP3*, which codes for the immunoregulatory protein A20 (Wertz et al., 2004, 2015) also confer susceptibility to IBD. The minor rs5029941 (alanine to valine substitution) allele is associated with increased risk for IBD with an OR of 3.75, the rs7753394, located upstream to the coding region, has an OR of 1.21 in heterozygotes and 1.48 in homozygotes for CD whereas the rs2327832 allele increases the OR for UC at 1.26 (K. Wang et al., 2010). Interestingly, the rs6927172 variant was associated with increased A20 expression, decreased tumor necrosis factor (TNF) levels and non-response to anti-TNF therapy in both CD and UC (Bank et al., 2014) whereas the rs6927210, rs7753394 and rs7773904 variants were linked to improved response to anti-TNF drugs (Vereecke et al., 2014).

SNPs in the *TNFSF15*, a Th-1 polarizing cytokine involved in systemic inflammation, confer huge susceptibility to CD in the Japanese population (OR 2.17) however, when this analysis was replicated in occidental populations there was a weaker effect. This may have been due to different marker genotypes or differences in susceptibility genes between Asian and European cohorts (Yamazaki et al., 2005).

The *IL23R* gene also contains many variants associated with CD. However, unlike in other genes, the rs11209026 SNP has the strongest association with protection against CD (OR: 0.4) while other variants, such as rs1004819, have a very small association (Naser et al., 2012).

Other SNPs have been related to the nuclear factor-κB (NF-κB) signaling pathway with diverse correlations. For instance, there is a positive association between pancolitis and the $TNFR1^{A36G}$ polymorphism in UC (OR: 5.341) (Pierik et al., 2004) and a really mild association between UC and the −94delATTG allele located in the promoter of the *NFBK1* gene (p=0.047-0.052) (Karban et al., 2004)

Of the 163 identified loci in the Caucasian population, 110 appear to be relevant to both CD and ulcerative colitis (*TNFAIP3, IRGM, TNFSF15*), whereas 23 appear to be specifically related to UC (*ATG16L1, NOD2*) and 30 to CD (*IRF5, NFKB1*), respectively. Interestingly, Burton et al. have shown that 50% of IBD susceptibility markers are shared with other immune-mediated diseases, such as psoriasis, ankylosing spondylitis, and primary immunodeficiency (Burton et al., 2007). Such amount of susceptibility loci with low hereditary factors suggest that each loci confers small susceptibility and IBD etymology probably depends on the addition and synergy of those together with environmental factors (Duerr et al., 2006; Franke et al., 2007; Libioulle et al., 2007; Parkes et al., 2007; Rioux et al., 2007).

1.1.2. EPITHELIAL BARRIER DEFECTS

The intestinal epithelium forms the protective barrier and host defense against the harmful luminal microenvironment with selective permeability and absorption of nutrients.

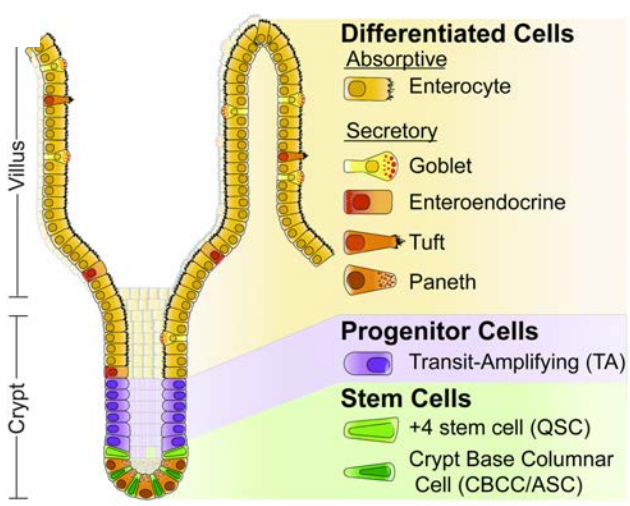

Figure 2. Intestinal structure. The intestinal epithelium is organized into crypt and villus regions, with the stem and progenitor zone localized in the crypt. Current models favor the existence of two stem cell populations, the +4 stem cell and the crypt base columnar cell (CBCC), which are thought to be quiescent and active stem cells, respectively. Transit-amplifying (TA) progenitors arise from the stem cell compartment and differentiate into absorptive enterocytes or secretory goblet, enteroendocrine, tuft, or Paneth cells. Most of the differentiated cell populations migrate up the villi, but, uniquely, the Paneth cells move downward and reside between the CBCCs. Adapted from (Carulli, Samuelson, & Schnell, 2014).

The epithelium is covered by a single-cell layer composed of different subtypes of specialized intestinal epithelial cells (IECs) including absorptive cells, goblet cells, enteroendocrine cells, Paneth cells, M cells, cup cells, and Tuft cells, all of which differentiate from common epithelial stem cells (Figure 2). These subsets of IECs are

functionally different and essential for maintaining intestinal homeostasis by separating the intestinal lumen from the underlying lamina propria and by controlling the crosstalk between luminal microbiota and subjacent immune cells. Thus, IEC defects and dysregulation of the epithelial barrier homeostasis play a crucial role in IBD pathogenesis (Coskun, 2014).

In normal conditions, the intestinal epithelium provides a selective permeable barrier that, on one hand, acts prophylactically through the secretion of anti-microbial peptides from Paneth cells, located at the base of the crypt, and a mucus layer, secreted by goblet cells, that coats the mucosa as a sticky gel. In addition, the intestinal epithelium directly prevents a detrimental invasion of foreign antigens, microorganisms and their toxins that could activate the immune system from the lamina propria. In IBD, both UC and CD patients have similar increased levels of human β-defensin 1 (HBD1) whereas HBD2, 3 and 4 are increased in UC compared to CD (Fahlgren, Hammarstrom, Danielsson, & Hammarstrom, 2004; Fahlgren, Hammarström, Danielsson, & Hammarström, 2003; O'Neil et al., 1999; Jan Wehkamp et al., 2002, 2003). Also, human α-defensin 5 and 6 (HD5 and HD6) were increased just in UC patients (Cunliffe et al., 2001; J Wehkamp et al., 2002) while decreased in CD patients (Jan Wehkamp et al., 2005). This increase in defensins expression is probably a result of Paneth cell metaplasia (PCM) that occurs during colonic inflammation to produce additional antibacterial peptides and counteract a bacterial attack towards the epithelium (Simmonds, Furman, Karanika, Phillips, & Bates, 2014). Overall, unlike UC patients, CD patients have decreased mucosal antimicrobial activity (Nuding, Fellermann, Wehkamp, & Stange, 2007). In contrast, whereas in CD the mucus layer is normal or even thicker than in controls, in UC the mucosal layer is thinner, more variable and in part denuded (McCormick, Horton, & Mee, 1990; Pullan et al., 1994; Rhodes, 1997). Although IBD cases have less goblet cells, UC patients have even lower numbers compared to CD specially in the upper part of the crypt (Gersemann et al., 2009). Interestingly, Ardesjö et al. showed a serum reactivity of IBD patients against goblet cells (Ardesjö et al., 2008).

IECs ability to act as a protective physical barrier is mediated by formation of a web of tight junctions (TJs) that seal the paracellular space between epithelial cells and separate the cell membrane into apical and basolateral domains. Altered expression and organization of TJ are typical features seen in IBD (H. Schmitz et al., 1999; Zeissig et al., 2007). In fact, different cytokines present in IBD such as TNF and interferon-γ, have been shown to increase TJ permeability (Marini et al., 2003; Nava et al., 2010). Interestingly, patients that respond to anti-TNF therapy show a restoration of the intestinal permeability (Suenaert et al., 2002; Toedter et al., 2012).

Another key aspect of intestinal homeostasis is the fast turnover of IECs. This is regulated by loss of senescent cells from the epithelial monolayer as well as a renewal from the stem cells in the crypt compartment. In humans, approximately 10^{10} IEC are shed every day (Blander, 2016). The rapid nature of the process, including clearance of dead cells by phagocytes, precludes its visualization. Apoptosis as a mechanism of shedding emerged with studies showing the presence of cells positive for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) at the tips of the villi in rodent and human intestinal sections (Gavrieli, Sherman, & Ben-Sasson, 1992; Hall, Coates, Ansari, & Hopwood, 1994; Shibahara et al., 1995). These early studies set the stage for debates to come decades later as to whether apoptosis precedes or follows IEC shedding. Although most of the dying cells are shed to the intestinal lumen, some are phagocytized by macrophages and dendritic cells.

In IBD, the numbers of dead cells in the gut epithelium is notably increased compared with controls (Di Sabatino et al., 2003; Dourmashiin et al., 1983; Hagiwara, Tanaka, & Kudo, 2002; Iwamoto, Koji, Makiyama, Kobayashi, & Nakane, 1996). Interestingly, patients with active UC, who ultimately require surgery, had higher apoptotic indices than UC patients that were receiving medication. It is important to note that, although most of the dying IECs are apoptotic cells, mice lacking caspase-3 (and caspase-8 and FADD as well) display limited apoptotic phenotype with no impact on gastrointestinal homeostasis (Brinkman et al., 2011; Günther et al., 2011a; Lakhani, 2006). Finally, electron microscopy on rectal biopsies of patients with CD and UC compared with normal controls showed patches of necrotic cells in four out of seven CD patients (Dourmashiin et al., 1983) and receptor-interacting serine/threonine-protein kinase-3 (RIPK3), a key necroptotic protein, is notably expressed at high levels in the terminal ileum of patients with CD (Günther et al., 2011b). A key regulatory pathway that prevents cell death is the NF-κB pathway. Numerous works have shown its importance in suppressing cell death in IECs and defects in it induce epithelial damage. Therefore, studying the role of NF-κB in IECs and its interactions with cell death processes are important for better understanding of IBD pathogenesis.

1.1.3. DYSREGULATED IMMUNE RESPONSES

Under physiological conditions, a large number of innate and immune cells are located in the intestinal lamina propria, such as T and B lymphocytes, natural killer (NK), NKT cells, macrophages (Mø), dendritic cells (DCs), mast cells, neutrophils, eosinophils, as well as stromal cells (such as fibroblasts) (Xu, Liu, Feng, & Liu, 2014). Those are important to maintain the intestine homeostasis and clear bacterial and other stress particles that can translocate in an acute and localized manner.

CD has been thought to be characterized by a Th1 immune response as it has been observed that mucosal T cells from CD patients produce higher amounts of IL-2 and interferon-γ (IFN-γ) than T cells from UC patients or controls (Breese, Braegger, Corrigan, Walker-Smith, & MacDonald, 1993; I J Fuss et al., 1996; Ivan J. Fuss et al., 2004; Noguchi, Hiwatashi, Liu, & Toyota, 1995), while UC has been considered as a Th2-mediated disease, with excessive production of IL-5 and IL-13 (Ivan J. Fuss et al., 2004; Heller et al., 2005). However, there have also been different observations about mucosal Th1 and Th2 cytokines in IBD. Both UC and CD biopsies cultured *ex vivo* release high and comparable amounts of IFN-γ and lower amounts of IL-13 were found in the colonic mucosa of UC patients compared to CD patients and control subjects (Kadivar et al., 2004; Rovedatti et al., 2009; Vainer, Nielsen, Hendel, Horn, & Kirman, 2000). The involvement of Th17, another T cell subset that secretes IL-17A, in intestinal inflammation has been extensively studied. High transcript levels of IL-17A have been detected both in CD and UC mucosa in comparison to normal gut, and it has been observed by immunohistochemistry that IL-17A is overexpressed in the lamina propria of IBD patients (Fujino et al., 2003; Kobayashi et al., 2008; Sugihara et al., 2010)

Dendritic cells and macrophages cells detect molecular patterns on microbes (pathogen-associated molecular patterns, PAMPs) via pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR) receptors (NLRs), C-type lectin receptors (CLRs), and retinoic acid-inducible

gene 1 (RIG-I)-like receptors (RLRs). Upon engagement of PRRs, cells produce inflammatory cytokines, chemokines, and antimicrobial peptides. As a result, neutrophils are recruited and macrophages are activated, leading to the direct killing and clearance of microbes. Additionally, these inflammatory products induce the maturation of DCs, promoting the induction of adaptive immune responses (Kawai & Akira, 2011; Steinbach & Plevy, 2014). Interestingly, a common 'suppression of inflammation' signature was noted on phagocytic cells after clearance of apoptotic cells, although the specific genes and pathways involved varied amongst dendritic cells and macrophages, reflecting specialized functions. Of note, several of the genes that were expressed by phagocytes bearing apoptotic IECs are IBD susceptibility genes (such as *Il12b*, *Lsp1, Fos, Cd40* and *Tnfaip3* among others) suggesting that a disruption of the phagocyte immunosuppressive response would have consequences in the gut homeostasis (Cummings et al., 2016). Dysregulation of these pathways can lead to both enhanced susceptibility to infections and development of chronic inflammatory diseases.

Chronic activation of immune cells from the lamina propria induces a broad spectrum of cytokines (including IL-1, IL-6, IL-12, IL-22, IL-23, and TNF) with pleiotropic effects that drive intestinal inflammation and associated symptoms, such as diarrhea (Markus F. Neurath, 2014). Studies in mouse models of IBD have shown that the modulation of cytokine function can be used for therapy and have identified new cytokines as potential therapeutic targets for chronic intestinal inflammation (Markus F. Neurath, Finotto, & Glimcher, 2002; Powrie et al., 1994).

- **IL-1**

IL-1 is represented by IL-1α and IL-1β, which signal through IL-1R. IEC produce IL-1Ra (IL-1 receptor antagonist) which functions to inhibit IL-1 pro-inflammatory actions by binding to target cells IL-1 receptor and competitively counteracting the effects of IL-1. In fact, a markedly significant decrease in the intestinal mucosal IL-1Ra/IL-1 ratio was found in both CD and UC patients when compared with control subjects, suggesting its pathologic importance in chronic intestinal inflammation (Casini-Raggi et al., 1995). Animal models suggest that IL-1 could play an important role at earlier stages. IL-1β promoted innate immune pathology in *Helicobacter hepaticus*-triggered intestinal inflammation by augmenting the recruitment of granulocytes and the activation of innate lymphoid cells (Balzola, Cullen, Ho, Russell, & Wehkamp, 2012). In the T cell transfer model of colitis, IL-1R signaling in T cells controlled the early accumulation and survival of pathogenic $CD4^+$ T cells in the colon. However, in chronic models its role is controversial as treatment with an IL-1R antagonist suppressed acute immune complex-induced colitis in rabbits (Cominelli et al., 1990) whereas TNF blockade, but not IL-1 blockade, was effective in treating chronic dextran sodium sulphate (DSS)-induced colitis in mice (Kojouharoff et al., 1997).

- **IL-6**

IL-6 is mostly produced by stimulated monocytes, macrophages, T- and B-lymphocytes. In fact, lamina propria macrophages and CD4+ T cells produce high amounts of IL-6 in experimental colitis and in IBD patients (Atreya et al., 2000; Kai et al., 2005). IL-6 binds to the soluble IL-6R (sIL-6R), and the IL-6–sIL-6R complex then activates intestinal target cells by binding to the gp130 surface molecule (also known as IL-6R subunit-β) and induce pro-

inflammatory responses in antigen presenting cells (APC) but also antiapoptotic functions in mucosal T cells (Atreya et al., 2000). Blockade of IL-6 signaling with monoclonal antibodies proved to be effective in suppressing chronic intestinal inflammation in mouse models (Atreya et al., 2000; Yamamoto, Yoshizaki, Kishimoto, & Ito, 2000). Such results provided the rational to use an IL-6R-specific antibody to block IL-6 signaling in patients with Crohn's disease that led to clinical responses in subgroups of patients (Ito et al., 2004) although one rare adverse event was gastrointestinal perforation in patients with a history of diverticulitis (Emery et al., 2008; Gout, Östör, & Nisar, 2011). These effects could be explained as IL-6 protects IECs from apoptosis and enhances their proliferation and tissue repair after intestinal damage (Grivennikov et al., 2009; Kuhn, Manieri, Liu, & Stappenbeck, 2014; Markus F. Neurath, 2017).

- **IL-12**

An initial role of IL-12 in IBD was suggested in 1995 when neutralizing antibodies targeting IL-12 resulted in an amelioration of TNBS-induced colitis in mice (M F Neurath, Fuss, Kelsall, Stüber, & Strober, 1995). It is important to note, however, that IL-12 is composed of p35 and p40 subunits but that p40 can also form heterodimers together with p19 to form IL-23. IL-12 and IL-23 are both expressed by DC and Mø in CD but not in UC, which suggests that activated phagocytes may favor Th1 cell differentiation and activation in CD (Z. Liu et al., 2011; Monteleone et al., 1997; Ng et al., 2011). Anti-IL-12 treatment showed efficacy in patients with CD in two different studies (Mannon, Fuss, Mayer, Elson, Sandborn, Present, Dolin, Goodman, Groden, Hornung, Quezado, Neurath, Salfeld, Veldman, Schwertschlag, & Strober, 2004; William J. Sandborn et al., 2008). Of note, those antibodies were directed against the p40 subunit, blocking the action of both IL-12 and IL-23. Later studies in several models of experimental colitis have suggested that IL-23 rather than IL-12 drives chronic intestinal inflammation (Uhlig et al., 2006; Yen et al., 2006). Subsequently, various antibodies against IL-12–IL-23 p40 and IL-23 p19 subunits were developed for clinical trials (Mannon, Fuss, Mayer, Elson, Sandborn, Present, Dolin, Goodman, Groden, Hornung, Quezado, Neurath, Salfeld, Veldman, Schwertschlag, Strober, et al., 2004; William J. Sandborn et al., 2008; William J. Sandborn, Gasink, et al., 2012). So far, Ustekinumab, a p40 blocker, has been approved for CD therapy, as it has been particularly effective for therapy in patients with CD who had previously taken anti-TNF agents (Feagan et al., 2016; William J. Sandborn et al., 2008; William J. Sandborn, Gasink, et al., 2012). Potentially, p19 blockers such as risankizumab will also be available for CD therapy in the future, as it has shown efficiency in patients with active CD in a phase II clinical trial (Markus F. Neurath, 2017).

- **IL-22**

The IL-22 signaling pathway is activated through a heterotrimeric receptor composed of IL-22R1 and IL-10R2. Although IL-10R2 is widely expressed on almost all cell types, the expression of IL-22R1 is restricted to the surfaces of non-hematopoietic cells such as IEC, hepatocytes and keratinocytes. IL-22 is thought to have a protective role in IBD and it is mostly secreted by Th17, Th22, $CD8^+$ T lymphocytes, lymphoid tissue inducer (LTi) cells, NK and DCs. In the DSS-induced colitis model, IL-22 knockout mice had extensive epithelial destruction and inflammation in the colon, more severe weight loss, and more impaired recovery compared to wild-type (WT) treated mice. In addition, T cells from IL-$22^{-/-}$ mice

cause a more severe colitis in the T cell transfer model of IBD (Zenewicz et al., 2008). Correspondingly, in a Th2-mediated colitis model, local IL-22 gene delivery rapidly attenuated colitis, while delivery of IL-22-binding protein (IL-22BP), a soluble single chain receptor that specifically prevents the binding of IL-22 to IL-22R, increased ulcerations and inflammation in DSS colitis (Sugimoto et al., 2008). This could be a consequence of the protective effect of IL-22 in the gut mucosa, as it directly stimulates proliferation of intestinal epithelial cells via STAT3 activation and induces production of protective barrier proteins such as REG proteins (Lindemans et al., 2015; Pickert et al., 2009).

- **IL-23 and Th17**

Interleukin-23 (IL-23) is a heterodimeric cytokine that belongs to the IL-12 family cytokines and shares both ligand and receptor subunits with IL-12. IL-23 heterodimer is made of p19 (IL-23A) and the shared beta chain, p40 (IL12β) subunit, which also dimerizes with IL-12p35 forming the IL-12 cytokine. Initial genetic deletion or antibody-mediated neutralization of IL-12 led to amelioration of intestinal inflammation in a number of different models (M F Neurath, Fuss, Kelsall, Stüber, & Strober, 1995; Simpson et al., 1998) and therefore it was thought to play an essential role in the pathology. However, the discovery in 2000 of IL-23 led to reevaluating the role of IL-12 as the approaches used were targeting the p40 subunit (Oppmann et al., 2000) and in 2006, four reports identified IL23 but not IL12 as an essential mediator of intestinal inflammation (Hue et al., 2006; Kullberg et al., 2006; Uhlig et al., 2006; Yen et al., 2006). In those studies, IL-23 was found to orchestrate an inflammatory cytokine cascade involving increased levels of TNF, IL-6, interferon-γ and IL-17 in the intestine.

IL-23 is expressed and secreted by DC, Mø and monocytes. Intestinal epithelial cells were also shown to contribute to IL-23 production (Macho-Fernandez et al., 2015), while IL-23 receptor is expressed by both innate and adaptive immune cells. The most studied IL-23 responsive cells are Th17 cells. Th17 cells require IL-23 for maintenance, maturation, expansion and to fully acquire a pathogenic character, but IL-23 is not necessary for differentiation from naïve CD4 T cells (Gaffen, Jain, Garg, & Cua, 2014; Korn, Bettelli, Oukka, & Kuchroo, 2009).

Th17 cells, and their key secreted cytokine IL-17A, have been reported to play a key pathogenic role in chronic inflammatory conditions such as psoriasis, rheumatoid arthritis, systemic lupus erythematous and multiple sclerosis (Fouser, Wright, Dunussi-Joannopoulos, & Collins, 2008). IL-17A and IL-17F are closely related (50% homology) Th17 signature cytokines that are produced in an IL-23-dependent manner (Eken & Oukka, 2016).

In fact, both IL-23 and IL-17 are expressed at higher levels in CD (Fujino et al., 2003; Rovedatti et al., 2009; Schmidt et al., 2005) and Th17 cells are needed for the pathogenesis of IBD in several murine models (Ahern et al., 2010; Durant et al., 2010; Izcue et al., 2008; Rovedatti et al., 2009). Because IL-17 stimulates production of various inflammatory mediators, through epithelial or endothelial cells, that recruit neutrophils, monocytes and dendritic cells, IL-17 involvement as a pathogenic molecule was studied in the IBD context. Unexpectedly, IL-17A neutralization or IL17KO mice in DSS model resulted in exacerbation of colitis (Ogawa, Andoh, Araki, Bamba, & Fujiyama, 2004; Yang et al., 2008). Similarly, adaptive colitis induced by naïve CD4+ T cells also developed more aggressively when il17$^{-/-}$ or il17r$^{-/-}$ T cells were transferred as compared with WT T cells (O'Connor Jr et al., 2009). This protective role of IL-17 in murine models has been confirmed in CD patients.

Monoclonal anti-IL-17A secukinumab treatment exacerbated the disease and adverse effects (high incidences of fungal infection) have been reported (Hueber et al., 2012; J. S. Lee et al., 2015; Maxwell et al., 2015). Therefore, the critical effect of IL-23 in mediating intestinal inflammation through Th17 cells is not through IL-17 but maybe through other Th17 secreted cytokines as GMCSF (Buonocore et al., 2010; Griseri et al., 2015; Griseri, McKenzie, Schiering, & Powrie, 2012; Ono et al., 2012).

- **TNF and the TNF superfamily**

Almost 40 years ago, an initial project on trypanosome-infected cattle gave rise to the study of a mediator of cachexia. TNF was discovered as a protein that mediated anemia and cachexia named cachexin (Carswell et al., 1975). At the moment, 19 structurally related cytokines of the TNF superfamily (TNFSF) and their receptors (TNF receptor superfamily, TNFRSF) have been described (Figure 3).

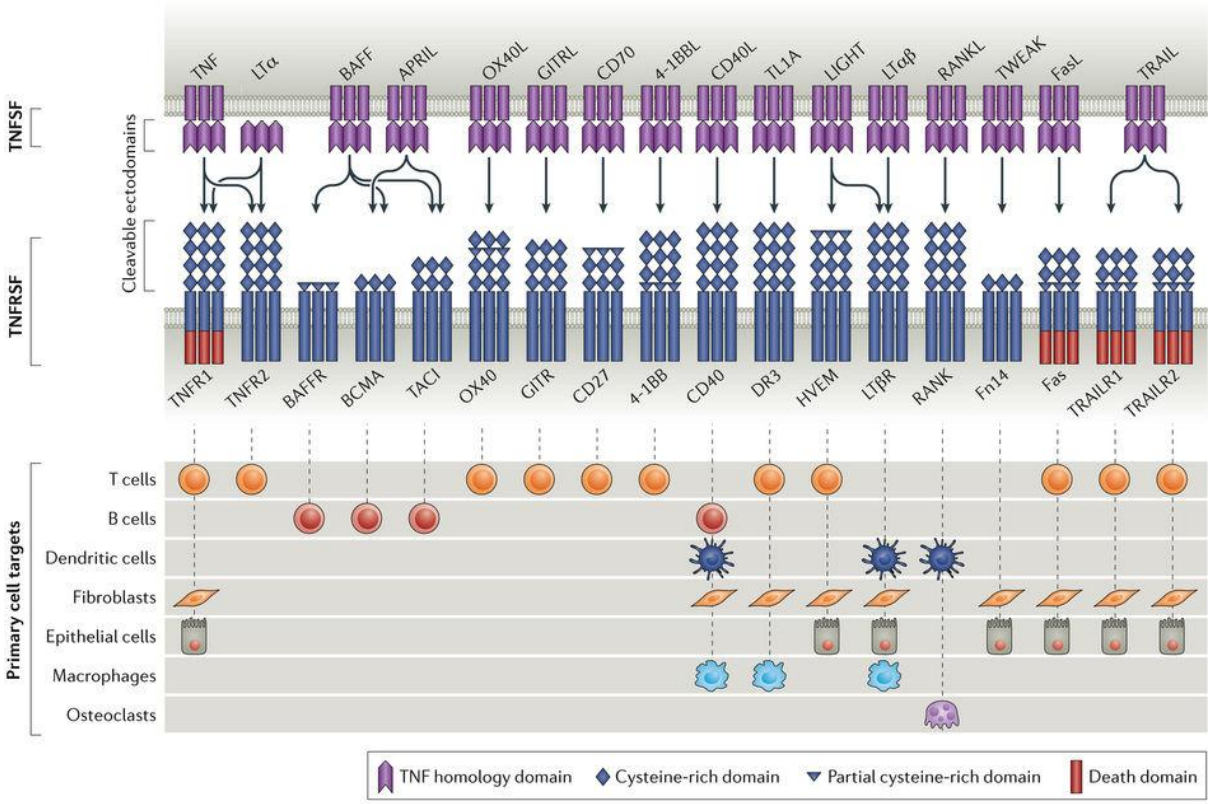

Figure 3 Overview of the TNFSF ligands and receptors. The TNF superfamily ligands, receptors and primary cell targets. Adapted from Michael Croft et al., Nature Review Reumatology, 2017 (Croft & Siegel, 2017).

TNFSF cytokines are trimeric molecules initially synthesized as type-II transmembrane proteins characterized by C-terminal TNF homology domains that can be cleaved by metalloproteinases (Bodmer, Schneider, & Tschopp, 2002). Binding to its receptors, which are type I transmembrane proteins containing varying numbers of extracellular ligand-binding cysteine-rich domains, leads them to trimerization and further assembly of intracellular signaling complexes (Bodmer et al., 2002). Although the similarity among TNFSF cytokines is not high, once engaged with its receptor, similar pathways will be

activated like NF-κB, MAPK, AKT or cell death. Of note, membrane-bound TNFSF cytokines can reverse signal, to the ligand-expressing cell, after engaging its receptor, with various effects as production of IL-4 or IL-6 (Eissner, Kolch, & Scheurich, 2004).

Multiple polymorphisms in the genes encoding TNFSF cytokines and their receptors have been associated with susceptibility to autoimmune diseases (Croft, Benedict, & Ware, 2013). For instance, DR3-TL1A has been related to both CD and UC, CD40 to CD and HVEM/LTβ-Light to UC (Croft et al., 2013).

TNF is one of the most important cytokines of the TNFSF in inducing IBD pathogenesis as it enhances hypervascularization and angiogenesis, augments pro-inflammatory cytokine production by Mø and T cells, causing barrier alterations and promoting death of IEC. TNF can be produced by a broad range of cells as a membrane bound precursor, which can become soluble after cleavage by TNF-converting enzyme (TACE, also named ADAM17) (Black et al., 1997; Moss et al., 1997). TNF trimers are able to bind to two receptors: tumor necrosis factor receptor 1 (TNFR1) and TNFR2. TNFR1 is ubiquitously expressed, contains death-domain motives and can be activated by soluble or membrane bound TNF. TNFR2 is restricted to specific cell types (neurons, immune cells and endothelial cells), lacks death-domain motifs and is thought to be mainly activated by transmembrane bound TNF (Grell et al., 1995). The notion that TNF plays an important role in IBD comes from 1991 when it was shown to be elevated in the serum of IBD patients (Murch, Lamkin, Savage, Walker-Smith, & MacDonald, 1991). Later studies confirmed that TNF was increased in the intestinal mucosa and that it correlated with the disease activity (Dionne, Hiscott, D'Agata, Duhaime, & Seidman, 1997; Maeda et al., 1992; Matsuda et al., 2009; Owczarek, Cibor, Głowacki, Cieśla, & Mach, 2012). The production of TNF in the colon mucosa of IBD patients was later localized to lamina propria Mø (Murch, Braegger, Walker-Smith, & MacDonald, 1993). Supporting the importance of TNF in IBD, mice with deletion of the 3' regulatory element from the TNF transcript have increased, sustained production of TNF, resulting in CD-like inflammation and immune profile (Kontoyiannis, Pasparakis, Pizarro, Cominelli, & Kollias, 1999). Similarly, TNF has been shown to be pathogenic in a variety of mouse models of IBD (Holm, Poulsen, Markholst, & Reedtz-Runge, 2012; Mueller, 2002; M F Neurath et al., 1997).

Although in excess TNF can contribute to GI pathology, TNF is also a critical protective factor that promotes GI homeostasis following injury and inflammation by inducing epithelial regeneration and wound healing (Dubé, Punit, & Polk, 2015). For example, neutralization of TNF with a monoclonal antibody aggravated the severity of acute colitis when the epithelial layer was damaged with DSS (Kojouharoff et al., 1997). Similarly, TNF knockout mice are highly sensitive to DSS-induced acute colitis (Noti, Corazza, Mueller, Berger, & Brunner, 2010).

Two main strategies have been developed to neutralize TNF: a monoclonal antibody against TNF and a TNF receptor II-Fc fusion protein. One important difference between the anti-TNF antibodies is that the soluble receptors are not specific for TNF as they are also able to bind lymphotoxin-α(LTα). Although TNF has some protective effects in the intestinal mucosa, clinically, treatment of IBD with antibodies that neutralize both soluble and membrane-bound TNF (such as infliximab and adalimumab) is highly effective inducing clinical response, remission, and mucosal healing, increasing quality of life (J. Colombel et al., 2014; Deeks, 2016; William J. Sandborn, Feagan, Marano, Zhang, Strauss, Johanns, Adedokun, Guzzo, Colombel, Reinisch, Gibson, Collins, Järnerot, Hibi, et al., 2014, 2014; William J. Sandborn,

Feagan, Marano, Zhang, Strauss, Johanns, Adedokun, Guzzo, Colombel, Reinisch, Gibson, Collins, Järnerot, Rutgeerts, et al., 2014; William J. Sandborn, van Assche, et al., 2012). In 2015 and 2016, infliximab biosimilars (biological products that are highly similar in structure and clinical efficacy to an already FDA-approved biological reference product) were approved for clinical therapy and adalimumab biosimilars are currently under development (Blair & Deeks, 2016). Anti-TNF therapy can also be combined with immunosuppressive agents, such as azathioprine, showing that combination therapy with infliximab and azathioprine is superior for inducing corticosteroid-free clinical remission than monotherapy with either agent in both Crohn's disease and ulcerative colitis (J. F. Colombel et al., 2010; Panaccione et al., 2014). Surprisingly, agents that preferentially block soluble TNF (for example, etanercept, a TNF receptor II-Fc fusion protein) had no therapeutic effect (Markus F. Neurath, 2014; W J Sandborn et al., 2001). This can be due to multiple reasons: first, different matrix metalloproteinases, such as MMP3 and MMP12, can induce a fast proteolytic degradation and inactivation of etanercept whereas cleaved infliximab and adalimumab functioned as F(ab')2 fragments that are still able to block TNF (Biancheri et al., 2015). Second, only full IgG1 monoclonal anti-TNF antibodies such as infliximab and adalimumab were found to induce regulatory Mø with wound-healing and active T-cell proliferation inhibition via Fc receptor signaling, suggesting that etanercept might fail to regulate macrophage function in IBD (Vos et al., 2011). Third, in comparison to other TNF blockers, etanercept has a lower affinity to transmembrane TNF, an important co-stimulatory signal for TNFR2 on mucosal T cells that mediates their resistance to apoptosis (Scallon et al., 2002). Therefore, other anti-TNF antibodies might be more effective in inducing T-cell apoptosis, as such apoptosis induction has been shown to correlate with clinical responses to anti-TNF therapy (Van den Brande et al., 2007). Finally, the inability of etanercept to bind LTα suggest that LTα could play a role in IBD.

1.1.4. THE MICROBIOME

It is believed that an inappropriate response against commensal gut microbiota, blamed on a 'loss of tolerance' occurs, but it has been difficult to determine whether this process is secondary to an altered microbiota, a defective immune response or some other factors (Manichanh, Borruel, Casellas, & Guarner, 2012). It is interesting to note that serum reactivity against selected components of the gut microbiota is common, even in healthy individuals, and that some CD associated serological markers against microbial antigens are present years before clinical manifestations in patients with CD as well as healthy individuals (Choung et al., 2016). Additionally, various microorganisms have been identified that are claimed to exert aggressive or protective functions relevant to Crohn's disease, such as adherent-invasive *Escherichia coli* and *Faecalibacterium prausnitzii*, respectively (Darfeuille-Michaud et al., 2004; Machiels et al., 2013).

The clinical observation that IBD can respond to antibiotic treatment (Khan et al., 2011; S.-L. Wang, Wang, & Yang, 2012) and the effectiveness of faecal stream as a treatment for CD (Harper, Lee, Kettlewell, Bennett, & Jewell, 1985; Janowitz, Croen, & Sachar, 1998) is also consistent with the idea that intestinal bacteria contribute to the inflammatory response. Despite these correlations, there is not enough information to answer whether dysbiosis in IBD is a primary or secondary phenomenon.

1.2. CELL DEATH

IEC form a protective barrier that separates the organism from the contents of the intestinal lumen. The lumen is loaded with a large number of microbes and bacteria, largely exceeding that of total cells in the human body, as well as xenobiotics and food. All these molecules contain antigens that can trigger an immune response, however the epithelial barrier prevents them from translocating into the lamina propria. In IBD, increased areas of epithelial cell death are seen, increasing the chances of antigen translocation and subsequently trigger of an inflammatory response (Di Sabatino et al., 2003; Dourmashiin et al., 1983; Hagiwara, Tanaka, & Kudo, 2002; Iwamoto, Koji, Makiyama, Kobayashi, & Nakane, 1996). Death cells in the epithelium present features of apoptosis and necrosis but little is known about the underlying mechanism.

The initial description of apoptosis as a programed cell death led to a dichotomous view of how cells die: they either undergo programmed cell death (apoptosis) or die 'accidentally' in response to overwhelming chemical or physical insult by the passive process of necrosis. Morphological changes were a way to distinguish between apoptosis and necrosis: apoptotic cells shrink and display nuclear condensation and membrane blebbing, whereas necrotic cells swell and rupture. Biochemical analysis of apoptosis revealed that it depends on the activity of one or more members of a family of cysteine proteases called caspases. This caspase-dependent cell death is triggered by the ligation of specific cell surface receptors, DNA damage, excess reactive oxygen species (ROS) and multiple types of cellular stress. Depending on the origin of the trigger, apoptosis is classified in intrinsic or extrinsic. While apoptosis has been studied for a long time, regulated necrosis has been characterized recently. Regulated necrosis includes multiple cell death sub-classes such as necroptosis, parthanatos, ferroptosis, (n)etosis, pyroptosis, and ischemia reperfusion injury (IRI)-mediated necrosis each of them depending on different biochemical mechanisms. It is still unclear whether shared pathways or converging pathways underline the common morphological features of these multiple forms of cell death, with necroptosis being the best-characterized form of regulated necrosis.

1.2.1. APOPTOSIS

Apoptosis is the major type of regulated cell death. This evolutionarily conserved process has crucial roles that range from tissue sculpting during embryonic development to maintaining intestinal homeostasis. It is characterized by an extensive plasma membrane blebbing followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called “budding.” Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment and can be seen as small bubbles surrounding the apoptotic cell. We can distinguish between intrinsic and extrinsic apoptosis depending on the triggering event location (Figure 4).

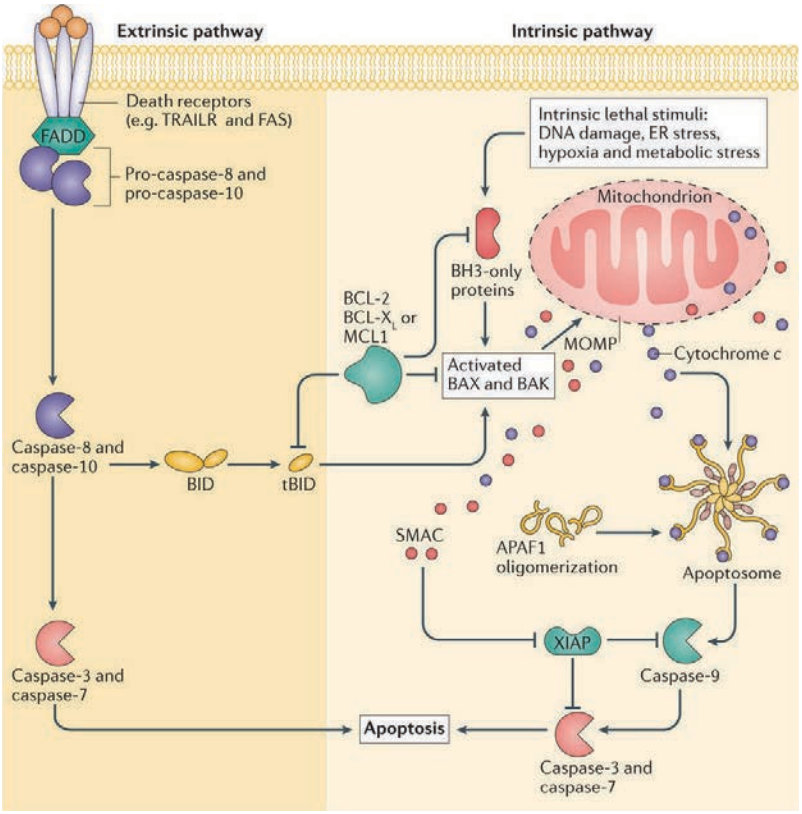

Figure 4 Overview of the apoptotic pathways. Upon TNF, TRAIL or FASL binding to their cognate receptor, the extrinsic apoptotic pathway is triggered. Initiator caspase-8 and caspase-10 are activated through dimerization mediated by FADD. Active caspase-8 and caspase-10 then cleave and activate the effector caspase-3 and caspase-7, leading to apoptosis. The intrinsic apoptotic pathway requires mitochondrial outer membrane permeabilization (MOMP). Cell stresses engage BH3-only protein activation, leading to BAX and BAK activity that triggers MOMP. Anti-apoptotic BCL-2 family proteins counteract this. Following MOMP, mitochondrial intermembrane space proteins such as SMAC and cytochrome c are released into the cytosol. Cytochrome c interacts with APAF1, triggering apoptosome assembly, which activates caspase-9. Active caspase-9, in turn, activates caspase-3 and caspase-7, leading to apoptosis (Ichim & Tait, 2016).

1.2.1.1. Extrinsic apoptosis

Extrinsic apoptosis is initiated by the binding of death ligands and death receptors. Death receptors belong to the TNF receptor superfamily, including TNF-R1 (DR1/CD120a/p55/p60), Fas (Apo-1/CD95), DR3 (APO-3/LARD/TRAMP), TRAILRR1 (DR4/APO-2), TRAILR2 (DR5), DR6, ectodysplasin A receptor (EDAR), and nerve growth factor receptor (NGFR)(Jing Li et al., 2016). Similar biochemical pathways are triggered where Fas-associated death domain-containing protein (FADD) and the initiator caspases, pro-caspase-8/10 (c8/c10) are recruited to the complex. FADD, through its dead domain, mediates the recruitment and dimerization of caspase-8 resulting in autoproteolytic cleavage and activation of caspase-8/10 (cC8, cleaved caspase-8). Activated caspase-8/10 cleave and activate effector caspases (caspase-3, -6 and -7), which subsequently target a range of cellular substrates that lead to apoptosis.

1.2.1.2. Intrinsic apoptosis

In intrinsic apoptosis, the mitochondria plays a central role releasing cytochrome-c, which will activate pro-caspase-9 and further downstream caspases. The intrinsic apoptotic pathway, which is often deregulated in cancer, is engaged by a wide array of stimuli that are sensed intracellularly, including cytokine deprivation, DNA damage and endoplasmic reticulum (ER) stress (Czabotar, Lessene, Strasser, & Adams, 2013). These diverse apoptotic stresses converge to trigger one crucial event: mitochondrial outer membrane permeabilization (MOMP). MOMP can also be achieved by cleavage of BID into tBID by caspase-8, enabling a crosstalk between the extrinsic and intrinsic apoptotic pathways (H. Li, Zhu, Xu, & Yuan, 1998; Luo, Budihardjo, Zou, Slaughter, & Wang, 1998). MOMP is usually considered the point of no return in the apoptotic pathway allowing the release of pro-apoptotic factors like cytochrome-c and SMAC/DIABLO from the mitochondria into the cytosol to activate the caspase cascade. SMAC will act by inhibiting apoptotic inhibitors while cytochrome-c binds to and oligomerizes Apaf1, a caspase-activating protein, recruiting pro-caspase-9 via interaction with its caspase recruitment domain (CARD) inducing its activation. Active cleaved caspase-9, in turn, activates caspase-3 and caspase-7, leading to apoptosis (Ichim & Tait, 2016).

Bcl-2 family proteins are central regulators of the intrinsic pathway, which either suppress or promote changes in mitochondrial membrane permeability required for release of cytochrome-c and other apoptogenic proteins. Bcl-2 proteins are classified into three groups according to their function in apoptosis and the number of Bcl-2 homology (BH) domains they possess (Czabotar, Lessene, Strasser, & Adams, 2013; García-Sáez, 2012; Ichim & Tait, 2016; Youle & Strasser, 2008):

(a) The anti-apoptotic or pro-survival Bcl-2 proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl1, and A1), which contain four BH domains BH1-BH4 and suppress cell death by binding and inhibiting the pro-apoptotic Bcl-2 proteins

(b) The pro-apoptotic effector proteins Bax and Bak, which present BH1-BH4 and directly promote MOMP

(c) The BH3-only proteins, which, except Bid, only contain a highly conserved BH3 domain and are very heterogeneous. The BH3-only proteins can be further classified as ‘direct activators’ if they directly interact and activate Bax and Bak or ‘sensitizers’ if they bind antiapoptotic proteins and displace the direct activators from them.

1.3.2. NECROPTOSIS

In the early 2000, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), a molecule already recognized as an important regulator of cell survival, inflammation and disease, was assigned an additional function: the regulation of a novel cell death pathway, that came to be known as necroptosis, downstream of FAS, TRAIL and TNF ligands (Holler et al., 2000). Subsequently, RIPK1 was identified as the target of a small-molecule inhibitor, necrostatin-1 (Nec-1), which suppressed cell death triggered by caspase inhibition (Degterev et al., 2005,

2008). These findings led to the coining of the term 'necroptosis' to refer to a necrosis-like cell death mode that depends on RIPK1 and RIPK3 kinase activity (Figure 5).

Necroptosis can be triggered through different stimuli. Most of the studies have been performed after TNF, FAS or TLRs stimulation but it can also be triggered by intracellular events, such as viral infection through Z-DNA or Z-RNA sensing via Z-DNA binding protein 1 (ZBP1/DAI) (Upton, Kaiser, & Mocarski, 2012). For instance, downstream of TNF, when caspases are not fully activated or their activity is blocked, for example by viral inhibitors, the protein kinase RIPK3 is recruited and forms the necrosome, which will lead to necroptotic cell death (Cho et al., 2009; S. He et al., 2009). Once engaged, another kinase, RIPK1, and RIPK3 undergo auto and transphosphorylation leading to their activation. Interestingly, whereas RIPK3 can also phosphorylate RIPK1, RIPK1 does not phosphorylate RIPK3 (Cho et al., 2009). The requirement for RIPK1 and RIPK3 trans and autophosphorylation can explain the formation, through RIPK1 and RIPK3 RHIM domains of RIPK1/3 amyloid structures to induce necroptosis, a required step for RIPK3 autophosphorylation (Jixi Li et al., 2012; Orozco et al., 2014). All those signals converge in mixed lineage kinase domain-like (MLKL) phosphorylation and activation and subsequent cell death (Sun et al., 2012; Zhao et al., 2012). Phosphorylated MLKL binds to the inner leaflet of the plasma membrane and forms the necroptotic pore, executing necroptosis (Cai et al., 2014; Yves Dondelinger et al., 2014; H. Wang et al., 2014).

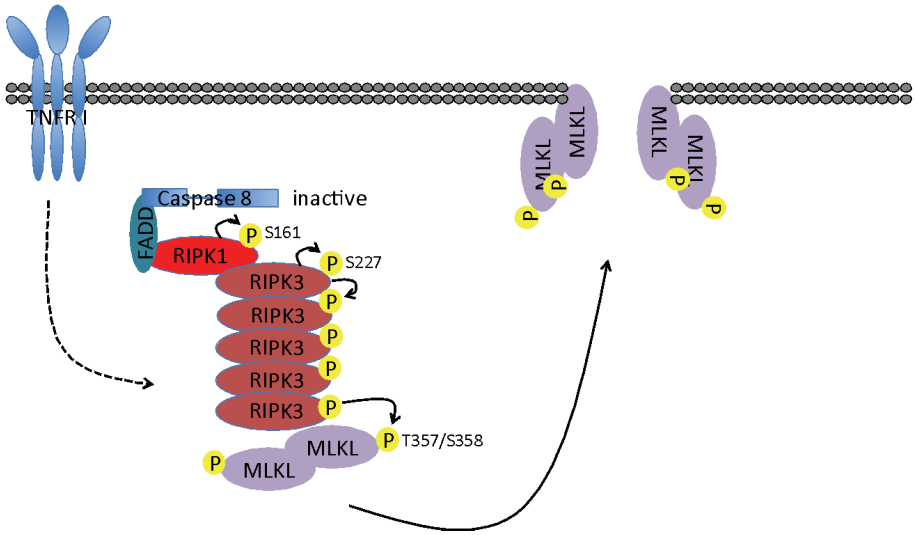

Figure 5. Necroptotic pathway. Upon necrosome formation, RIPK1 and RIPK3 oligomerize and are authophosphorylated at S161 and S227 respectively. These phosphorylations allow RIPK3 phosphorylation of MLKL at T357 and S358. Subsequently, MLKL binds the inner part of the cellular membrane creating the necroptotic pore and inducing necroptosis.

RIPK1 can be autophosphorylated at S14/15, S20, S161 and S166, (Degterev et al., 2008) although only S161 is required to induce necroptosis (Y. Zhang et al., 2017). RIPK1 phosphorylation on S89 or MK2 mediated phosphorylation of S321 impair RIPK1 mediated cell death (Jaco et al., 2017; McQuade, Cho, & Chan, 2013). Phosphorylation of S227 in RIPK3 allows the binding of RIPK3 to MLKL (Sun et al., 2012). In addition, MLKL is phosphorylated by RIPK3 at T357 and S358 residues in human, and S345, S347, and T349 residues in mouse. These phosphorylation sites are necessary for necroptosis since mutation of both sites inhibits necroptotic cell death (J. M. Murphy et al., 2013; Sun et al., 2012).

Nonetheless, under normal conditions, TNFR1 activation will trigger NF-κB activation. The NF-κB pathway will, in turn, induce the expression of anti-apoptotic genes that will block cell death. Necroptosis will also be inhibited as RIPK1 needs to bind first to caspase-8, which in turn can cleave RIPK1 and RIPK3 (Feng et al., 2007; Y. Lin, Devin, Rodriguez, & Liu, 1999). Of note, NF-κB activation is a typical feature of IBD.

1.3. NF-κB SIGNALING

In 1986 Sen and Baltimore discovered NF-κB. It was first characterized as a transcription factor binding to a sequence in the κ immunoglobulin enhancer and thought to be restricted to B cells (Sen & Baltimore, 1986). Since then, NF-κB expression has been found in every cell type and five transcription factors have been characterized in mammal cells: RelA (p65), RelB, c-Rel, and the two precursors NF-κB1 and NF-κB2 (p105, p100) that can be processed through the proteasome to p50 and p52, respectively. While all five NF-κB molecules contain the N-terminal Rel homology domain, which is essential for DNA binding, dimerization, inhibitor of κB (IκB) interaction, and nuclear localization (Durand & Baldwin, 2017), Rel proteins (RelA, RelB and c-Rel) also contain a C-terminal transcriptional activation domain (M. L. Schmitz & Baeuerle, 1991). Translocation of the NF-κB dimers to the nucleus will induce the transcription of pro-inflammatory genes, including *TNF, IL1, IL6* as well as anti-apoptotic genes, such as *BFL1/A1, BCLXL, BCL2, NR13, FLIP, IAPs* and *XIAP* (Figure 6).

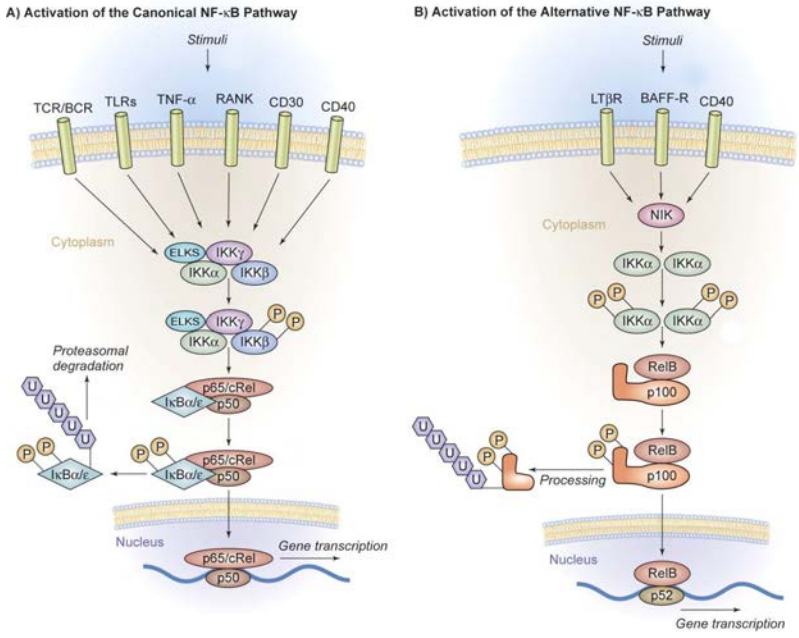

Figure 6. NF-κB pathway overview. Schematic canonical (A) and alternative (B) NF-κB pathway activation. Adapted from Jost *et al.*, Blood, 2007 (Jost & Ruland, 2007).

The canonical NF-κB pathway depends on the degradation of the IκB proteins, which are preventing nuclear entry and DNA binding of RelA, RelB and c-Rel. Of note, the

translocation of p52;RelB dimers does not depend on the degradation of IκB but the proteosomal processing of p100 into p52, induced by an alternative NF-κB pathway.

1.3.1. CANONICAL NF-κB SIGNALING

The canonical NF-κB dimer is composed of a p50 and a RelA (p65) subunit that in basal conditions is sequestered in the cytosol as part of a complex together with IκBα (Baeuerle & Baltimore, 1988). The IκB family is composed of seven proteins containing an ankyrin repeat domain (ARD), IκBα, IκBβ, IκBε and the unusual Bcl3, IκBζ and IκBNS. IκBα, IκBβ, and IκBε bind to NF-κB dimers under basal conditions and their phosphorylation leads to IκB degradation, releasing NF-κB dimers to translocate to the nucleus and bind DNA. Bcl3 and IκBζ can be induced upon stimulation and regulate the activity of NF-κB dimers in the nucleus. IκBNS participates in the development of Tregs.

IκBs are phosphorylated by the IκB kinase (IKK) complex, at two N-terminal serines triggering IκB polyubiquitination and proteasome-mediated degradation (DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997). The IKK complex is composed of the two catalytic subunits IKKα/IKK1 and IKKβ/IKK2 and two regulatory subunits, NEMO/IKKγ and ELKS. Activation of the kinases in the IKK complex depends on the phosphorylation at two different sites for IKKα and IKKβ (S176, S180 and S177, S181 respectively). Phosphorylation of IKKβ by transforming growth factor-β activated kinase 1 (TAK1) will induce the canonical NF-κB pathway whereas phosphorylation of IKKα by NF-κB-inducing kinase (NIK) determines the signaling through the alternative NF-κB pathway. Although IKKα is located in the IKK complex, its kinase activity is dispensable for the activation of the canonical NF-κB (Hu et al., 1999; Takeda et al., 1999) (Figure 6 A).

Multiple intracellular or extracellular stimuli such as TNF, IL-1, lipopolysaccharide (LPS), viral double-stranded RNA, and ionizing radiation can induce the activation of the IKK complex (Brach et al., 1991; Osborn, Kunkel, & Nabel, 1989) (Brach et al., 1991; Chow, Young, Golenbock, Christ, & Gusovsky, 1999; Gil, Alcamí, & Esteban, 2000; Hoshino et al., 1999; Osborn et al., 1989). Among them, TNFα interaction with its receptor is the most widely studied mechanism for the canonical NF-κB activation.

1.3.2. ALTERNATIVE NF-κB SIGNALING AND THE LYMPHOTOXIN RECEPTOR

Different receptors can activate the alternative NF-κB pathway including the LTβ receptor (LTβR), CD40, B cell activating factor (BAFF), Fibroblast growth factor-inducible 14 (Fn14), TNFR2, receptor activator of NF-κB (RANK) and several other TNFR family members (Claudio, Brown, Park, Wang, & Siebenlist, 2002; Coope et al., 2002; Dejardin et al., 2002; Kayagaki et al., 2002; Rauert et al., 2010; Saitoh et al., 2003) (Figure 6 B). Of note, these receptors not only trigger the non-canonical NF-κB pathway, but simultaneously also the canonical pathway.

Different receptors can activate the alternative NF-κB pathway, among LTβR, which is mainly expressed in non-haematopoietic and myeloid lineage cells whereas its cell-surface ligand LTαβ is expressed by activated lymphocytes and a subset of resting B cells (J L

Browning et al., 1997; Jeffrey L Browning & French, 2002). Two are the ligands for LTβR: LTα1β2 heterotrimers and lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells (LIGHT). LTα is a secreted protein, but when co-expressed with a second related protein known as LTβ, it forms a heteromeric TNF-family ligand that remains tethered to the cell surface through the transmembrane domain of LTβ (Ware, VanArsdale, Crowe, & Browning, 1995). The secreted LTα forms homotrimers that can bind TNFR1 and TNFR2. LIGHT binds not only to LTβR but also to the additional receptors herpes-virus entry mediator (HVEM) and decoy receptor 3 (DCR3) (Gommerman & Browning, 2003).

NF-κB inducing kinase (NIK) is the most important kinase of the non-canonical pathway and in in resting cells it is kept at very low levels by basal proteosomal degradation. (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Upon ligand binding to its receptor, NIK is accumulated and activated via trans or auto-phosphorylation (X. Lin et al., 1998). NIK activation leads to IKKα phosphorylation on its activation loop, that in turn causes IKKα to bind and phosphorylate p100 within its C-terminal ankyrin repeat domain (Liang, Zhang, & Sun, 2006). This phosphorylation stimulates recruitment of the $SCF^{\beta TrCP}$ E3 ligase complex that polyubiquinates p100 and causes its partial degradation, resulting in release of the N-terminal p52 fragment bound to RelB. RelB/p52 dimers are able to translocate to the nucleus and regulate a different group of genes induced by the canonical NF-κB pathway such as *IL8*, *IL2, CD28RE* or cyclin D1 (*CCND1*) (Bonizzi et al., 2004; Dejardin et al., 2002; Fusco et al., 2009; Leung, Hoffmann, & Baltimore, 2004).

1.3.3. NF-κB TARGET GENES AND GENE REGULATION

The NF-κB transcription factor family contains a conserved sequence of 300 aminoacids in the amino-terminal region, the Rel homology domain (RHD), which is required for dimerization, nuclear translocation, interaction with IκBs and DNA binding. The amino-terminal part of the RHD mediates specific DNA binding to the NF-κB consensus sequence present in regulatory elements of NF-κB target genes (5'GGGPuNNPyPyCC-3') (Ghosh, Van Duyne, Ghosh, & Sigler, 1995; Müller, Rey, Sodeoka, Verdine, & Harrison, 1995). Whereas the dimeric complex p50/p65 is the most abundant being found in almost every cell type, other dimeric complexes of dimeric complexes of p65/p65, p65/c-Rel, p65/p52, c-Rel/c-Rel, p52/c-Rel, p50/c-Rel, p50/p50, RelB/p50, and RelB/p52 have been described, some of them only in limited subsets of cells (Hayden & Ghosh, 2004). It is interesting to note that not all of them are transcriptionally active, for instance, p50 and p52 homo and heterodimers have been found to repress κB-dependent transcription, most probably by preventing transcriptionally active NF-κB dimers from binding to κB sites, or through recruitment of deacetylases to promoter regions (Zhong, May, Jimi, & Ghosh, 2002)

The NF-κB family of proteins can be further divided into two groups based on their transactivation potential because only p65, RelB, and c-Rel contain carboxy-terminal transactivation domains (TAD). RelB is unique in that it requires an amino-terminal leucine zipper region in addition to its TAD to be fully active. p50 and p52 are generated by processing of the precursor molecules p105 and p100, respectively. The amino-termini of these precursors contain the RHDs of p50 or p52, followed by a glycine rich region (GRR) and multiple copies of ankyrin repeats that are characteristic for the IκB protein family and

need to be partially degraded to allow the translocation to the nucleus.

Biochemical analysis revealed preferential affinities of individual dimers for particular sites *in vitro*. However, knockout studies demonstrated that biochemical affinity data do not fully explain the specificity of NF-κB dimers *in vivo* and it has become obvious that dimer selection cannot be reduced to the κB sequence alone (Britanova, Makeev, & Kuprash, 2008; Hoffmann, Leung, & Baltimore, 2003; J. Schreiber et al., 2006; Udalova, Mott, Field, & Kwiatkowski, 2002). Therefore, NF-κB dimer's binding ability to the DNA depends also on the chromatin configuration and the recruitment of other transcription factors on modifying enzymes.

1.4. TNF RECEPTOR SIGNALING: A DOUBLE EDGE SWORD

TNF is able to bind two receptors: TNFR1 and TNFR2, which differ in their structure and expression pattern, as well as in the signaling pathways that they induce once they are engaged. TNFR1 is expressed in all cell types whereas TNFR2 is restricted to immune cells and endothelial cells. Both are able to activate the NF-κB pathway through different signaling cascades as a result of strikingly different intracellular domains. TNFR1 contains a cytoplasmic death domain (DD), which is a conserved sequence of 80 amino acids that forms a distinctive fold (Lavrik, Golks, & Krammer, 2005; Tartaglia, Ayres, Wong, & Goeddel, 1993) and allows the recruitment of TRADD. TNFR2 lacks the death domain and recruits TNFR-associated factor 1 (TRAF1) and TRAF2 rather than TRADD (Hsu, Xiong, & Goeddel, 1995; Rothe, Sarma, Dixit, & Goeddel, 1995; Tartaglia et al., 1993). Both TNFR1–TRADD signaling and TNFR2 signaling through TRAF1 and TRAF2 can lead to NF-κB activation, but whereas TNFR2 engagement promotes cell survival via this pathway, TNFR1–TRADD signaling can result in either cell survival or cell death depending on downstream signaling events and cellular context, and therefore this work focuses on the downstream events of TNFR1 (Figure 7).

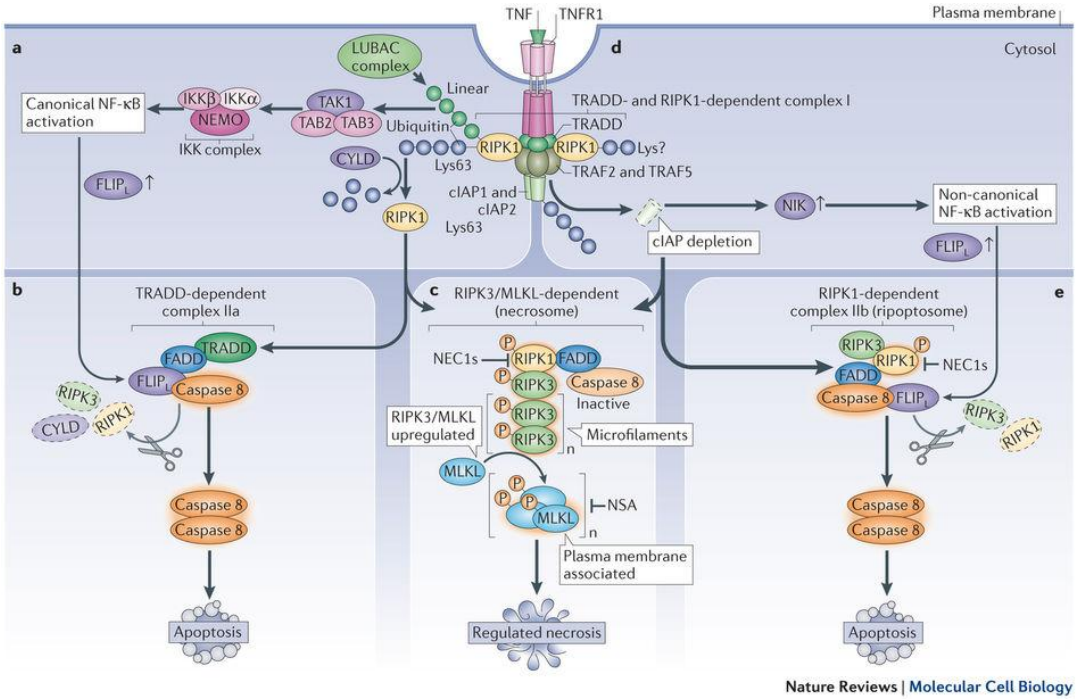

Figure 7 Overview of the downstream events triggered by TNF. Upon TNF engagement to the TNFR1, TNFR1 complex I is formed leading to the activation of the canonical NF-κB pathway (A). In certain conditions, such as inhibition of NF-κB signaling, complex IIa if formed inducing apoptosis (B). When caspases are inhibited, a regulated form of necrosis, termed necroptosis, takes place. The necrosome is formed where the kinase activity of both RIPK1 and RIPK3 is required. Necrostatin-1s (Nec1s), a RIPK1 inhibitor, and necrosulfonamide, an inhibitor of human MLKL, prevent this cell death (C). When cIAP1/2 levels are depleted, NIK can get accumulated in the cytoplasm and activate the alternative NF-κB pathway (D). cIAP1/2 depleted conditions or certain alterations in the ubiquitin status of RIPK1 induce to the formation of complex IIb or Ripoptosome. The Ripoptosome depends on the kinase activity of RIPK1 to induce RIPK1-dependent apoptosis (E) (Berghe, Linkermann, Jouan-Lanhouet, Walczak, & Vandenabeele, 2014).

1.4.1. TNFR1 COMPLEX I

Upon TNF binding to homotrimers of TNFR1, the adaptor molecule TNFR1-associated death domain protein (TRADD) is recruited to the cytoplasmatic TNFR1 domain. In a step-wise process, RIPK1, TNFR-associated factor 2 (TRAF2), cellular inhibitor of apoptosis protein 1 (cIAP1) or cIAP2, and linear ubiquitin chain assembly complex (LUBAC) are recruited to form signaling complex I (Figure 8). TRAF2 and cIAP1/2 mediate K63-linked ubiquitination of the complex. In this situation, the kinase RIPK1 acts as a scaffold protein that allow the docking of the adaptor proteins TAK1-binding protein 2 (TAB2) and (TAB3) and the kinase TAK1 through RIPK1 K63-ubiquitins (Kanayama et al., 2004). Meanwhile, the LUBAC complex, consisting of heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1), HOIP (HOIL-1 interacting protein, also known as E3 ubiquitin-protein ligase RNF31) and SHANK-associated RH domain-interacting protein (SHARPIN), mediates M1-ubiquitination of some components in the complex I, as RIPK1, or NEMO (Haas et al., 2009; Ikeda et al., 2011a). The IKK complex is also recruited to the complex and, after phosphorylation of IKKβ by TAK1, mediates the activation of the canonical NF-κB pathway and cell survival. TAK1 also

triggers mitogen-activated kinase (MAPK) signaling cascades that lead to activation of downstream JUN N-terminal kinase (JNK) and p38, as well as AP1 transcription factors (Kalliolias & Ivashkiv, 2015).

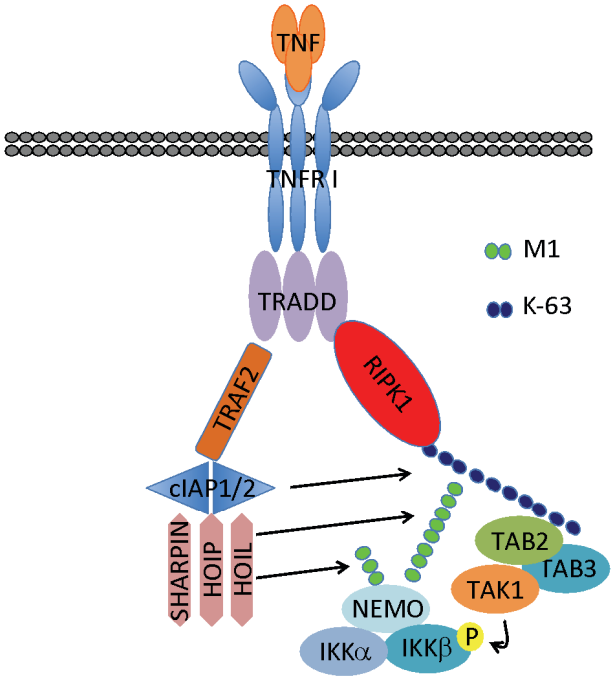

Figure 8.TNFR complex I. TNF binding to TNFR1 induces recruitment of TRADD and RIPK1. RIPK1 is lineary ubiquitinated by LUBAC (SHARPIN, HOIP and HOIL) and K63-ub by cIAP1/2. This allow the recruitment of the IKK complex through linear ubiquitins and its activation by TAK1, which is bound to K63 ubiquitins through TAB2/3.

As TNF induces a very potent signaling cascade, fine-tuning regulation of kinetics is required to prevent chronic inflammation. These processes take place at different levels by affecting the assembly of complex I, preventing the activation of the IKK complex or regulating downstream checkpoints. TNFR cleavage is an upstream regulatory mechanism that desensitizes cells from TNF by depleting TNFR1 from the cell surface but also by increasing the pool of soluble TNFR that could function as an inhibitor by competing for the binding of the ligand to the cell surface receptors. However, soluble receptors could also bind and stabilize TNF acting as an agonist (Xanthoulea et al., 2004). Downstream events also target the formation of complex I. Optineurin, a homolog of NEMO, competes with NEMO for binding K63 ubiquitins in RIPK1 displacing the IKK complex from the TNFR (Zhu, Wu, Zhao, & Ashwell, 2007). Other described mechanisms for IKK complex inactivation involve p47 mediated lysosomal degradation of NEMO (Y. Shibata et al., 2012) or dephosphorylation of the IKK complex by PP2A and PP1 (H.-Y. Li et al., 2008; S. Li, Wang, Berman, Zhang, & Dorf, 2006). Post-translational modifications of NF-κB subunits and have been shown to participate in shutting off the NF-κB response by altering cofactor binding or mediating displacement and degradation of NF-κB dimers (Chew et al., 2009; Kiernan et al., 2003; Tanaka, Grusby, & Kaisho, 2007). TNF target genes also include negative feedback regulators. Newly resynthesized IκB proteins can enter the nucleus remove NF-κB from the DNA, and re-localize it to the cytosol (Hayden & Ghosh, 2004; Hoffmann, 2002).

Finally, alterations in the ubiquitination patterns in complex I by newly synthesized or already

present proteins can terminate the TNF signaling (Skaug, Jiang, & Chen, 2009).

1.4.2. TNF-INDUCED APOPTOTSIS OR COMPLEX IIa

TNF is a major activator of the pro-survival pathway NF-κB (complex I). However, in special circumstances, complex I is converted to complex IIa, IIb or the necrosome to induce different types of cell death.

Upon stimulation of TNFR1 by TNF, a RIPK1- and TRADD-dependent receptor-bound complex I is formed, which is pivotal for the activation of NF-κB and the resulting upregulation of anti-apoptotic genes such as *BCL2* and *FLIP* (FLICE-like inhibitory protein). In a negative feedback loop, the deubiquitinating activity of A20 is believed to restrict TNF-induced NF-κB signaling by removing K63-linked polyubiquitin chains from RIPK1 (Wertz et al., 2004). Moreover, CYLD also removes polyubiquitin chains from RIPK1, which results in the dissociation of RIPK1 from TNFR1 and formation of a cytosolic death-inducing signaling complex (DISC) (Figure 9) (Hitomi et al., 2008; Moquin, McQuade, & Chan, 2013; O'Donnell et al., 2011; Schlicher et al., 2016). Ubiquitin removal from RIPK1, through deubiquitination by CYLD, or ubiquitination-impairment by cIAP1/2 depletion (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008), alter formation of complex I allowing its disassembly and TNFR1 internalization (Schneider-Brachert et al., 2004). TRADD, FADD, pro-caspase-8 (caspase-8) and FLIPs are then recruited to the TNFR1. The long isoform of FLIP ($FLIP_L$) and pro-caspase-8 form a heterodimeric caspase that cleaves and inactivates RIPK1 and RIPK3, as well as CYLD, to prevent necroptosis.

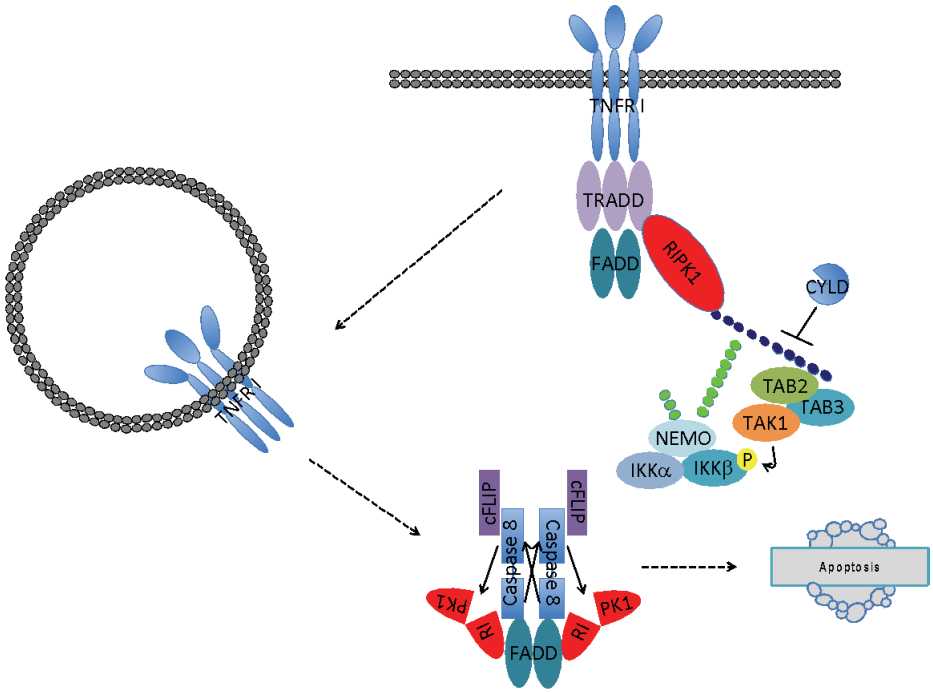

Figure 9. TNFR complex IIa.TNFR1 internalization, after RIPK1 deubiquitination and complex I destabilization, allows the creation of complex IIa. Caspase-8 in complex IIa will undergo homocleavage to induce its activation as well as cleavage of RIPK1 and will execute apoptosis.

This TRADD-dependent complex IIa also allows caspase-8 homodimerization and activation, resulting in activation of the executioner caspases caspase-3 and caspase-7, which trigger apoptosis, although in normal conditions this process will be inhibited as a consequence of previous NF-κB activation and expression of anti-apoptotic genes (Feng et al., 2007; Micheau et al., 2002; O'Donnell et al., 2011).

1.4.3 TNF-INDUCED RIPOPTOSOME OR COMPLEX IIb

TNFR complex IIb or Ripoptosome (Figure 10) has been described to occur downstream of TNF when cIAP1/2 is depleted through SMAC mimetics (SM) (Petersen et al., 2007; Tenev et al., 2011; L. Wang et al., 2008), however it does not necessary require TNF if triggered by etoposide (Tenev et al., 2011). The exact mechanism that triggers the formation of complex IIb instead of IIa is unknown, although in this case the activation of NF-κB does not prevent apoptosis (Y Dondelinger et al., 2013). TNF treatment together with TAK1 pharmacological inhibition also triggers RIPK1-dependent apoptosis, in a similar manner as TNF+SM treatment, suggesting that TAK1 recruitment to cIAP1/2-ubiquitinated RIPK1 inhibits RIPK1-dependent apoptosis (Y Dondelinger et al., 2013). In fact, IKKα and IKKβ, the downstream kinases of the kinase TAK1, inhibit RIPK1 association with the Ripoptosome through direct phosphorylation of RIPK1 (Legarda-Addison, Hase, O'Donnell, & Ting, 2009).

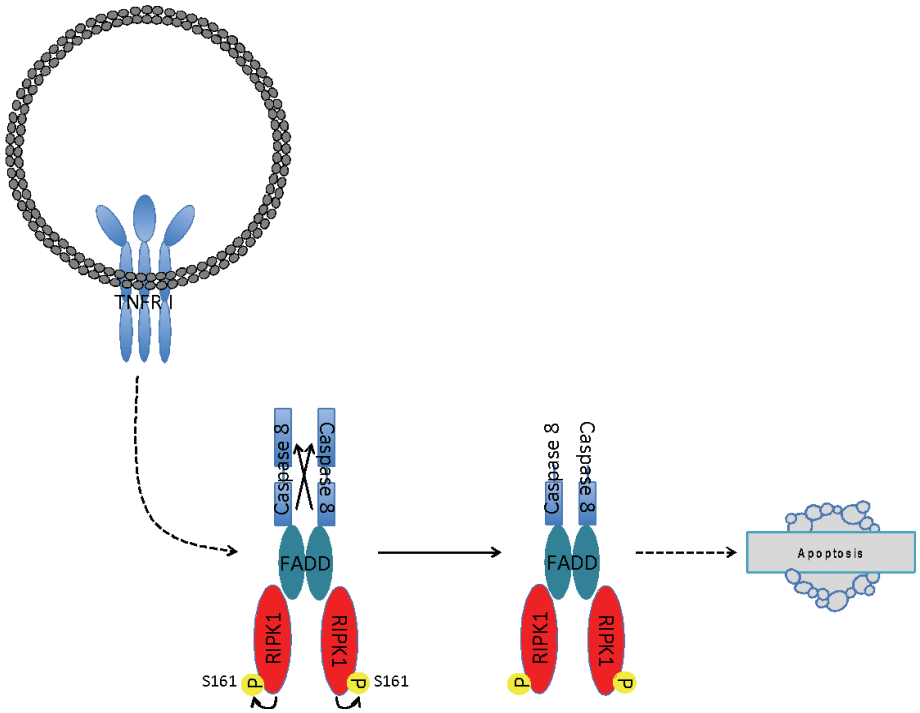

Figure 10.TNFR complex IIB or Ripoptosome. Complex IIb or Ripoptosome is composed of caspase-8, FADD and RIPK1. RIPK1 needs to be activated by autophosphorylation in S161 to induce RIPK1-dependent apoptosis mediated by caspase-8.

Complex IIb or Ripoptosome is composed of RIPK1, FADD and Caspase-8 and is independent of TRAILR1/DR4, TRAILR2/DR5, and Fas/CD95 activation (Petersen et al., 2007). TLR3 could also potentially induce complex IIb. TLR3 activation can induce apoptotic

cell death downstream of TRIF that depends on a complex formed also by RIPK1, caspase-8 and FADD, although whether it requires RIPK1 kinase activity is unknown (Han et al., 2004; Kaiser & Offermann, 2005). Of note, TNFR1 activation is dispensable if cell death is triggered by etoposide, a genotoxic stress inducer that also depletes cIAPs, although in this case complex IIb formation occurs 6h after the treatment, 4 hours later than when triggered downstream of TNF. Complex IIb requires the kinase activity of RIPK1 to induce cell death although the exact mechanism of its activation or its role as a kinase is unknown (Tenev et al., 2011).

1.4.4. TNF-INDUCED NECROPTOSIS

It is well established that engagement of death receptors as Fas, TRAILR1/2 and specially TNF can lead to programmed cell death via apoptosis, through recruitment of the caspase-8 precursor to the respective receptor complexes and activation of the latter protease as a consequence. However, in the presence of viral or synthetic caspase inhibitors, the signaling can be shifted a much more rapid and necrotic mode of cell death that requires activation of RIPK3 and the downstream pore-forming protein MLKL (Figure 5) (S. He et al., 2009; Holler et al., 2000; Sun et al., 2012; Vercammen et al., 1998; D.-W. Zhang et al., 2009). Blockage of caspases, specially caspase-8, is of vital importance as presumable they mediate the cleavage of RIPK1 and RIPK3 and prevent necroptosis (Feng et al., 2007; Y. Lin, Devin, Rodriguez, & Liu, 1999; Ofengeim & Yuan, 2013; van Raam, Ehrnhoefer, Hayden, & Salvesen, 2013). When caspases are inhibited, activated RIPK1, which gets autophosphorylated at different residues (Degterev et al., 2008), heterodimerizes through the RHIM domain with RIPK3 (Cho et al., 2009). RIPK3 autophosphorylation and MLKL recruitment through its RHIM domain are required for further phosphorylation by RIPK3 of MLKL and downstream execution of necroptosis (Orozco et al., 2014; Sun et al., 2012; Wu et al., 2014).

1.4.4. UBIQUITIN ROLE IN TNF SIGNALING AND THE NF-κB PATHWAY

Upon TNF engagement to its receptor, different pathways can be triggered. NF-κB induction or cell death execution depends on activation of antiapoptotic genes, protein synthesis or protease activation, such as caspases, among others. However, they all depend on different complex formation, which is tightly regulated by ubiquitination.

Two independent studies in the 1980s showed how a 76-aminoacid molecule, termed ubiquitin (Ub), upon binding to proteins induced their degradation through an ATP-dependent protease (Ciehanover, Hod, & Hershko, 1978; Hershko, Ciechanover, Heller, Haas, & Rose, 1980; Wilkinson, Urban, & Haas, 1980). The protease was characterized by several laboratories much later, in the 1990s, and is now termed the 26S proteasome (Hough, Pratt, & Rechsteiner, 1986; Varshavsky, 2017). Since then, we have learned that ubiquitins can link in different combinations to perform a wide variety of functions. The c-terminal glycine of ubiquitin is linked via lysine or methionine residues of substrate proteins to by ubiquitin E3 ligases. Ubiquitin contains seven lysine residues and the free N-terminal methionine forming M1-, K6-, K11-, K27-, K29-, K33-, K48- and K63-linked ubiquitin chains.

Single ubiquitin molecules can also be attached to target proteins and this is termed mono- or multi mono-ubiquitylation (Feltham & Silke, 2017). It is interesting to note that M1-linked ubiquitin chains (or linear ubiquitin chains) depend on the presence of K63-linked chains forming hybrid chains (M1-K63-linked ubiqutins), or independent chains within the same protein (Emmerich et al., 2013). This is thought to allow the recruitment of different complexes to mediate its activation.

K48-ubiquitin chains are recognized by the 26S proteasome and usually trigger degradation of the protein substrate. K63-ubiquitination generally does not trigger protein degradation and instead it regulates non-proteolytic functions including protein trafficking, kinase activation, the DNA damage response and signal transduction (Yau & Rape, 2016). M1-linked conjugates are rapidly synthesized following activation of inflammatory signaling cascades (Gerlach et al., 2011; Tokunaga et al., 2009). K11-ubiquitin chains are usually synthesized during mitosis and early G1 phase targeting cell cycle regulators for degradation (L. Jin, Williamson, Banerjee, Philipp, & Rape, 2008; Song & Rape, 2010). K6 linkages were observed during removal of damaged mitochondria from cells (Ordureau et al., 2015), K27 linkages have been implicated in regulating DNA repair and autoimmunity (Gatti et al., 2015; J. Liu et al., 2014), K29 linkages have been attributed roles in proteasomal degradation (Johnson, Ma, Ota, & Varshavsky, 1995), and K33-linked chains were proposed to regulate trafficking through the trans-Golgi network (Yuan et al., 2014).

1.4.4.1. Ubiquitin ligases

Ubiquitination is a reversible covalent modification catalyzed by three enzymatic steps. In the first step, ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. In the second step, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2 or Ubc), forming an E2-Ub thioester. Finally, in the presence of a ubiquitin-protein ligase (E3), ubiquitin is attached to a target protein through an isopeptide bond between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue in the target protein (Rieser, Cordier, & Walczak, 2013). Although different E3 ligases are recruited to the TNFR1 complex, not all of them are directly mediating an ubiquitin ligase activity. For instance, although the E3 TRAF2 is recruited to the TNFR1 complex I and TRAF2 RNAi or gene ablation decreases RIPK1 ubiquitination (T. H. Lee, Shank, Cusson, & Kelliher, 2004; Wertz et al., 2004) it indirectly acts through recruiting cIAP1/2 (Mahoney et al., 2008; Tada et al., 2001). Two are the main ubiquitin ligases that control NF-κB activation and further downstream cell death events after TNFR1 activation: cIAP1/2 and the LUBAC complex.

- **cIAP1/2**

The cIAP1 and cIAP2 (cIAP1/2) proteins are members of the Inhibitors of Apoptosis (IAP) family, RING E3 ubiquitin ligases that associate with activated TNFR1, They are recruited to the TNFR1 through TRAF2, promoting the polyubiquitination of K63-RIPK1 through binding to the E2 UBCH5 and complex I formation. *In vitro* and *in vivo* experiments have shown that cIAP1/2 have redundant roles in TN-induced NF-κB activation and deletion of both is required to severely impair its activation and induce cell death (Mahoney et al., 2008;

Varfolomeev et al., 2008).

- **LUBAC complex**

So far, only one complex has been described to generate linear ubiquitin chains called the linear ubiquitin chain assembly complex. Initially is was thought to be just formed by two subunits termed HOIP/RNF31 and HOIL-1L/RBCK1 (Kirisako et al., 2006). Later on, an additional non-catalytic subunit called SHARPIN was described as part of the complex (Ikeda et al., 2011a, 2011b; Tokunaga et al., 2011). The HOIP subunit of LUBAC contains all the catalytic machinery required to synthesize linear ubiquitin chains. HOIP also acts as an anchoring molecule where HOIL-1L and SHARPIN bind. Interestingly, HOIP also binds to OTULIN, a deubiquitinase specific for the hydrolysis of linear polyubiquitin chains (Keusekotten et al., 2013; Rivkin et al., 2013). It was known that CYLD played a role in the LUBAC complex, but no direct interaction between both proteins could be detected *in vitro* (Draber et al., 2015; Takiuchi et al., 2014). Recently, SPATA2 has been described to interact with HOIP and bring CYLD, adding a degree of complexity to the LUBAC complex (Elliott et al., 2016; Kupka et al., 2016).

1.4.4.2. Deubiquitinases

Deubiquitinases (DUBs) are proteases that cleave ubiquitin from target proteins and therefore oppose the function of E3 ligases. The removal of ubiquitins from complex I induces its disassembly and termination of NF-κB signaling. CYLD and A20 are two deubiquitinases that play an important role in NF-κB signaling.

- **CYLD**

Cylindromatosis (CYLD) mediates the cleavage of various polyubiquitin linkages with a preference for K63 and M1 polyubiquitin, and to a lesser extent K11 and K48 polyubiquitin *in vitro* (Ritorto et al., 2014). In the TNFR complex I, it mediates the removal of K63 and M1 polyubiquitin chains from TRAF2, NEMO, TNFR1, TRADD and RIPK1 (Brummelkamp, Nijman, Dirac, & Bernards, 2003; Draber et al., 2015; Moquin et al., 2013; J. Zhang et al., 2006). However, it has been recently shown that K48–K63 branching protect K63 linkages from CYLD-mediated deubiquitination (Ohtake, Saeki, Ishido, Kanno, & Tanaka, 2016). In agreement with its role in deubiquitinating several key NF-κB signaling proteins, CYLD deficiency leads to constitutive NF-κB activation resulting in proinflammatory gene expression (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). A siRNA screen for regulators of necroptosis showed CYLD as a mediator or of necroptosis, a caspase-independent form of programmed cell death (Hitomi et al., 2008). Furthermore, CYLD is cleaved by caspase-8 and expression of an uncleavable CYLD mutant facilitates TNF-induced necroptosis as well as destabilization of TNFR complex I (O'Donnell et al., 2011). Finally, Moquin et al. (Moquin et al., 2013) demonstrated that CYLD regulates RIPK1 ubiquitination not only at the TNFR1 complex but also at the necrosome and thereby facilitates RIPK1 kinase activation and necroptosis stressing out the function of CYLD in both regulating NF-κB activation and cell death.

- **OTULIN**

OTU DUB with linear linkage specificity (OTULIN) was discovered in 2013 as an exclusive M1-ubiquitin chain deubiquitinase (Keusekotten et al., 2013; Rivkin et al., 2013). It has been reported to antagonize the activity of LUBAC and LUBAC-mediated NF-κB activation (Fiil et al., 2013; Keusekotten et al., 2013; Rivkin et al., 2013). In the context of TNFR1 signaling, removal of M1 polyubiquitin by OTULIN inhibits the association of NEMO with polyubiquitinated RIPK1 (Keusekotten et al., 2013). Counter-intuitively, expression of a catalytically inactive OTULIN mutant leads to decreased NF-κB activation (Keusekotten et al., 2013). M1 ubiquitination protects from TNF-induced cell death by stabilization of complex I (Haas et al., 2009) and stable OTULIN overexpression sensitizes cells to TNF-induced cell death (Keusekotten et al., 2013). Surprisingly, knockdown of OTULIN, and hence M1 ubiquitin accumulation, also induced cell death. Draber et al. (Draber et al., 2015) showed that OTULIN is not recruited to the TNFR1 signaling complex and does not regulate the amount of M1 ubiquitination at the signaling complex itself but rather the amount of cytosolic M1-ubiquitinated proteins including LUBAC components themselves. However, Wagner et al. found low levels of endogenous OTULIN in the TNFR1 complex upon TNF stimulation (Wagner, Satpathy, Beli, & Choudhary, 2016). Together it suggests that OTULIN might have an indirect effect in the TNFR complex I through the modulation of other complexes as LUBAC.

- A20

A20 is encoded by the *TNFAIP3* (TNFα-induced protein 3), a NF-κB-responsive gene that is thought to be involved in negative feedback regulation of NF-κB activation in response to many proinflammatory stimuli (Opipari, Boguski, & Dixit, 1990; Osborn et al., 1989). A20 contains an ovarian tumor (OTU) domain with deubiquitinating activity (DUB) in the amino-terminal region and seven carboxy-terminal zinc finger (ZnF) domains. A20's functions differ between *in vitro* and *in vivo* studies. *In vitro* studies have shown that A20 has the ability to hydrolyze K48 and, to a lesser extent, K63 through its OTU domain, as the mutation of the residue C103, located in the domain, prevents its activity (Evans et al., 2004; Komander & Barford, 2008). Another *in vitro* study has shown that ZnF4 is responsible for K48 ubiquitin ligase activity replacing K63-linked chains in RIPK1 and targeting it for proteosomal degradation (Wertz et al., 2004). However, cellular studies have shown that the *in vivo* E3 K48 ubiquitinase activity of A20 might depend on Itch and RNF11 (Shembade et al., 2008; Shembade, Parvatiyar, Harhaj, & Harhaj, 2009). Biochemical studies have shown that ZnF4 has three ubiquitin binding sites for K63-linked chains (Bosanac et al., 2010) and that ZnF7 has the ability to bind linear ubiquitin chains (Draber et al., 2015; Tokunaga et al., 2012; Verhelst et al., 2012). Interestingly, A20 binding to linear-ubiquitin chains prevents CYLD mediated cleavage of M1 chains and induces stabilization of the TNFR complex I (Draber et al., 2015). Finally, Lu et al. (Lu et al., 2013) have mapped a homodimerization domain in the OTU domain of A20. In fact, structural analysis of the OTU revealed that there are six or four molecules, per crystallographic asymmetric unit (Komander & Barford, 2008; S. C. Lin et al., 2008).

A20-deficient mice have a severe inflammatory phenotype, with hypersensitivity to TNF and

die prematurely due to severe multiorgan inflammation and cachexia (E. G. Lee, 2000). Although several reports describe that A20 terminates the NF-κB pathway through its DUB activity, A20 knock-in mice bearing an inactivating mutation in DUB (C103A) or ZnF4 domains do not exhibit the severe inflammatory phenotype of full A20-knockout mice (De, Dainichi, Rathinam, & Ghosh, 2014; Lu et al., 2013). Interestingly, macrophages of wild type and A20 C103A mice have similar amounts of ubiquitinated RIPK1 after TNF stimulation (De et al., 2014) and Timothy et al. found just a slightly decrease in the activation of NF-κB in MEFs from OTU or ZnF4 mutant knock in mice (Lu et al., 2013). This suggest that previous reports showing inhibition of NF-κB mediated by A20 could be as a consequence of massive overexpression of mutant proteins that may change the stoichiometry ratios between interacting proteins. Indeed, multiple examples show how A20 can modulate the NF-κB signaling via non-catalytic mechanisms through binding of A20 to K63 and M1 polyubiquitin via its ZnF4 and ZnF7, respectively, competing with other ubiquitin binding proteins (Bosanac et al., 2010; Draber et al., 2015; Shembade, Ma, & Harhaj, 2010; Verhelst et al., 2012).

A20 role in cell death seems to be more dependent on the cell type than its NF-κB regulatory function. A20 specific deletion in B and T cells protected them from FAS and TCR induced cell death (Onizawa et al., 2015; Tavares et al., 2010) whereas MEFs are more susceptible to TNF induced cell death (E. G. Lee, 2000). In IEC, deletion of A20 on those cells renders the mice more susceptible to the DSS colitis model with higher amounts of apoptotic cells in the epithelial colon (Vereecke et al., 2010). Whereas the previous study did not show spontaneous intestinal inflammation, combined deletion of A20 in IEC and the myeloid compartment induces spontaneous colitis and ileitis with the presence of apoptotic cells in crypt compartment (Vereecke et al., 2014). Overexpression of A20 in the IEC protects the intestinal epithelial barrier after LPS challenge and prevents colitis induced by DSS but not TNBS (Kolodziej et al., 2011; Rhee et al., 2012).

The mechanism by which A20 regulates cell death remains largely unclear. In Jurkat T cells, it was proposed that A20 prevents the recruitment of TRADD and RIPK1 to the TNFR1, subsequently impairing the recruitment of FADD and caspase-8 (C8) (K.-L. He & Ting, 2002). In c-Jun N-terminal kinase (JNK) dependent apoptosis, A20 has been described to bind ASK1 mediating ASK1 degradation, leading to suppression of JNK activation and eventually blockage of apoptosis (Won et al., 2010). Two studies talk about the relation between ZnF7 and cell death as they show that MEFs, an immortalized cell line, knockout for A20 reconstituted with a ZnF7 mutant form are more susceptible to TNF induced cell death (Draber et al., 2015; Yamaguchi & Yamaguchi, 2015). Another study shows how A20 induces the deubiquitination of RIPK3 in necroptotic conditions in MEF and 293T cells, preventing the formation of the necrosome (Onizawa et al., 2015).

Several studies have linked SNPs of *TNFAIP3* with susceptibility to multiple autoimmune human diseases. These diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, type 1 diabetes, coeliac disease, Crohn's disease, coronary artery disease in type 2 diabetes and systemic sclerosis (Ma & Malynn, 2012). Most of the SNPs related with IBD are located in non-exon areas, implying that probably they play a role in RNA synthesis or maturation. Given that A20 SNPs in other diseases, as SLE, have been related to lower expression or function (Adrianto et al., 2011; Musone et al., 2008) and that A20 deletion in the whole mice or in different compartments, including the intestine, induces spontaneous inflammation (Chu et al., 2011; E. G. Lee, 2000; Tavares et al., 2010;

Vereecke et al., 2014) it is thought (but not proven) that SNPs in the *TNFAIP3* gene associated with IBD decrease A20 expression. Two independent works have looked into the RNA expression of A20 in IBD. Whereas Arsenescu et al. found a decrease in the RNA levels of A20, as well as other typical proinflammatory markers of IBD in non-inflamed IBD tissue compared with control samples (Arsenescu et al., 2008), Vereecke et al. found the opposite (Vereecke et al., 2014). Interestingly, Vereecke et al. found that A20 levels of non-responder patients to anti-TNF therapy was higher both before and after treatment compared to controls and responders. In line with this, patients that responded to anti-TNF drugs had higher levels of A20, compared with controls, before the treatment that diminished to basal levels after therapy.

1.5. NF-κB IN THE REGULATION OF INTESTINAL EPITHELIAL HOMEOSTASIS AND CELL DEATH

The intestinal lumen is covered by a single cell layered epithelium forming a surface of several hundred square meters and is best known for its life-sustaining digestive functions. In addition, the intestinal epithelium creates a physical barrier separating the luminal contents and microbiota from the cells constituting the intestinal mucosa. The intestinal lumen hosts trillions of commensal bacteria that normally do not cause any pathology and provide essential digestive support to the host. Considering that the intestinal mucosa contains also numerous immune cells, it becomes clear that the interactions of the bacteria 39 with the mucosal immune system and epithelium must be tightly regulated to maintain immune homeostasis and prevent intestinal inflammation. In fact, the intestinal epithelium expresses several PRRs, including TLRs, both at their basolateral and their apical cell membrane (Abreu, 2010). On encountering their microbial ligands, these receptors initiate signaling cascades, leading to the activation of NF-κB and other proinflammatory pathways. Therefore, commensal bacteria are believed to regulate the level of NF-κB activity at the intestinal epithelial interface and thereby affect the mucosal immune balance (Abreu, 2010; Artis, 2008; Wullaert, Bonnet, & Pasparakis, 2010). Multiple evidences suggest that the NF-κB pathway plays a major role in the regulation of intestinal homeostasis contributing to the development and maintenance of intestinal inflammation.

1.5.1. ROLE OF THE CANONICAL NF-κB PATHWAY

First, NF-κB was found to be activated in mucosal cells of IBD patients (S. Schreiber, Nikolaus, & Hampe, 1998) and administration of antisense oligonucleotides to p65 or a peptide that binds to NEMO and inhibits IKK activation reduced the severity of colon inflammation in both chemical-induced models and in the Il-10$^{-/-}$ mouse model of colitis (Davé et al., 2007; M F Neurath, Pettersson, Meyer zum Büschenfelde, & Strober, 1996; W. Shibata et al., 2007). Those experiments showed a correlation between excessive intestinal inflammation and NF-κB activation, However, pharmacological inhibition could not address whether the pathogenic effect of NF-κB was due to NF-κB activation in epithelial or in mucosal immune cells.

The first study addressing the role of the IKK/NF-κB pathway in the intestinal epithelium came through the specific deletion of IKKβ from IECs ($IKK\beta^{IEC\text{-}KO}$). Although there was no defect in the development or function of the gut, IKKβ deficiency severely compromised the survival of intestinal epithelial cells after ischemia–reperfusion injury and increased the susceptibility to intestinal damage and inflammation caused by a model of colitis (Chen et al., 2003; Greten et al., 2004). Interestingly, p65 deletion in IECs induced a spontaneous intestinal pathology in about 10% of these mice resulting in their death during the first three weeks of life, while adult $p65^{IEC\text{-}KO}$ mice did not show signs of severe colitis observed in $NEMO^{IEC\text{-}KO}$ and $IKK\alpha/\beta^{IEC\text{-}KO}$ animals (Nenci et al., 2007; Steinbrecher, Harmel-Laws, Sitcheran, & Baldwin, 2008). These differences could be explained as a result of a partial gain of function of the other NF-κB transcription factors when p65 is not present or IKKα activation when IKKβ is deleted. These results demonstrate a protective function of the NF-κB pathway in the homeostasis maintenance of the intestine.

Unexpectedly, the generation, by two independent groups, of a IEC-specific overexpression of constitutively active mutant IKKβ induced mild mucosal inflammation but strongly sensitized the intestine to experimental challenges inducing intestinal injury (Guma et al., 2011; Vlantis et al., 2011). In Guma et al, a mouse model expressing a human constitutively active IKKβ under the villin promoter was generated. This IKKβ has two mutations (S177E, S181E) mimic its phosphorylation and, therefore, generated a constitutively active protein ($IKK\beta^{(EE)}$). $IKK\beta^{(EE)}$ mice exhibited NF-κB activation in IECs and expressed copious amounts of inflammatory chemokines, including CCL20, CCL2, and M-CSF, but only small amounts of TNF. Although $IKK\beta^{(EE)}$ mice exhibited inflammatory cell infiltration in the lamina propria (LP) of their small intestine, they did not manifest tissue damage. Yet, upon challenge with relatively mild immune and microbial stimuli, $IKK\beta^{(EE)}$ mice succumbed to destructive acute inflammation accompanied by enterocyte apoptosis, intestinal barrier disruption, and bacterial translocation. Inflammation was driven by massive TNF production, which required additional activation of p38 and extracellular-signal-regulated kinase mitogen-activated protein kinases (MAPKs). This was demonstrated to depend on TNF as deletion of both TNF and TNFR1 protected from the challenging stimuli. However, the mechanism though which a constitutive activation of a protective pathway, NF-κB, renders mice susceptible to cell death has remained elusive. Vlantis et al., through another strategy, showed how constitutively active form of IKKβ (IKKβca) in IEC induced spontaneous tumors in aged mice, and also strongly enhanced chemical and Apc mutation–mediated carcinogenesis through increasing Wnt signaling, affecting cell-intrinsic and stromal alterations required for intestinal tumorigenesis (Vlantis et al., 2011).

1.5.2. ROLE OF THE ALTERNATIVE NF-κB PATHWAY

In the intestinal mucosa, lymphoid cells are organized in gut-associated lymphoid tissue (GALT) structures. Those can be classified into effector sites, which consist of lymphocytes scattered throughout the epithelium and lamina propria of the mucosa, and organized tissues, that are responsible for the induction phase of the immune response. In this second group we find Peyer's patches (PP) and mesenteric lymph nodes (MLNs), as well as smaller, isolated lymphoid follicles, which have the appearance of microscopic Peyer's patches and are distributed throughout the wall of the small and large intestines (Hamada et al., 2002; Mowat, 2003). The formation of both PP and MLNs is tightly linked to activation of different

members of the TNFSF upstream of the alternative NF-κB pathway through LTBR.

The presence of LTα1β2 in B cells from PP, for instance, induces the differentiation of $Lgr5^{+}$ stem cells into a specialized epithelial cell named microfold cells (M cells) (Debard, Sierro, Browning, & Kraehenbuhl, 2001; Golovkina, Shlomchik, Hannum, & Chervonsky, 1999; Kerneis, 1997). M cells are specialized phagocytic epithelial cells with several adaptations that facilitate their ability to efficiently sample particulate antigens. LTα1β2, LTβR and LIGHT have also complementary roles in MLN development (Cuff, Sacca, & Ruddle, 1999; Debard, Sierro, & Kraehenbuhl, 1999; Scheu et al., 2002).

A deregulated immune response is a key component in the pathogenesis of IBD. Given the pivotal role of LTβR and its ligands in the proper homeostasis of the GALT, it is not surprising that LTβR blocking by an antagonist antibody was shown to attenuate the development of both clinical and histological manifestations of the disease in two murine models of colitis. Interestingly, blockade of TNF and LTβR in $CD45RB^{hi}$ $CD4^{+}$–reconstituted SCID mice and bone marrow–transplanted tgε26 mice (which express 30-35 copies of human CD3E in the thymus) models achieved similar efficacies (Mackay et al., 1998). Depletion of LTβR or LIGHT protected mice from DSS induced colitis (Krause et al., 2014; Macho-Fernandez et al., 2015), although another study was not able to reproduce LIGHT protective role (Jungbeck et al., 2009). Later studies determined the role of LTβR in the secretion of IL-22 by innate lymphoid cells, a IEC tissue repair mediator cytokine (Ota et al., 2011; Spits, 2011; Tumanov et al., 2011; Y. Zheng et al., 2008). Finally, Wang et al. showed how adoptive transfer of LIGHT transgenic mesenteric lymph node cells into $RAG^{-/-}$ mice rapidly induced a disease similar to the key pathologic features and cytokine characterization observed in CD (J. Wang et al., 2005).

Chapter 2:
AIMS

Inflammatory bowel diseases are characterized by a dysregulated inflammatory process and the presence of intestinal epithelial cell death, with the subsequent increase in pro-inflammatory cytokines. The objective of this work is:

- To determine how chronic NF-κB activation in intestinal epithelial cells is affecting the pathogenesis of IBD.

- To understand the controversial role of A20, a key NF-κB regulatory protein, in IBD.

- To determine if common IBD cytokines synergize to induce intestinal epithelial cell death.

- To understand the mechanism of TNF-induced cell death in intestinal epithelial cells.

Chapter 3:
MATERIALS AND METHODS

3.1 IMMUNOBLOTS

Whole cell extracts were obtained by lysing enteroids in ice-cold RIPA lysis buffer (Cell Signaling) containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and 1% deoxycholate, or NP-40 buffer (containin 10mM Tris-HCl pH 7.6, 140mM NaCl, 1% NP-40, 5mM EDTA, 50mM NaF and 0.4mM Na3VO4) supplemented with a protease and phosphatase inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE and transferred to PVDF membranes that were incubated with antibodies against IκB(371 Santa Cruz), RIPK1 (610459, BD Biosciences), phospho-RIPK1 (31122, Cell Signaling), CYLD (12797, Cell Signaling), cC-3 (9611, Cell Signaling), cC-8 (8592, Cell Signaling), A20 (5630, Cell Signaling), cIAP1 (4952, Cell Signaling), cIAP1 (AF8171, R&D Systems), C-8 (804-447, Alexis), FADD (AAM-212, Stressgen) and tubulin (T5168, Sigma).

Sample preparation:

Lysate organoids in RIPA buffer, cell lines and tissue samples in NP-40 buffer, for 20 minutes

Assess protein concentration by Bradford analysis.

Add Laemmli buffer 4x with 2-mercaptoethanol.

Boil for 7 minutes at 96ºC.

Load between 15 to 25 g of protein per condition.

Sample running:

Run the samples at 120 volts for 1:30h in an 8% polyacrylamide gel or a Biorad precast gel (Mini-PROTEAN® TGX™ Gels)

Sample transfer:

Incubate the polyacrylamide gel in transfer buffer for 5 minutes.

Transfer the proteins to a PVDF membrane at 400mA for 1 hour.

Blocking and blotting:

Incubate the membrane in 5% milk in TBST for 1 hour.

Incubate over night (O/N) the membrane with the proper primary antibody.

Incubate with secondary antibody HRP-linked for 1:30h.

Develop using clarity ECL (Biorad) or Supersignal West Femto ECL (Thermofisher).

Expose with X-ray film and develop.

3.2 IMMUNOHISTOCHEMISTRY (IHC)

Intestines were removed, opened longitudinally, cleaned, processed as "Swiss rolls" and fixed in 10% phosphate-buffered formalin for 24 hours. Fixed tissues were paraffin embedded and 5 µm sections were prepared and stained with H&E. For IHC, mouse and human tissue sections were incubated overnight at 4ºC with anti-cC-3 antibodies (9661, Cell Signaling, Danvers, MA) at a 1:200 dilution or p65 (372X, Santa Cruz) at a 1:100 dilution. Antigen retrieval was performed with citrate buffer pH 6.0 at 96ºC for 20 min. For double staining IHC, antigen retrieval was achieved by boiling the samples for 20 mins in citrate buffer at pH 6.0. Tissue sections were incubated overnight with an anti-A20 primary antibody (ab111192) in PBS plus 3% BSA, and then incubated with secondary alkaline phosphatase linked antibody (1:200, AP-9500) at room temperature for 1 hr. After washing, slides were developed using VectorRed (SK-5100, Vector Laboratories, Burlingame, CA). Slides were further washed and incubated overnight with the second primary antibody against cC-3 (9661, Cell Signaling, Danvers, MA). Samples were then incubated with the secondary HRP conjugated antibody (MP-7401, Vector Laboratories, Burlingame, CA) and developed with diaminobenzidine (DAB) solution plus nickel (SK-4100, Vector Laboratories, Burlingame, CA). All sections were counterstained with hematoxylin and photographed using an Axioplan 200 microscope with AxioVision Release 4.5 software (Zeiss).

Deparaffinization:

Xylene: 3 minutes (x3 times)

100% Ethanol: 3 minutes (x3 times)

95% Ethanol: 3 minutes (x3 times)

70% Ethanol: 3 minutes

ddH_2O: 5 minutes (x3 times)

Perform antigen retrieval using citrate buffer pH6 for 20 minutes at 96ºC. In the case of p65, incubate the samples O/N at 86ºC in citrate buffer pH 6.

Let it cool down to room temperature (RT).

Wash in PBS.

Block peroxidases in 3% H2O2 in PBS for 7 minutes.

Perform blocking in 3% BSA in PBS (IHC blocking buffer).

Incubate the samples O/N in IHC blocking buffer at 4ºC with the primary antibody.

Wash in PBS.

Incubate the samples with the secondary antibody (HRP or alkaline phosphatase linked) (ImPRESS, Vector Labs).

Develop using DAB peroxidase substrate kit (Vector Labs) for HRP substrates or ImmPACT Vector Red Alkaline Phosphatase (AP) Substrate (Vector Labs) for alkaline phosphatase substrates.

For double IHC, incubate with new primary antibody and develop as before.

Counterstain with haematoxylin.

Dehydrate the slides as follows:

70% Ethanol: 3 minutes

95% Ethanol: 3 minutes (x3 times)

100% Ethanol: 3 minutes (x3 times)

Xylene: 3 minutes (x3 times)

Mount in mounting media.

3.3. BACTERIA TRANSFORMATION

MAX Efficiency™ Stbl2™ Competent Cells were heat shocked, as described below, to transform with our plasmid of interest. Bacteria were grown at 37ºC O/N and colonies were screened.

Thaw MAX Efficiency™ Stbl2™ Competent Cells (ThermoFisher) bacteria in ice for 10 minutes.

Add your vector of interest up to a maximum volume of 10% of the bacteria volume (50μl).

Incubate in ice for 30 minutes.

Heat-shock at 42ºC for 25 seconds.

Incubate in ice for 2 minutes.

Add 0.5ml of RT S.O.C. medium.

Shake tube at 225rpm at 30ºC for 90 minutes.

Plate 100 μl into antibiotic agar plates and spread with glass beads.

Incubate at 37ºC at 225rpm O/N.

Screen colonies.

3.4. INDUCIBLE VECTORS

Inducible vectors were generated from A20 and IKKβ previously cloned constructs using the Gateway cloning system into a pInducer20 construct (Meerbrey et al., 2011).

Perform PCR from the donor constructs using an *att*B-flanked PCR primer containing:

Primer forward: GGGG ACA AGT TTG TAC AAA AAA GCA GGC TGG

Primer Reverse: GGGG AC CAC TTT GTA CAA GAA AGC TGG GTA

Add the following components to a 1.5 ml tube at room temperature and mix to perform BP reaction:

attB-PCR product 100ng

pDONOR vector 150 ng

TE buffer, pH 8.0 to 8 µl

Thaw on ice the BP Clonase™ II enzyme mix for about 2 minutes. Vortex the BP Clonase™ II enzyme mix briefly.

To each sample, add 2 µl of BP Clonase™ II enzyme mix to the reaction.

Incubate reactions at 25°C for 1 hour.

Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Transform bacteria and purify plasmid.

Add the following components to a 1.5 ml tube at room temperature and mix to perform LR reaction:

pDONOR 100ng

pInducer20 150ng

TE buffer, pH 8.0 to 8 µl

Thaw on ice the LR Clonase ™ II enzyme mix for about 2 minutes. Vortex the LR Clonase ™ II enzyme mix.

To each sample, add 2 µl of LR Clonase ™II enzyme mix to the reaction.

Incubate reactions at 25°C for 1 hour.

Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Transform bacteria.

3.5. SITE-DIRECTED MUTAGENESIS

A PCR from original vector with primers carrying the point mutation and overlapping sequences from adjacent sites was performed. We subsequently assembled the PCR products using the Gibson assembly method, transformed bacteria and screened for positive colonies.

Primer design (number of primers = initial + final + 2*(n mutagenesis sites)):

Design an initial primer at the beginning of the insert containing a single cut restriction enzyme site. Repeat with a final primer at the end of the insert. The restriction enzyme site must be located in the center of the primer with 18 base pairs (bp) upstream and downstream of it.

Design a forward and reverse primer per mutation site that:

Do not have more than 6bp overhanging in the targeted mutation site.

Contain 12bp that will anneal in the PCR reaction.

Contain 6 bp complementary to the other primer pair.

All primers must have a melting temperature between 55ºC to 60ºC.

Perform PCR reactions:

Mix:

5µl Primestar 2x (Takara)

0.2 µM primer mix

0.5 ng vector

PCR protocol:

98ºC for 10 seconds

55ºC for 5 seconds

72ºC for (10 seconds/kb)

Repeat 35 times

Vector digestion

Digest vector using the restriction enzymes located at the first and last plasmid.

Run the product in an agar gel.

Cut and purify the expected band.

Vector annealing (NEBuilder® HiFi DNA Assembly Cloning Kit)

Mix:

For 2 to 3 fragments use a molar ratio vector:insert = 1:2

For 4 to 6 fragments use a molar ratio vector:insert = 1:1

Up to 5µl (with ddH_2O)

5µl NEBuilder

Incubate samples in a thermocycler at 50°C for 15 minutes (when 2 or 3 fragments are being assembled) or 60 minutes (when 4 to 6 fragments are being assembled).

Transform bacteria with the product.

3.6. cDNA

RNA was isolated from 2 wells from a 24 well/plate per condition using RNeasy plus mini kit (Qiagen) as described below. Reverse transcription was performed using iScript™ Reverse Transcription Supermix (Biorad) as below.

Obtaining RNA (RNeasy plus mini kit, Qiagen):

Organoids: harvest two wells from a 24 well/plate per condition and lysate with 350µl of RLT buffer plus supplemented with β-mercaptoethanol. Vortex for 30 seconds.

Tissue: Homogenize the tissue in 350µl of RLT plus supplemented with β-mercaptoethanol.

Transfer the lysate to a gDNA Eliminator spin column placed in a 2ml collection tube to eliminate genomic DNA.

Centrifuge for 30 seconds at maximum speed. Discard the column and save the flow-through.

Add 350µl of 70% ethanol and mix well by pipetting.

Transfer 700 µl of the sample to an RNeasy spin column placed in a 2ml collection tube to capture RNA.

Centrifuge for 15 seconds at maximum speed and discard the flow-through.

Add 700 µl buffer RW1 to the RNeasy mini spin column and centrifuge for 15 seconds at maximum speed to wash biomolecules such as carbohydrates, proteins, fatty acids etc., which are non-specifically bound to the silica membrane.

Discard the flow-through

Add 700 µl buffer RPE to the RNeasy mini spin column and centrifuge for 2 minutes at maximum speed to remove traces of salts, which are still on the column due to buffers used earlier in the protocol.

Place the RNeasy mini spin column in a new 2ml collection tube and centrifuge for 1 minute at maximum speed.

Place the RNeasy mini spin column in a new 1.5ml collection tube. Add 30µl of RNase-free water. Let it sit for 1 minute.

Centrifuge for 1 minute at full speed.

Measure the RNA concentration using a nanodrop spectrophotometer.

Retrotranscription assay (iScript™ Reverse Transcription Supermix for RT-qPCR):

In a PCR tube mix:

4µl of iScript RT supermix

1µg of RNA

ddH_2O to 20µl

Perform reaction in a PCR machine as follows:

25ºC for 10 minutes

42ºC for 1 hour

85ºC for 5 minutes

4ºC indefinitely

Dilute in ddH_2O 10 times to a final volume of 200µl.

3.7 qPCR

RNA expression was assessed through qPCR from cDNA using SsoAdvanced™ Universal SYBR Green Supermix (Biorad).

Per well, mix 10µl of SsoAdvanced™ Universal SYBR Green Supermix (Biorad), 2µl of 10µM forward and reverse primer mix and 8µl of cDNA.

Perform the reaction in a qRT-PCR machine using the following steps:

95ºC for 3 minutes

Repeat 45 times:

95ºC for 10 seconds

55ºC for 30 seconds

95ºC for 10 seconds

65ºC to 95ºC through 0.5ºC increments every 5 seconds

Murine primers:

TNFAIP3	Forward	TTCCTCCGGACCAGGTCAGT
	Reverse	AAGCTCGTGGCTCTGAAAAC
TNF	Forward	GCACAGAAAGCATGACCCG
	Reverse	GCCCCCCATCTTTTGGG
XIAP	Forward	CGCCTTAGCTGCTCTTCAGT
	Reverse	GGTCCTGATTGCAGATCTTGT
CYLD	Forward	CTCCTTTCCTGTGTCACGCT
	Reverse	GGGATGGAAGGTTTGATGG
cFLIP	Forward	ACTGCACAACTCACCCAGAA
	Reverse	CCACTGTTCCACGCATACAC
BIRC3	Forward	TCTGGGGATGTAGTTTTGTGC
	Reverse	CCGGAGATCAGAGGTCATTG

Human primers:

IL-1b	Forward	AAGCCCTTGCTGTAGTGGTG
	Reverse	GAAGCTGATGGCCCTAAACA
IL-6	Forward	TTTCCCATCAATAGCATCCA
	Reverse	CATCTCCTACTGTCCCACGG
IL-23p19	Forward	CCACACTGGATATGGGGAAC
	Reverse	AGAAGCTCTGCACACTGGC
LTB	Forward	GCTTCTGAAACCCCAGTCCT
	Reverse	TATCACTGTCCTGGCTGTGC
LIGHT	Forward	GGTTGACCTCGTGAGACCTT
	Reverse	CTGGCGTCTAGGAGAGATGG
TNF	Forward	AGATGATCTGACTGCCTGGG
	Reverse	CTGCTGCACTTTGGAGTGAT

3.8. ORGANOID ISOLATION

Small intestinal organoids (enteroids) were cultured as described (Sato et al., 2009). Briefly, crypts were collected from mouse small intestine after 30 min incubation in PBS (pH 7.4)

containing 2 mM EDTA in 6°C. Enteroids were plated in Matrigel (BD Bioscience) and maintained in DMEM/F12 (Life Technologies) containing B27 and N2 supplements (Life Technologies), 1.25 mM N-acetyl L-cysteine (Sigma), 100 ng/ml noggin (GoldBio), 50 ng/ml mEGF (Biosource), and 10% Rspo1-Fc-conditioned medium (the Rspo1-Fc-expressing cell line was a generous gift from Dr. Calvin Kuo, Stanford).

Collect the small intestine (SI) from the murine genotype desired.

Discard the duodenum and the terminal ilium.

Flush with PBS.

Cut open the SI longitudinally.

Scrape the villi with a coverslip.

Wash several times in a 50ml tube with PBS by vigorous shaking until no foam is visible.

Chop the intestine transversally in 1mm thick pieces.

Wash several times in a 50ml tube with PBS by vigorous shaking until the PBS is clean.

Incubate at 4ºC for 45 minutes rocking in PBS/2mM EDTA.

Aspirate the supernatant.

Repeat 4 times:

Vigorously shake the intestine in 10% FBS in PBS (10 ml).

Filter the supernatant in a 40μm cell strainer into 4 different 50ml tubes.

Centrifuge at 600rpm for 5 minutes.

Pool together the pellets in 10ml of advanced DMEM/F12 (Invitrogen) supplemented with 5ml HEPES 1M (Invitrogen), Glutamax (Invitrogen) and penicillin/streptomycin (+++ medium).

Centrifuge at 800rpm for 3 minutes.

Aspirate the supernatant and resuspend the pellet in 10ml of +++ medium.

Centrifuge at 800rpm for 5 minutes.

Resuspend the pellet in 70% matrigel phenolred free, reduced growth factor (Corning) with CCM.

Plate 25μl per well in a pre-warmed 24 well plate.

Incubate at 37ºC for 15 minutes until the matrigel is solidified.

Add 500μl of CCM per well.

For 40ml of CCM (crypt culture media):

36ml of +++ medium

4ml of RspoI condition media

B27 (Life Technologies)

N2 (Life Technologies)

50ng/ml mEGF (Biosource)

100ng/ml Noggin (GoldBio)

1.25mM N-acetylcysteine (Sigma)

3.9 ORGANOID CULTURE

Briefly, organoids were changed media every two days. Every 4 days they were split using mechanical disaggregation and plated again as follows.

Day 0:

Seed organoids. 25 μl of 70% matrigel in CCM per well in a pre-warmed 24 well plate.

Incubate at 37ºC for 15 minutes until the matrigel is solidified.

Add 500μl of CCM per well.

Day 2:

Aspirate medium.

Add fresh medium.

Day 4/0:

Collect organoids in 1ml of ice-cold +++ medium.

Vigorously resuspend to mechanically split and break organoids.

Collect broken organoids into 5ml of ice-cold +++ medium.

Centrifuge at 600rpm for 5 minutes.

Aspirate supernatant.

Resuspend pellet in 70% matrigel in CCM.

Plate 25μl per well in a pre-warmed 24 well plate.

Incubate at 37ºC for 15 minutes until the matrigel is solidified.

Add 500μl of CCM per well.

3.10. ORGANOID INFECTION

Single cells were obtained by digesting the enteroids with Trypsin-EDTA 0.25% (Life Technologies) for 5 min at 37°C. Cells were mixed with high titer lentivirus plus polybrene (8 µg/ml, Santa Cruz) and Y-27632 (10 u µM, Apexbio) in a 48-well culture plate and centrifuged at 600g at 32ºC for 60 min, followed by 5 hours incubation at 37ºC. Cells were then plated in matrigel and cultured in media with 50% media for two days, followed by selection with puromycin (2 µg/ml) or G418 (800 ng/ml) in regular media for 1 week.

Organoid preparation

Day 0:

Change medium to 50% medium supplemented with Nicotinamide 10 µM. Use 2 wells per condition.

Day 2-3:

Collect organoids in 1ml of ice-cold +++ medium.

Vigorously resuspend to mechanically split and break organoids.

Collect broken organoids into 5ml of ice-cold +++ medium.

Centrifuge at 600rpm for 5 minutes.

Resuspend cells in 0.25% trypsin/EDTA (Gibco).

Incubate at 37ºC for 4 minutes.

Resuspend cells.

Add 5ml of “for 50% medium”.

Centrifuge at 800rpm for 7 minutes.

Aspirate supernatant.

Lentivirus preparation

Day 0:

Plate 10ml of 293FT cells at a concentration of $5x10^5$ cells/ml per condition.

Day 1:

Aspirate 3ml of medium.

Transfect as follows:

Solution A:

600 µl OPTIMEM (Gibco) + 54 µl PEI (1g/ml)

Solution B:

600µl OPTIMEM (Gibco) + 2.25 µg p.MD2.G (Addgene, Plasmid 12259) + 6.75 µg psPAX2 (Addgene, Plasmid 12260) + 9 µg lentivector of interest

Mix solutions A + B and incubate for 15 minutes at room temperature.

Add mixure to 293FT cells.

Day 2:

Change medium to 7ml of DMEM supplemented with 4mM caffeine.

Day 3:

Collect and filter through a 0.45µm syringe filter the medium.

Centrifuge O/N at 4ºC at 9200rpm.

Spinoculation

Aspirate medium from the virus tubes (a white pellet should be visible).

Resuspend virus in 150µl of 50% medium supplemented with 10mM Nicotinamide, 10µM Y-27632 and 8µg/ml polybrene.

Resuspend single cell organoids with the previous solution.

Centrifuge at 600g for 1 hour at 32ºC.

Incubate at 37ºC for 4 to 6 hours.

Resuspend cells in 5ml of “for 50% medium”.

Centrifuge at 800rpm for 7 minutes.

Resuspend cells in 100% matrigel.

Plate 25 µl per well in a pre-warmed 24 well plate.

Incubate at 37ºC for 15 minutes until the matrigel is solidified.

Add 500µl of 50% medium per well.

Two days later change medium to CCM with selection (2µg/ml Puromycin).

Select for 5 to 7 days.

3.11 ORGANOID IMMUNOFLUORESCENCE (IF)

Enteroids in matrigel were fixed with 4% paraformaldehyde overnight at 4°C. Fixed enteroids were blocked with PBS containing 0.2% Triton X-100, 0.05% Tween-20 and 0.1% bovine serum albumin for 90 min, immunostained with primary antibodies overnight at 4°C and with Alexa Fluor IgG secondary antibody for 1 hr at room temperature. Immunostained enteroids were gently mounted on the slide glass and imaged under the Zeiss confocal microscope.

Fix organoids in 4% paraformaldehyde O/N at 4ºC

Wash with 100mM glycine in PBS.

Block in IF buffer for 1:30 hours at room temperature.

Dilute primary antibody (1:200) in IF buffer and incubate with sample for 2 hours at room temperature.

Wash with IF buffer for 20 minutes at room temperature (x3 times).

Dilute Alexa Fluor IgG secondary antibody (1:400) in IF buffer and incubate with sample for 60 minutes at room temperature.

Wash with IF buffer for 20 minutes at room temperature (x3 times).

Wash with PBS for 10 minutes at room temperature (x2 times).

Counterstain with DAPI for 10 minutes.

Mount in Fluorsave™ (Calbiochem)

IF buffer:

0.2% triton X-100

0.1% BSA

0.05% Tween-20

in PBS

3.12. 50% MEDIUM FOR ORGANOID CULTURE

This media was used for organoid infection and can also be used for human spheroids.

Seed L-WRN cells.

After one day, change media and add G418 (500μg/ml) and hygromycin (500μg/ml).

Grow cells until they become confluent.

Wash thoroughly and split into 150 cm^2 flasks.

Incubate until cells become over-confluent without G418 or hygromycin.

Add 20ml of “for 50% medium”.

Every 24 hours and for 4 days collect and filter the supernatant pooling it together.

Repeat to have two batches.

Mix at a 1:1 ratio condition media with “for 50% medium”.

Store at -80ºC.

50% medium working solution:

Supplement 50% medium with Y-26732 (10mM, Apexbio) and A-8301 (10mM, Sigma).

“for 50% medium”:

Advanced DMEM/F12 (Invitrogen)

FCS (20 %, vol/vol)

Penicillin (100 U/ml)

Streptomycin (100 µg/ml)

L-glutamine (2 mM)

3.13. IMMUNOPRECIPITATIONS (IP)

For immunoprecipitation (IP) experiments, enteroids were incubated with 100 µg/ml avitag-mTNF for 1 hr on ice, then at 37°C for 10, 30 and 60 minutes before collection. Cell extracts were immunoprecipitated with biotinylated magnetic beads (Cell Signaling). In other experiments, we used anti-FADD antibody and Protein G and A sepharose beads overnight at 4°C. Immunecomplexes were washed with lysis buffer and analyzed by immunoblotting.

3.13.1. TNFR IP

Incubate the organoids with avitag-TNF (100ng/ml) rocking over ice for 1h. Use 12 wells from a 24 well/plate per condition.

Place them in the cell incubator at 37ºC for 10, 30 and 60 minutes.

Collect them in ice-cold advanced DMEM/F12 (Invitrogen).

Centrifuge at 300g for 5 minutes.

Lysate in TNFR lysis buffer supplemented with protease and phosphatase inhibitors (Pierce) and NEM (50mM) for 20 minutes.

Spin 13200 rpm for 10 minutes.

Assess protein concentration by Bradford assay.

Use 200 to 300 μg of protein per condition.

Add TNFR lysis buffer to 250 μl final volume.

Add 5μl of biotinylated magnetic beads (Cell Signaling Technologies).

Incubate O/N at 4ºC rotating.

Place eppendorfs into a magnetic rack.

Wash beads with ice-cold TNFR lysis buffer rotating for 5 minutes (x3 times).

Add 50 μl of laemli buffer 2X.

Resuspend and boil for 7 minutes.

Load 20μl of sample per condition.

TNFR lysis buffer:

50mM Tris pH 7.4

150mM NaCl

1% NP-40

0.25% sodium deoxycholate

1% triton X-100

1mM EDTA

in ddH_20

3.13.2. FADD IP

Incubate the organoids with TNF (20ng/ml) and Z-VAD-FMK (50 μM) at 37ºC for 2 hours. Use 12 wells from a 24 well/plate per condition.

Mix protein A and protein G sepharose beads (use 15 μl of each per sample).

Wash sepharose beads with PBS.

Centrifuge at 3000rpm for 4 minutes.

Wash sepharose beads in 0,1% BSA in PBS.

Incubate sepharose beads with anti-FADD antibody (5μl per sample) at 4ºC for 1 hour, rotating.

Wash with PBS.

Wash with wash buffer (0.2M triethanolamine in PBS).

Repeat 3 times:

Crosslink antibody to sepharose beads using 13mg/ml of dimethyl pimelimidate dihydrochloride in wash buffer.

Rotate at room temperature (RT) for 30 minutes.

Wash with wash buffer for 5 minutes.

Repeat 2 times:

Wash with 500ml of quench buffer (50mM ethanolamine in PBS) for 5 minutes.

Repeat 2 times:

Wash with 1M glycine pH3 for 10 minutes.

Wash with PBS 3 times.

Resuspend in complex II lysis buffer.

Collect organoids in ice-cold advanced DMEM/F12 (Invitrogen).

Centrifuge at 300g for 5 minutes.

Lysate in complex II lysis buffer supplemented with protease and phosphatase inhibitors for 20 minutes.

Centrifuge at 13200rpm for 10 minutes.

Assess protein concentration by Bradford assay.

Use between 200 and 300 μg of protein per sample.

Add more complex II lysis buffer to a final volume of 250 μl.

Add 10μl of sepharose beads per sample.

Incubate O/N at 4ºC rotating.

Centrifuge at 3000rpm for 4 minutes.

Wash with complex II lysis buffer for 5 minutes (x3 times).

Resuspend in 50μl of 2X Laemli buffer and boil for 7 minutes.

Load 20 μl of sample per condition.

Complex II lysis buffer:

150 mM NaCl

20 mM Tris pH 7.5

1% NP-40

1 mM EDTA

3 mM NaF

1 mM β-glycerophosphate

1 mM sodium orthovanadate

in ddH_20

3.14. KINASE ASSAY

Organoids were lysated after TNF stimulation and NEMO was immunoprecipitated. Kinase assay was performed as below using GST-IκBα as a substrate.

Collect organoids in ice-cold advanced DMEM/F12 (Invitrogen).

Centrifuge at 300g for 5 minutes.

Lysate in KA lysis buffer supplemented with protease and phosphatase inhibitors for 20 minutes.

Centrifuge at 13200rpm for 10 minutes.

Assess protein concentration by Bradford assay.

Use between 200 and 300 μg of protein per sample.

Add more KA lysis buffer to a final volume of 250 μl.

Add 50μl of sepharose beads + 1μl of NEMO antibody (Pharmingen) + 9 μl NaCl (5M) to a concentration of 400mM NaCl per sample.

Incubate O/N at 4ºC rotating.

Centrifuge at 3000rpm for 4 minutes.

Wash with KA lysis buffer 400mM NaCl for 5 minutes.

Wash with KA buffer 1X (x2 times).

Add 30 μl of reaction KA buffer 1X.

Incubate at 30ºC for 30 minutes.

Boil 5 minutes at 96ºC with laemli buffer.

Centrifuge at 3000rpm for 4 minutes.

Run samples.

KA Lysis Buffer:

50 mM Tris-HCl pH 7.5

250 mM NaCl

3 mM EDTA

1% Triton X-100

0.5% Igepal

10% glycerol

This buffer is kept at 4ºC. Just before use add:

2 mM DTT

0.1 mM sodium orthovanadate

2 mM PnPP

Protease inhibitors

KA buffer 10X:

200 mM HEPES, pH 7.5

100 mM $MgCl_2$

This buffer is kept at room temperature. Just before use add:

20 mM β-glycerolphosphate

10 mM PNPP

1 mM DTT

50 μM sodium orthovanadate

20 μM cold ATP

1.5 μg GST-IκBα (1-54) substrate (per reaction)

1μl ^{32}PγATP (per reaction)

3.15. MICE STRAINS

A20-Tg mice were previously described (Kolodziej et al., 2011). A20-Tg mice x $Rag^{+/-}$ were used in the *in vivo* experiments. $RIPK1^{D138N}$ and $RIPK3^{-/-}$ were obtained from Dr. Vishva Dixit (Genentech) (Newton et al., 2014a; Newton, Sun, & Dixit, 2004), $IKK\beta^{EE(IEC)}$ (Guma et al., 2011), *TNF*$^{-/-}$ (Pasparakis, Alexopoulou, Episkopou, & Kollias, 1996), *MYD88*$^{\Delta IEC}$ (Kawai, Adachi, Ogawa, Takeda, & Akira, 1999), *NIK*$^{-/-}$ (Amgen, Yin et al., 2001). Mice used in the experiments were 8-12 weeks old and were of the C57BL/6J genetic background and maintained under SPF conditions at a UCSD animal facility, accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols were approved by the institutional review board, following NIH guidelines. Mice were fed autoclaved standard chow and all of the different strains were co-housed to minimize microbiome fluctuations.

3.16. MICE EXPERIMENTS

- mTNF (R&D) (from 1μg to 5μg/mouse) was delivered through retro-orbital (i.v.) injection in a volume of 100μl in PBS after isoflurane induced anesthesia.
- LPS (5 mg/kg) was delivered through intra-peritoneal (i.p.) injection in a volume of 150μl in PBS.
- 4H8 (100 μg/mouse) was delivered through intra-peritoneal (i.p.) injection in a volume of 150μl in PBS one hour prior to mTNF or PBS injection.
- GSK'963 (50 mg/kg) was delivered through intra-peritoneal (i.p.) injection in a volume of 150μl in PBS and 6% cavitron.
- BHA (20 mg/kg) was delivered through intra-peritoneal (i.p.) injection in a volume of 150μl in PBS and 5% DMSO.

3.17. ANTIBODIES

	Company	Catalog number	IHC dilution	WB dilution
Cleaved caspase-3	Cell Signaling	9661	1:200	1:1000
Cleaved caspase-8	Cell Signaling	8592	1:200	1:1000
p65	Santa Cruz	372X	1:100	1:1000
Tubulin	Sigma	T5168	N/A	1:2000
IKKβ	Upstate	05-535	N/A	1:1000
A20	Cell Signaling	5630	N/A	1:500
A20	Abcam	ab111192	1:50	N/A
FADD (IP)	Santa Cruz	6036	N/A	N/A
FADD	Stressgen	AAM-212	N/A	1:1000
RIPK1	BD Biosciences	610459	N/A	1:500
p-RIPK1	Cell Signaling	31122	N/A	1:500
Caspase-8	Alexis	804-447	N/A	1:1000
CYLD	Cell Signaling	12797	N/A	1:500
TNFR1	Cell Signaling	13377	N/A	1:1000
cIAP1	Cell Signaling	4952	N/A	1:500
cIAP2	R&D Systems	AF8171	N/A	1:500
c-FLIP	Santa Cruz	5276	N/A	1:500
Survivin	Cell Signaling	71G4B7	N/A	1:1000
Bcl-2	Cell Signaling	3498	N/A	1:500
Bcl-xL	Cell Signaling	2764	N/A	1:1000
NIK	Cell Signaling	4994	N/A	1:500
HSP90	BD	610419	N/A	1:1000
IκBα	Santa Cruz	371	N/A	1:500

Chapter 4:
RESULTS

4.1. NF-κB AND CASPASE-3 ACTIVATION IN HUMAN IBD

We first conducted IHC analysis of human tissue specimens from healthy individuals, patients suffering from either ileal or colonic CD or UC to determine the correlation between NF-κB activation and cell death. In total, we examined 10 normal colon sections, 10 samples with active UC and 10 samples with colonic CD, as well as 4 active ileitis samples and 5 inactive ileal CD samples, all of which were stained for p65/RelA and cC-3. In general, normal colonic or ileal specimens contained hardly any IEC that were positive for cC-3 or nuclear p65 (Figure 11). Active IBD specimens contained more cC-3-positive cells than control samples and the areas containing these cells overlapped with areas containing cells with nuclear p65 (Figure 11) in agreement with previous reports (Kaser, Zeissig, & Blumberg, 2010; Rogler et al., 1998).

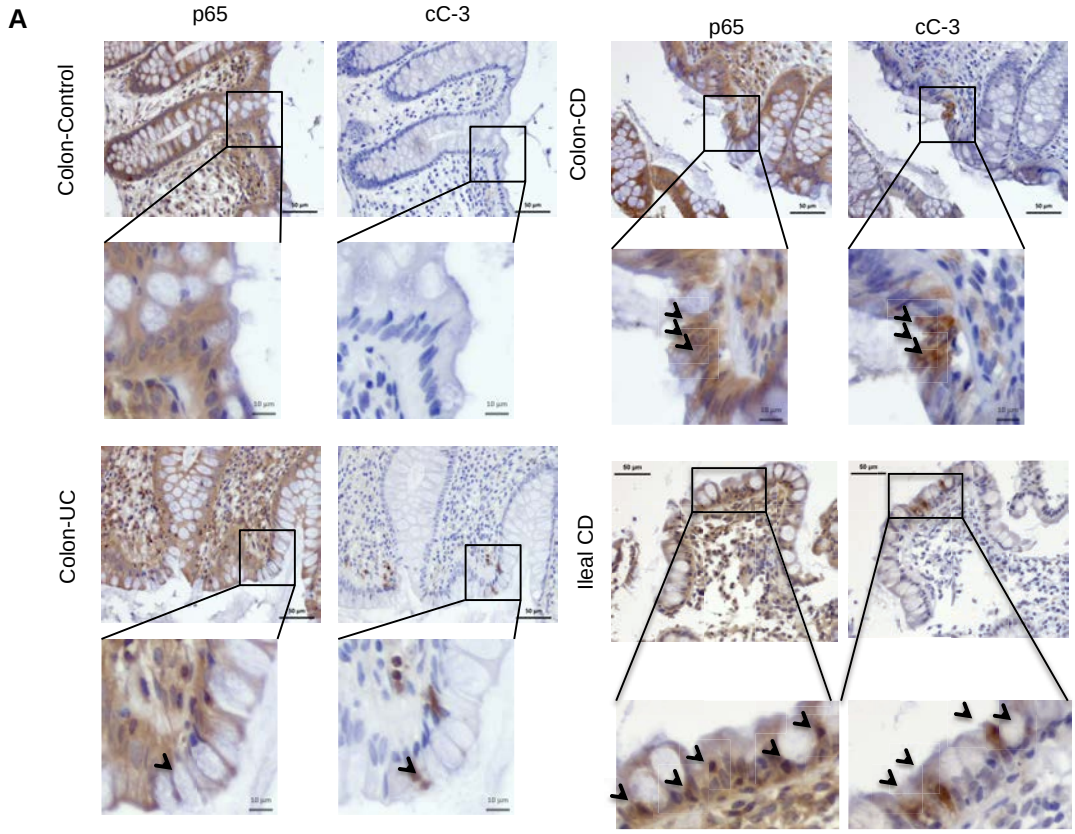

Figure 11. Active IBD tissue displays spatially adjacent NF-κB and caspase-3 activation in epithelial cells. (A) Human healthy colonic, ileal CD and colonic CD and UC sections were stained with antibodies to cC-3 and p65/RelA. Arrows: positive cells.

Active UC specimens contained more cC-3- and p65-positive cells than CD specimens and hardly any differences were observed between colonic and ileal CD (Table 2).

A. Number of samples and the corresponding percentages of nuclear p65 expression level in IEC of control tissue and active IBD specimens.

Expression level	C ileum	C colon	CD ileum	CD colon	UC colon
0	4 (80)	7 (70)	1 (25)	0	0
1	1 (20)	3 (30)	1 (25)	2 (20)	1 (10)
2	0	0	2 (50)	5 (50)	4 (40)
3	0	0	0	3 (30)	5 (50)
Total	5	10	4	10	10

IEC: intestinal epithelial cells, IBD: inflammatory bowel disease, C: control, CD: Crohn's disease, UC: ulcerative colitis

B. Number of samples and the corresponding percentages of the caspase-3 expression level in IEC of control tissue and active IBD specimens.

Expression level	C ileum	C colon	CD ileum	CD colon	UC colon
0	2 (40)	5 (50)	1 (25)	0	0
1	3 (60)	5 (50)	1 (25)	3 (30)	2 (20)
2	0	0	2 (50)	5 (50)	4 (40)
3	0	0	0	2 (20)	4 (40)
Total	5	10	4	10	10

IEC: intestinal epithelial cells, IBD: inflammatory bowel disease, C: control, CD: Crohn's disease, UC: ulcerative colitis

Table 2. Number of samples and the corresponding percentages of nuclear p65 and cleaved caspase-3 expression level in IEC of control tissue and active IBD specimens.

We also compared the transcriptome of active human IBD (from ileal and colon CD) to that of healthy ileal and colonic tissue. The results revealed strong upregulation of TNF mRNA and numerous other NF-κB target genes including *NOS2*, *CXCL10*, *CXCL11*, *CCL2*, *LTB*, *CCL8*, *CCL7*, *IL10*, *CSF1*, *CCL11* and *CCL20* and several anti-apoptotic genes including *BIRC3* in disease tissue (Figure 12A).

Figure 12. IBD samples have activated NF-κB target genes. (A) mRNA extracted from one ileal and one colonic biopsy specimens from healthy individuals and from three ileal and three colonic biopsy specimens from CD patients was subjected to transcriptomic profiling. Shown is differential expression of innate immune and inflammatory response genes in CD relative to normal tissue both in ileal and colonic specimens. (B) Comparison between genes that are differentially expressed between WT and IKKβ$^{(EE)}$ enterocytes (Guma et al., 2011) and those that are differentially expressed between CD and normal human ileum (left). EE, IKKβ$^{(EE)}$.

4.2. TNF-INDUCED APOPTOSIS IN IKKβ$^{(EE)}$ MICE

To determine the pathogenic function of persistent NF-κB activation which occurs in IBD (Kaser et al., 2010), we used IKKβ$^{(EE)}$ mice in which a constitutively active IKKβ$^{(EE)}$ variant is expressed in IEC from the villin promoter (Guma et al., 2011). IKKβ$^{(EE)}$ mice instead of being resistant to TNF-induced mucosal erosion, display severe TNF-dependent epithelial layer destruction when challenged with various stimuli that promote Tnf mRNA stabilization and translation or given exogenous TNF (Guma et al., 2011). The mechanism by which constitutive IKKβ/NF-κB activation renders mouse IEC susceptible to TNF-induced killing, rather than prevent it, is unknown, but is likely to be relevant to the effect of chronic activation of NF-κB in IEC of active IBD lesions. Of note, many of these genes upregulated in IBD samples were also upregulated in the intestinal epithelium of IKKβ$^{(EE)}$ mice relative to WT mouse epithelium (Figure 12B). The few genes that were upregulated in CD but not in the IKKβ$^{(EE)}$ epithelium could represent either CD-specific genes or genes that are

predominantly expressed in immune cells. Despite ample TNF mRNA expression, IKKβ$^{(EE)}$ intestinal tissue does not express TNF protein unless exposed to additional stimuli that promote Tnf mRNA stabilization and translation(Guma et al., 2011). That is the reason why unchallenged IKKβ$^{(EE)}$ mice do not exhibit mucosal erosion despite having elevated Tnf mRNA.

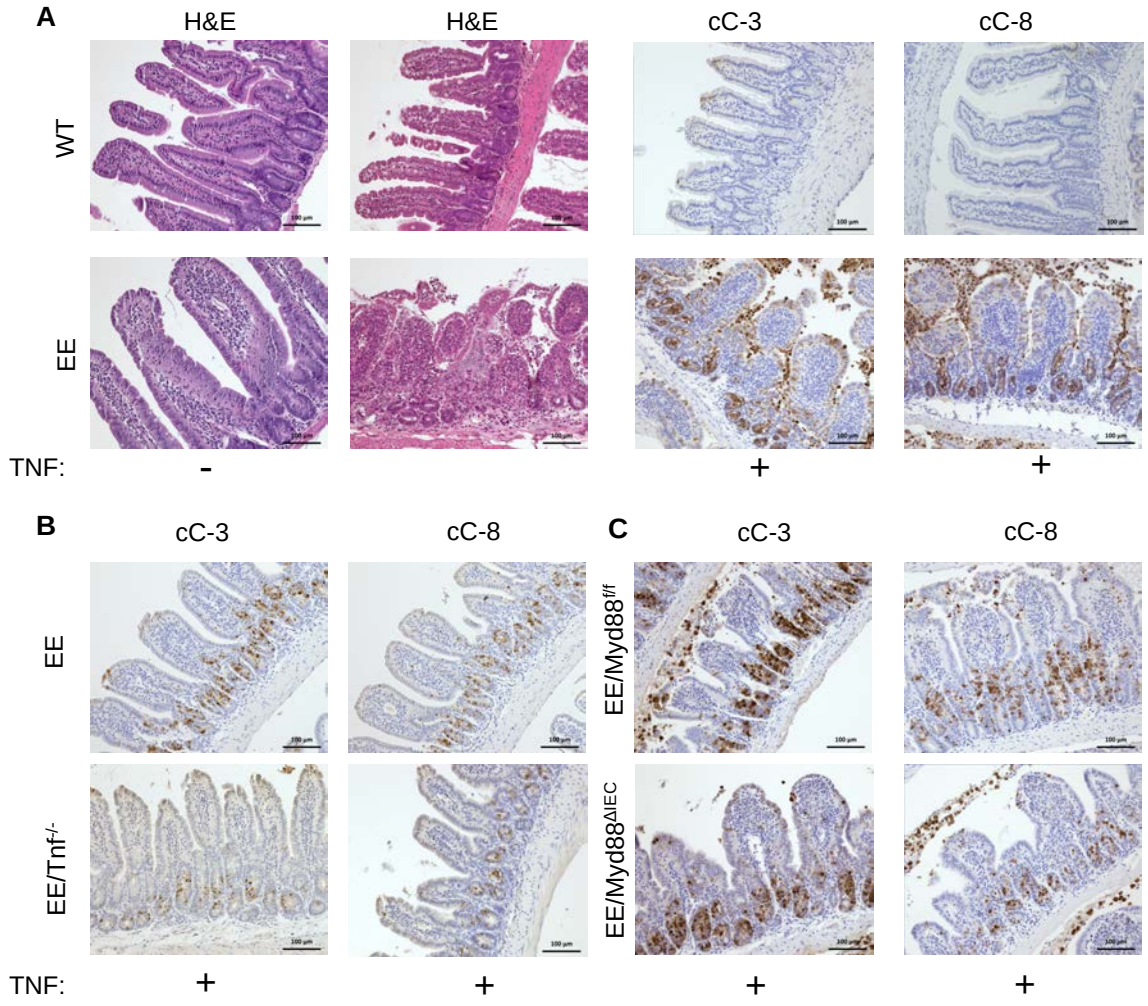

Figure 13. IKKβ$^{(EE)}$-expressing intestinal crypts are susceptible to TNF-induced apoptosis independently of TLR signaling. (A) WT and IKKβ$^{(EE)}$ mice were analyzed 4 hours after TNF injection (2 µg i.v.). Jejunal sections were stained with either H&E or antibodies to cleaved caspase-3 (cC-3) or -8 (cC-8). (B) IKKβ$^{(EE)}$ and IKKβ$^{(EE)}$/Tnf$^{-/-}$ mice were injected with TNF (2 µg i.v.) Jejunal sections were prepared 4 hours later and stained with antibodies to either cC-3 or cC-8. (C) IKKβ$^{(EE)}$/Myd88$^{\Delta IEC}$ and IKKβ$^{(EE)}$/Myd88$^{F/F}$ mice were analyzed 4 hours after TNF injection as above. EE, IKKβ$^{(EE)}$.

Since TNF can trigger either apoptosis or necrosis, we used immunohistochemistry (IHC) with antibodies specific for activated/cleaved caspase-8 (cC-8) or cleaved caspase-3 (cC-3) to determine the type of cell death affecting the IKKβ$^{(EE)}$ intestinal epithelium after TNF or LPS administration. Treatment of IKKβ$^{(EE)}$ mice with either agent activated both caspases in villi and especially within crypt compartments, leading to cell shedding and tissue damage (Figures 13A). LPS-treated WT mice showed only transient caspase-3 activation near the villi tips but not within the crypt compartment and hardly any caspase-8 activation, resulting

in very little mucosal damage. IKKβ$^{(EE)}$/Tnf$^{-/-}$ mice remained hypersensitive to exogenous TNF and showed caspase-3 and -8 activation after its administration (Figure 13B). Similar results were obtained in TNF-treated IKKβ$^{(EE)}$/Myd88$^{\Delta IEC}$ mice (Figure 13C), suggesting an inherent ability of persistent IKKβ activation to sensitize IEC to TNF-induced apoptosis independently of IEC-autonomous TLR signaling.

To further investigate this phenomenon and determine whether or not it depends on microbiota and immune cells, we established enteroid (intestinal organoid) cultures in which intestinal stem cell (ISC) proliferation, differentiation and three dimensional tissue organization into crypts and villi are retained(Sato et al., 2009). Whereas WT enteroids exhibited minimal TNF-induced death, IKKβ$^{(EE)}$ and IKKβ$^{(EE)}$/Tnf$^{-/-}$ enteroids underwent extensive cell death, especially within crypt domains, after incubation with TNF (Figures 14A and 13B), leading to their rapid disintegration. As expected, WT enteroids exhibited extensive apoptosis only after incubation with TNF plus cyclohexamide (CHX), a protein synthesis inhibitor that mimics NF-κB impaired activation (Figure 14C) and cC-3 immunofluorescent (IF) staining (Figure 14D) demonstrating that IKKβ$^{(EE)}$ enteroids underwent TNF-induced apoptosis mainly within crypt domains.

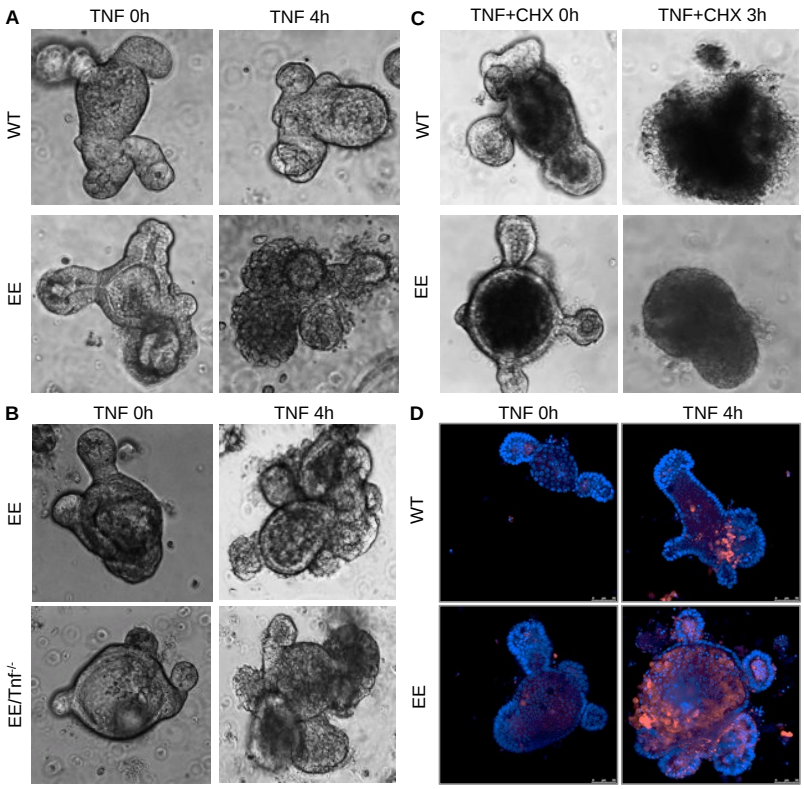

Figure 14. IKKβ$^{(EE)}$-expressing enteroids are susceptible to TNF-induced apoptosis. (A) WT and IKKβ$^{(EE)}$ enteroids were photographed under brightfield 4 hours after TNF stimulation (40 ng/ml). Magnification 200×. (B) IKKβ$^{(EE)}$ and IKKβ$^{(EE)}$/Tnf$^{-/-}$ enteroids were photographed under brightfield 4 hours after incubation with TNF (40 ng/ml). Magnification 200×. (C) WT and IKKβ$^{(EE)}$ enteroids were incubated as indicated with or without TNF (40 ng/ml) plus cycloheximide (10 μg/ml) and photographed under bright field. Magnification 200×. (D) WT and IKKβ$^{(EE)}$ enteroids were stained with cC-8 antibody and visualized by confocal microscopy at indicated times after TNF (40 ng/ml) addition. (E) Lysates of WT and IKKβ$^{(EE)}$ enteroids were IB analyzed at different times after TNF addition. (F) Lysates of Tnf$^{-/-}$ and IKKβ$^{(EE)}$/Tnf$^{-/-}$ enteroids incubated with or without TNF were analyzed by IB. EE, IKKβ$^{(EE)}$.

IB analysis of that IKKβ$^{(EE)}$ or IKKβ$^{(EE)}$/Tnf$^{-/-}$ enteroids confirmed caspase-3 and -8 cleavage (Figure 15A and 15B), providing an independent confirmation of the *in vivo* observations and indicating that immune cells and gut microbiota are not needed for TNF-induced apoptosis of IEC that express activated IKKβ.

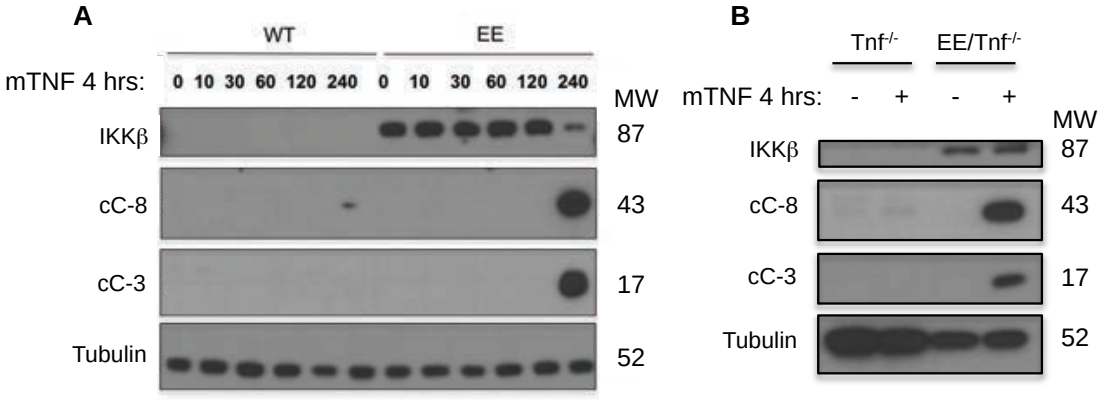

Figure 15. IKKβ(EE)-expressing enteroids are susceptible to TNF-induced apoptosis.(A) Lysates of WT and IKKβ$^{(EE)}$ enteroids were IB analyzed at different times after TNF addition. (B) Lysates of Tnf$^{-/-}$ and IKKβ$^{(EE)}$/Tnf$^{-/-}$ enteroids incubated with or without TNF were analyzed by IB. EE, IKKβ$^{(EE)}$.

To determine whether the elevated susceptibility of IKKβ$^{(EE)}$ enteroids to TNF-induced death was NF-κB-dependent, we transduced them with a lentivirus expressing IκBα super-repressor (IκBαSR). Enteroids containing IκBαSR no longer exhibited TNF-induced crypt apoptosis (Figures 16A and 16B), but unexpectedly IκBαSR expression led to reduced IKKβ$^{(EE)}$ expression (Figure 16B). NF-κB inhibition correlated with downregulation of A20, which is encoded by an NF-κB target gene. We had also transduced WT enteroids with a doxycycline-inducible(i) IKKβ$^{(EE)}$ construct to determine how long NF-|B needs to be activated to confer susceptibility to TNF-induced death. TNF-induced apoptosis and caspase activation were observed after 6 hours of doxycycline treatment and only in enteroids receiving the iIKKβ $^{(EE)}$ construct (Figure 16C and 16D). Doxycyline itself did not cause cell death, neither did it sensitize WT enteroids to TNF-induced apoptosis (Figure 16C and 16D).

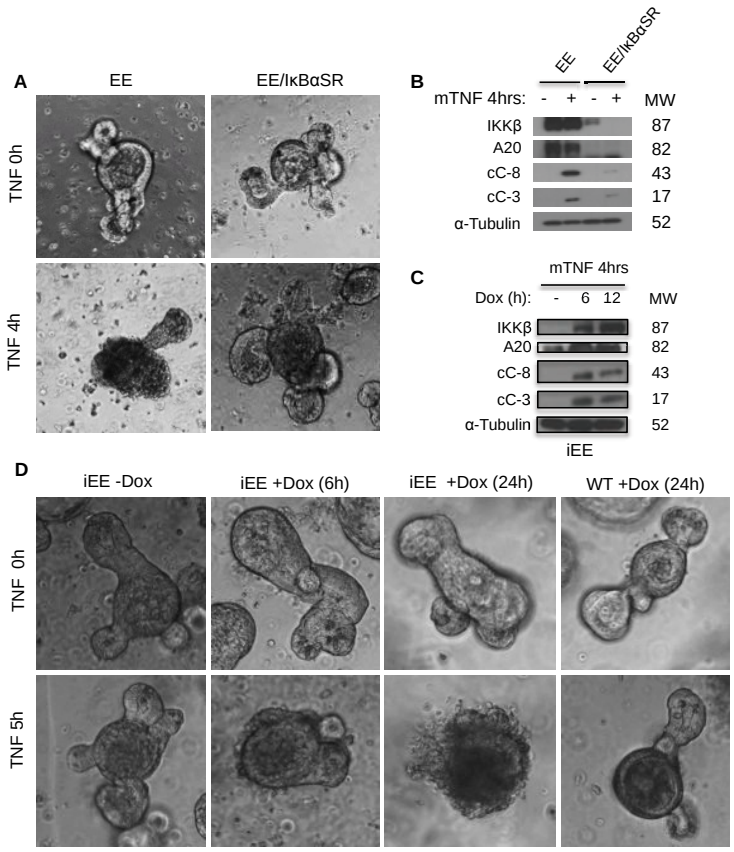

Figure 16. Susceptibility of IKKβ$^{(EE)}$ enteroids to TNF-induced death is NF-κB-dependent.(A) Control and IκBαSR transduced IKKβ$^{(EE)}$ enteroids were incubated with TNF as indicated and photographed under bright field. Magnification 200×. (B) Control and IκBαSR-transduced IKKβ$^{(EE)}$ enteroids incubated with or without TNF were lysed and IB analyzed. (C) WT enteroids transduced with inducible IKKβ$^{(EE)}$ construct were pre-incubated with or without doxycycline as indicated, lysed and IB analyzed 4 hours after addition of TNF. (D) Control and inducible-IKKβ$^{(EE)}$ transduced WT enteroids were incubated for 24 hours with doxycycline prior to TNF stimulation as indicated. Magnification 200×. EE, IKKβ$^{(EE)}$.

4.3. RIPK1 KINASE ACTIVITY IS REQUIRED FOR TNF-INDUCED TISSUE DAMAGE

TNFR1 engagement triggers assembly of several distinct signaling complexes, the first of which is complex I, that includes TRADD, RIPK1, TRAF2 and cIAP1/2, and is responsible for IKK and NF-κB activation and inhibition of apoptosis. The latter is mediated by induction of anti-apoptotic genes, including those coding for c-FLIP, a caspase-8 inhibitor (Kreuz, Siegmund, Scheurich, & Wajant, 2001), and survivin, a caspase-3 inhibitor (Kawakami et al., 2005). Notably and despite its susceptibility to TNF-induced apoptosis, the intestinal

epithelium of IKKβ$^{(EE)}$ mice exhibited elevated expression of anti-apoptotic molecules, including BIRC3, BCL-2 and BCL2L1 (Guma et al., 2011). Persistent TNFR1 engagement, however, results in receptor internalization, which can promote cell death through either RIPK1-dependent apoptosis or necroptosis (Ofengeim & Yuan, 2013; Weinlich & Green, 2014). To query the involvement of RIPK1 kinase activity in the observed phenotype, we used pharmacological inhibitors of RIPK1 (Necrostain-1 and GSK'963) as well as RIPK3 (GSK'843 and GSK'872) (Berger et al., 2015; Degterev et al., 2008; Mandal et al., 2014) and examined the ability of RIPK1 inhibitors to block the TNF-induced death of IKKβ$^{(EE)}$ expressing IEC. Indeed, GSK'963 and Nec-1 effectively blocked the TNF-induced apoptosis of IKKβ$^{(EE)}$ enteroids (Figures 17A and 17B), without any effect on crypt proliferation (Figures 17C and 17D).

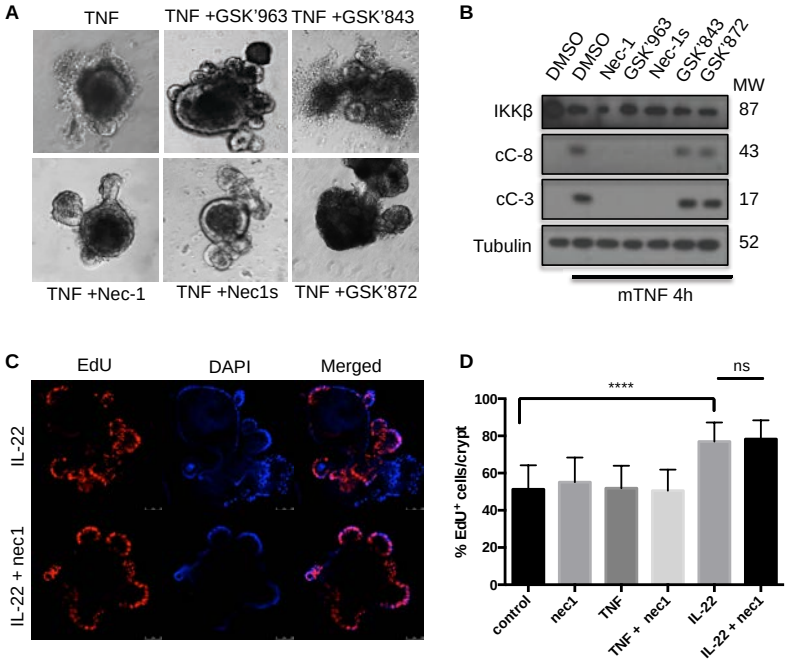

Figure 17. RIPK1 kinase activity is required for TNF-induced enteroids death. (A) IKKβ$^{(EE)}$ enteroids were incubated with TNF (40 ng/ml) in the absence or presence of RIPK1 or RIPK3 inhibitors and photographed 4 hours later. Magnification 200×. (B) Lysates of IKKβ$^{(EE)}$ enteroids treated as above were subjected to IB analysis after 4 hours. (C) WT enteroids were treated for 24 hours with IL-22 (10ng/ml) in the absence or presence of nec-1, incubated with EdU and stained 2hours later. (D) Percentages of EdU positive cells per enteroids are shown.

To further validate the role of RIPK1 in TNF-induced apoptosis and tissue damage in IKKβ$^{(EE)}$ mice, we crossed the latter to Ripk1$^{D138N/D138N}$ homozygous knockin mice, which express a catalytically inactive version of RIPK1 that retains its scaffold function (Polykratis et al., 2014). We also crossed IKKβ$^{(EE)}$ mice with Ripk3$^{-/-}$ mice (Newton et al., 2014a) to genetically rule out a role for RIPK3-dependent necroptosis in TNF-induced mucosal erosion in our model. Notably, RIPK3 ablation had no significant effect in enteroids isolated from these mice that were incubated with TNF (Figures 18A and 18C). By contrast, RIPK1 kinase inactivation completely blocked TNF-induced crypt cell death and caspase-3 activation in IKKβ$^{(EE)}$ enteroids (Figures 18B and 18C). As expected (Vandenabeele, Declercq, Van Herreweghe, & Vanden Berghe, 2010; D.-W. Zhang et al., 2009), *Ripk3* gene ablation prevented necroptosis in enteroids incubated with TNF + zVAD (Figure 18E).

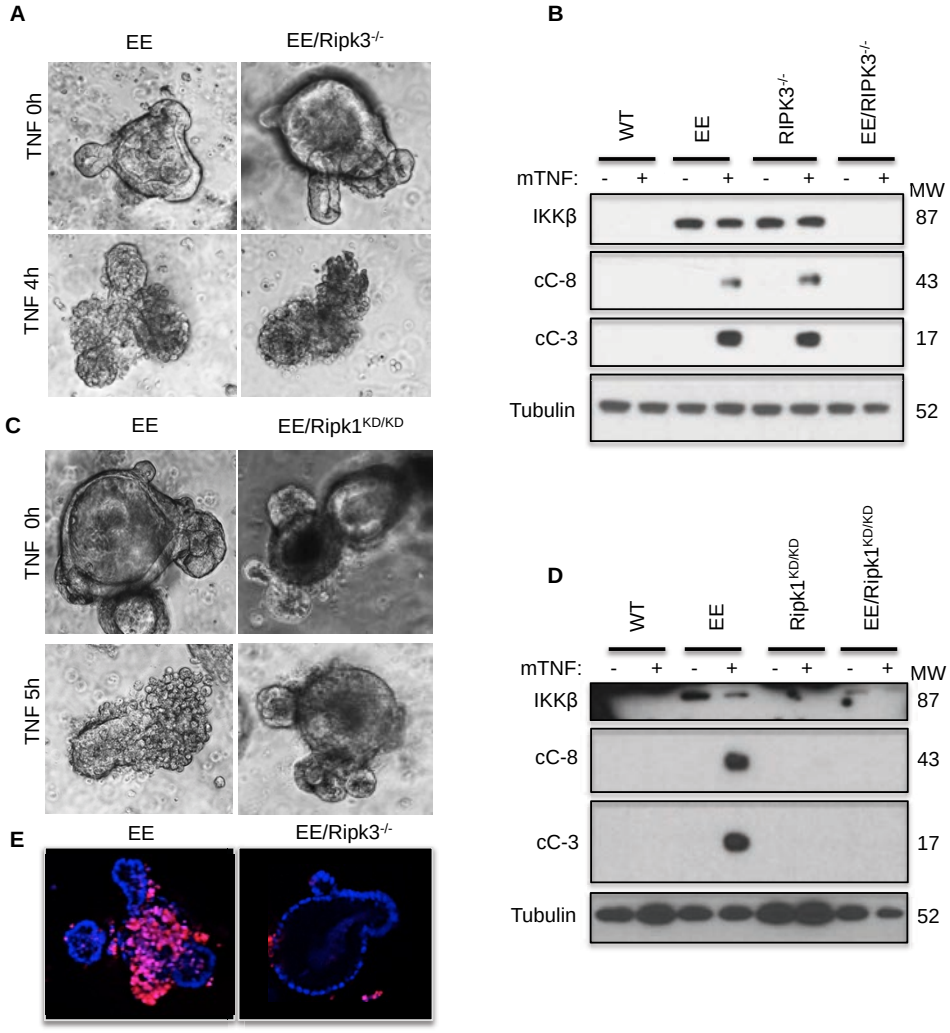

Figure 19. Genetic impairment of the kinase activity of RIPK1 impairs TNF-induced epithelial cell death.(A) IKKβ$^{(EE)}$ and IKKβ$^{(EE)}$/Ripk3$^{-/-}$ enteroids were photographed under brightfield before and 4 hours after incubation with TNF (40 ng/ml). Magnification 200×. (B) WT, IKKβ$^{(EE)}$, Ripk3-/- and IKKβ$^{(EE)}$/Ripk3$^{-/-}$ enteroids were incubated with or without TNF, lysed and analyzed by IB 5 hours later. (C) IKKβ$^{(EE)}$ and IKKβ$^{(EE)}$/Ripk1$^{D138N/D138N}$ enteroids were photographed under brightfield before and 5 hours after TNF addition. Magnification 200×. (D) WT, IKKβ$^{(EE)}$, Ripk1$^{D138N/D138N}$ and IKKβ$^{(EE)}$/Ripk1$^{D138N/D138N}$ enteroids were incubated with or without TNF, lysed and analyzed by IB 5 hours later. (E) IKKβ$^{(EE)}$ and IKKβ$^{(EE)}$/Ripk3$^{-/-}$ enteroids were incubated with TNF (40 ng/ml and zVAD (10 μg/ml) for 4 hours, stained with Hoechst 33342 and propidium iodide and examined by confocal microscopy. Magnification 200×. EE, IKKβ$^{(EE)}$.

IKKβ $^{(EE)}$ mice are extremely sensitive to TNF-induced mucosal damage and even a low dose of LPS, which induces TNF expression, causes their rapid death (Guma et al., 2011). Whereas Ripk3 ablation did not decrease LPS-induced mortality, inactivation of RIPK1 was fully protective (Figure 19A). The specific RIPK1 inhibitor GSK'963 also prevented LPS-induced death in IKKβ $^{(EE)}$ mice challenged with LPS (Figure 19B).

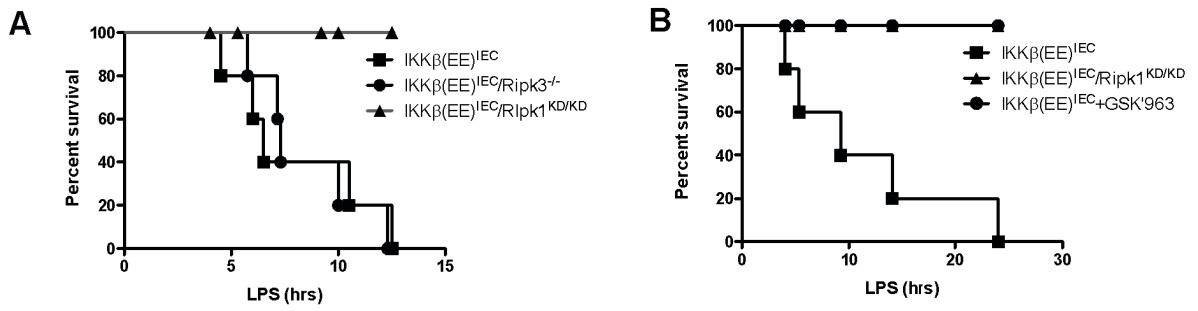

Figure 19. RIPK1 kinase activity is required for mice survival LPS challenge. (A) IKKβ$^{(EE)}$, IKKβ$^{(EE)}$/Ripk$^{1D138N/D138N}$ and IKKβ$^{(EE)}$/Ripk3$^{-/-}$ mice were injected with LPS (0.5 mg/kg E. coli O111:B4) and their survival was monitored over a 36 hour period (n=5 per group). (B) IKKβ$^{(EE)}$ were injected with LPS in the absence or presence of GSK'963 (50 mg/kg) and their survival analyzed over a 36 hour period and compared to that of LPS-injected IKKβ$^{(EE)}$/Ripk1$^{D138N/D138N}$ mice (n=5 per group).

LPS-induced caspase-3 activation in IKKβ$^{(EE)}$ intestinal crypts was not prevented by Ripk3 deletion (9.2 ± 0.4 cC-3+cells per crypt in IKKβ$^{(EE)}$ vs 7.6 ± 0.9 in IKKβ$^{(EE)}$/Ripk3$^{-/-}$ mice, p=0.2). However, RIPK1 inactivation inhibited TNF- and LPS-induced caspase-3 activation in IKKβ $^{(EE)}$ intestinal villi and crypts (5.25 ± 1 cC-3+ cells per crypt in IKKβ $^{(EE)}$ vs 1.1 ± 0.35 in IKKβ$^{(EE)}$/ Ripk1$^{D138N/D138N}$ mice, 4 hours after LPS administration p<0.001), as assessed by IHC (Figures 20A and 20B). Furthermore, the specific RIPK1 inhibitor GSK'963 prevented LPS-induced tissue damage in IKKβ $^{(EE)}$ mice challenged with LPS (5.5 ± 0.3 cC-3+ cells per crypt in IKKβ$^{(EE)}$ vs 3.2 ± 0.6 in GSK'963-treated IKKβ$^{(EE)}$ mice, p=0.002) (Figure 20C). Curiously, within 1 hr after TNF or LPS administration WT mice contained a few cC-3 positive IEC in their villi, whose number was also reduced upon RIPK1 inactivation (4.5 ± 0.8 cC-3+ cells per villi in WT vs 1.5 ± 0.5 in Ripk1$^{D138N/D138N}$ mice, p=0.01; Figure 20B).

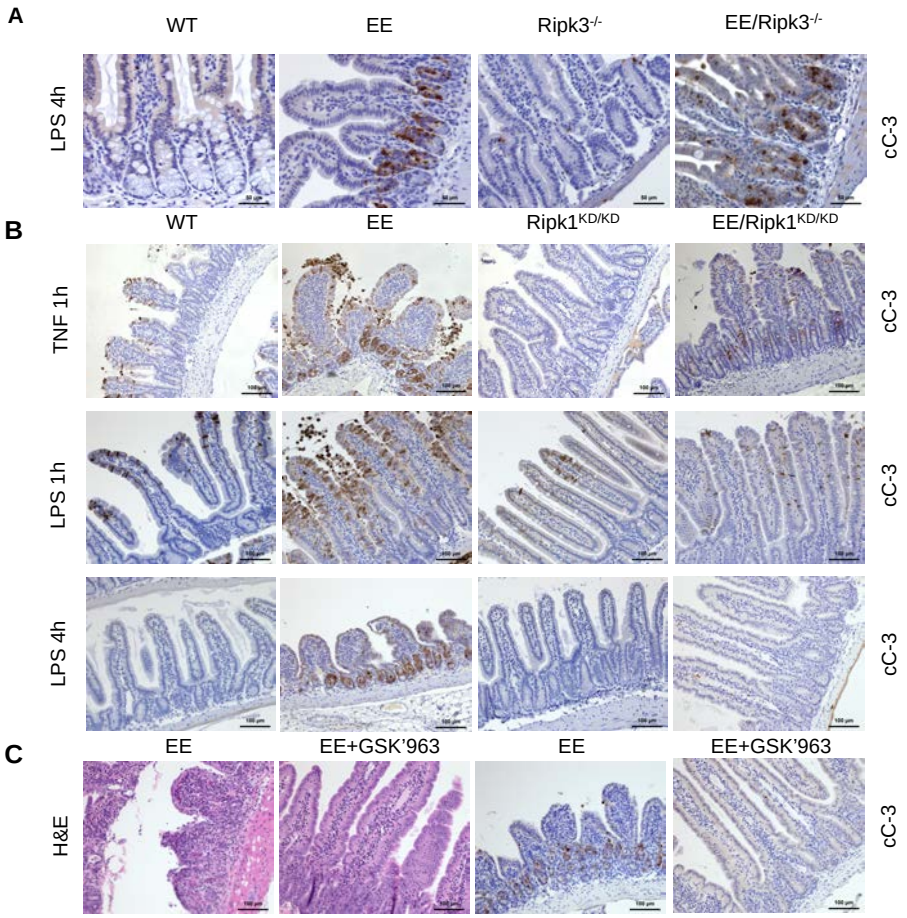

Figure 20. RIPK1 kinase activity is required for TNF-induced death in intestinal crypts. (A) WT, IKKβ$^{(EE)}$, Ripk3$^{-/-}$ and IKKβ$^{(EE)}$/Ripk3$^{-/-}$ mice were analyzed 4 hours after LPS (0.5 μg/gr i.p.) injection. Jejunal sections were stained with antibody to cC-3 and photographed (B) IKKβ$^{(EE)}$, IKKβ$^{(EE)}$/Ripk1$^{D138N/D138N}$ and WT mice were injected with TNF (2 μg i.v.) or LPS (0.5 mg/kg i.p.). Jejunal sections were collected at indicated times and stained for cC-3. (C) IKKβ$^{(EE)}$ were injected with LPS (0.5 mg/kg i.p) in the absence or presence of GSK'963 (50 mg/kg). Jejunal sections were collected after 4 hours of LPS injection and stained for H&E or cC-3. EE, IKKβ$^{(EE)}$.

4.4. RIPOPTOSOME FORMATION AND PROTECTION BY ANTIOXIDANTS

RIPK1 activation as a kinase and RIPK1-dependent apoptosis depend on formation of the Ripoptosome, or complex IIb, which also contains FADD and caspase-8 (Berghe, Linkermann, Jouan-Lanhouet, Walczak, & Vandenabeele, 2014; L. Wang et al., 2008). Although RIPK1 and FADD basal levels were the same in WT and IKKβ$^{(EE)}$ enteroids (Figure 21A), FADD immunocomplexes isolated from IKKβ$^{(EE)}$-expressing enteroids stimulated with TNF contained much more RIPK1 than FADD complexes from TNF-stimulated WT enteroids (Figure 21B). Treatment of TNF-stimulated cells with the caspase inhibitor zVAD allowed the recovery of intact caspase-8 in FADD immunocomplexes from either WT or IKKβ$^{(EE)}$ enteroids and increased the amount of RIPK1 present in the latter. These results suggest that IKKβ$^{(EE)}$ expression facilitates Ripoptosome formation and that caspase activation may be responsible for the decline in RIPK1 that is seen after some time in TNF-stimulated cells.

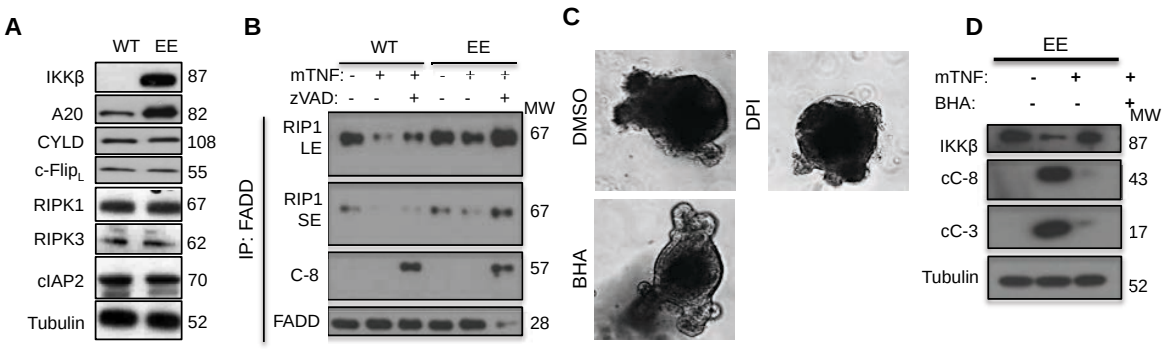

Figure 21. TNF-induced apoptosis of IKKβ$^{(EE)}$-expressing IEC involves Ripoptosome formation and can be inhibited by BHA *in vitro*. (A) WT and IKKβ$^{(EE)}$ enteroids were stimulated without or with TNF alone or together with zVAD (10 µg/ml) for 4 hours. Complex IIb was isolated by FADD immunoprecipitation and IB analyzed with indicated antibodies (B) IKKβ$^{(EE)}$ enteroids were incubated with DMSO, BHA (10 µM) or diphenylene iodonium (DPI, 5 µM) together with TNF (40 ng/ml) andphotographed under brightfield 4 hours later. Magnification 200×. (C) Lysates of IKKβ$^{(EE)}$ enteroids treated with BHA and/or TNF as indicated were IB analyzed after 4 hours. SE, short exposure. LE, long exposure

Inflammation is intimately related to accumulation of reactive oxygen and nitrogen species (ROS/RNS) and oxidative stress was proposed to underlie the pathophysiology of IBD (Alzoghaibi, 2013). Moreover, RIPK1 activation increases ROS production after TNF stimulation (Kim, Morgan, Choksi, & Liu, 2007) and the antioxidant butylated hydroxyanisole (BHA) can inhibit Ripoptosome formation and RIPK1-dependent apoptosis (Shindo, Kakehashi, Okumura, Kumagai, & Nakano, 2013). We therefore examined the effect of BHA and other antioxidants on TNF-induced death of IKKβ$^{(EE)}$-expressing IEC.

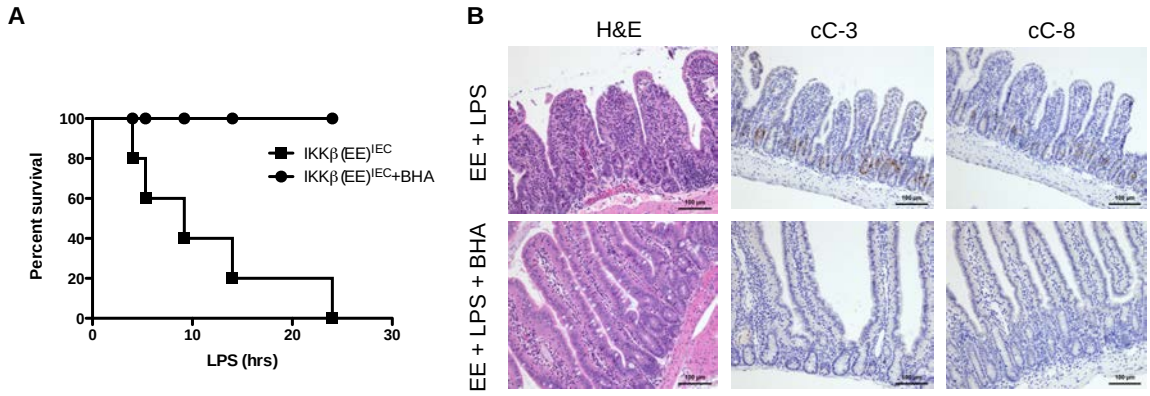

Figure 22. TNF-induced apoptosis of IKKβ(EE)-expressing IEC inhibited by BHA *in vivo*. (A) IKKβ$^{(EE)}$ mice were treated with or without BHA (20 mg/kg), challenged with LPS (0.5 mg/kg E. coli O111:B4) and their survival was monitored over a 36 hr period (n=5 mice per group). (B) IKKβ$^{(EE)}$ mice treated as above were analyzed 4 hours after LPS injection. Jejunal sections were stained with either H&E or antibodies to cC-8 or cC-3. EE, IKKβ$^{(EE)}$.

We incubated IKKβ$^{(EE)}$ enteroids with either diphenyleneiodonium (DPI), a NOX inhibitor, which did not protect the enteroids from TNF-cell death, or BHA, which was fully protective

(Figure 21C). BHA treatment also prevented TNF-induced caspase-8 and -3 cleavage in IKKβ$^{(EE)}$-expressing enteroids (Figure 21D). BHA administration to IKKβ$^{(EE)}$ mice protected them from LPS-induced mortality and IEC apoptosis (Figures 22A and 22B).

4.5. A20 AND CASPASE-3 ACTIVATION IN HUMAN IBD

In order to further understand the molecular mechanism enhancing the formation of complex IIb in IKKβ$^{(EE)}$-expressing IECs, we assessed the expression levels of different candidates known to modulate the TNF pathway. Interestingly, A20 was increased both by RNA and protein levels (Figures 12B and 21A). As previously mentioned, the *TNFAIP3* gene, which codes for the putative anti-inflammatory protein A20, contains three intronic single nucleotide polymorphisms that associate with responsiveness to anti-TNF drugs, however its effect in the mRNA expression or protein function is unknown. Published data is misleading as some authors have reported a decrease in A20 mRNA levels in IBD (Arsenescu et al., 2008) while other have described the opposite (Vereecke et al., 2014). Arsenescu et al. used non-inflamed IBD samples while Vereecke et al. looked at the correlation between non-responders versus responders before and after anti-TNF treatment. Interestingly, while both groups had higher levels of A20 before the treatment, with non-responders having even higher levels than responders, after the treatment responder's levels went similar to controls while non-responders kept A20 upregulated. Only one paper has looked at A20 protein expression by IHC showing increased levels of A20 in IBD and other inflammatory cases compared with control samples(C. F. Zheng & Huang, 2011). In order to shed some light into this dichotomy, we examined A20 expression in our IBD specimens by comparing the transcriptome of active ileal and colonic CD to that of healthy tissue. The results revealed strong upregulation of A20 mRNA and other NF-κB target genes and inflammatory cytokines including *TNF* and *LTB* (Figures 23A, 12A and 12B). A20 was also upregulated in UC (Figure 23A).

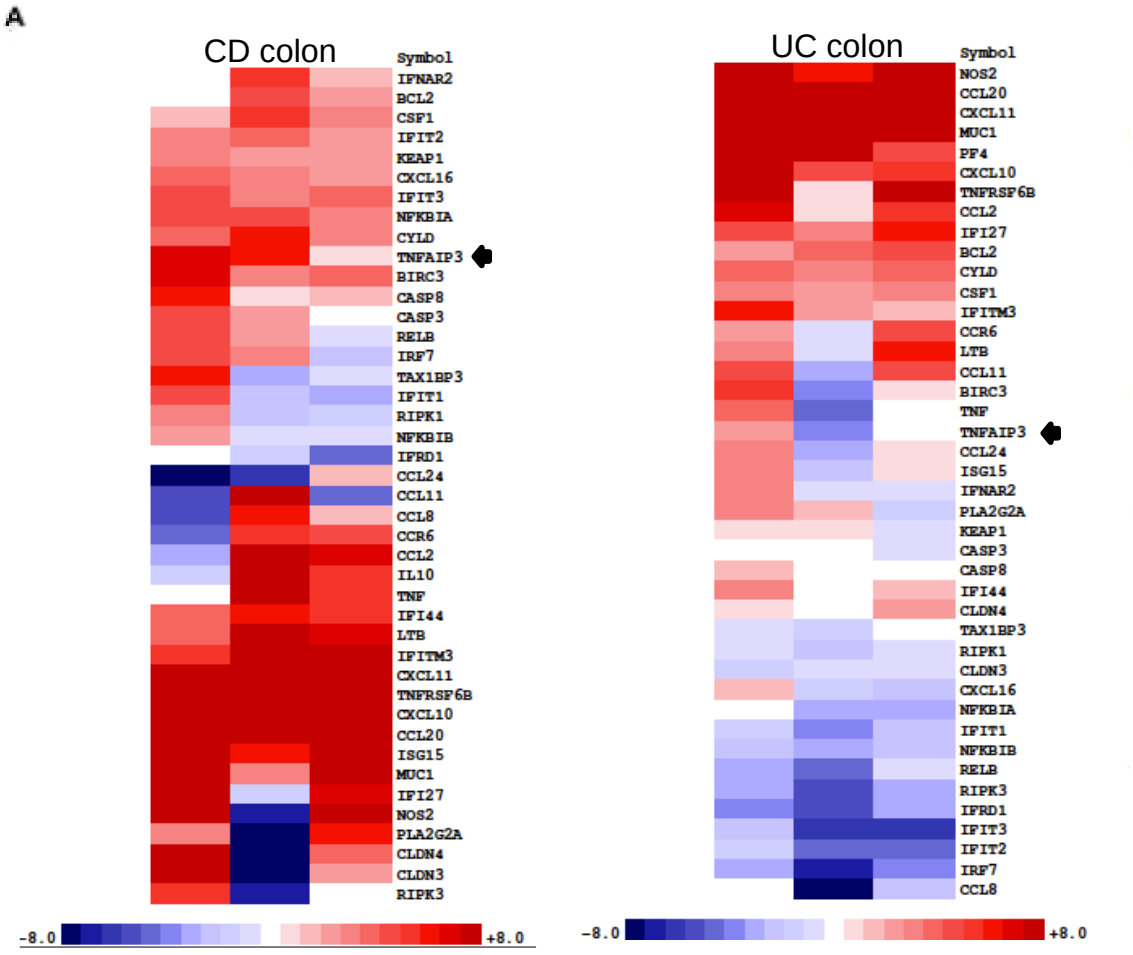

Figure 23. A20 expression is increased in our IBD specimens. (A) Heat maps showing differentially expressed IBD-related genes between colonic ulcerative colitis (UC) or colonic Crohn disease (CD) and healthy tissue biopsies (3 different IBD individuals compared with 1 healthy control).

Mining of publically available data sets showed a strong positive correlation between *TNFAIP3*, *TNF* and *BIRC3* transcripts (Figure 24A), suggesting that A20 may not inhibit NF-κB mediated transcription in IBD tissues. Furthermore, most patients with high *TNF* mRNA content also showed high levels of *TNFAIP3* mRNA (Figure 24B).

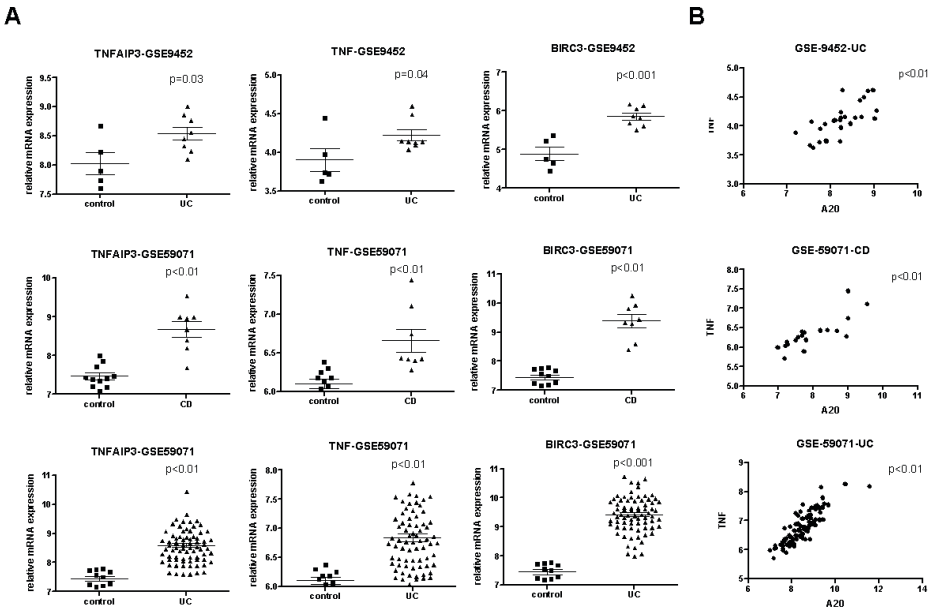

Figure 24. A20 expression is increased in different IBD datasets. (A) Expression of *TNFAIP3*, *TNF* and *BIRC3* mRNAs in CD and UC specimens from Array GSE9452 and GSE59071. (C) Correlation between *TNFAIP3* and *TNF* in the indicated Arrays.

Immunohistochemical (IHC) analysis revealed that A20 was upregulated in both lamina propria cells and IEC of active IBD tissue and that its expression in IEC correlated with cleaved caspase-3 (cC-3; p<0.01, r=0.51) (Figure 25A and Table 3).

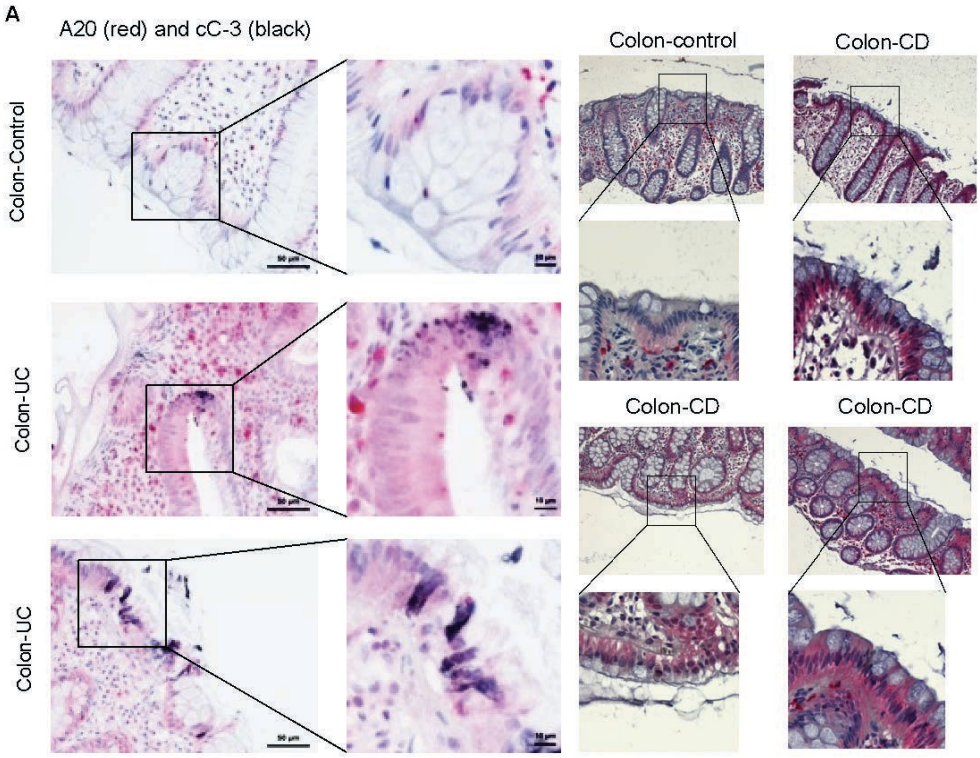

Figure 25. Increased A20 expression in human IBD specimens correlates with IEC apoptosis. (A) IHC double-staining of cleaved caspase-3 (cC-3) (black) and A20 (red) in human control, CD and UC colonic sections

Number of samples with corresponding cleaved caspase-3 (cC-3) and A20 expression levels in IEC of control tissue (C) and active UC and CD specimens.

Expression level	C colon (cC-3)	C colon (A20)	UC colon (cC-3)	UC colon (A20)	CD colon (cC-3)	CD colon (A20)
0	4	0	0	0	3	0
1	0	4	0	0	2	2
2	1	1	5	6	4	3
3	0	0	4	3	2	6
Total	5	5	9	9	11	11

IEC: intestinal epithelial cells, C: control, UC: ulcerative colitis, CD: Crohn's Disease

Table 3. Number of samples of cleaved caspase-3 and A20 expression level in IEC of control tissue and active IBD specimens.

Moreover, the level of inflammation reported in these samples also correlated with cC-3 ($p<0.05$, $r=0.26$) and a20 ($p<0.05$, $r=0.29$) expression (Figure 26A and 26B), confirming A20 upregulation in inflamed IBD tissues.

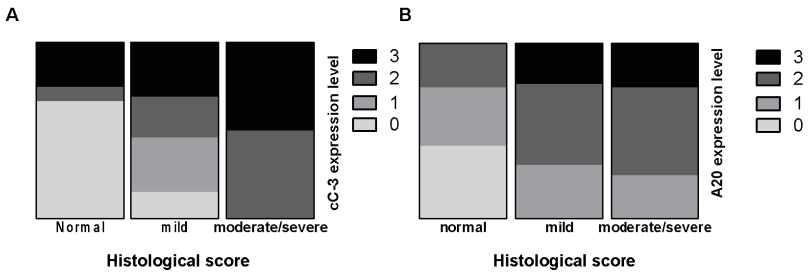

Figure 26. A20 expression and apoptosis in human IBD specimens correlates with inflammation. (A) Expression levels of cC-3 and (B) A20 (from 0 to 3) are plotted according histological score (mild, moderate and severe) in human IBD specimens.

4.6. TNF-INDUCED APOPTOSIS IN A20 TG MICE

A20 expression in IEC was reported to either restrain apoptosis and maintain barrier function (Kolodziej et al., 2011; Vereecke et al., 2010) or promote the development of colitis (S. F. Murphy et al., 2014), two contradictory outcomes neither of which is consistent with A20-mediated inhibition of NF-κB signaling. To determine how elevated A20 affects the viability of TNF-exposed IEC, we used villin-*Tnfaip3* (hereafter A20) Tg mice (Kolodziej et al., 2011). These mice are healthy and display normal growth and intestinal development (Rhee et al., 2012), but after retro-orbital injection of 1 µg of murine TNF, all A20 Tg mice died whereas WT controls survived (Figure 27A).

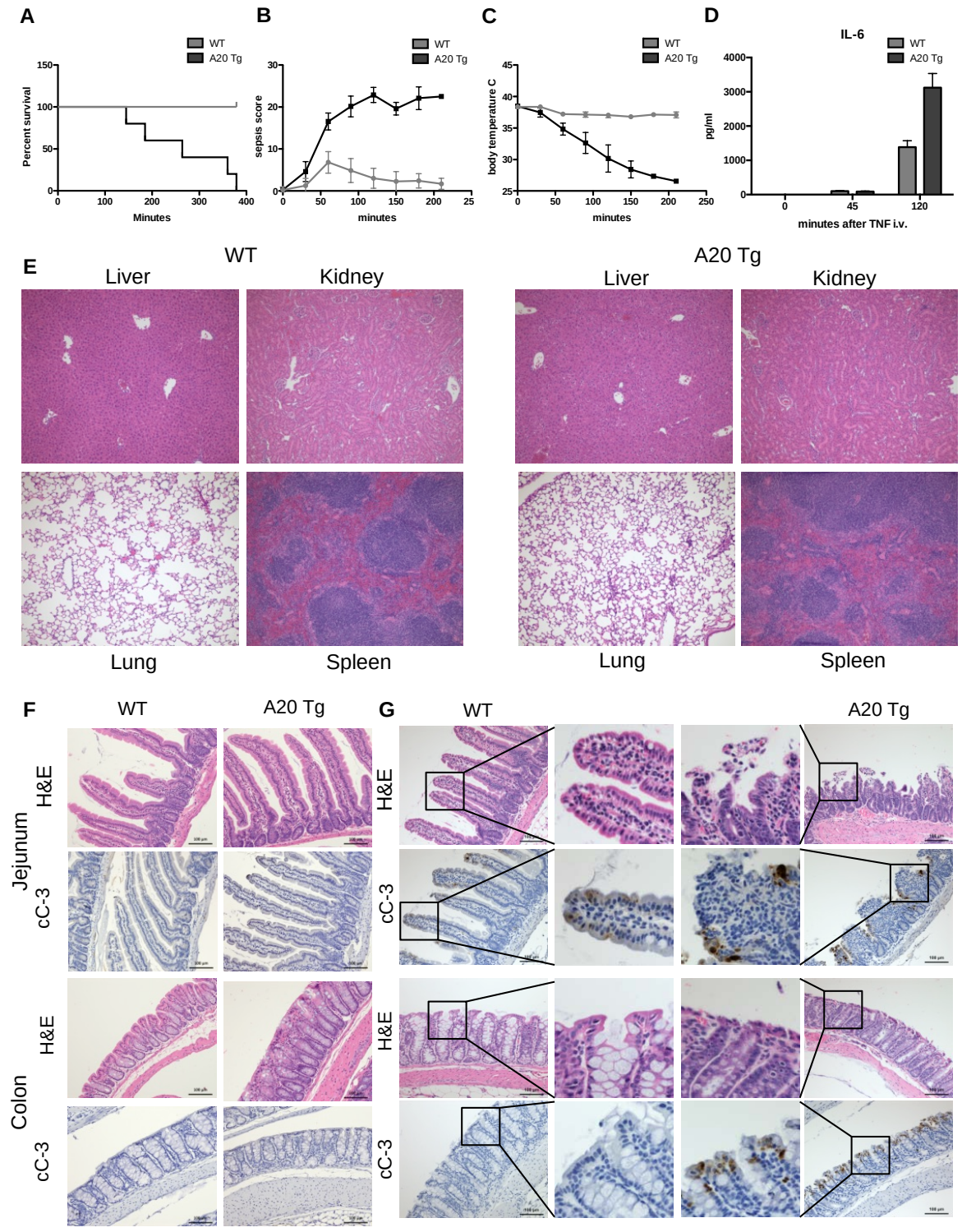

Figure 27. Increased A20 expression induce mortality and intestinal epithelial damage in mice. (A) Survival, (B) sepsis score, and (C) Rectal temperature of WT (grey lines) and A20-tg (black lines) mice after retro-orbital TNF (1µg) injection. Results are means ± s.e.m. (n=5). (D) Circulating IL-6 after TNF administration at different time points after TNF injection. Results are means ± s.e.m. (n=5). (E) Lungs, spleen and liver were stained with H&E in samples obtained after 2hours of TNF injection. (F) Representative hematoxylin and eosin (H&E) and cC-3 IHC staining of jejunal and colonic sections from WT and A20 Tg mice at time 0 and (G) 4 hours after TNF injection (1 µg retro-orbital injection, n=5).

TNF challenged A20 Tg mice exhibited a markedly elevated septic score and a dramatic drop in body temperature (Figures 27B and 27C). Inflammation in TNF challenged A20 Tg mice was accompanied by enhanced IL-6 production but no obvious damage to other organs (Figures 27D and 27E), other than extensive mucosal erosion, with complete loss of villi in the small bowel, and caspase-3 activation in the small bowel and colon (Figures 27F and 27G).

4.7. TNF-INDUCED APOPTOSIS IN A20-EXPRESSING ENTEROIDS

A20 Tg mice that are IL-10 deficient exhibit defective expression of mucosal cytokines and antimicrobial peptides, resulting in microbial colonization of the inner mucus layer, along with microbial dysbiosis (S. F. Murphy et al., 2014).

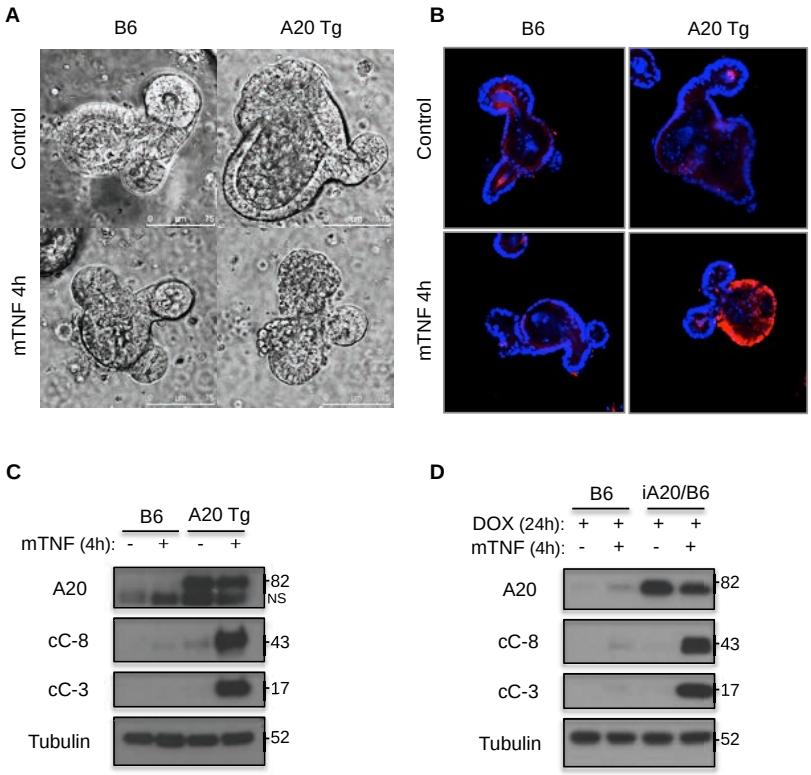

Figure 28. A20 overexpression sensitizes enteroids to TNF-induced apoptosis. (A) Brightfield images of WT and A20 Tg enteroids at 0 and 4 hours after TNF (40 ng/ml) addition. Magnification 200×. Brightfield. (B) Confocal images of cC-3-stained enteroids after TNF addition as in a. Scale bars, 50 µm. (C) Immunoblots of WT and A20 Tg enteroids with or without TNF stimulation (D). IB of iA20 enteroids incubated with or without doxycycline (DOX, 1 µg/ml for 24 hours) before TNF stimulation.

To assess IEC-autonomous A20 effects we used enteroid (intestinal organoids) cultures. Whereas WT enteroids exhibited minimal cell death, A20 Tg enteroids underwent extensive TNF-induced cell death, especially within villi domains (Figure 28A). Four hours after TNF challenge, immunofluorescence analysis revealed cC-3 in villi domains of A20 Tg enteroids (Figure 28B), consistent with TNF-induced villi erosion seen *in vivo* (Figure 27G). A20 Tg enteroids also showed strong expression of cC-3/cC-8 by immunoblotting (IB), whereas

control enteroids were unaffected (Figure 28C), indicating that immune cells and gut microbiota are not needed for TNF-induced apoptosis of A20-expressing IEC. We validated these findings by transducing control enteroids with a doxycycline-inducible (i) A20 construct. A20 induction strongly enhanced TNF-induced caspase activation (Figure 28D), indicating that the results are not due to any type of reprogramming that is peculiar to A20 Tg mice.

4.8. A20 HAS A MINOR EFFECT ON NF-κB ACTIVATION AND TRANSCRIPTIONAL ACTIVITY

As found in human IBD specimens, elevated A20 did not result in reduced mRNA and protein expression of antiapoptotic genes and NF-κB-related molecules in small bowel and colon IEC from A20 Tg mice (Figures 29A and 29B). A20 Tg and iA20 enteroids also did not show any defect in antiapoptotic genes and NF-κB-related molecules (Figure 29C). TNF-induced IκB⟨ degradation and IKK kinase activity were only marginally affected by A20 expression (Figures 29D and 29E) suggesting that TNF-induced apoptosis in A20-expressing cells was not secondary to NF-κB inhibition and decreased expression of anti-apoptotic genes. Thus, unlike its effect in other cell types (Vereecke, Beyaert, & van Loo, 2009), A20 has only a minor effect on NF-κB signaling in IEC.

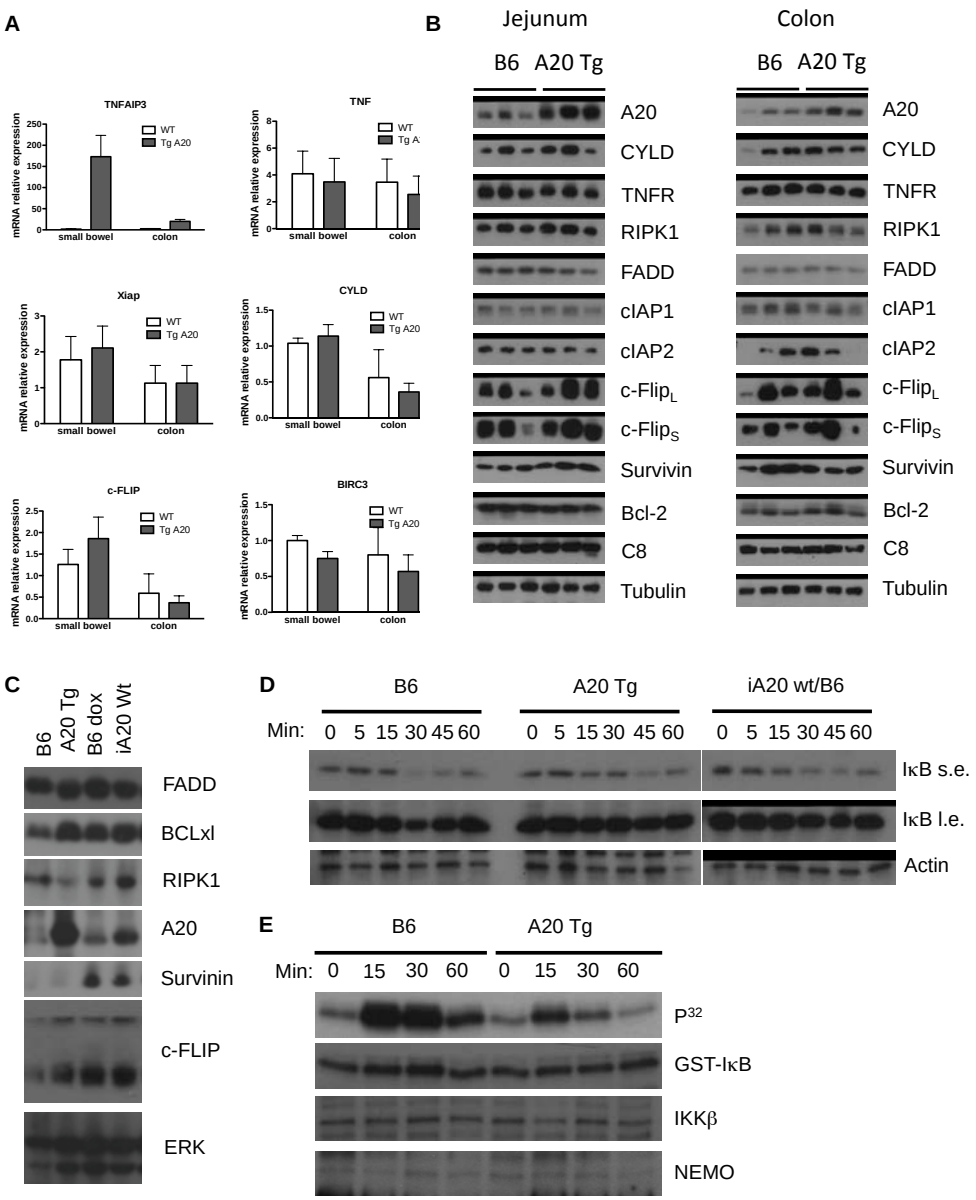

Figure 29. A20 overexpression does not impair NF-κB response in iEC and enteroids. (A) Quantitative real-time PCR (qPCR) analysis of *Tnfaip3*, *Tnf*, *Birc3*, *Xiap*, *Flip* and *Cyld* in RNA from jejunal and colonic IEC from WT and A20 Tg mice. (B) IB of whole jejunum and colon from WT and A20 mice. (C) IB of WT, A20 Tg, and WT and iA20 enteroids incubated with doxycycline (DOX, 1 µg/ml for 24 hours) at baseline. (D) The indicated proteins were analyzed in WT, A20 and iA20 enterocytes by immunoblotting after TNF stimulation at the indicated time points. (E) IKK activity was measured by immunecomplex kinase assay using GST-IκBα(1-79) as a substrate in lysates of WT and A20 Tg enteroids stimulated with TNF at the indicated time points.

4.9. RIPK1 KINASE ACTIVITY IS REQUIRED FOR TNF-INDUCED TISSUE DAMAGE

TNF-induced cell death depends on formation of death inducing signaling complexes. Whereas complex IIa mediates classical apoptosis in cells where synthesis of anti-apoptotic

proteins is inhibited, complex IIb, also known as the Ripoptosome, which requires RIPK1 kinase activity and leads to apoptosis, is engaged in cells treated with SMAC mimetics, which induce cIAP1/2 degradation (L. Wang et al., 2008). By contrast, the necrosome, whose activity depends on both RIPK1 and RIPK3 and caspase inhibition, mediates programmed necrosis (Wallach, Kang, Dillon, & Green, 2016). We used pharmacological inhibitors of RIPK1 (Necrostain-1 and GSK'963) and RIPK3 (GSK'843 and GSK'872) (Berger et al., 2015; Degterev et al., 2008; Mandal et al., 2014) to discern the involvement of RIPK1 and RIPK3 in TNF-induced death of A20-expressing IEC. Whereas RIPK1 inhibitors prevented TNF-induced cell death and caspase activation, RIPK3 inhibitors did not (Figures 30A and 30B).

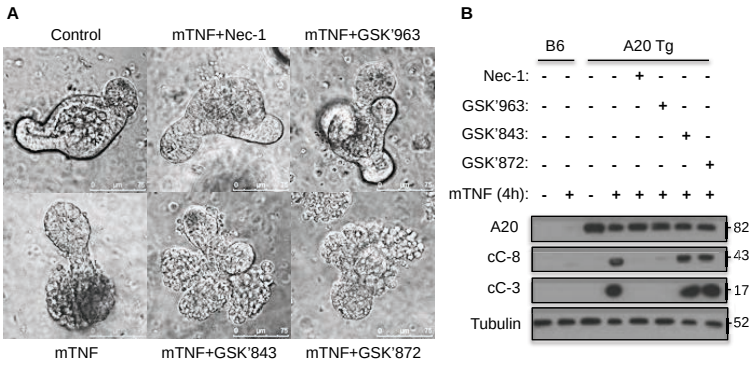

Figure 30. TNF-induced apoptosis of A20-expressing IEC depends on RIPK1 kinase activity. (A) Brightfield images of A20 Tg enteroids incubated with TNF for 4 hours without or with RIPK1 or RIPK3 inhibitors. Magnification 200×. (B) IB of A20 Tg enteroids treated as above.

Similar results were obtained in iA20 enteroids, and iA20 expression in enteroids derived from *Ripk1*$^{D138N/D138N}$ mice (Polykratis et al., 2014) did not sensitize the cells to TNF-induced apoptosis (Figures 31A and 31B).

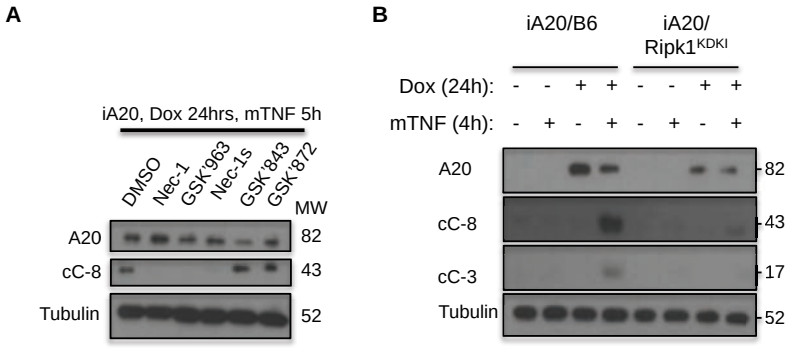

Figure 31. TNF-induced apoptosis of A20-expressing IEC enteroids depends on RIPK1 kinase activity. (A) IB of iA20-transduced enteroids after DOX pretreatment and TNF stimulation for 5 hours without or with RIPK1 or RIPK3 inhibitors. (B) IB of iA20 transduced WT or Ripk1$^{D138N/D138N}$ enteroids after DOX treatment and TNF stimulation.

Furthermore, administration of the RIPK1 inhibitor GSK'963 by intra-peritoneal injection to A20 Tg mice 1 hour prior to TNF challenge, greatly improved the septic score and inhibited

mucosal erosion (Figures 32A and 32B). Of note, the RIPK1 inhibitor did not block cytokine and inflammatory gene induction on TNF-challenge (data not shown).

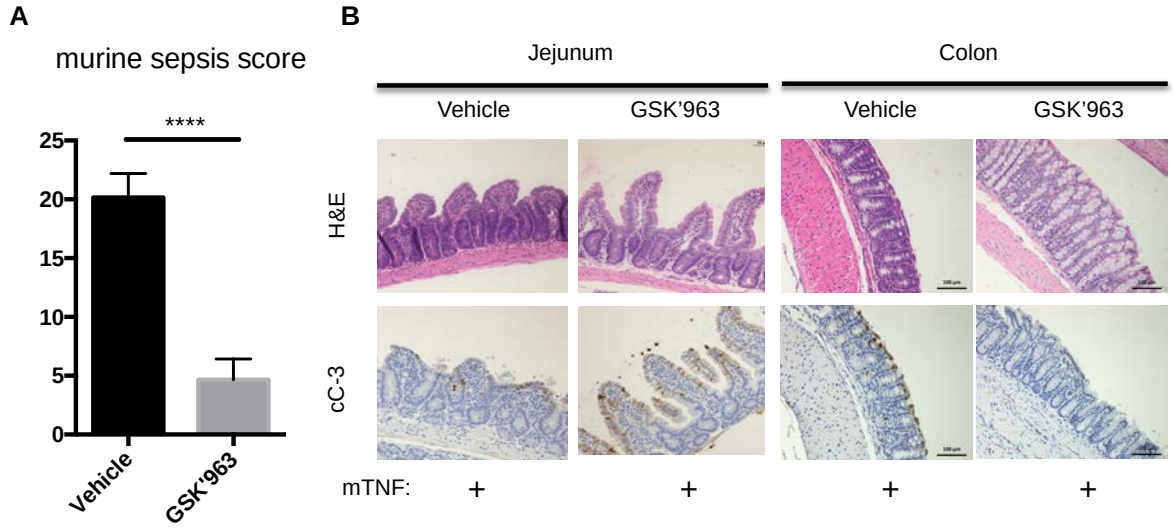

Figure 32. TNF-induced apoptosis of A20-expressing IEC depends on RIPK1 kinase activity. (A) Murine sepsis scores of 5 A20 Tg mice after TNF (2 µg) injection without or with GSK'963 (50 mg/kg). (B) H&E or cC-3 staining of jejunal and colonic sections from TNF-injected A20 Tg mice treated without or with GSK'963 (50 mg/kg).

4.10. A CRITICAL ROLE FOR ZNF7 IN TNF-INDUCED RIPK1-DEPENDENT IEC DEATH

We examined whether A20 expression activates RIPK1 upon TNF stimulation. Notably, TNF treatment together with the pan-caspase inhibitor zVAD stimulated RIPK1 phosphorylation on S166 in A20 Tg, but not in WT enteroids (Figure 33A). This modification likely represents RIPK1 autophosphorylation and it was absent in Nec-1-treated TNF-incubated enteroids. In A20 Tg enteroids treated with TNF alone, a weaker ~37 kDa band, which corresponds to N-terminally cleaved phospho-RIPK1, was observed, consistent with reports that activated RIPK1 is rapidly cleaved by caspase-8 (Ofengeim & Yuan, 2013; L. Zhang et al., 2015). RIPK1 activation as a kinase and its pro-apoptotic activity depend on formation of the Ripoptosome, which also contains FADD and caspase-8 (Berghe, Linkermann, Jouan-Lanhouet, Walczak, & Vandenabeele, 2014; L. Wang et al., 2008). Indeed, FADD immunocomplexes from TNF-stimulated A20-expressing enteroids contained much more p-RIPK1 and caspase-8 than FADD complexes from TNF-stimulated WT enteroids (Figure 32B), confirming that A20 enhances Ripoptosome formation. Even more important, we detected A20 in FADD immunecomplexes from TNF-stimulated A20-expressing enteroids, (Figure 33B). WT enteroids treated with cyclohexamide and then stimulated with TNF, which also trigger TNF-induced cell death, did not induce RIPK1 phosphorylation or A20 recruitment in the Ripoptosome (Figure 33B).

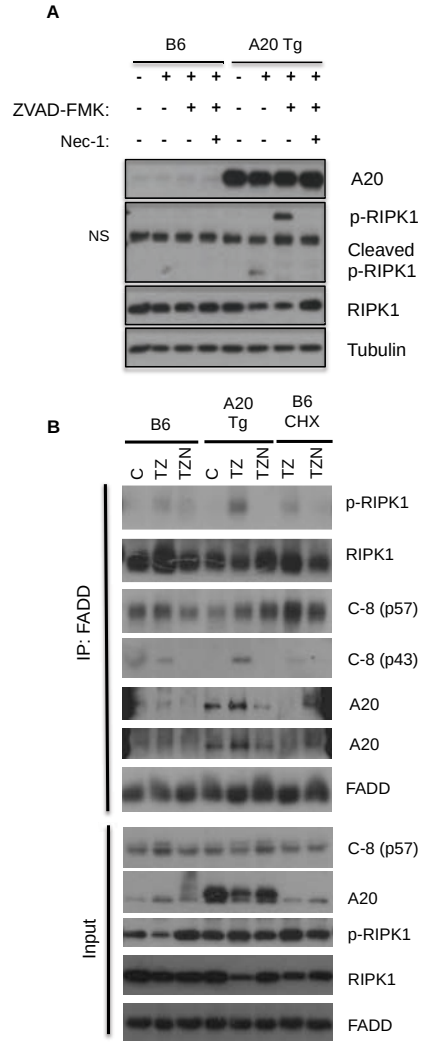

Figure 33. A20 enhances Ripoptosome formation. (A) IB of WT and A20 Tg entoroids stimulated with TNF alone or TNF + zVAD (10 µg/ml) or Nec1 (50 µM). (B) Complex IIb immunoprecipitation with anti-FADD antibody from WT in the presence or absence of cyclohexamide (CHX; 5 µM) and A20 Tg enteroids, stimulated with TNF alone (T) or TNF + zVAD (10 µg/ml) (TZ) or Nec1 (50 µM), or TNF + zVAD (10 µg/ml) (TZ) + Nec1 (50 µM) (TZN).

RIPK1 is modified by K63-linked polyubiquitin (Wertz et al., 2015), whose removal by the DUB CYLD promotes the transition from complex I to complex IIB (L. Wang et al., 2008). As A20 may also remove K63-linked polyubiquitin from RIPK1 (Wertz et al., 2004), we examined the ubiquitination status of TNFR1-associated RIPK1, expecting to find less ubiquitinated RIPK1 in TNF-stimulated A20 Tg enteroids. Much to our surprise, A20 expression prevented deubiquitination of TNFR1-bound RIPK1, which was readily observed in TNF-stimulated WT enteroids (Figures 34A and 34B). Furthermore, A20 expression increased the amount of TNFR1-bound RIPK1. In contrast, WT enteroids treated with CHX and then stimulated with TNF did not prevent deubiquitination of TNFR1-bound RIPK1 (Figure 34B). As reported (Wertz et al., 2004), A20 itself was incorporated into the TNFR1 signaling complex (Figures 34A and 34B). Of note, CYLD mRNA and protein expression were similar in WT and A20-expressing IECs (Figures 29A and 29B).

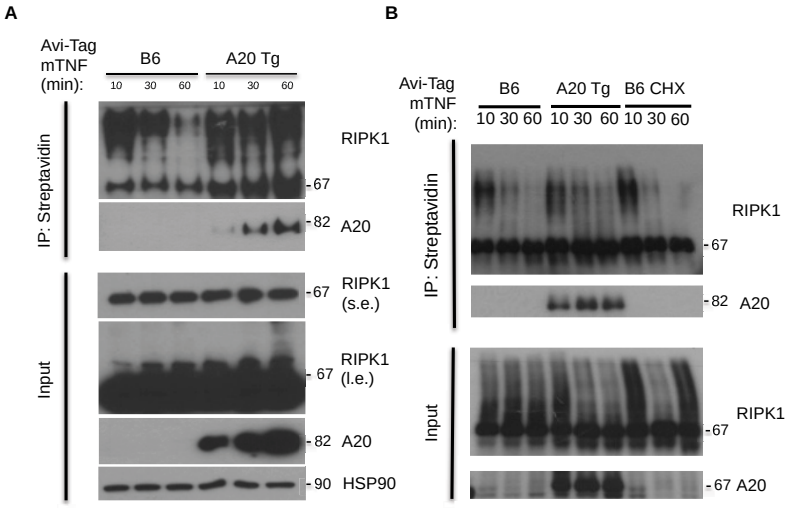

Figure 34. A20 prevents deubiquitination of TNFR1-bound RIPK1. (A) TNFR1 immunoprecipitation from WT and A20 Tg enteroids stimulated with AviTag mTNF followed by IB analysis with indicated antibodies. (B) TNFR1 immunoprecipitation from WT with or without cyclohexamide (CHX; 5 μM) and A20 Tg enteroids stimulated with AviTag mTNF followed by IB analysis with indicated antibodies.

We conducted mutational analysis to identify the functional domain of A20 required for enhancement of TNF-mediated caspase activation. Inducible expression of A20 variants lacking either its DUB activity, due to a C103A OTU domain substitution (Wertz et al., 2004), or its K48-linked E3 ligase function (C609A/C612A), which resides in ZnF4 (Wertz et al., 2015), were as effective as WT A20 in sensitizing IEC to TNF-induced apoptosis (Figure 35A). Next, we examined the role of ZnF7, which is required for binding of linear polyubiquitin (Catrysse et al., 2016), using a C764A/C767A double substitution mutant. Notably, expression of the ZnF7(C794A/C767A) mutant failed to enhance TNF-induced caspase activation, whereas the OTU-deficient mutant was fully functional (Figure 35B).

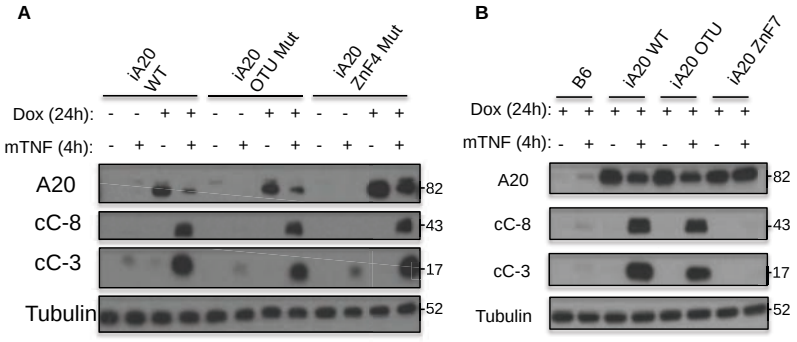

Figure 35. A20 requires its ZnF7 domain to activate RIPK1-dependent apoptosis. (A) IB of WT enteroids transduced with full length A20 and indicated A20 mutants after DOX treatment and TNF stimulation. (B) IB of WT enteroids transduced with full length A20 and indicated A20 after DOX treatment and TNF stimulation.

ZnF7 mutant also did not prevent deubiquitination of TNFR1-bound RIPK1 (Figure 36A) and failed to enhance Ripoptosome formation and RIPK1 phosphorylation in S166 after TNF treatment together with the pan-caspase inhibitor zVAD (Figure 36B).

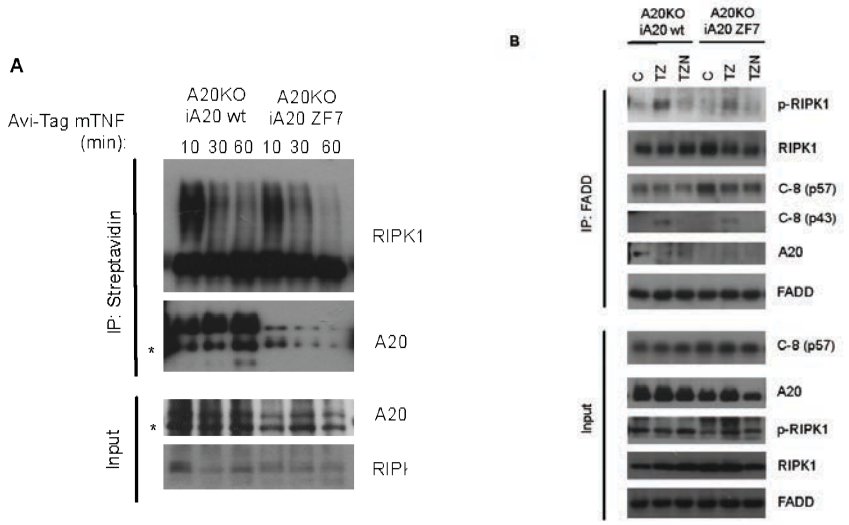

Figure 36. A20 requires its ZnF7 to prevent deubiquitination of TNFR1-bound RIPK1 and enhance Ripoptosome activation. (A) TNFR1 immunoprecipitation from full length iA20 or iA20ZnF7 mutant enteroids stimulated with AviTag mTNF followed by IB analysis with indicated antibodies. (B) Complex IIb immunoprecipitation with anti-FADD antibody from of A20-/- enteroids transduced with full length iA20 or iA20ZnF7 mutant stimulated with with TNF alone (T) or TNF + zVAD (10 µg/ml) (TZ) or Nec1 (50 µM), or TNF + zVAD (10 µg/ml) (TZ) + Nec1 (50 µM) (TZN). (D) TNFR1 immunoprecipitation from of A20-/- enteroids transduced with full length A20 or iA20-ZnF7 mutant stimulated with AviTag mTNF followed by IB analysis with indicated antibodies (NS: non-specific band).

4.11. LTB AND LIGHT EXPRESSION IN HUMAN IBD

IBD arrays showed an increase of different proinflammatory cytokines, among them LTB (Figures 12A, 12B and 23A). We further validated the expression of several cytokines including LTB and LIGHT in active human IBD (from ileum and colon CD, and colon UC) to that of healthy ileum and colonic tissue. The results revealed an upregulation of TNF mRNA and numerous other cytokines including IL-1α, IL-6 and IL-23 in active disease tissue (Figure 37A). Of note, both LTβ and LIGHT cytokines were also elevated in human IBD samples.

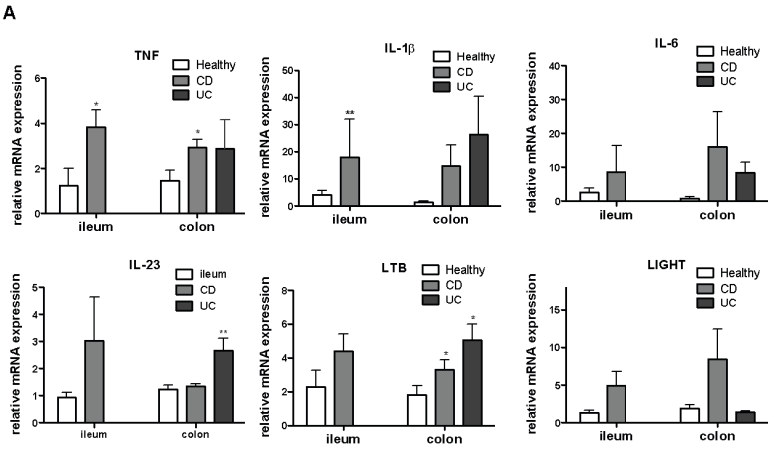

Figure 37. LTB and LIGHT are upregulated in IBD. (A) Real-time PCR for selected genes in human control (n=3 for ileum and n=5 for colon) or IBD specimens (n=5 for ileum CD and colonic UC, and n=8 for colonic CD). Data plotted with SD. *, P ≤ 0.05. **, P ≤ 0.01

4.12. TNF-LTβR ACTIVATION INDUCES APOPTOSIS IN BOTH HUMAN AND MURINE ENTEROIDS

To determine the pathogenic function of TNF and LTβR activation, we stimulated enteroid cultures. Whereas WT enteroids exhibited minimal TNF-induced death, WT enteroids underwent cell death after incubation with TNF and LTβR agonist as seen by the presence of cleaved caspase-3 and 8 by IB (Figure 38A), excluding requirement/involvement of other immune cells and gut microbiota. Both LTα1β2 and LIGHT signal through LTβR. LIGHT treatment together with TNF induced IEC death (Figure 38B). Unfortunately, recombinant LTα1β2 is not currently available. Of note, other cytokines highly expressed in inflamed IBD specimen including IL-1α or IL-6 did not trigger IEC apoptosis when combined with TNF (Figure 38C).

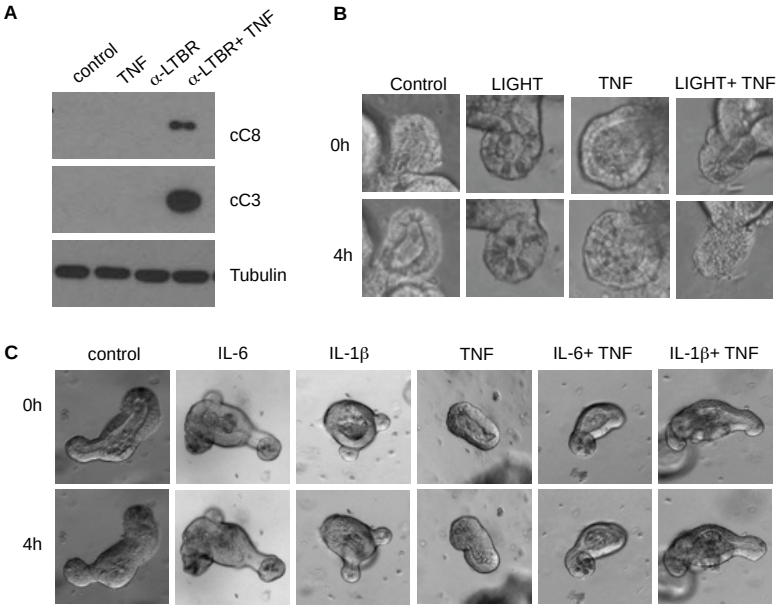

Figure 38. Lymphotoxin beta receptor activation induces cell death in intestinal epithelial cells. (A) Immunoblot from WT enteroids treated with murine TNF (40ng/ml), 4H8 (5µg/ml) or a combination. (B) Representative pictures from WT enteroids treated with murine TNF (40ng/ml), IL-6 (20ng/ml) and IL-1β (20ng/ml) or together with TNF. Pictures were taken at time 0 and 4 hours post treatment. (C) Representative pictures from WT enteroids treated with murine LIGHT (50ng/ml), TNF (40ng/ml) or a combination.

4.13. TNF-LTβR ACTIVATION INDUCED APOPTOSIS IS RIPK1 DEPENDENT BUT NIK INDEPENDENT

To query the involvement of RIPK1 kinase activity in the observed phenotype, we first examined the ability of Nec-1 to block the TNF-LTβR induced death of WT enteroids. Nec-1 effectively blocked the TNF-LTβR induced apoptosis of WT enteroids (Figure 39A). To further validate the role of RIPK1 in TNF-LTβR-induced IEC apoptosis, we established enteroids from *Ripk1*$^{D138N/D138N}$ homozygous mice. RIPK1$^{D138N/D138N}$ enteroids were fully protected against TNF-LTβR stimulation (Figure 39B).

As NIK is the critical kinase in LTβR signaling and has been reported that NIK triggers RIPK1-dependent apoptosis, we then studied NIK involvement in epithelial cell death. Unexpectedly, NIK-deficient enteroids also exhibited TNF-LTβR induced death susceptibility comparable to WT enteroids (Figure 39C), suggesting the TNF-LTβR induced apoptosis of WT enteroids is RIPK1 dependent but NIK independent.

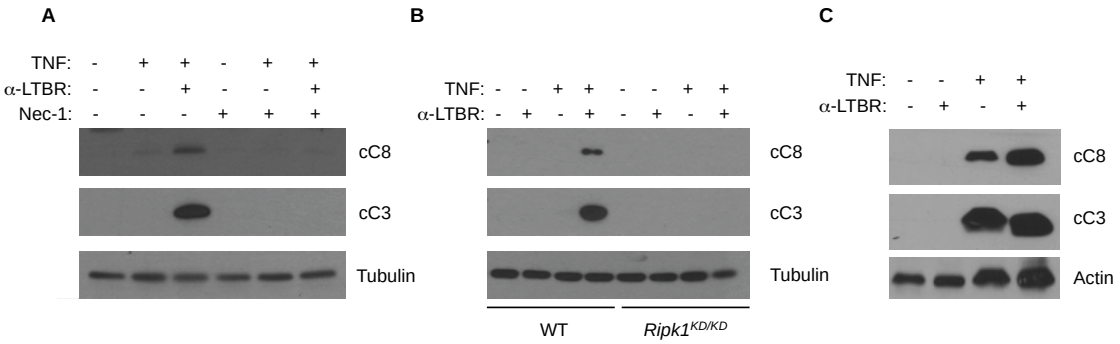

Figure 39. Lymphotoxin beta receptor activation together with TNF induces RIPK1 dependent apoptosis. (A) IB from WT enteroids treated with murine TNF (40ng/ml) and 4H8 (5µg/ml) together with nec-1 (50µM). (B) IB from WT or RIPK1$^{D138N/D138N}$ enteroids treated with murine TNF (40ng/ml) and 4H8 (5µg/ml). (C) IB from NIK knockout enteroids treated with murine TNF (40ng/ml) and 4H8 (5µg/ml).

4.14. TNF-LTβR INDUCED IEC APOPTOSIS IS RIPK1 DEPENDENT

To further validate the role of TNF-LTβR in IEC apoptosis, we compared IEC response after TNF injection with or without concomitant LTβR agonist stimulation in WT mice. We observed some apoptotic signal localized at the villus tips in TNF injected WT mice. Interestingly, both stimuli injected simultaneously triggered an apoptotic response beyond the villi affecting the crypt compartment, which was not observed in TNF-injected WT mice (Figure 40A). We only observed the apoptotic response shortly after the administration of the stimuli and repeated injection of TNF and LTβR did not elicited chronic damage in the intestinal epithelium (Figure 40B). Consistent with *in vitro* data on enteroids, Ripk1$^{D138N/D138N}$ homozygous knockin mice were protected against TNF-LTβR induced IEC apoptosis (Figure 39C).

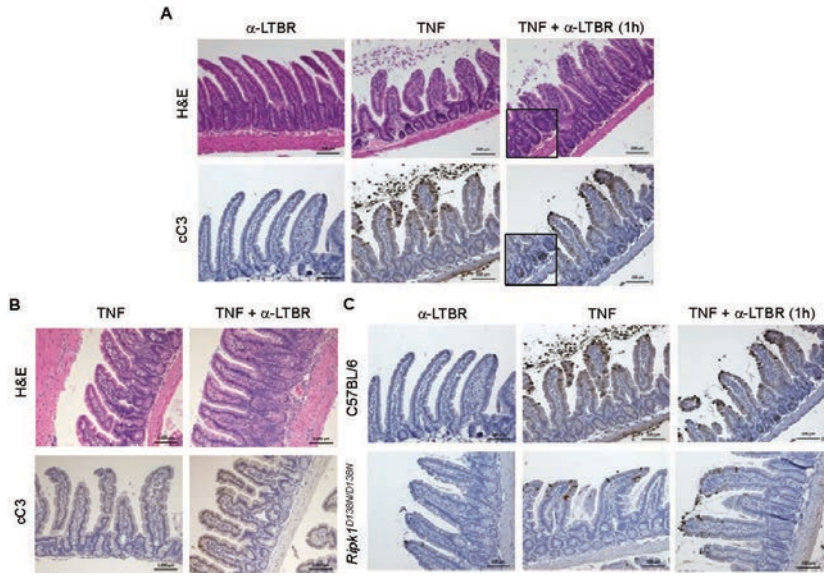

Figure 40. RIPK1$^{D138N/D138N}$ mice are protected from LTβR activation and TNF induced cell death. **(**A) Representative images of WT mice intestine sections stained with hematoxilyn and eosin or an antibody to cleaved caspase-3 after i.p. treatment with 4H8 (100µg/mice) one hour prior to i.v. injection of murine TNF (5 µg/mice) and sacrificed at 1 post TNF injection (N=6). (B) Representative images of WT mice intestine sections stained with hematoxilyn and eosin or an antibody to cleaved caspase-3 after i.p. treatment with 4H8 (10µg/mice i.p.) and i.v. murine TNF (5µg/mouse i.v.) for 5 consecutive days (N=5). (C) Representative images of WT or RIPK1$^{D138N/D138N}$ mice intestine sections stained an antibody to cleaved caspase-3 after i.p. treatment with 4H8 (100µg/mice) one hour prior to i.v. injection of murine TNF (5µg/mice) and sacrificed 1 hour post TNF injection (N=3 per strain). WT mice from panel A and C were from the same experiment.

Chapter 5:
DISCUSSION

A striking feature of UC and CD is an extensive epithelial cell death, which leads to ulceration, mucosal erosion, loss of barrier integrity and other lesions, including crypt abscess and fistulas (Khor, Gardet, & Xavier, 2011). This is thought to occur downstream of TNF, a cytokine whose expression is highly increased in IBD patients. In fact, blockade of TNF is one of the most effective therapeutic options. TNF blockade inhibits intestinal epithelial cell death in CD (Zeissig, 2004) and accelerates mucosal repair (J.-F. Colombel, Feagan, Sandborn, Van Assche, & Robinson, 2012). However, how TNF triggers intestinal epithelial cell death and mucosal erosion remains unknown.

IKKβ activation is required to induce TNF-dependent apoptosis in intestinal cells.

TNF is a poor IEC killer *in vitro*, and actually prevents intestinal injury due to its ability to activate NF-κB in WT mice (Chen et al., 2003). However, IKK- and NF-κB-deficient IEC are highly susceptible to TNF-induced killing via conventional caspase-8-dependent apoptosis (Chen et al., 2003; Egan et al., 2004; Greten et al., 2004; Nenci et al., 2007). Nonetheless, IKKα/β or NF-κB deficiencies have never been reported in IBD. On the contrary, a common feature of IECs in both UC and CD is harboring activated NF-κB (Kaser, Zeissig, & Blumberg, 2010; Rogler et al., 1998). We found that areas with activated NF-κB co-localized with apoptotic cells, a surprising finding because NF-κB is anti-apoptotic and promotes IEC survival (Eckmann et al., 2008; Egan et al., 2004; Nenci et al., 2007).

To study the correlation between NF-κB activation and cell death, we used transgenic mice that express a constitutively active form of IKKβ (IKKβ$^{(EE)}$) in IECs to induce chronic activation of the NF-κB pathway (Guma et al., 2011). Unexpectedly, these mice are highly sensitive to different damaging stimuli that leads to IEC death in the villi and the base of the crypt. Of note, intestinal stem cells are located at the base of the crypt (Shyer, Huycke, Lee, Mahadevan, & Tabin, 2015), so it will be interesting to study if the apoptotic cells at the base of the crypt are, in fact, stem cells.

A possible explanation for the observed cell death could have been that it was as a consequence of the microbiome. A conditional deletion of MyD88 in IECs did not protect IKKβ$^{(EE)}$ mice from cell death (although TRIF, the other TLR adaptor protein, was not tested) while TNF deletion was protective. Furthermore, experiments using IKKβ$^{(EE)}$ organoids showed that chronically active NF-κB in IECs also enhanced cell death after TNF treatment, proving that TNF-induced IEC apoptosis downstream of chronically active NF-κB is independent of the microbiota or lamina propria immune cells. Interestingly, prolonged NF-κB activation might not be required to induce IEC death. When an inducible vector expressing IKKβ$^{(EE)}$ was introduced in WT organoids, treatment with doxycycline for 6 hours was enough to render those enteroids susceptible to TNF. These results counterintuitively suggest that NF-κB enhances cell death after TNF treatment. To understand if NF-κB target gene expression is required to promote cell death downstream of IKKβ activation, we infected IKKβ$^{(EE)}$ organoids with a lentiviral construct that encodes the IκBα super-repressor to suppress NF-κB transcriptional activation. Although that was able to protect IKKβ$^{(EE)}$ organoids from TNF-induced cell death, closer analysis revealed that IKKβ$^{(EE)}$ levels were reduced upon expression of the IκBα super-repressor, probably because the promoter of IKKβ$^{(EE)}$ transgene, the villin promoter, contains two putative p65 binding sites with a 5%

dissimilarity as predicted by PROMO (Farré et al., 2003; Messeguer et al., 2002). This hampered our conclusion about the requirement of transcriptional NF-κB activation to induce cell death. The protection seen upon NF-κB inhibition in IKKβ$^{(EE)}$ organoids can be either because IKKβ$^{(EE)}$ was no longer present or because, in fact, it requires NF-κB transcriptional activation to induce TNF-dependent apoptosis. Regardless, we conclude that IKKβ activation is required to induce TNF-dependent apoptosis in IEC.

A20 overexpression renders intestinal epithelial cells more sensible to TNF-induced apoptosis though its ZnF7 domain.

Genome-wide association studies have identified *TNFAIP3*, the gene encoding A20, as a disease susceptibility locus in several inflammatory diseases, including IBD (Catrysse et al., 2016). However, most disease-associated SNPs within the *TNFAIP3* locus are located in its non-coding regions and their effects on A20 expression and IBD pathogenesis are unknown and controversial (Catrysse et al., 2016). For example, a study correlated the A20 SNPs rs6927210, rs7753394 and rs7773904 with improved response to anti-TNF drugs (Vereecke et al., 2014) whereas another study showed that rs6927172 was associated with increased A20 expression, decreased TNF levels and non-response to anti-TNF therapy in both CD and UC (Bank et al., 2014).

A similar dichotomy is observed in A20 mRNA expression in IBD. Whereas some groups showed that A20 mRNA is increased in IBD and that it negatively correlates with anti-TNF response (Vereecke et al., 2014), others showed the opposite when checking IBD non-inflamed areas (Arsenescu et al., 2008). In 2010, a mouse strain lacking A20 in IEC was developed and showed increased susceptibility to DSS colitis (Vereecke et al., 2010) but not spontaneous epithelial damage. Double deletion of A20 in both IEC and the myeloid compartment was shown to be required for spontaneous intestinal damage (Vereecke et al., 2014). Nevertheless, the increased susceptibility of the A20$^{\Delta IEC}$ mice to DSS and the decreased A20 mRNA levels in non-inflamed IBD tissue led to the conclusion that probably most of the SNPs in A20 decreased its expression in IBD. Subsequently, it was though that decreased A20 expression had detrimental effects on the pathogenesis of IBD and the homeostasis of IECs. However, in 2012, the A20 Tg mouse was generated. Although as expected, it showed protection from DSS colitis, it did not protect against TNBS (Rhee et al., 2012). In addition, when these mice were crossed with *IL10*$^{-/-}$ mice, which develops spontaneous colitis, A20 Tg/IL10$^{-/-}$ mice presented worse colitis accompanied by altered expression of mucosal antimicrobial peptides and altered organization of the mucus layer, as is typical in IBD. Only one paper has studied protein levels, which found that inflamed IBD and non-IBD intestinal pediatric biopsies showed increased levels of A20 (C. F. Zheng & Huang, 2011). Thus, consistently with previous work that suggested that A20 might be indeed elevated in IBD, our work is the first to demonstrate a positive correlation between A20 expression levels and cell death and inflammation in IBD IECs.

Since A20 levels are indeed increased in IEC of IBD patients, and correlate with inflammation score and cell death, we used transgenic mice that express A20 in IEC (A20 Tg) (Kolodziej et al., 2011). Consistently with the correlation observed in IBD patients, we found that A20 Tg mice were highly susceptible to TNF-induced apoptosis. These results challenge the view of A20 as an anti-inflammatory protein (Wertz et al., 2004, 2015).

In some cells, A20 removes K63-linked polyubiquitin chains from RIPK1 via its deubiquitinase activity (Afonina, Zhong, Karin, & Beyaert, 2017; Vereecke, Beyaert, & van Loo, 2009). Subsequently, A20 induces RIPK1 degradation though K48-linked polyubiquitin via the E3 ligase activity associated with zinc finger (ZnF) 4 (Wertz et al., 2004, 2015), resulting in NF-κB inhibition (Afonina et al., 2017; Vereecke et al., 2009). In IEC, A20 expression had little effect on NF-κB signaling and was instead found to prevent deubiquitination of TNFR1-bound RIPK1 and enhanced its recruitment to the FADD-associated IIb/Ripoptosome complex, thereby promoting TNF-induced cell death. This effect depends on the ability of ZnF7 of A20 to bind linear ubiquitin (Wertz et al., 2015). Overall, we have shown that A20 is increased in IBD samples and that it enhances TNF-dependent apoptosis through ZnF7, its linear ubiquitin binding domain.

LTβR activation enhances TNF-induced cell death of intestinal epithelial cells

To understand whether cytokines that are typically present in inflamed areas of IBD synergize with TNF to induce cell death, we treated organoids with IL-6, IL-1β and an agonistic antibody for LTβR. IL-6 did not promote TNF-dependent cell death, probably due to its pro-survival potential (Grivennikov et al., 2009; Kuhn, Manieri, Liu, & Stappenbeck, 2014; Markus F. Neurath, 2017). Neither did IL-1β, although we cannot discard an effect IL-1Ra secreted by the organoids, since all the treatments were done in fresh media neglecting the effect of potential accumulation of secreted molecules. LTβR activation enhanced IEC death both *in vitro* and *in vivo* upon TNF stimulation. However, WT mice treated with LTβR agonist and TNF transiently presented apoptotic cells without succumbing to the injection of this cytokines and chronic challenge did not induce colitis. LTβR can be activated by various ligands, including $LT\alpha_1\beta_2$, LIGHT and BAFF. Recombinant LIGHT was able to induce apoptosis upon TNF stimulation as well as BAFF, although BAFF induced a delayed and milder cell death compared to LIGHT that could potentially speed up if the BAFF concentration is increased. Unfortunately, recombinant $LT\alpha_1\beta_2$is not currently available so we could not test it. We can conclude that LTβR activation enhances TNF-dependent intestinal epithelial cell death.

TNF induces RIPK1-dependent apoptosis in intestinal epithelial cells in the studied conditions.

To understand what type of cell death was TNF triggering in IECs, we used pharmacological as well as genetic mouse models approaches. TNF can induce three different types of cell death: RIPK1-independent apoptosis, RIPK1-dependent apoptosis and necroptosis. While necroptosis is independent of caspase activation, apoptosis requires caspase activity. IKKβ activation, A20 overexpression and LTβR activation promoted TNF-dependent cell death, with the vast majority of dead cells stained by cleaved caspase 3, a converging caspase in the apoptotic pathway. To further clarify if cell death was apoptotic or necroptotic, we inhibited RIPK3 with either pharmacological agents or by genetic ablation. RIPK3, a master regulator of necroptosis, associates with RIPK1 and allows formation of the necroptotic pore in the cell membrane by MLKL (Cai et al., 2014; Yves Dondelinger et al., 2014; H. Wang et

al., 2014; Weinlich & Green, 2014). The dependence of cell death on RIPK3 is used to classify non-apoptotic forms of cell death, although it has been demonstrated that RIPK3 is likely involved in additional pathways beyond necroptosis such as inflammasome formation (Newton et al., 2016; X. Wang et al., 2014). To understand if this apoptotic process was dependent on RIPK1 activity, we used a small molecule that blocks its kinase activity. As complete RIPK1 ablation mice is lethal within few days after birth (Rickard et al., 2014), we used a knock in model in which residue 161 of the catalytic DFG motif in RIPK1 was mutated from Asp to Arg (Newton et al., 2014b). Our results show that TNF stimulation in both A20 and IKK$\beta^{(EE)}$ Tg mice and TNF+ LTβR stimulation in WT mice trigger apoptosis that is RIPK1-dependent.

RIPK1 kinase-dependent apoptosis occurs downstream of TNF when cIAP1/2 are depleted, independent of NF-κB activation (Petersen et al., 2007; Tenev et al., 2011; L. Wang et al., 2008). RIPK1 then forms the Ripoptosome, or complex IIb, that also contains FADD and caspase 8, upon TNFR1 activation. The exact mechanism that triggers the formation of complex IIb is unknown.

Previous papers have suggested that cIAP1/2 deletion is required for RIPK1-dependent apoptosis, however neither IKK$\beta^{(EE)}$ nor A20 Tg organoids showed a decrease in cIAP1/2 levels. In fact, cIAP1/2 are expressed downstream of the IKK complex and NF-κB activation and they act as deubiquitinases of RIPK1. Of note, complex I immunoprecipitation from A20 Tg enteroids showed a persistent RIPK1 ubiquitination over time that was not seen in WT organoids, suggesting that A20 is preventing RIPK1 deubiquitination through its ubiquitin binding domain, ZnF7. Of note, although A20 is a downstream target of NF-κB, IKK$\beta^{(EE)}$ enteroids are not likely dying through this mechanism because preliminary experiments do not show a maintenance of RIPK1 ubiquitination after TNF stimulation. In fact, K63 and linear (M1) ubiquitination of RIPK1 are required events for necroptosis. The linear ubiquitin assembly complex (LUBAC) is the only complex so far that mediates M1 ubiquitination. Linear ubiquitin chains are often added onto K63 ubiquitin chains, resulting in branched K63-linear ubiquitin chains (Emmerich et al., 2013). RIPK1 itself is subject to K63 and M1 ubiquitination within complex II (M C de Almagro, Goncharov, Newton, & Vucic, 2015; Lafont et al., 2017) and a K115R mutation decreased both linear and K63 ubiquitination of RIPK1 (M Cristina de Almagro et al., 2016). Expression of RIPK1^{K115R} mutant inhibited RIPK1 phosphorylation, necrosome assembly and necroptotic cell death. A20 is able to bind linear ubiquitin chains through its ZnF7 domain and protect them from degradation by other deubiquitinases (Draber et al., 2015; Verhelst et al., 2012). Moreover A20 is not recruited to complex I when HOIP, the ubiquitin ligase responsible for linear ubiquitination, is absent or inactivated (Draber et al., 2015). Deletion of A20 or its ZnF7 resulted in a marked reduction in linear ubiquitination of RIPK1 (Draber et al., 2015). We therefore suggest that A20 overexpression prevents other deubiquitinases, such as CYLD, from removing linear ubiquitin chains from RIPK1, thereby facilitating RIPK1 activation as a kinase. Of note, A20 contains a dimerization domain near its N-terminus end and Lu et al. have elegantly described how A20 dimerizes in the TNFR complex I (Lu et al., 2013).

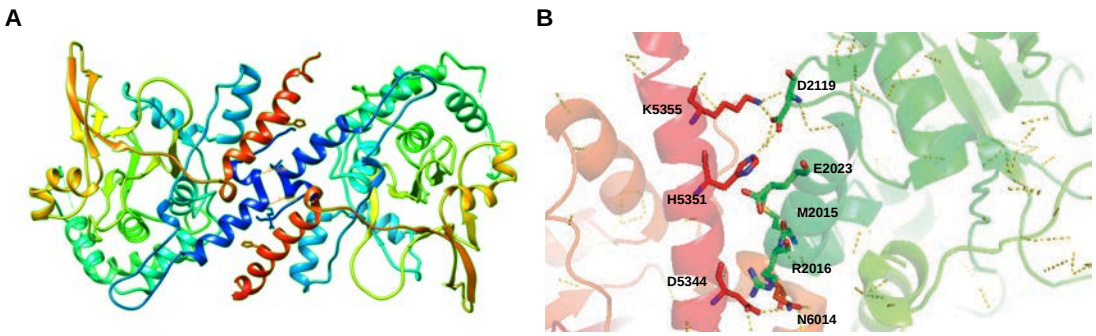

Figure 41. (A) Two interacting dimers of the A20 OTU domain (PDB: 3DKB) through the blue and red α-helix (B) Interface region between two interacting dimers (PDB: 3DKB) with possible interacting residues highlighted.

Analysis of the crystalized A20 OTU domain from two different groups (Komander & Barford, 2008; S. C. Lin et al., 2008) revealed that there are six and four molecules, respectively, per crystallographic asymmetric unit. Further analysis shows that two molecules, in both crystallographic asymmetric units, interact in a very similar manner (Figure 41A and 41B). The affinity of those interactions are by far higher than the interactions between other subunits (ΔG = -5.1kcal/mol as predicted for structures F + C by PDBePISA, PDB:3dkb) strongly suggesting that A20 homodimerizes through the OTU domain. Lin et al. showed that M15A, R16E and H351A substitutions prevented this dimerization, although the effect on NF-κB signaling and cell death has not been assessed. We propose that A20 dimerization could further facilitate activation of RIPK1 via transautophosphorylation by stabilizing RIPK1 dimers (Figure 42A). A similar model has been used to explain TRAIL-induced caspase-8 activation. By itself the caspase-8 dimer is too unstable once released from the death inducing signaling complex (Donepudi, Sweeney, Briand, & Grütter, 2003; Pop, Fitzgerald, Green, & Salvesen, 2007). However, binding of p62/SQSTM1 to caspase-8-linked K63 polyubiquitin facilitates caspase-8 oligomerization and activation (Z. Jin et al., 2009). In order to test this hypothesis, we will immunoprecipitate RIPK1 from TNFR1 and blot it with different anti-ubiquitin chain antibodies to check the ubiquitination status of RIPK1. Also, we will infect $A20^{KO}$ organoids with an inducible A20 construct mutated at residues essential for dimerization and study its effects on RIPK1-dependent apoptosis downstream of TNF as well as on NF-κB activation. We do not expect to see any alteration of the NF-κB activation pathway but a protection from RIPK1 dependent-apoptosis downstream of TNF.

cIAP1/2 as a possible mechanism for TNF-induced cell death after LTβR activation.

Although cIAP1/2 are not probably mediating the outcome in $IKK\beta^{(EE)}$ and A20 Tg intestinal epithelial cells, they might be playing a role in the LTβR model. A possible mechanism that could explain the different outcomes in cell death induced by TNF and other cytokines could be the recruitment of cIAP1/2 to LTβR but not IL-6 nor IL-1β. Upon recruitment of cIAP1/2 to LTβR, the availability of cIAP1/2 for binding to TNFR would decrease (Figure 42B). This would act as a SMAC mimetic inducing RIPK1 dependent cell death. It is important to mention that although RIPK1 needs to be linearly and K63 ubiquitinated to induce RIPK1

dependent cell death (M C de Almagro, Goncharov, Newton, & Vucic, 2015; M Cristina de Almagro et al., 2016), RIPK1 is modified in multiple residues while in complex I (Wertz & Dixit, 2010). It is plausible that formation of complex IIb requires the deubiquitination of certain residues while keeping others intact. cIAP1/2 decrease in complex I might mediate this effect. In order to answer this, we will immunoprecipitate TNFRI and blot for cIAP1/2 presence in complex I as well as the ubiquitin status of RIPK1 after TNF treatment with or without LTβR activation.

RIPK1 activation downstream of ROS accumulation

Other mechanisms were described to enhance Ripoptosome formation. Reactive oxygen species (ROS) have been long suggested as potential regulators of necroptosis (Y. Lin et al., 2004; Schenk & Fulda, 2015), and recently it has been shown that ROS enhance RIPK1 activation through cysteine modifications (Y. Zhang et al., 2017). Specifically, ROS directly acts on RIPK1 C257, C268 and C586, promoting disulfide bonds between RIPK1 molecules. This allowed the formation of an amyloid structure that enhances RIPK1 S161 autophosphorylation, facilitating its activation and downstream necrosome formation (Y. Zhang et al., 2017). Importantly, the antioxidant capacity of patients with IBD is reduced, even in the asymptomatic phase of the disease (Achitei et al., 2013; Alzoghaibi, 2013; Genser, Kang, Vogelsang, & Elmadfa, 1999) and IKKβ$^{(EE)}$ mice show increased markers of oxidative species due to a chronic NF-κB activation (Shaked et al., 2012). In fact, in IKKβ$^{(EE)}$ IEC, ROS is indispensable for RIPK1-dependent apoptosis. When organoids were treated with DPI, an inhibitor of NADPH oxidases (NOXes) and dual oxidases (DUOXes), it did not protect from cell death, however the ROS scavenger BHA was protective. BHA is known to prevent the necrosome formation (Shindo, Kakehashi, Okumura, Kumagai, & Nakano, 2013; Y. Zhang et al., 2017) and although it is used as a ROS scavenger, it has been known to inhibit other proteins. For instance, BHA can block cell respiration by inhibiting the mitochondrial activity of complex I (NADH-CoQ reductase), complex II (succinate-CoQ oxidoreductase) and complex III (cytochrome c-ubiquinole reductase) (Ferreira, 1990; Festjens et al., 2006; Nakagawa, Nakajima, Moore, & Moldéus, 1994; Okubo, Yokoyama, Kano, & Kano, 2004; Uslu & Bonavida, 1996) being complex I a major ROS-producing site in the electron transport chain. Therefore, it is plausible that ROS-induced IEC death in IKKβ$^{(EE)}$ comes from mitochondria. To further support this idea MitoVit-E, MitoQ or SkQ1, different mitochondrial ROS (mtROS) scavengers, should be tested for their ability to prevent cell death in IKKβ$^{(EE)}$ enteroids after TNF treatment as well as Ripoptosome formation. We would expect to see less active caspases and less RIPK1 autophosphorylation and complex IIb formation as a result of mtROS decrease. Overall, it is possible that basal levels of oxidative species in IKKβ$^{(EE)}$ intestinal epithelial cells enhance the activation of RIPK1 favoring TNF-induced apoptosis (Figure 42C).

Interestingly, the Ripoptosome is described to be negatively regulated by the kinase activity of TAK1 and its downstream substrate, the IKK complex (Y Dondelinger et al., 2013; Yves Dondelinger et al., 2015; Legarda-Addison, Hase, O'Donnell, & Ting, 2009). In fact, it was suggested how IKKα and IKKβ directly phosphorylate RIPK1, inhibiting the Ripoptosome formation in MEFs. In organoids, however, this does not seem to occur as the activated form of IKKβ is promoting RIPK1-dependent apoptosis and preliminary kinase assays do not show IKKβ phosphorylation of RIPK1 using recombinant proteins obtained from mammal

cells. This also does not occur in the case of A20 overexpression as A20 does not completely abolish IKK kinase activity after TNF stimulation. A possible explanation could be that Legarda-Addison et al. used recombinant proteins generated in bacteria, which lack some eukaryotic posttranslational protein modifications that could potentially affect their function. The use of different buffers could also explain those differences. Finally, Legarda-Addison et al. used a wild type form whereas we used a constitutively active form of IKKβ that might interact differently with RIPK1 as a consequence of the added glutamic acids.

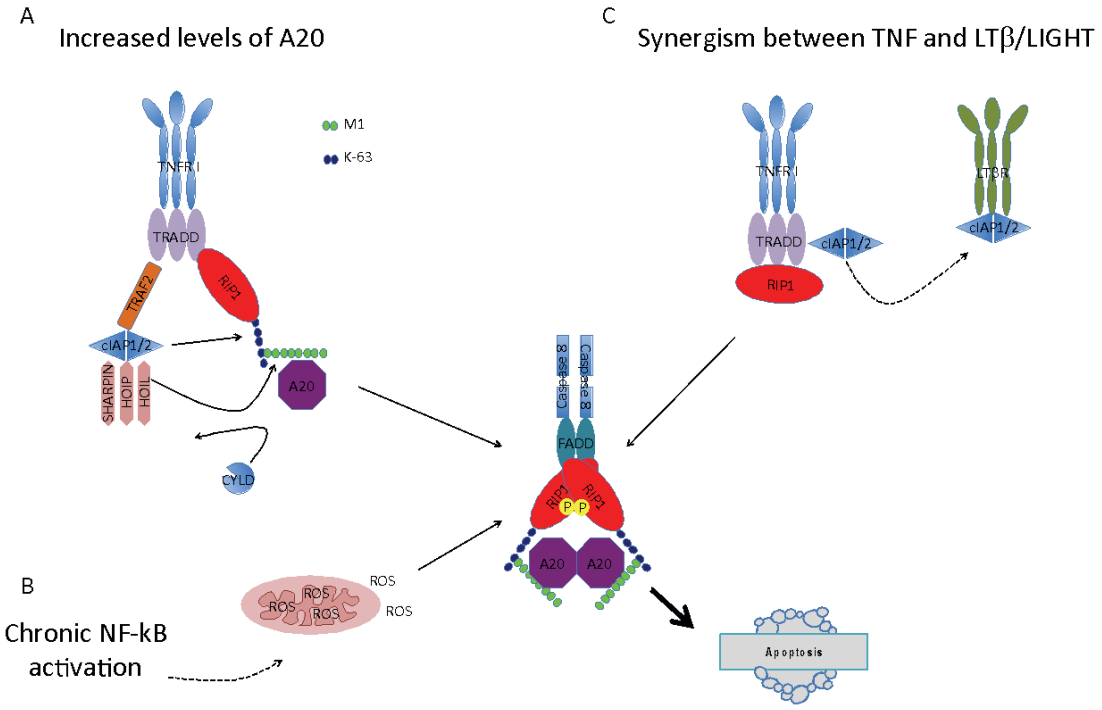

Figure 42. Proposed models. (A) A20 binding through ZnF7 to linear ubiquitins in RIPK1 protects them from degradation through other deubiquitinases and allows the formation of complex IIb. Through A20 dimerization the complex is stabilized and RIPK1 interaction is enhanced to induce RIPK1 apoptosis. (B) Chronic NF-κB enhances ROS production that allows RIPK1 interaction and formation of the ripoptosome to induce cell death. (C) cIAP1/2 recruitment to LTβR reduces cIAP1/2 pool to bind to the TNFR1 inducing the formation of the ripoptosome and subsequent apoptosis.

We examined the effect of RIPK1 inhibitors on TNF induced IEC death, and we are currently testing its interference with cell differentiation and migration, necessary steps to heal damaged mucosa. This is of vital importance as RIPK1 is required to supports IKK/NF-κB and MAPK activation downstream of TNF leading to induction of cell survival and proliferation. Thus, targeting the kinase activity of RIPK1 without interfering with its scaffolding function presents a way to selectively inhibit TNF's destructive properties (which depend on kinase activity) while preserving its survival and proliferative properties (which are kinase-independent).

This work has shown that NF-κB activation in IBD correlates with cell death and that it enhances TNF-dependent cell death in a ROS dependent manner. It has also shown that A20 is increased in IBD and how A20, through its ZnF7, favors the formation of complex II

and TNF-induced apoptosis. LTβR activation, a receptor from different cytokines upregulated in IBD, also enhances TNF-dependent apoptosis. Finally, we deciphered the mechanisms of TNF-induced cell death in intestinal epithelial cells downstream of NF-κB activation, A20 overexpression and LTβR activation and how the kinase activity of RIPK1 plays a major role to induce apoptosis. These results might be fundamental for the development of new therapeutic tools for ulcerative colitis and Crohn's disease.

Chapter 6:
CONCLUSIONS

There is a positive correlation between activated NF-κB, A20 levels and apoptosis in intestinal epithelial cells in IBD.

IKKβ activation, A20 overexpression and lymphotoxin β receptor activation promotes RIPK1-dependent TNF-induced intestinal epithelial cell death.

TNF-induced intestinal epithelial cell apoptosis occurs independently of gut microbiome and lamina propria inflammation.

RIPK1-dependent TNF-induced death in intestinal epithelial cells with chronic IKKβ activation in both crypt and villi compartment occurs in a ROS dependent manner.

RIPK1-dependent TNF-induced death in intestinal epithelial cells with A20 overexpression is at villi compartment, due to a lack of RIPK1 deubiquitination in complex I.

A20 induced cell death depends on its linear-ubiquitin binding zinc finger 7 domain.

A20 overexpression does not completely abrogate NF-κB activation in intestinal epithelial cells.

LTβR activation, but not IL-6 or IL-1β, enhances TNF-dependent cell death in intestinal epithelial cells.

Chapter 7:
REFERENCES

Abreu, M. T. (2010). Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nature Reviews Immunology*, *11*(3), 215–215. https://doi.org/10.1038/nri2728

Achitei, D., Ciobica, A., Balan, G., Gologan, E., Stanciu, C., & Stefanescu, G. (2013). Different profile of peripheral antioxidant enzymes and lipid peroxidation in active and non-active inflammatory bowel disease patients. *Digestive Diseases and Sciences*, *58*(5), 1244–1249. https://doi.org/10.1007/s10620-012-2510-z

Adrianto, I., Wen, F., Templeton, A., Wiley, G., King, J. B., Lessard, C. J., … Gaffney, P. M. (2011). Association of a functional variant downstream of TNFAIP3 with systemic lupus erythematosus. *Nature Genetics*, *43*(3), 253–258. https://doi.org/10.1038/ng.766

Afonina, I. S., Zhong, Z., Karin, M., & Beyaert, R. (2017). Limiting inflammation—the negative regulation of NF-κB and the NLRP3 inflammasome. *Nature Immunology*, *18*(8), 861–869. https://doi.org/10.1038/ni.3772

Ahern, P. P., Schiering, C., Buonocore, S., McGeachy, M. J., Cua, D. J., Maloy, K. J., & Powrie, F. (2010). Interleukin-23 Drives Intestinal Inflammation through Direct Activity on T Cells. *Immunity*, *33*(2), 279–288. https://doi.org/10.1016/j.immuni.2010.08.010

Alzoghaibi, M. A. (2013). Concepts of oxidative stress and antioxidant defense in Crohn's disease. *World Journal of Gastroenterology*, *19*(39), 6540. https://doi.org/10.3748/wjg.v19.i39.6540

Anderson, C. A., Boucher, G., Lees, C. W., Franke, A., D'Amato, M., Taylor, K. D., … Rioux, J. D. (2011). Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nature Genetics*, *43*(3), 246–252. https://doi.org/10.1038/ng.764

Ardesjö, B., Portela-Gomes, G. M., Rorsman, F., Gerdin, E., Lööf, L., Grimelius, L., … Ekwall, O. (2008). Immunoreactivity against goblet cells in patients with inflammatory bowel disease. *Inflammatory Bowel Diseases*, *14*(5), 652–661. https://doi.org/10.1002/ibd.20370

Arsenescu, R., Bruno, M. E. C., Rogier, E. W., Stefka, a T., McMahan, a E., Wright, T. B., … Kaetzel, C. S. (2008). Signature biomarkers in Crohn's disease: toward a molecular classification. *Mucosal Immunology*, *1*(5), 399–411. https://doi.org/10.1038/mi.2008.32

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature Reviews Immunology*, *8*(6), 411–420. https://doi.org/10.1038/nri2316

Atreya, R., Mudter, J., Finotto, S., Müllberg, J., Jostock, T., Wirtz, S., … Neurath, M. F. (2000). Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in Crohn disease and experimental colitis in vivo. *Nature Medicine*, *6*(5), 583–588. https://doi.org/10.1038/nm1110-1341

Baeuerle, P. A., & Baltimore, D. (1988). Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. *Cell*, *53*(2), 211–217. https://doi.org/10.1016/0092-8674(88)90382-0

Balzola, F., Bernstein, C., Ho, G. T., & Lees, C. (2011). Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum: Commentary. *Inflammatory Bowel Disease Monitor*. https://doi.org/10.1073/pnas.1003363107

Balzola, F., Cullen, G., Ho, G. T., Russell, R. K., & Wehkamp, J. (2012). IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4+ Th17 cells. *Inflammatory Bowel Disease Monitor*. https://doi.org/10.1084/jem.20111453

Bank, S., Andersen, P. S., Burisch, J., Pedersen, N., Roug, S., Galsgaard, J., … Andersen, V. (2014). Associations between functional polymorphisms in the NF kappa B signaling pathway and response to anti-TNF treatment in Danish patients with inflammatory bowel disease. *PHARMACOGENOMICS JOURNAL*, *14*(6), 526–534. https://doi.org/10.1038/tpj.2014.19

Berger, S. B., Harris, P., Nagilla, R., Kasparcova, V., Hoffman, S., Swift, B., … Gough, P. J. (2015). Characterization of GSK′963: a structurally distinct, potent and selective inhibitor of RIP1 kinase. *Cell Death Discovery*, *1*, 15009. https://doi.org/10.1038/cddiscovery.2015.9

Berghe, T. Vanden, Linkermann, A., Jouan-Lanhouet, S., Walczak, H., & Vandenabeele, P. (2014). Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nature Reviews Molecular Cell Biology*, *15*(2), 135–147. https://doi.org/10.1038/nrm3737

Bertrand, M. J. M., Milutinovic, S., Dickson, K. M., Ho, W. C., Boudreault, A., Durkin, J., … Barker, P. A. (2008). cIAP1 and cIAP2 Facilitate Cancer Cell Survival by Functioning as E3 Ligases that Promote RIP1 Ubiquitination. *Molecular Cell*, *30*(6), 689–700. https://doi.org/10.1016/j.molcel.2008.05.014

Biancheri, P., Brezski, R. J., Di Sabatino, A., Greenplate, A. R., Soring, K. L., Corazza, G. R., … MacDonald, T. T. (2015). Proteolytic Cleavage and Loss of Function of Biologic Agents That Neutralize Tumor Necrosis Factor in the Mucosa of Patients With Inflammatory Bowel Disease. *Gastroenterology*, *149*(6), 1564–1574.e3. https://doi.org/10.1053/j.gastro.2015.07.002

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., … Cerretti, D. P. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature*, *385*(6618), 729–733. https://doi.org/10.1038/385729a0

Blair, H. A., & Deeks, E. D. (2016). Infliximab Biosimilar (CT-P13; Infliximab-dyyb): A Review in Autoimmune Inflammatory Diseases. *BioDrugs*, *30*(5), 469–480. https://doi.org/10.1007/s40259-016-0193-2

Blander, J. M. (2016). Death in the intestinal epithelium-basic biology and implications for inflammatory bowel disease. *The FEBS Journal*, *283*(14), 2720–30. https://doi.org/10.1111/febs.13771

Bodmer, J.-L., Schneider, P., & Tschopp, J. (2002). The molecular architecture of the TNF superfamily. *Trends in Biochemical Sciences*, *27*(1), 19–26. https://doi.org/10.1016/S0968-0004(01)01995-8

Bonizzi, G., Bebien, M., Otero, D. C., Johnson-Vroom, K. E., Cao, Y., Vu, D., … Karin, M. (2004). Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *The EMBO Journal*, *23*(21), 4202–4210. https://doi.org/10.1038/sj.emboj.7600391

Bosanac, I., Wertz, I. E., Pan, B., Yu, C., Kusam, S., Lam, C., … Hymowitz, S. G. (2010). Ubiquitin Binding to A20 ZnF4 Is Required for Modulation of NF-κB Signaling. *Molecular Cell*, *40*(4), 548–557. https://doi.org/10.1016/j.molcel.2010.10.009

Brach, M. A., Hass, R., Sherman, M. L., Gunji, H., Weichselbaum, R., & Kufe, D. (1991). Ionizing radiation induces expression and binding activity of the nuclear factor kappa B. *The Journal of Clinical Investigation*, *88*(2), 691–5. https://doi.org/10.1172/JCI115354

Breese, E., Braegger, C. P., Corrigan, C. J., Walker-Smith, J. A., & MacDonald, T. T. (1993). Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. *Immunology*, *78*(1), 127–131.

Brinkman, B. M., Hildebrand, F., Kubica, M., Goosens, D., Del Favero, J., Declercq, W., … Vandenabeele, P. (2011). Caspase deficiency alters the murine gut microbiome. *Cell Death and Disease*, *2*(10), e220. https://doi.org/10.1038/cddis.2011.101

Britanova, L. V., Makeev, V. J., & Kuprash, D. V. (2008). In vitro selection of optimal RelB/p52 DNA-binding motifs. *Biochemical and Biophysical Research Communications*, *365*(3), 583–588. https://doi.org/10.1016/j.bbrc.2007.10.200

Browning, J. L., & French, L. E. (2002). Visualization of lymphotoxin-beta and lymphotoxin-beta receptor expression in mouse embryos. *Journal of Immunology (Baltimore, Md.: 1950)*, *168*(10), 5079–5087. https://doi.org/10.4049/jimmunol.168.10.5079

Browning, J. L., Sizing, I. D., Lawton, P., Bourdon, P. R., Rennert, P. D., Majeau, G. R., … Hochman, P. S. (1997). Characterization of lymphotoxin-alpha beta complexes on the surface of mouse lymphocytes. *Journal of Immunology (Baltimore, Md.: 1950)*, *159*(7), 3288–98.

Brummelkamp, T. R., Nijman, S. M. B., Dirac, A. M. G., & Bernards, R. (2003). Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-κB. *Nature*, *424*(6950), 797–801. https://doi.org/10.1038/nature01811

Buonocore, S., Ahern, P. P., Uhlig, H. H., Ivanov, I. I., Littman, D. R., Maloy, K. J., & Powrie, F. (2010). Innate lymphoid cells drive IL-23 dependent innate intestinal pathology. *Nature*, *464*(7293), 1371–1375. https://doi.org/10.1038/nature08949

Burton, P. R., Clayton, D. G., Cardon, L. R., Craddock, N., Deloukas, P., Duncanson, A., … Worthington, J. (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, *447*(7145), 661–678. https://doi.org/10.1038/nature05911

Cadwell, K., Liu, J. Y., Brown, S. L., Miyoshi, H., Loh, J., Lennerz, J. K., … Virgin IV, H. W. (2008). A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature*, *456*(7219), 259–263. https://doi.org/10.1038/nature07416

Cai, Z., Jitkaew, S., Zhao, J., Chiang, H.-C., Choksi, S., Liu, J., … Liu, Z.- G. (2014). Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nature Cell Biology*, *16*(1), 55–65. https://doi.org/10.1038/ncb2883

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(9), 3666–70. https://doi.org/10.1073/pnas.72.9.3666

Casini-Raggi, V., Kam, L., Chong, Y. J., Fiocchi, C., Pizarro, T. T., & Cominelli, F. (1995). Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. *Journal of Immunology (Baltimore, Md.: 1950)*, *154*(5), 2434–2440.

Catrysse, L., Farhang Ghahremani, M., Vereecke, L., Youssef, S. A., Mc Guire, C., Sze, M., … van Loo, G. (2016). A20 prevents chronic liver inflammation and cancer by protecting hepatocytes from death. *Cell Death and Disease*, *7*(6), e2250. https://doi.org/10.1038/cddis.2016.154

Chen, L. W., Egan, L., Li, Z. W., Greten, F. R., Kagnoff, M. F., & Karin, M. (2003). The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nat Med*, *9*(5), 575–581. https://doi.org/10.1038/nm849\rnm849 [pii]

Chew, J., Biswas, S., Shreeram, S., Humaidi, M., Wong, E. T., Dhillion, M. K., … Tergaonkar, V. (2009). WIP1 phosphatase is a negative regulator of NF-kappaB signalling. *Nature Cell Biology*, *11*(5), 659–66. https://doi.org/10.1038/ncb1873

Cho, Y. S., Challa, S., Moquin, D., Genga, R., Ray, T. D., Guildford, M., & Chan, F. K. M. (2009). Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. *Cell*, *137*(6), 1112–1123. https://doi.org/10.1016/j.cell.2009.05.037

Choung, R. S., Princen, F., Stockfisch, T. P., Torres, J., Maue, A. C., Porter, C. K., … Colombel, J. F. (2016). Serologic microbial associated markers can predict Crohn's disease behaviour years before disease diagnosis. *Alimentary Pharmacology & Therapeutics*, *43*(12), 1300–1310. https://doi.org/10.1111/apt.13641

Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., & Gusovsky, F. (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *Journal of Biological Chemistry*, *274*(16), 10689–10692. https://doi.org/10.1074/jbc.274.16.10689

Chu, Y., Vahl, J. C., Kumar, D., Heger, K., Bertossi, A., Wójtowicz, E., … Schmidt-Supprian, M. (2011). B cells lacking the tumor suppressor TNFAIP3/A20 display impaired differentiation and hyperactivation and cause inflammation and autoimmunity in aged mice. *Blood*, *117*(7), 2227–2236. https://doi.org/10.1182/blood-2010-09-306019

Ciehanover, A., Hod, Y., & Hershko, A. (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochemical and Biophysical Research Communications*, *81*(4), 1100–1105. https://doi.org/10.1016/0006-291X(78)91249-4

Claudio, E., Brown, K., Park, S., Wang, H., & Siebenlist, U. (2002). BAFF-induced NEMO-independent processing of NF-κB2 in maturing B cells. *Nature Immunology*, *3*(10), 958–965. https://doi.org/10.1038/ni842

Colombel, J.-F., Feagan, B. G., Sandborn, W. J., Van Assche, G., & Robinson, A. M. (2012). Therapeutic Drug Monitoring of Biologics for Inflammatory Bowel Disease. *Inflammatory Bowel Diseases*, *18*(2), 349–358. https://doi.org/10.1002/ibd.21831

Colombel, J. F., Sandborn, W. J., Reinisch, W., Mantzaris, G. J., Kornbluth, A., Rachmilewitz, D., … SONIC Study Group. (2010). Infliximab, Azathioprine, or Combination Therapy for Crohn's Disease. *New England Journal of Medicine*, *362*(15), 1383–1395. https://doi.org/10.1056/NEJMoa0904492

Colombel, J., Rutgeerts, P. J., Sandborn, W. J., Yang, M., Camez, A., Pollack, P. F., … Chao, J. (2014). Adalimumab Induces Deep Remission in Patients With Crohn's Disease. *Clinical Gastroenterology and Hepatology*, *12*(3), 414–422.e5. https://doi.org/10.1016/j.cgh.2013.06.019

Cominelli, F., Nast, C. C., Clark, B. D., Schindler, R., Llerena, R., Eysselein, V. E., … Dinarello, C. A. (1990). Interleukin 1 (IL-1) gene expression, synthesis, and effect of specific IL-1 receptor blockade in rabbit immune complex colitis. *Journal of Clinical Investigation*, *86*(3), 972–980. https://doi.org/10.1172/JCI114799

Coope, H. J., Atkinson, P. G. P., Huhse, B., Belich, M., Janzen, J., Holman, M. J., … Ley, S. C. (2002). CD40 regulates the processing of NF-??B2 p100 to p52. *EMBO Journal*, *21*(20), 5375–5385. https://doi.org/10.1093/emboj/cdf542

Coskun, M. (2014). Intestinal epithelium in inflammatory bowel disease. *Frontiers in Medicine*, *1*, 1–5. https://doi.org/https://doi.org/10.3389/fmed.2014.00024

Croft, M., Benedict, C. A., & Ware, C. F. (2013). Clinical targeting of the TNF and TNFR superfamilies. *Nature Reviews Drug Discovery*, *12*(2), 147–168. https://doi.org/10.1038/nrd3930

Croft, M., & Siegel, R. M. (2017). Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases. *Nature Reviews Rheumatology*, *13*(4), 217–233. https://doi.org/10.1038/nrrheum.2017.22

Cuff, C. A., Sacca, R., & Ruddle, N. H. (1999). Differential induction of adhesion molecule and chemokine expression by LTalpha3 and LTalphabeta in inflammation elucidates potential mechanisms of mesenteric and peripheral lymph node development. *Journal of Immunology (Baltimore, Md : 1950)*, *162*(10), 5965–5972. Retrieved from http://www.jimmunol.org/content/162/10/5965.long%5Cnpapers://99cf836b-0208-4bf7-a35c-5506d5268000/Paper/p22214

Cummings, R. J., Barbet, G., Bongers, G., Hartmann, B. M., Gettler, K., Muniz, L., … Blander, J. M. (2016). Different tissue phagocytes sample apoptotic cells to direct distinct homeostasis programs. *Nature*, *539*(7630), 565–569. https://doi.org/10.1038/nature20138

Cunliffe, R. N., Rose, F. R., Keyte, J., Abberley, L., Chan, W. C., & Mahida, Y. R. (2001). Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut*, *48*(2), 176–85. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11156637

Czabotar, P. E., Lessene, G., Strasser, A., & Adams, J. M. (2013). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nature Reviews Molecular Cell Biology*, *15*(1), 49–63. https://doi.org/10.1038/nrm3722

Darfeuille-Michaud, A., Boudeau, J., Bulois, P., Neut, C., Glasser, A. L., Barnich, N., … Colombel, J. F. (2004). High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology*, *127*(2), 412–421. https://doi.org/10.1053/j.gastro.2004.04.061

Davé, S. H., Tilstra, J. S., Matsuoka, K., Li, F., Karrasch, T., Uno, J. K., … Plevy, S. E. (2007). Amelioration of chronic murine colitis by peptide-mediated transduction of the IkappaB kinase inhibitor NEMO binding domain peptide. *Journal of Immunology (Baltimore, Md. : 1950)*, *179*(11), 7852–7859. https://doi.org/179/11/7852 [pii]

De, A., Dainichi, T., Rathinam, C. V., & Ghosh, S. (2014). The deubiquitinase activity of A20 is dispensable for NF-kappaB signaling. https://doi.org/10.15252/embr

de Almagro, M. C., Goncharov, T., Izrael-Tomasevic, A., Duttler, S., Kist, M., Varfolomeev, E., … Vucic, D. (2016). Coordinated ubiquitination and phosphorylation of RIP1 regulates necroptotic cell death. *Cell Death and Differentiation*, *24*(1), 1–12. https://doi.org/10.1038/cdd.2016.78

de Almagro, M. C., Goncharov, T., Newton, K., & Vucic, D. (2015). Cellular IAP proteins and LUBAC differentially regulate necrosome-associated RIP1 ubiquitination. *Cell Death and Disease*, *6*(6), e1800. https://doi.org/10.1038/cddis.2015.158

de Souza, H. S. P., & Fiocchi, C. (2015). Immunopathogenesis of IBD: current state of the art. *Nature Reviews Gastroenterology & Hepatology*, *13*(1), 13–27. https://doi.org/10.1038/nrgastro.2015.186

Debard, N., Sierro, F., Browning, J., & Kraehenbuhl, J. (2001). Effect of mature lymphocytes and lymphotoxin on the development of the follicle-associated epithelium and M cells in mouse Peyer's patches. *Gastroenterology*, *120*(5), 1173–1182. https://doi.org/10.1053/gast.2001.22476

Debard, N., Sierro, F., & Kraehenbuhl, J.-P. (1999). Development of Peyer's patches, follicle-associated epithelium and M cell: Lessons from immunodeficient and knockout mice. *Seminars in Immunology*, *11*(3), 183–191. https://doi.org/10.1006/smim.1999.0174

Deeks, E. D. (2016). Certolizumab Pegol: A Review in Inflammatory Autoimmune Diseases. *BioDrugs*, *30*(6), 607–617. https://doi.org/10.1007/s40259-016-0197-y

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I. L., Korkina, O., Teng, X., … Yuan, J. (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nature Chemical Biology*, *4*(5), 313–321. https://doi.org/10.1038/nchembio.83

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., … Yuan, J. (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nature Chemical Biology*, *1*(2), 112–119. https://doi.org/10.1038/nchembio711

Dejardin, E., Droin, N. M., Delhase, M., Haas, E., Cao, Y., Makris, C., … Green, D. R. (2002). The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity*, *17*(4), 525–35. https://doi.org/10.1016/S1074-

7613(02)00423-5

Di Sabatino, A., Ciccocioppo, R., Luinetti, O., Ricevuti, L., Morera, R., Cifone, M. G., … Corazza, G. R. (2003). Increased Enterocyte Apoptosis in Inflamed Areas of Crohn's Disease. *Diseases of the Colon and Rectum*, *46*(11), 1498–1507. https://doi.org/10.1007/s10350-004-6802-z

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., & Karin, M. (1997). A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature*, *388*(6642), 548–554. https://doi.org/10.1038/41493

Dionne, S., Hiscott, J., D'Agata, I., Duhaime, A., & Seidman, E. G. (1997). Quantitative PCR analysis of TNF-alpha and IL-1 beta mRNA levels in pediatric IBD mucosal biopsies. *Digestive Diseases and Sciences*, *42*(7), 1557–66. https://doi.org/10.1023/A:1018895500721

Dondelinger, Y., Aguileta, M. A., Goossens, V., Dubuisson, C., Grootjans, S., Dejardin, E., … Bertrand, M. J. M. (2013). RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition. *Cell Death and Differentiation*, *20*(10), 1381–1392. https://doi.org/10.1038/cdd.2013.94

Dondelinger, Y., Declercq, W., Montessuit, S., Roelandt, R., Goncalves, A., Bruggeman, I., … Vandenabeele, P. (2014). MLKL Compromises Plasma Membrane Integrity by Binding to Phosphatidylinositol Phosphates. *Cell Reports*, *7*(4), 971–981. https://doi.org/10.1016/j.celrep.2014.04.026

Dondelinger, Y., Jouan-Lanhouet, S., Divert, T., Theatre, E., Bertin, J., Gough, P. J. J., … Bertrand, M. J. M. J. M. (2015). NF-κB-Independent Role of IKKα/IKKβ in Preventing RIPK1 Kinase-Dependent Apoptotic and Necroptotic Cell Death during TNF Signaling. *Molecular Cell*, *60*(1), 63–76. https://doi.org/10.1016/j.molcel.2015.07.032

Donepudi, M., Sweeney, A. Mac, Briand, C., & Grütter, M. G. (2003). Insights into the regulatory mechanism for caspase-8 activation. *Molecular Cell*, *11*(2), 543–549. https://doi.org/10.1016/S1097-2765(03)00059-5

Dourmashiin, R. R., Davies, H., Wells, C., Shah, D., Price, A., O'Morain, C., & Levi, J. (1983). Epithelial patchy necrosis in Crohn's disease. *Human Pathology*, *14*(7), 643–648. https://doi.org/10.1016/S0046-8177(83)80207-X

Draber, P., Kupka, S., Reichert, M., Draberova, H., Lafont, E., de Miguel, D., … Walczak, H. (2015). LUBAC-Recruited CYLD and A20 Regulate Gene Activation and Cell Death by Exerting Opposing Effects on Linear Ubiquitin in Signaling Complexes. *Cell Reports*, *13*(10), 2258–2272. https://doi.org/10.1016/j.celrep.2015.11.009

Dubé, P. E., Punit, S., & Polk, D. B. (2015). Redeeming an old foe: protective as well as pathophysiological roles for tumor necrosis factor in inflammatory bowel disease. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *308*(3), G161–G170. https://doi.org/10.1152/ajpgi.00142.2014

Duerr, R. H., Taylor, K. D., Brant, S. R., Rioux, J. D., Silverberg, M. S., Daly, M. J., … Cho, J. H. (2006). A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science (New York, N.Y.)*, *314*(5804), 1461–3. https://doi.org/10.1126/science.1135245

Durand, J. K., & Baldwin, A. S. (2017). Targeting IKK and NF-κB for Therapy. In *Advances in Protein Chemistry and Structural Biology* (Vol. 107, pp. 77–115). https://doi.org/10.1016/bs.apcsb.2016.11.006

Durant, L., Watford, W. T., Ramos, H. L., Laurence, A., Vahedi, G., Wei, L., … O'Shea, J. J. (2010). Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity*, *32*(5), 605–615. https://doi.org/10.1016/j.immuni.2010.05.003

Eckmann, L., Nebelsiek, T., Fingerle, A. A., Dann, S. M., Mages, J., Lang, R., … Greten, F. R. (2008). Opposing functions of IKKbeta during acute and chronic intestinal inflammation. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(39), 15058–63. https://doi.org/10.1073/pnas.0808216105

Egan, L. J., Eckmann, L., Greten, F. R., Chae, S., Li, Z.-W., Myhre, G. M., … Kagnoff, M. F. (2004). IkappaB-kinasebeta-dependent NF-kappaB activation provides radioprotection to the intestinal epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(8), 2452–7. https://doi.org/10.1073/pnas.0306734101

Eissner, G., Kolch, W., & Scheurich, P. (2004). Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. *Cytokine & Growth Factor Reviews*, *15*(5), 353–366. https://doi.org/10.1016/j.cytogfr.2004.03.011

Eken, A., & Oukka, M. (2016). Interleukin 23 in IBD Pathogenesis. In *New Insights into Inflammatory Bowel Disease*. InTech. https://doi.org/10.5772/64882

Elliott, P. R., Leske, D., Hrdinka, M., Bagola, K., Fiil, B. K., McLaughlin, S. H., … Gyrd-Hansen, M. (2016). SPATA2 Links CYLD to LUBAC, Activates CYLD, and Controls LUBAC Signaling. *Molecular Cell*, *63*(6), 990–1005. https://doi.org/10.1016/j.molcel.2016.08.001

Emery, P., Keystone, E., Tony, H. P., Cantagrel, A., van Vollenhoven, R., Sanchez, A., … Kremer, J. (2008). IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial. *Annals of the Rheumatic Diseases*, *67*(11), 1516–1523. https://doi.org/10.1136/ard.2008.092932

Emmerich, C. H., Ordureau, A., Strickson, S., Arthur, J. S. C., Pedrioli, P. G. A., Komander, D., & Cohen, P. (2013). Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proceedings of the National Academy of Sciences*, *110*(38), 15247–15252. https://doi.org/10.1073/pnas.1314715110

Evans, P. C., Ovaa, H., Hamon, M., Kilshaw, P. J., Hamm, S., Bauer, S., … Smith, T. S. (2004). Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity. *The Biochemical Journal*, *378*(Pt 3), 727–734. https://doi.org/10.1042/BJ20031377

Everhart, J. E., & Ruhl, C. E. (2009). Burden of Digestive Diseases in the United States Part I: Overall and Upper Gastrointestinal Diseases. *Gastroenterology*, *136*(2), 376–386. https://doi.org/10.1053/j.gastro.2008.12.015

Fahlgren, A., Hammarstrom, S., Danielsson, A., & Hammarstrom, M.-L. (2004). beta-Defensin-3 and -4 in intestinal epithelial cells display increased mRNA expression in ulcerative colitis. *Clinical and Experimental Immunology*, *137*(2), 379–85. https://doi.org/10.1111/j.1365-2249.2004.02543.x

Fahlgren, A., Hammarström, S., Danielsson, A., & Hammarström, M.-L. (2003). Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clinical and Experimental Immunology*, *131*(1), 90–101. https://doi.org/10.1046/j.1365-2249.2003.02035.x

Farré, D., Roset, R., Huerta, M., Adsuara, J. E., Roselló, L., Albà, M. M., & Messeguer, X. (2003). Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. *Nucleic Acids Research*, *31*(13), 3651–3653. https://doi.org/10.1093/nar/gkg605

Feagan, B. G., Sandborn, W. J., Gasink, C., Jacobstein, D., Lang, Y., Friedman, J. R., … UNITI–IM-UNITI Study Group. (2016). Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease. *New England Journal of Medicine*, *375*(20), 1946–1960. https://doi.org/10.1056/NEJMoa1602773

Feltham, R., & Silke, J. (2017). The small molecule that packs a punch: ubiquitin-mediated regulation of RIPK1/FADD/caspase-8 complexes. *Cell Death and Differentiation*, *24*(7), 1196–1204. https://doi.org/10.1038/cdd.2017.67

Feng, S., Yang, Y., Mei, Y., Ma, L., Zhu, D. e., Hoti, N., … Wu, M. (2007). Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. *Cellular Signalling*, *19*(10), 2056–2067. https://doi.org/10.1016/j.cellsig.2007.05.016

Ferran, C. (n.d.). *The multiple therapeutic targets of A20*. Retrieved from https://books.google.es/books?id=61ejBAAAQBAJ&pg=PA176&lpg=PA176&dq=tnfaip3+a20+susceptibility+ibd+cd+uc&source=bl&ots=MiZjk95xFS&sig=6pfVHNHML3XLcgiNowbNXpZfKSk&hl=es&sa=X&ved=0ahUKEwj_zZ6VpLfWAhXHBZoKHU6_BWEQ6AEIMTAB#v=onepage&q=tnfaip3 a20 susceptibility ibd cd uc&f=false

Ferreira, J. (1990). Effect of butylated hydroxyanisole on electron transport in rat liver mitochondria. *Biochemical Pharmacology*, *40*(4), 677–684. https://doi.org/10.1016/0006-2952(90)90301-Z

Festjens, N., Kalai, M., Smet, J., Meeus, A., Van Coster, R., Saelens, X., & Vandenabeele, P. (2006). Butylated hydroxyanisole is more than a reactive oxygen species scavenger. *Cell Death and Differentiation*, *13*(1), 166–169. https://doi.org/10.1038/sj.cdd.4401746

Fiil, B. K., Damgaard, R. B., Wagner, S. A., Keusekotten, K., Fritsch, M., Bekker-Jensen, S., … Gyrd-Hansen, M. (2013). OTULIN Restricts Met1-Linked Ubiquitination to Control Innate Immune Signaling. *Molecular Cell*, *50*(6), 818–830. https://doi.org/10.1016/j.molcel.2013.06.004

Fouser, L. A., Wright, J. F., Dunussi-Joannopoulos, K., & Collins, M. (2008). Th17 cytokines and their emerging roles in inflammation and autoimmunity. *Immunological Reviews*. https://doi.org/10.1111/j.1600-065X.2008.00712.x

Franke, A., Hampe, J., Rosenstiel, P., Becker, C., Wagner, F., Häsler, R., … Schreiber, S. (2007). Systematic association mapping identifies NELL1 as a novel IBD disease gene. *PLoS ONE*, *2*(8). https://doi.org/10.1371/journal.pone.0000691

Franke, A., McGovern, D. P. B., Barrett, J. C., Wang, K., Radford-Smith, G. L., Ahmad, T., … Parkes, M. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nature Genetics*, *42*(12), 1118–1125. https://doi.org/10.1038/ng.717

Fujino, S., Andoh, A., Bamba, S., Ogawa, A., Hata, K., Araki, Y., … Fujiyama, Y. (2003). Increased expression of interleukin 17 in inflammatory bowel disease. *Gut*, *52*(1), 65–70. https://doi.org/10.1136/gut.52.1.65

Fusco, A. J., Huang, D.-B., Miller, D., Wang, V. Y.-F., Vu, D., & Ghosh, G. (2009). NF-κB p52:RelB heterodimer recognizes two classes of κB sites with two distinct modes. *EMBO Reports*, *10*(2), 152–159. https://doi.org/10.1038/embor.2008.227

Fuss, I. J., Heller, F., Boirivant, M., Leon, F., Yoshida, M., Fichtner-Feigl, S., … Strober, W. (2004). Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *Journal of Clinical Investigation*, *113*(10), 1490–1497. https://doi.org/10.1172/JCI200419836

Fuss, I. J., Neurath, M., Boirivant, M., Klein, J. S., de la Motte, C., Strong, S. A., … Strober, W. (1996). Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *Journal of Immunology (Baltimore, Md.: 1950)*, *157*(3), 1261–70. https://doi.org/10.4049/jimmunol.1100043

Gaffen, S. L., Jain, R., Garg, A. V., & Cua, D. J. (2014). The IL-23–IL-17 immune axis: from mechanisms to therapeutic testing. *Nature Reviews Immunology*, *14*(9), 585–600. https://doi.org/10.1038/nri3707

García-Sáez, A. J. (2012). The secrets of the Bcl-2 family. *Cell Death and Differentiation*, *19*(11), 1733–1740. https://doi.org/10.1038/cdd.2012.105

Gatti, M., Pinato, S., Maiolica, A., Rocchio, F., Prato, M., Aebersold, R., & Penengo, L. (2015). RNF168 promotes noncanonical K27ubiquitination to signal DNA damage. *Cell Reports*, *10*(2), 226–238. https://doi.org/10.1016/j.celrep.2014.12.021

Gavrieli, Y., Sherman, Y., & Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *Journal of Cell Biology*, *119*(3), 493–501. https://doi.org/10.1083/jcb.119.3.493

Genser, D., Kang, M. H., Vogelsang, H., & Elmadfa, I. (1999). Status of lipidsoluble antioxidants and TRAP in patients with Crohn's disease and healthy controls. *European Journal of Clinical Nutrition*, *53*(9), 675–9. https://doi.org/10.1038/sj.ejcn.1600764

Gerlach, B., Cordier, S. M., Schmukle, A. C., Emmerich, C. H., Rieser, E., Haas, T. L., … Walczak, H. (2011). Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature*, *471*. https://doi.org/10.1038/nature09816

Gersemann, M., Becker, S., Kübler, I., Koslowski, M., Wang, G., Herrlinger, K. R., … Stange, E. F. (2009). Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation*, *77*(1), 84–94. https://doi.org/10.1016/j.diff.2008.09.008

Ghosh, Van Duyne, G., Ghosh, S., & Sigler, P. B. (1995). Structure of NF-kB p50 homodimer bound to akB site. *Nature*, *378*, 287–292. https://doi.org/065/0

Gil, J., Alcamí, J., & Esteban, M. (2000). Activation of NF-κB by the dsRNA-dependent protein kinase, PKR involves the IκB kinase complex. *Oncogene*, *19*(11), 1369–1378. https://doi.org/10.1038/sj.onc.1203448

Gillen, C. D., Walmsley, R. S., Prior, P., Andrews, H. a, & Allan, R. N. (1994). Ulcerative colitis and Crohn's disease: a comparison of the colorectal cancer risk in extensive colitis. *Gut*, *35*, 1590–1592. https://doi.org/10.1136/gut.35.11.1590

Golovkina, T. V, Shlomchik, M., Hannum, L., & Chervonsky, a. (1999). Organogenic role of B lymphocytes in mucosal immunity. *Science (New York, N.Y.)*, *286*(5446), 1965–1968. https://doi.org/10.1126/science.286.5446.1965

Gommerman, J. L., & Browning, J. L. (2003). Lymphotoxin/LIGHT, lymphoid microenvironments and autoimmune disease. *Nature Reviews Immunology*, *3*(8), 642–655. https://doi.org/10.1038/nri1151

Gout, T., Östör, A. J. K., & Nisar, M. K. (2011). Lower gastrointestinal perforation in rheumatoid arthritis patients treated with conventional DMARDs or tocilizumab: A systematic literature review. *Clinical Rheumatology*, *30*(11), 1471–1474. https://doi.org/10.1007/s10067-011-1827-x

Grell, M., Douni, E., Wajant, H., Löhden, M., Clauss, M., Maxeiner, B., … Scheurich, P. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell*, *83*(5), 793–802. https://doi.org/10.1016/0092-8674(95)90192-2

Greten, F. R., Eckmann, L., Greten, T. F., Park, J. M., Li, Z. W., Egan, L. J., … Karin, M. (2004). IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell*, *118*(3), 285–296. https://doi.org/10.1016/j.cell.2004.07.013

Griseri, T., Arnold, I. C., Pearson, C., Krausgruber, T., Schiering, C., Franchini, F., … Powrie, F. (2015). Granulocyte Macrophage Colony-Stimulating Factor-Activated Eosinophils Promote Interleukin-23 Driven Chronic Colitis. *Immunity*, *43*(1), 187–200. https://doi.org/10.1016/j.immuni.2015.07.008

Griseri, T., McKenzie, B. S., Schiering, C., & Powrie, F. (2012). Dysregulated Hematopoietic Stem and Progenitor Cell Activity Promotes Interleukin-23-Driven Chronic Intestinal Inflammation. *Immunity*, *37*(6), 1116–1129. https://doi.org/10.1016/j.immuni.2012.08.025

Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G.-Y., Vallabhapurapu, S., … Karin, M. (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell*, *15*(2), 103–13. https://doi.org/10.1016/j.ccr.2009.01.001

Guma, M., Stepniak, D., Shaked, H., Spehlmann, M. E., Shenouda, S., Cheroutre, H., … Karin, M. (2011). Constitutive intestinal NF-κB does not trigger destructive inflammation unless accompanied by MAPK activation. *The Journal of Experimental Medicine*, *208*(9), 1889–900. https://doi.org/10.1084/jem.20110242

Günther, C., Martini, E., Wittkopf, N., Amann, K., Weigmann, B., Neumann, H., … Becker, C. (2011a). Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. *Nature*, *477*(7364), 335–339. https://doi.org/10.1038/nature10400

Günther, C., Martini, E., Wittkopf, N., Amann, K., Weigmann, B., Neumann, H., … Becker, C. (2011b). Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. *Nature*, *477*(7364), 335–339. https://doi.org/10.1038/nature10400

Haas, T. L., Emmerich, C. H., Gerlach, B., Schmukle, A. C., Cordier, S. M., Rieser, E., … Walczak, H. (2009). Recruitment of the Linear Ubiquitin Chain Assembly Complex Stabilizes the TNF-R1 Signaling Complex and Is Required for TNF-Mediated Gene Induction. *Molecular Cell*, *36*(5), 831–844. https://doi.org/10.1016/j.molcel.2009.10.013

Hagiwara, C., Tanaka, M., & Kudo, H. (2002). Increase in colorectal epithelial apoptotic cells in patients with ulcerative colitis ultimately requiring surgery. *Journal of Gastroenterology and Hepatology (Australia)*, *17*(7), 758–764. https://doi.org/10.1046/j.1440-1746.2002.02791.x

Hall, P. a, Coates, P. J., Ansari, B., & Hopwood, D. (1994). Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *Journal of Cell Science*, *107 (Pt 1*, 3569–3577. https://doi.org/10.1002/path.1711670106

Hamada, H., Hiroi, T., Nishiyama, Y., Takahashi, H., Masunaga, Y., Hachimura, S., … Ishikawa, H. (2002). Identification of Multiple Isolated Lymphoid Follicles on the Antimesenteric Wall of the Mouse Small Intestine. *The Journal of Immunology*, *168*(1), 57–64. https://doi.org/10.4049/jimmunol.168.1.57

Han, K. J., Su, X., Xu, L. G., Bin, L. H., Zhang, J., & Shu, H. B. (2004). Mechanisms of the TRIF-induced Interferon-stimulated Response Element and NF-κB Activation and Apoptosis Pathways. *Journal of Biological Chemistry*, *279*(15), 15652–15661. https://doi.org/10.1074/jbc.M311629200

Harper, P. H., Lee, E. C., Kettlewell, M. G., Bennett, M. K., & Jewell, D. P. (1985). Role of the faecal stream in the maintenance of Crohn's colitis. *Gut*, *26*(3), 279–284. https://doi.org/10.1136/gut.26.3.279

Hayden, M. S., & Ghosh, S. (2004). Signaling to NF-kappaB. *Genes & Development*, *18*(18), 2195–224. https://doi.org/10.1101/gad.1228704

He, K.-L., & Ting, A. T. (2002). A20 inhibits tumor necrosis factor (TNF) alpha-induced apoptosis by disrupting recruitment of TRADD and RIP to the TNF receptor 1 complex in Jurkat T cells. *Molecular and Cellular Biology*, *22*(17), 6034–45. https://doi.org/10.1128/MCB.22.17.6034–6045.2002

He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., & Wang, X. (2009). Receptor Interacting Protein Kinase-3 Determines Cellular Necrotic Response to TNF-α. *Cell*, *137*(6), 1100–1111. https://doi.org/10.1016/j.cell.2009.05.021

Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., … Schulzke, J. D. (2005). Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology*, *129*(2), 550–564. https://doi.org/10.1016/j.gastro.2005.05.002

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., & Rose, I. A. (1980). Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proceedings of the National Academy of Sciences*, *77*(4), 1783–1786. https://doi.org/10.1073/pnas.77.4.1783

Hitomi, J., Christofferson, D. E., Ng, A., Yao, J., Degterev, A., Xavier, R. J., & Yuan, J. (2008). Identification of a Molecular Signaling Network that Regulates a Cellular Necrotic Cell Death Pathway. *Cell*, *135*(7), 1311–1323. https://doi.org/10.1016/j.cell.2008.10.044

Hoffmann, A. (2002). The IκB-NF-κB Signaling Module: Temporal Control and Selective Gene Activation. *Science*, *298*(5596), 1241–1245. https://doi.org/10.1126/science.1071914

Hoffmann, A., Leung, T. H., & Baltimore, D. (2003). Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *The EMBO Journal*, *22*(20), 5530–5539. https://doi.org/10.1093/emboj/cdg534

Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, a, Valitutti, S., … Tschopp, J. (2000). Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nature Immunology*, *1*(6), 489–495. https://doi.org/10.1038/82732

Holm, T. L., Poulsen, S. S., Markholst, H., & Reedtz-Runge, S. (2012). Pharmacological Evaluation of the SCID T Cell Transfer Model of Colitis: As aModel of Crohn's Disease. *International Journal of Inflammation*, 1–11. Retrieved from http://10.0.4.131/2012/412178%5Cnhttp://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=87287147&site=ehost-live

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., … Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *Journal of Immunology (Baltimore, Md.: 1950)*, *162*(7), 3749–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10201887

Hough, R., Pratt, G., & Rechsteiner, M. (1986). Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. *Journal of Biological Chemistry*, *261*(5), 2400–2408.

Hsu, H., Xiong, J., & Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell*, *81*(4), 495–504. https://doi.org/10.1016/0092-8674(95)90070-5

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., … Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science (New York, N.Y.)*, *284*(April), 316–320. https://doi.org/10.1126/science.284.5412.316

Hue, S., Ahern, P., Buonocore, S., Kullberg, M. C., Cua, D. J., McKenzie, B. S., … Maloy, K. J. (2006). Interleukin-23 drives innate and T cell–mediated intestinal inflammation. *The Journal of Experimental Medicine*, *203*(11), 2473–2483. https://doi.org/10.1084/jem.20061099

Hueber, W., Sands, B. E., Lewitzky, S., Vandemeulebroecke, M., Reinisch, W., Higgins, P. D. R., … Secukinumab in Crohn's Disease Study Group. (2012). Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut*, *61*(12), 1693–700. https://doi.org/10.1136/gutjnl-2011-301668

Hugot, J. P., Chamaillard, M., Zouali, H., Lesage, S., Cézard, J. P., Belaiche, J., … Thomas, G. (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*, *411*(6837), 599–603. https://doi.org/10.1038/35079107

Ichim, G., & Tait, S. W. G. (2016). A fate worse than death: apoptosis as an oncogenic process. *Nature Reviews Cancer*, *16*(8), 539–548. https://doi.org/10.1038/nrc.2016.58

Ikeda, F., Deribe, Y. L., Skånland, S. S., Stieglitz, B., Grabbe, C., Franz-Wachtel, M., … Dikic, I. (2011a). SHARPIN forms a linear ubiquitin ligase complex regulating NF-kB activity and apoptosis. https://doi.org/10.1038/nature09814

Ikeda, F., Deribe, Y. L., Skånland, S. S., Stieglitz, B., Grabbe, C., Franz-Wachtel, M., … Dikic, I. (2011b). SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. *Nature*, *471*(7340), 637–641. https://doi.org/10.1038/nature09814

Ito, H., Takazoe, M., Fukuda, Y., Hibi, T., Kusugami, K., Andoh, A., … Kishimoto, T. (2004). A Pilot Randomized Trial of a Human Anti-Interleukin-6 Receptor Monoclonal Antibody in Active Crohn's Disease. *Gastroenterology*, *126*(4), 989–996. https://doi.org/10.1053/j.gastro.2004.01.012

Iwamoto, M., Koji, T., Makiyama, K., Kobayashi, N., & Nakane, P. K. (1996). Apoptosis of crypt epithelial cells in ulcerative colitis. *Journal of Pathology*, *180*(2), 152–159. https://doi.org/10.1002/(SICI)1096-9896(199610)180:2<152::AID-PATH649>3.0.CO;2-Y

Izcue, A., Hue, S., Buonocore, S., Arancibia-Cárcamo, C. V., Ahern, P. P., Iwakura, Y., … Powrie, F. (2008). Interleukin-23 Restrains Regulatory T Cell Activity to Drive T Cell-Dependent Colitis. *Immunity*, *28*(4), 559–570. https://doi.org/10.1016/j.immuni.2008.02.019

Jaco, I., Annibaldi, A., Lalaoui, N., Wilson, R., Tenev, T., Laurien, L., … Meier, P. (2017). MK2 Phosphorylates RIPK1 to Prevent TNF-Induced Cell Death. *Molecular Cell*, *66*(5), 698–710.e5. https://doi.org/10.1016/j.molcel.2017.05.003

Janowitz, H. D., Croen, E. C., & Sachar, D. B. (1998). The role of the fecal stream in Crohn's disease: an historical and analytic review. *Inflammatory Bowel Diseases*, *4*(1), 29–39. https://doi.org/10.1002/ibd.3780040107

Jin, L., Williamson, A., Banerjee, S., Philipp, I., & Rape, M. (2008). Mechanism of Ubiquitin-Chain Formation by the Human Anaphase-Promoting Complex. *Cell*, *133*(4), 653–665. https://doi.org/10.1016/j.cell.2008.04.012

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V. C., Lill, J. R., … al., et. (2009). Cullin3-Based Polyubiquitination and p62-Dependent Aggregation of Caspase-8 Mediate Extrinsic Apoptosis Signaling. *Cell*, *137*(4), 721–735. https://doi.org/10.1016/j.cell.2009.03.015

Johnson, E. S., Ma, P. C. M., Ota, I. M., & Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. *Journal of Biological Chemistry*, *270*(29), 17442–17456. https://doi.org/10.1074/jbc.270.29.17442

Jungbeck, M., Daller, B., Federhofer, J., Wege, A. K., Wimmer, N., Männel, D. N., & Hehlgans, T. (2009). Neutralization of LIGHT ameliorates acute dextran sodium sulphate-induced intestinal inflammation. *Immunology*, *128*(3), 451–458. https://doi.org/10.1111/j.1365-2567.2009.03131.x

Kadivar, K., Ruchelli, E. D., Markowitz, J. E., Defelice, M. L., Strogatz, M. L., Kanzaria, M. M., … Brown, K. a. (2004). Intestinal interleukin-13 in pediatric inflammatory bowel disease patients. *Inflammatory Bowel Diseases*, *10*(5), 593–8. https://doi.org/10.1097/00054725-200409000-00014

Kai, Y., Takahashi, I., Ishikawa, H., Hiroi, T., Mizushima, T., Matsuda, C., … Kiyono, H. (2005). Colitis in mice lacking the common cytokine receptor gamma chain is mediated by IL-6-producing CD4+ T cells. *Gastroenterology*, *128*(4), 922–34. https://doi.org/10.1053/j.gastro.2005.01.013

Kaiser, W. J., & Offermann, M. K. (2005). Apoptosis Induced by the Toll-Like Receptor Adaptor TRIF Is Dependent on Its Receptor Interacting Protein Homotypic Interaction Motif. *The Journal of Immunology*, *174*(8), 4942–4952. https://doi.org/10.4049/jimmunol.174.8.4942

Kalliolias, G. D., & Ivashkiv, L. B. (2015). TNF biology, pathogenic mechanisms and emerging therapeutic strategies. https://doi.org/10.1038/nrrheum.2015.169

Kanayama, A., Seth, R. B., Sun, L., Ea, C. K., Hong, M., Shaito, A., … Chen, Z. J. (2004). TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Molecular Cell*, *15*(4), 535–548. https://doi.org/10.1016/j.molcel.2004.08.008

Karban, A. S., Okazaki, T., Panhuysen, C. I. M., Gallegos, T., Potter, J. J., Bailey-Wilson, J. E., … Brant, S. R. (2004). Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. *Human Molecular Genetics*. https://doi.org/10.1093/hmg/ddh008

Kaser, A., Zeissig, S., & Blumberg, R. S. (2010). Inflammatory Bowel Disease. *Annual Review of Immunology*, *28*(1), 573–621. https://doi.org/10.1146/annurev-immunol-030409-101225

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., & Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*, *11*(1), 115–122. https://doi.org/10.1016/S1074-7613(00)80086-2

Kawai, T., & Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*, *34*(5), 637–50. https://doi.org/10.1016/j.immuni.2011.05.006

Kawakami, H., Tomita, M., Matsuda, T., Ohta, T., Tanaka, Y., Fujii, M., … Mori, N. (2005). Transcriptional activation of survivin through the NF-κB pathway by human T-cell leukemia virus type I tax. *International Journal of Cancer*, *115*(6), 967–974. https://doi.org/10.1002/ijc.20954

Kayagaki, N., Yan, M., Seshasayee, D., Wang, H., Lee, W., French, D. M., … Dixit, V. M. (2002). BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-??B2. *Immunity*, *17*(4), 515–524. https://doi.org/10.1016/S1074-7613(02)00425-9

Kerneis, S. (1997). Conversion by Peyer's Patch Lymphocytes of Human Enterocytes into M Cells that Transport Bacteria. *Science*, *277*(5328), 949–952. https://doi.org/10.1126/science.277.5328.949

Keusekotten, K., Elliott, P. R., Glockner, L., Fiil, B. K., Damgaard, R. B., Kulathu, Y., … Komander, D. (2013). OTULIN antagonizes LUBAC signaling by specifically hydrolyzing met1-linked polyubiquitin. *Cell*, *153*(6). https://doi.org/10.1016/j.cell.2013.05.014

Khan, K. J., Ullman, T. A., Ford, A. C., Abreu, M. T., Abadir, A., Marshall, J. K., … Moayyedi, P. (2011). Antibiotic Therapy in Inflammatory Bowel Disease: A Systematic Review and Meta-Analysis. *The American Journal of Gastroenterology*, *106*(4), 661–673. https://doi.org/10.1038/ajg.2011.72

Khor, B., Gardet, A., & Xavier, R. J. (2011). Genetics and pathogenesis of inflammatory bowel disease. *Nature*, *474*(7351), 307–317. https://doi.org/10.1038/nature10209

Kiernan, R., Brès, V., Ng, R. W. M., Coudart, M. P., El Messaoudi, S., Sardet, C., … Benkirane, M. (2003). Post-activation turn-off of NF-κB-dependent transcription is regulated by acetylation of p65. *Journal of Biological Chemistry*, *278*(4), 2758–2766. https://doi.org/10.1074/jbc.M209572200

Kim, Y.-S., Morgan, M. J., Choksi, S., & Liu, Z. (2007). TNF-Induced Activation of the Nox1 NADPH Oxidase and Its Role in the Induction of Necrotic Cell Death. *Molecular Cell*, *26*(5), 675–687. https://doi.org/10.1016/j.molcel.2007.04.021

Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., … Iwai, K. (2006). A ubiquitin ligase complex assembles linear polyubiquitin chains. *The EMBO Journal*, *25*(20), 4877–4887. https://doi.org/10.1038/sj.emboj.7601360

Kobayashi, T., Okamoto, S., Hisamatsu, T., Kamada, N., Chinen, H., Saito, R., … Hibi, T. (2008). IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut*, *57*(12), 1682–1689. https://doi.org/10.1136/gut.2007.135053

Kojouharoff, G., Hans, W., Obermeier, F., Männel, D. N., Andus, T., Schölmerich, J., … Falk, W. (1997). Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clinical and Experimental Immunology*, *107*(2), 353–8. https://doi.org/10.1111/j.1365-2249.1997.291-ce1184.x

Kolodziej, L. E., Lodolce, J. P., Chang, J. E., Schneider, J. R., Grimm, W. A., Bartulis, S. J., … Boone, D. L. (2011). TNFAIP3 Maintains Intestinal Barrier Function and Supports Epithelial Cell Tight Junctions. *PLoS ONE*, *6*(10), e26352. https://doi.org/10.1371/journal.pone.0026352

Komander, D., & Barford, D. (2008). Structure of the A20 OTU domain and mechanistic insights into deubiquitination. *Biochemical Journal*, *409*(1), 77–85. https://doi.org/10.1042/BJ20071399

Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F., & Kollias, G. (1999). Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies. *Immunity*, *10*, 387–398. https://doi.org/10.1016/S1074-7613(00)80038-2

Korn, T., Bettelli, E., Oukka, M., & Kuchroo, V. K. (2009). IL-17 and Th17 Cells. *Annual Review of Immunology*, *27*(1), 485–517. https://doi.org/10.1146/annurev.immunol.021908.132710

Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israël, A., Wallach, D., & Courtois, G. (2003). The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. *Nature*, *424*(6950), 801–5. https://doi.org/10.1038/nature01802

Krause, P., Zahner, S. P., Kim, G., Shaikh, R. B., Steinberg, M. W., & Kronenberg, M. (2014). The tumor necrosis factor family member TNFSF14 (LIGHT) is required for resolution of intestinal inflammation in mice. *Gastroenterology*, *146*(7). https://doi.org/10.1053/j.gastro.2014.02.010

Kreuz, S., Siegmund, D., Scheurich, P., & Wajant, H. (2001). NF- B Inducers Upregulate cFLIP, a Cycloheximide-Sensitive Inhibitor of Death Receptor Signaling. *Molecular and Cellular Biology*, *21*(12), 3964–3973. https://doi.org/10.1128/mcb.21.12.3964-3973.2001

Kuhn, K. A., Manieri, N. A., Liu, T.-C., & Stappenbeck, T. S. (2014). IL-6 Stimulates Intestinal Epithelial Proliferation and Repair after Injury. *PLoS ONE*, *9*(12), e114195. https://doi.org/10.1371/journal.pone.0114195

Kullberg, M. C., Jankovic, D., Feng, C. G., Hue, S., Gorelick, P. L., McKenzie, B. S., … Sher, A. (2006). IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. *The Journal of Experimental Medicine*, *203*(11), 2485–94. https://doi.org/10.1084/jem.20061082

Kupka, S., De Miguel, D., Draber, P., Martino, L., Surinova, S., Rittinger, K., … Walczak, H. (2016). SPATA2-Mediated Binding of CYLD to HOIP Enables CYLD Recruitment to Signaling Complexes. *Cell Reports*, *16*(9), 2271–2280. https://doi.org/10.1016/j.celrep.2016.07.086

Lafont, E., Kantari-Mimoun, C., Draber, P., De Miguel, D., Hartwig, T., Reichert, M., … Walczak, H. (2017). The linear ubiquitin chain assembly complex regulates TRAIL-induced gene activation and cell death. *The EMBO Journal*, *36*(9), 1147–1166. https://doi.org/10.15252/embj.201695699

Lakhani, S. A. (2006). Caspases 3 and 7: Key Mediators of Mitochondrial Events of Apoptosis. *Science*, *311*(5762), 847–851. https://doi.org/10.1126/science.1115035

Lavrik, I., Golks, A., & Krammer, P. H. (2005). Death receptor signaling. *Journal of Cell Science*, *118*(Pt 2), 265–267. https://doi.org/10.1242/jcs.01610

Lee, E. G. (2000). Failure to Regulate TNF-Induced NF-kappa B and Cell Death Responses in A20-Deficient Mice. *Science*, *289*(5488), 2350–2354. https://doi.org/10.1126/science.289.5488.2350

Lee, J. S., Tato, C. M., Joyce-Shaikh, B., Gulan, F., Cayatte, C., Chen, Y., … Cua, D. J. (2015). Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability. *Immunity*, *43*(4), 727–738. https://doi.org/10.1016/j.immuni.2015.09.003

Lee, T. H., Shank, J., Cusson, N., & Kelliher, M. A. (2004). The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. *J Biol Chem*, *279*(32), 33185–33191. https://doi.org/10.1074/jbc.M404206200

Legarda-Addison, D., Hase, H., O'Donnell, M. A., & Ting, A. T. (2009). NEMO/IKKgamma regulates an early NF-kappaB-independent cell-death checkpoint during TNF signaling. *Cell Death and Differentiation*, *16*(9), 1279–88. https://doi.org/10.1038/cdd.2009.41

Leung, T. H., Hoffmann, A., & Baltimore, D. (2004). One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers. *Cell*, *118*(4), 453–64. https://doi.org/10.1016/j.cell.2004.08.007

Li, H.-Y., Liu, H., Wang, C.-H., Zhang, J.-Y., Man, J.-H., Gao, Y.-F., … Zhang, X.-M. (2008). Deactivation of the kinase IKK by CUEDC2 through recruitment of the phosphatase PP1. *Nature Immunology*, *9*(5), 533–541. https://doi.org/10.1038/ni.1600

Li, H., Zhu, H., Xu, C., & Yuan, J. (1998). Cleavage of BID by Caspase 8 Mediates the Mitochondrial Damage in the Fas Pathway of Apoptosis. *Cell*, *94*(4), 491–501. https://doi.org/10.1016/S0092-8674(00)81590-1

Li, J., McQuade, T., Siemer, A. B., Napetschnig, J., Moriwaki, K., Hsiao, Y. S., … Wu, H. (2012). The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell*, *150*(2), 339–350. https://doi.org/10.1016/j.cell.2012.06.019

Li, J., Yang, Z., Li, Y., Xia, J., Li, D., Li, H., … Zhang, Y. (2016). Cell apoptosis, autophagy and necroptosis in osteosarcoma treatment. *Oncotarget*, *7*(28), 44763–44778. https://doi.org/10.18632/oncotarget.8206

Li, S., Wang, L., Berman, M. A., Zhang, Y., & Dorf, M. E. (2006). RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-κB signaling. *Molecular Cell*, *24*(4), 497–509. https://doi.org/10.1016/j.molcel.2006.10.015

Liang, C., Zhang, M., & Sun, S. C. (2006). β-TrCP binding and processing of NF-κB2/p100 involve its phosphorylation at serines 866 and 870. *Cellular Signalling*, *18*(8), 1309–1317. https://doi.org/10.1016/j.cellsig.2005.10.011

Libioulle, C., Louis, E., Hansoul, S., Sandor, C., Farnir, F., Franchimont, D., … Georges, M. (2007). Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS Genetics*, *3*(4), 0538–0543. https://doi.org/10.1371/journal.pgen.0030058

Lin, S. C., Chung, J. Y., Lamothe, B., Rajashankar, K., Lu, M., Lo, Y. C., … Wu, H. (2008). Molecular Basis for the Unique Deubiquitinating Activity of the NF-κB Inhibitor A20. *Journal of Molecular Biology*, *376*(2), 526–540. https://doi.org/10.1016/j.jmb.2007.11.092

Lin, X., Mu, Y., Cunningham, E. T., Marcu, K. B., Geleziunas, R., & Greene, W. C. (1998). Molecular Determinants of NF-κB-Inducing Kinase Action. *Molecular and Cellular Biology*, *18*(10), 5899–5907. https://doi.org/10.1128/MCB.18.10.5899

Lin, Y., Choksi, S., Shen, H. M., Yang, Q. F., Hur, G. M., Kim, Y. S., … Liu, Z. G. (2004). Tumor Necrosis Factor-induced Nonapoptotic Cell Death Requires Receptor-interacting Protein-mediated Cellular Reactive Oxygen Species Accumulation. *Journal of Biological Chemistry*, *279*(11), 10822–10828. https://doi.org/10.1074/jbc.M313141200

Lin, Y., Devin, A., Rodriguez, Y., & Liu, Z. G. (1999). Cleavage of the death domain kinase RIP by Caspase-8 prompts TNF-induced apoptosis. *Genes and Development*, *13*(19), 2514–2526. https://doi.org/10.1101/gad.13.19.2514

Lindemans, C. A., Calafiore, M., Mertelsmann, A. M., O'Connor, M. H., Dudakov, J. A., Jenq, R. R., … Hanash, A. M. (2015). Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. *Nature*, *528*(7583), 560–564. https://doi.org/10.1038/nature16460

Liu, J., Han, C., Xie, B., Wu, Y., Liu, S., Chen, K., … Cao, X. (2014). Rhbdd3 controls autoimmunity by suppressing the production of IL-6 by dendritic cells via K27-linked ubiquitination of the regulator NEMO. *Nat Immunol*, *15*(7), 612–622. https://doi.org/10.1038/ni.2898

Liu, J. Z., van Sommeren, S., Huang, H., Ng, S. C., Alberts, R., Takahashi, A., … Weersma, R. K. (2015). Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nature Genetics*, *47*(9), 979–986. https://doi.org/10.1038/ng.3359

Liu, Z., Yadav, P. K., Xu, X., Su, J., Chen, C., Tang, M., … Wang, X. (2011). The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity. *Journal of Leukocyte Biology*, *89*(4), 597–606. https://doi.org/10.1189/jlb.0810456

Lu, T. T., Onizawa, M., Hammer, G. E., Turer, E. E., Yin, Q., Damko, E., … Ma, A. (2013). Dimerization and Ubiquitin Mediated Recruitment of A20, a Complex Deubiquitinating Enzyme. *Immunity*, *38*(5), 896–905. https://doi.org/10.1016/j.immuni.2013.03.008

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., & Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*, *94*(4), 481–490. https://doi.org/10.1016/S0092-8674(00)81589-5

Ma, A., & Malynn, B. A. (2012). A20: linking a complex regulator of ubiquitylation to immunity and human disease. *Nature Reviews Immunology*, *12*(11), 774–785. https://doi.org/10.1038/nri3313

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., … Vermeire, S. (2013). A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut*, *0*, 1–9. https://doi.org/10.1136/gutjnl-2013-304833

Macho-Fernandez, E., Koroleva, E. P., Spencer, C. M., Tighe, M., Torrado, E., Cooper, A. M., … Tumanov, A. V. (2015). Lymphotoxin beta receptor signaling limits mucosal damage through driving IL-23 production by epithelial cells. *Mucosal Immunology*, *8*(2), 403–413. https://doi.org/10.1038/mi.2014.78

Mackay, F., Browning, J. L., Lawton, P., Shah, S. A., Comiskey, M., Bhan, A. K., … Simpson, S. J. (1998). Both the lymphotoxin and tumor necrosis factor pathways are involved in experimental murine models of colitis. *Gastroenterology*, *115*(6), 1464–1475.

Maeda, M., Watanabe, N., Neda, H., Yamauchi, N., Okamoto, T., Sasaki, H., … Niitsu, Y. (1992). Serum Tumor Necrosis Factor Activity in Inflammatory Bowel Disease. *Immunopharmacology and Immunotoxicology*, *14*(3), 451–461. https://doi.org/10.3109/08923979209005404

Mahoney, D. J., Cheung, H. H., Mrad, R. L., Plenchette, S., Simard, C., Enwere, E., … Korneluk, R. G. (2008). Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(33), 11778–83. https://doi.org/10.1073/pnas.0711122105

Mandal, P., Berger, S. B., Pillay, S., Moriwaki, K., Huang, C., Guo, H., … Kaiser, W. J. (2014). RIP3 Induces Apoptosis Independent of Pronecrotic Kinase Activity. *Molecular Cell*, *56*(4), 481–495. https://doi.org/10.1016/j.molcel.2014.10.021

Manichanh, C., Borruel, N., Casellas, F., & Guarner, F. (2012). The gut microbiota in IBD. *Nature Reviews Gastroenterology & Hepatology*, *9*(10), 599–608. https://doi.org/10.1038/nrgastro.2012.152

Mannon, P. J., Fuss, I. J., Mayer, L., Elson, C. O., Sandborn, W. J., Present, D., … Anti-IL-12 Crohn's Disease Study Group. (2004). Anti–Interleukin-12 Antibody for Active Crohn's Disease. *New England Journal of Medicine*, *351*(20), 2069–2079. https://doi.org/10.1056/NEJMoa033402

Mannon, P. J., Fuss, I. J., Mayer, L., Elson, C. O., Sandborn, W. J., Present, D., … Strober, W. (2004). Anti–Interleukin-12 Antibody for Active Crohn's Disease. *New England Journal of Medicine*, *351*(20), 2069–2079. https://doi.org/10.1056/NEJMoa033402

Marini, M., Bamias, G., Rivera-Nieves, J., Moskaluk, C. A., Hoang, S. B., Ross, W. G., … Cominelli, F. (2003). TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 8366–71. https://doi.org/10.1073/pnas.1432897100

Matsuda, R., Koide, T., Tokoro, C., Yamamoto, T., Godai, T., Morohashi, T., … Umemura, S. (2009). Quantitive cytokine mRNA expression profiles in the colonic mucosa of patients with steroid Na??ve ulcerative colitis during active and quiescent disease. *Inflammatory Bowel Diseases*, *15*(3), 328–334. https://doi.org/10.1002/ibd.20759

Maxwell, J. R., Zhang, Y., Brown, W. A., Smith, C. L., Byrne, F. R., Fiorino, M., … Towne, J. E. (2015). Differential Roles for Interleukin-23 and Interleukin-17 in Intestinal Immunoregulation. *Immunity*, *43*(4), 739–750. https://doi.org/10.1016/j.immuni.2015.08.019

McCormick, D. a, Horton, L. W., & Mee, a S. (1990). Mucin depletion in inflammatory bowel disease. *Journal of Clinical Pathology*, *43*(2), 143–146. https://doi.org/10.1136/jcp.43.2.143

McQuade, T., Cho, Y., & Chan, F. K.-M. (2013). Positive and negative phosphorylation regulates RIP1- and RIP3-induced programmed necrosis. *Biochemical Journal*, *456*(3), 409–415. https://doi.org/10.1042/BJ20130860

Meerbrey, K. L., Hu, G., Kessler, J. D., Roarty, K., Li, M. Z., Fang, J. E., … Elledge, S. J. (2011). The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. *Proceedings of the National Academy of Sciences*, *108*(9), 3665–3670. https://doi.org/10.1073/pnas.1019736108

Messeguer, X., Escudero, R., Farré, D., Núñez, O., Martínez, J., & Albà, M. M. (2002). PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics (Oxford, England)*, *18*(2), 333–334. https://doi.org/10.1093/bioinformatics/18.2.333

Micheau, O., Thome, M., Schneider, P., Holler, N., Tschopp, J., Nicholson, D. W., … Grütter, M. G. (2002). The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *Journal of Biological Chemistry*, *277*(47), 45162–45171. https://doi.org/10.1074/jbc.M206882200

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., … Kaplan, G. G. (2012). Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology*, *142*(1). https://doi.org/10.1053/j.gastro.2011.10.001

Monteleone, G., Biancone, L., Marasco, R., Morrone, G., Marasco, O., Luzza, F., & Pallone, F. (1997). Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology*, *112*(4), 1169–1178. https://doi.org/10.1016/S0016-5085(97)70128-8

Moquin, D. M., McQuade, T., & Chan, F. K. M. (2013). CYLD Deubiquitinates RIP1 in the TNFalpha-Induced Necrosome to Facilitate Kinase Activation and Programmed Necrosis. *PLoS ONE*, *8*(10). https://doi.org/10.1371/journal.pone.0076841

Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Carter, H. L., Chen, W.-J., … Becherer, J. D. (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. *Nature*, *385*(6618), 733–736. https://doi.org/10.1038/385733a0

Mowat, A. M. (2003). Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Reviews. Immunology*, *3*(4), 331–41. https://doi.org/10.1038/nri1057

Mueller, C. (2002). Tumour necrosis factor in mouse models of chronic intestinal inflammation. *Immunology*. https://doi.org/10.1046/j.1365-2567.2002.01329.x

Müller, C. W., Rey, F. a, Sodeoka, M., Verdine, G. L., & Harrison, S. C. (1995). Structure of the NF-kappa B p50 homodimer bound to DNA. *Nature*, *373*, 311–317. https://doi.org/10.1038/373311a0

Murch, S. H., Braegger, C. P., Walker-Smith, J. a, & MacDonald, T. T. (1993). Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut*, *34*, 1705–1709. https://doi.org/10.1136/gut.34.12.1705

Murch, S. H., Lamkin, V. A., Savage, M. O., Walker-Smith, J. A., & MacDonald, T. T. (1991). Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut*, *32*(8), 913–7. https://doi.org/10.1136/gut.32.8.913

Murphy, J. M., Czabotar, P. E., Hildebrand, J. M., Lucet, I. S., Zhang, J. G., Alvarez-Diaz, S., … Alexander, W. S. (2013). The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity*, *39*(3), 443–453. https://doi.org/10.1016/j.immuni.2013.06.018

Murphy, S. F., Rhee, L., Grimm, W. A., Weber, C. R., Messer, J. S., Lodolce, J. P., … Boone, D. L. (2014). Intestinal epithelial expression of TNFAIP3 results in microbial invasion of the inner mucus layer and induces colitis in IL-10-deficient mice. *AJP: Gastrointestinal and Liver Physiology*, *307*(9), G871–G882. https://doi.org/10.1152/ajpgi.00020.2014

Musone, S. L., Taylor, K. E., Lu, T. T., Nititham, J., Ferreira, R. C., Ortmann, W., … Criswell, L. A. (2008). Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus. *Nature Genetics*, *40*(9), 1062–1064. https://doi.org/10.1038/ng.202

Nakagawa, Y., Nakajima, K., Moore, G., & Moldéus, P. (1994). On the mechanisms of 3-tert-butyl-4-hydroxyanisole- and its metabolites-induced cytotoxicities in isolated rat hepatocytes. *European Journal of Pharmacology*, *270*(4), 341–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7805783

Naser, S. A., Arce, M., Khaja, A., Fernandez, M., Naser, N., Elwasila, S., & Thanigachalam, S. (2012). Role of ATG16L, NOD2 and IL23R in Crohn's disease pathogenesis. *World Journal of Gastroenterology*. https://doi.org/10.3748/wjg.v18.i5.412

Nava, P., Koch, S., Laukoetter, M. G., Lee, W. Y., Kolegraff, K., Capaldo, C. T., … Nusrat, A. (2010). Interferon-gamma regulates intestinal epithelial homeostasis through converging beta-catenin signaling pathways. *Immunity*, *32*(3), 392–402. https://doi.org/10.1016/j.immuni.2010.03.001

Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., … Pasparakis, M. (2007). Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*, *446*(7135), 557–561. https://doi.org/10.1038/nature05698

Neurath, M. F. (2014). Cytokines in inflammatory bowel disease. *Nature Reviews Immunology*, *14*(5), 329–342. https://doi.org/10.1038/nri3661

Neurath, M. F. (2017). Current and emerging therapeutic targets for IBD. *Nature Reviews Gastroenterology & Hepatology*, *14*(5), 269–278. https://doi.org/10.1038/nrgastro.2016.208

Neurath, M. F., Finotto, S., & Glimcher, L. H. (2002). The role of Th1/Th2 polarization in mucosal immunity. *Nature Medicine*, *8*(6), 567–573. https://doi.org/10.1038/nm0602-567

Neurath, M. F., Fuss, I., Kelsall, B. L., Stüber, E., & Strober, W. (1995). Antibodies to interleukin 12 abrogate established experimental colitis in mice. *The Journal of Experimental Medicine*, *182*(5), 1281–90. https://doi.org/10.1084/jem.182.5.1281

Neurath, M. F., Fuss, I., Pasparakis, M., Alexopoulou, L., Haralambous, S., Meyer zum Büschenfelde, K. H., … Kollias, G. (1997). Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. *European Journal of Immunology*, *27*(7), 1743–1750. https://doi.org/10.1002/eji.1830270722

Neurath, M. F., Pettersson, S., Meyer zum Büschenfelde, K. H., & Strober, W. (1996). Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nature Medicine*, *2*(9), 998–1004. https://doi.org/10.1038/nm0996-998

Newton, K., Dugger, D. L., Maltzman, A., Greve, J. M., Hedehus, M., Martin-McNulty, B., … Vucic, D. (2016). RIPK3 deficiency or catalytically inactive RIPK1 provides greater benefit than MLKL deficiency in mouse models of inflammation and tissue injury. *Cell Death and Differentiation*, *23*(9), 1565–76. https://doi.org/10.1038/cdd.2016.46

Newton, K., Dugger, D. L., Wickliffe, K. E., Kapoor, N., de Almagro, M. C., Vucic, D., … Dixit, V. M. (2014a). Activity of Protein Kinase RIPK3 Determines Whether Cells Die by Necroptosis or Apoptosis. *Science*, *343*(6177), 1357–1360. https://doi.org/10.1126/science.1249361

Newton, K., Dugger, D. L., Wickliffe, K. E., Kapoor, N., de Almagro, M. C., Vucic, D., … Dixit, V. M. (2014b). Activity of Protein Kinase RIPK3 Determines Whether Cells Die by Necroptosis or Apoptosis. *Science*, *343*(6177), 1357–1360. https://doi.org/10.1126/science.1249361

Newton, K., Sun, X., & Dixit, V. M. (2004). Kinase RIP3 Is Dispensable for Normal NF- Bs, Signaling by the B-Cell and T-Cell Receptors, Tumor Necrosis Factor Receptor 1, and Toll-Like Receptors 2 and 4. *Molecular and Cellular Biology*, *24*(4), 1464–1469. https://doi.org/10.1128/mcb.24.4.1464-1469.2004

Ng, S. C., Benjamin, J. L., McCarthy, N. E., Hedin, C. R. H., Koutsoumpas, A., Plamondon, S., … Stagg, A. J. (2011). Relationship between human intestinal dendritic cells, gut microbiota, and disease activity in Crohn's disease. *Inflammatory Bowel Diseases*, *17*(10), 2027–2037. https://doi.org/10.1002/ibd.21590

Noguchi, M., Hiwatashi, N., Liu, Z., & Toyota, T. (1995). Enhanced interferon-gamma production and B7-2 expression in isolated intestinal mononuclear cells from patients with Crohn's disease. *Journal of Gastroenterology*, *30 Suppl 8*, 52–5. Retrieved from http://europepmc.org/abstract/med/8563891

Noti, M., Corazza, N., Mueller, C., Berger, B., & Brunner, T. (2010). TNF suppresses acute intestinal inflammation by inducing local glucocorticoid synthesis. *The Journal of Experimental Medicine*, *207*(5), 1057–1066. https://doi.org/10.1084/jem.20090849

Nuding, S., Fellermann, K., Wehkamp, J., & Stange, E. F. (2007). Reduced mucosal antimicrobial activity in Crohn's disease of the colon. *Gut*, *56*(9), 1240–1247. https://doi.org/10.1136/gut.2006.118646

O'Connor Jr, W., Kamanaka, M., Booth, C. J., Town, T., Nakae, S., Iwakura, Y., … Flavell, R. A. (2009). A protective function for interleukin 17A in T cell–mediated intestinal inflammation. *Nature Immunology*, *10*(6), 603–609. https://doi.org/10.1038/ni.1736

O'Donnell, M. A., Perez-Jimenez, E., Oberst, A., Ng, A., Massoumi, R., Xavier, R., … Ting, A. T. (2011). Caspase 8 inhibits programmed necrosis by processing CYLD. *Nature Cell Biology*, *13*(12), 1437–1442. https://doi.org/10.1038/ncb2362

O'Neil, D. a, Porter, E. M., Elewaut, D., Anderson, G. M., Eckmann, L., Ganz, T., & Kagnoff, M. F. (1999). Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *Journal of Immunology (Baltimore, Md.: 1950)*, *163*, 6718–6724. https://doi.org/ji_v163n12p6718 [pii]

Ofengeim, D., & Yuan, J. (2013). Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nature Reviews Molecular Cell Biology*, *14*(11), 727–736. https://doi.org/10.1038/nrm3683

Ogawa, A., Andoh, A., Araki, Y., Bamba, T., & Fujiyama, Y. (2004). Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clinical Immunology*, *110*(1), 55–62. https://doi.org/10.1016/j.clim.2003.09.013

Ogura, Y., Bonen, D. K., Inohara, N., Nicolae, D. L., Chen, F. F., Ramos, R., … Cho, J. H. (2001). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*, *411*(6837), 603–606. https://doi.org/10.1038/35079114

Ohtake, F., Saeki, Y., Ishido, S., Kanno, J., & Tanaka, K. (2016). The K48-K63 Branched Ubiquitin Chain Regulates NF-??B Signaling. *Molecular Cell*, *64*(2), 251–266. https://doi.org/10.1016/j.molcel.2016.09.014

Okubo, T., Yokoyama, Y., Kano, K., & Kano, I. (2004). Molecular Mechanism of Cell Death Induced by the Antioxidant tert-Butylhydroxyanisole in Human Monocytic Leukemia U937 Cells. *Biological & Pharmaceutical Bulletin*, *27*(3), 295–302. https://doi.org/10.1248/bpb.27.295

Onizawa, M., Oshima, S., Schulze-Topphoff, U., Oses-Prieto, J. A., Lu, T., Tavares, R., … Ma, A. (2015). The ubiquitin-modifying enzyme A20 restricts ubiquitination of the kinase RIPK3 and protects cells from necroptosis. *Nature Immunology*, *16*(6), 618–627. https://doi.org/10.1038/ni.3172

Ono, Y., Kanai, T., Sujino, T., Nemoto, Y., Kanai, Y., Mikami, Y., … Hibi, T. (2012). T-helper 17 and interleukin-17-producing lymphoid tissue inducer-like cells make different contributions to colitis in mice. *Gastroenterology*, *143*(5), 1288–1297. https://doi.org/10.1053/j.gastro.2012.07.108

Opipari, a W., Boguski, M. S., & Dixit, V. M. (1990). The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. *The Journal of Biological Chemistry*, *265*(25), 14705–14708. Retrieved from http://www.jbc.org/content/265/25/14705.long

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., … Kastelein, R. A. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity*, *13*(5), 715–725. https://doi.org/10.1016/S1074-7613(00)00070-4

Ordureau, A., Heo, J.-M., Duda, D. M., Paulo, J. A., Olszewski, J. L., Yanishevski, D., … Harper, J. W. (2015). Defining roles of PARKIN and ubiquitin phosphorylation by PINK1 in mitochondrial quality control using a ubiquitin replacement strategy. *Proceedings of the National Academy of Sciences*, *112*(21), 6637–6642. https://doi.org/10.1073/pnas.1506593112

Orozco, S., Yatim, N., Werner, M. R., Tran, H., Gunja, S. Y., Tait, S. W., … Oberst, A. (2014). RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death and Differentiation*, *21*(10), 1511–1521. https://doi.org/10.1038/cdd.2014.76

Osborn, L., Kunkel, S., & Nabel, G. J. (1989). Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(7), 2336–40. https://doi.org/10.1073/pnas.86.7.2336

Ota, N., Wong, K., Valdez, P. A., Zheng, Y., Crellin, N. K., Diehl, L., & Ouyang, W. (2011). IL-22 bridges the lymphotoxin pathway with the maintenance of colonic lymphoid structures during infection with Citrobacter rodentium. *Nature Immunology*, *12*(10), 941–948. https://doi.org/10.1038/ni.2089

Owczarek, D., Cibor, D., Głowacki, M. K., Cieśla, A., & Mach, P. (2012). TNF-α and soluble forms of TNF receptors 1 and 2 in the serum of patients with Crohn's disease and ulcerative colitis. *Polskie Archiwum Medycyny Wewnętrznej*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23160117

Palomino-Morales, R. J., Oliver, J., Gómez-García, M., López-Nevot, M. A., Rodrigo, L., Nieto, A., … Martín, J. (2009). Association of ATG16L1 and IRGM genes polymorphisms with inflammatory bowel disease: a meta-analysis approach. *Genes and Immunity*, *10*(4), 356–364. https://doi.org/10.1038/gene.2009.25

Panaccione, R., Ghosh, S., Middleton, S., Márquez, J. R., Scott, B. B., Flint, L., … Rutgeerts, P. (2014). Combination Therapy With Infliximab and Azathioprine Is Superior to Monotherapy With Either Agent in Ulcerative Colitis. *Gastroenterology*, *146*(2), 392–400.e3. https://doi.org/10.1053/j.gastro.2013.10.052

Parkes, M., Barrett, J. C., Prescott, N. J., Tremelling, M., Anderson, C. A., Fisher, S. A., … Mathew, C. G. (2007). Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nature Genetics*, *39*(7), 830–832. https://doi.org/10.1038/ng2061

Pasparakis, M., Alexopoulou, L., Episkopou, V., & Kollias, G. (1996). Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *The Journal of Experimental Medicine*, *184*(4), 1397–411. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8879212

Petersen, S. L., Wang, L., Yalcin-Chin, A., Li, L., Peyton, M., Minna, J., … Wang, X. (2007). Autocrine TNFα Signaling Renders Human Cancer Cells Susceptible to Smac-Mimetic-Induced Apoptosis. *Cancer Cell*, *12*(5), 445–456. https://doi.org/10.1016/j.ccr.2007.08.029

Pickert, G., Neufert, C., Leppkes, M., Zheng, Y., Wittkopf, N., Warntjen, M., … Becker, C. (2009). STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *The Journal of Experimental Medicine*, *206*(7), 1465–1472. https://doi.org/10.1084/jem.20082683

Pierik, M., Vermeire, S., Steen, K. V, Joossens, S., Claessens, G., Vlietinck, R., & Rutgeerts, P. (2004). Tumour necrosis factor-alpha receptor 1 and 2 polymorphisms in inflammatory bowel disease and their association with response to infliximab. *Alimentary Pharmacology & Therapeutics*, *20*(3), 303–310. https://doi.org/10.1111/j.1365-2036.2004.01946.x

Polykratis, A., Hermance, N., Zelic, M., Roderick, J., Kim, C., Van, T.-M., … Kelliher, M. A. (2014). Cutting Edge: RIPK1 Kinase Inactive Mice Are Viable and Protected from TNF-Induced Necroptosis In Vivo. *The Journal of Immunology*, *193*(4), 1539–1543. https://doi.org/10.4049/jimmunol.1400590

Pop, C., Fitzgerald, P., Green, D. R., & Salvesen, G. S. (2007). Role of proteolysis in caspase-8 activation and stabilization. *Biochemistry*, *46*(14), 4398–4407. https://doi.org/10.1021/bi602623b

Powrie, F., Leach, M. W., Mauze, S., Menon, S., Barcomb Caddle, L., & Coffman, R. L. (1994). Inhibition of Thl responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity*, *1*(7), 553–562. https://doi.org/10.1016/1074-7613(94)90045-0

Pullan, R. D., Thomas, G. A., Rhodes, M., Newcombe, R. G., Williams, G. T., Allen, A., & Rhodes, J. (1994). Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut*, *35*(3), 353–9. https://doi.org/10.1136/gut.35.3.353

Rauert, H., Wicovsky, A., Müller, N., Siegmund, D., Spindler, V., Waschke, J., … Wajant, H. (2010). Membrane Tumor Necrosis Factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). *Journal of Biological Chemistry*, *285*(10), 7394–7404. https://doi.org/10.1074/jbc.M109.037341

Rhee, L., Murphy, S. F., Kolodziej, L. E., Grimm, W. A., Weber, C. R., Lodolce, J. P., … Boone, D. L. (2012). Expression of TNFAIP3 in intestinal epithelial cells protects from DSS- but not TNBS-induced colitis. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *303*(2). Retrieved from http://ajpgi.physiology.org/content/303/2/G220.long

Rhodes, J. M. (1997). Colonic mucus and ulcerative colitis. *Gut*, *40*(6), 807–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9245940

Rickard, J. A., O'Donnell, J. A., Evans, J. M., Lalaoui, N., Poh, A. R., Rogers, T., … Silke, J. (2014). RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell*, *157*(5), 1175–1188. https://doi.org/10.1016/j.cell.2014.04.019

Rieser, E., Cordier, S. M., & Walczak, H. (2013). Linear ubiquitination: A newly discovered regulator of cell signalling. *Trends in Biochemical Sciences*. https://doi.org/10.1016/j.tibs.2012.11.007

Rioux, J. D., Xavier, R. J., Taylor, K. D., Silverberg, M. S., Goyette, P., Huett, A., … Brant, S. R. (2007). Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nature Genetics*, *39*(5), 596–604. https://doi.org/10.1038/ng2032

Ritorto, M. S., Ewan, R., Perez-Oliva, A. B., Knebel, A., Buhrlage, S. J., Wightman, M., … Trost, M. (2014). Screening of DUB activity and specificity by MALDI-TOF mass spectrometry. *Nature Communications*, *5*, 4763. https://doi.org/10.1038/ncomms5763

Rivkin, E., Almeida, S. M., Ceccarelli, D. F., Juang, Y.-C., MacLean, T. A., Srikumar, T., … Cordes, S. P. (2013). The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis. *Nature*, *498*(7454), 318–324. https://doi.org/10.1038/nature12296

Rogler, G., Brand, K., Vogl, D., Page, S., Hofmeister, R., Andus, T., … Gross, V. (1998). Nuclear factor κB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology*, *115*(2), 357–369. https://doi.org/10.1016/s0016-5085(98)70202-1

Rothe, M., Sarma, V., Dixit, V. M., & Goeddel, D. V. (1995). TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science (New York, N.Y.)*, *269*(5229), 1424–1427. https://doi.org/10.1126/science.7544915

Rovedatti, L., Kudo, T., Biancheri, P., Sarra, M., Knowles, C. H., Rampton, D. S., … Macdonald, T. T. (2009). Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut*, *58*(12), 1629–36. https://doi.org/10.1136/gut.2009.182170

Saitoh, T., Nakayama, M., Nakano, H., Yagita, H., Yamamoto, N., & Yamaoka, S. (2003). TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation. *The Journal of Biological Chemistry*, *278*(38), 36005–12. https://doi.org/10.1074/jbc.M304266200

Sandborn, W. J., Feagan, B. G., Fedorak, R. N., Scherl, E., Fleisher, M. R., Katz, S., … Rutgeerts, P. (2008). A Randomized Trial of Ustekinumab, a Human Interleukin-12/23 Monoclonal Antibody, in Patients With Moderate-to-Severe Crohn's Disease. *Gastroenterology*, *135*(4), 1130–1141. https://doi.org/10.1053/j.gastro.2008.07.014

Sandborn, W. J., Feagan, B. G., Marano, C., Zhang, H., Strauss, R., Johanns, J., … PURSUIT-Maintenance Study Group. (2014). Subcutaneous Golimumab Maintains Clinical Response in Patients With Moderate-to-Severe Ulcerative Colitis. *Gastroenterology*, *146*(1), 96–109.e1. https://doi.org/10.1053/j.gastro.2013.06.010

Sandborn, W. J., Feagan, B. G., Marano, C., Zhang, H., Strauss, R., Johanns, J., … PURSUIT-SC Study Group. (2014). Subcutaneous Golimumab Induces Clinical Response and Remission in Patients With Moderate-to-Severe Ulcerative Colitis. *Gastroenterology*, *146*(1), 85–95. https://doi.org/10.1053/j.gastro.2013.05.048

Sandborn, W. J., Gasink, C., Gao, L.-L., Blank, M. A., Johanns, J., Guzzo, C., … CERTIFI Study Group. (2012). Ustekinumab Induction and Maintenance Therapy in Refractory Crohn's Disease. *New England Journal of Medicine*, *367*(16), 1519–1528. https://doi.org/10.1056/NEJMoa1203572

Sandborn, W. J., Hanauer, S. B., Katz, S., Safdi, M., Wolf, D. G., Baerg, R. D., … Zinsmeister, A. R. (2001). Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology*, *121*(5), 1088–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11677200

Sandborn, W. J., van Assche, G., Reinisch, W., Colombel, J., D'Haens, G., Wolf, D. C., … Thakkar, R. B. (2012). Adalimumab Induces and Maintains Clinical Remission in Patients With Moderate-to-Severe Ulcerative Colitis. *Gastroenterology*, *142*(2), 257–265.e3. https://doi.org/10.1053/j.gastro.2011.10.032

Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., … Clevers, H. (2009). Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche. *Nature*, *459*(7244), 262–265. https://doi.org/10.1038/nature07935

Scallon, B., Cai, A., Solowski, N., Rosenberg, A., Song, X.-Y., Shealy, D., & Wagner, C. (2002). Binding and functional comparisons of two types of tumor necrosis factor antagonists. *The Journal of Pharmacology and Experimental Therapeutics*, *301*(2), 418–26. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11961039

Schenk, B., & Fulda, S. (2015). Reactive oxygen species regulate Smac mimetic/TNFα-induced necroptotic signaling and cell death. *Oncogene*, *34*(47), 5796–5806. https://doi.org/10.1038/onc.2015.35

Scheu, S., Alferink, J., Pötzel, T., Barchet, W., Kalinke, U., & Pfeffer, K. (2002). Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin beta in mesenteric lymph node genesis. *Journal of Experimental Medicine*, *195*(12), 1613–1624. https://doi.org/10.1084/jem.20020215

Schlicher, L., Wissler, M., Preiss, F., Brauns‐Schubert, P., Jakob, C., Dumit, V., … Maurer, U. (2016). SPATA2 promotes CYLD activity and regulates TNF‐induced NF‐κB signaling and cell death. *EMBO Reports*, *17*(10), 1485–1497. https://doi.org/10.15252/embr.201642592

Schmidt, C., Giese, T., Ludwig, B., Mueller-Molaian, I., Marth, T., Zeuzem, S., … Stallmach, A. (2005). Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflammatory Bowel Diseases*, *11*(1), 16–23. https://doi.org/00054725-200501000-00003 [pii]

Schmitz, H., Barmeyer, C., Fromm, M., Runkel, N., Foss, H. D., Bentzel, C. J., … Schulzke, J. D. (1999). Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology*, *116*(2), 301–309. https://doi.org/10.1016/S0016-5085(99)70126-5

Schmitz, M. L., & Baeuerle, P. A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *The EMBO Journal*, *10*(12), 3805–3817. https://doi.org/10.1002/j.1460-2075.1991.tb04950.x

Schneider-Brachert, W., Tchikov, V., Neumeyer, J., Jakob, M., Winoto-Morbach, S., Held-Feindt, J., … Schütze, S. (2004). Compartmentalization of TNF receptor 1 signaling: Internalized TNF receptosomes as death signaling vesicles. *Immunity*, *21*(3), 415–428. https://doi.org/10.1016/j.immuni.2004.08.017

Schreiber, J., Jenner, R. G., Murray, H. L., Gerber, G. K., Gifford, D. K., & Young, R. a. (2006). Coordinated binding of NF-kappaB family members in the response of human cells to lipopolysaccharide. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(15), 5899–5904. https://doi.org/10.1073/pnas.0510996103

Schreiber, S., Nikolaus, S., & Hampe, J. (1998). Activation of nuclear factor kappa B inflammatory bowel disease. *Gut*, *42*(4), 477–84. https://doi.org/10.1136/gut.42.4.477

Sen, R., & Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*, *46*(5), 705–716. https://doi.org/10.1016/0092-8674(86)90346-6

Shaked, H., Hofseth, L. J., Chumanevich, A., Chumanevich, A. A., Wang, J., Wang, Y., … Karin, M. (2012). Chronic epithelial NF-κB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(35), 14007–12. https://doi.org/10.1073/pnas.1211509109

Shembade, N., Harhaj, N. S., Parvatiyar, K., Copeland, N. G., Jenkins, N. A., Matesic, L. E., & Harhaj, E. W. (2008). The E3 ligase Itch negatively regulates inflammatory signaling pathways by controlling the function of the ubiquitin-editing enzyme A20. *Nature Immunology*, *9*(3), 254–262. https://doi.org/10.1038/ni1563

Shembade, N., Ma, A., & Harhaj, E. W. (2010). Inhibition of NF-κB Signaling by A20 Through Disruption of Ubiquitin Enzyme Complexes. *Science*, *327*(5969). Retrieved from http://science.sciencemag.org/content/327/5969/1135

Shembade, N., Parvatiyar, K., Harhaj, N. S., & Harhaj, E. W. (2009). The ubiquitin-editing enzyme A20 requires RNF11 to downregulate NF-κB signalling. *The EMBO Journal*, *28*(5), 513–522. https://doi.org/10.1038/emboj.2008.285

Shibahara, T., Sato, N., Waguri, S., Iwanaga, T., Nakahara, a, Fukutomi, H., & Uchiyama, Y. (1995). The fate of effete epithelial cells at the villus tips of the human small intestine. *Archives of Histology and Cytology*, *58*(2), 205–219. https://doi.org/10.1679/aohc.58.205

Shibata, W., Maeda, S., Hikiba, Y., Yanai, A., Ohmae, T., Sakamoto, K., … Omata, M. (2007). Cutting edge: The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks inflammatory injury in murine colitis. *Journal of Immunology (Baltimore, Md : 1950)*, *179*(5), 2681–2685. https://doi.org/179/5/2681 [pii]

Shibata, Y., Oyama, M., Kozuka-Hata, H., Han, X., Tanaka, Y., Gohda, J., & Inoue, J. (2012). p47 negatively regulates IKK activation by inducing the lysosomal degradation of polyubiquitinated NEMO. *Nature Communications*, *3*, 1061. https://doi.org/10.1038/ncomms2068

Shindo, R., Kakehashi, H., Okumura, K., Kumagai, Y., & Nakano, H. (2013). Critical contribution of oxidative stress to TNFα-induced necroptosis downstream of RIPK1 activation. *Biochemical and Biophysical Research Communications*, *436*(2), 212–216. https://doi.org/10.1016/j.bbrc.2013.05.075

Shyer, A. E., Huycke, T. R., Lee, C., Mahadevan, L., & Tabin, C. J. (2015). Bending Gradients: How the intestinal stem cell gets its home. *Cell*, *161*(3), 569–580. https://doi.org/10.1016/j.cell.2015.03.041

Simmonds, N., Furman, M., Karanika, E., Phillips, A., & Bates, A. W. (2014). Paneth cell metaplasia in newly diagnosed inflammatory bowel disease in children. *BMC Gastroenterology*, *14*(1), 93. https://doi.org/10.1186/1471-230X-14-93

Simpson, S. J., Shah, S., Comiskey, M., de Jong, Y. P., Wang, B., Mizoguchi, E., … Terhorst, C. (1998). T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin 12/Signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon gamma expression by T cells. *The Journal of Experimental Medicine*, *187*(8), 1225–34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9547334

Skaug, B., Jiang, X., & Chen, Z. J. (2009). The role of ubiquitin in NF-kappaB regulatory pathways. *Annual Review of Biochemistry*, *78*, 769–796. https://doi.org/10.1146/annurev.biochem.78.070907.102750

Song, L., & Rape, M. (2010). Regulated Degradation of Spindle Assembly Factors by the Anaphase-Promoting Complex. *Molecular Cell*, *38*(3), 369–382. https://doi.org/10.1016/j.molcel.2010.02.038

Spehlmann, M. E., Begun, A. Z., Burghardt, J., Lepage, P., Raedler, A., & Schreiber, S. (2008). Epidemiology of inflammatory bowel disease in a German twin cohort: Results of a nationwide study. *Inflammatory Bowel Diseases*, *14*(7), 968–976. https://doi.org/10.1002/ibd.20380

Spits, H. (2011). Another armament in gut immunity: Lymphotoxin-mediated crosstalk between innate lymphoid and dendritic cells. *Cell Host and Microbe*. https://doi.org/10.1016/j.chom.2011.07.002

Steinbach, E. C., & Plevy, S. E. (2014). The Role of Macrophages and Dendritic Cells in the Initiation of Inflammation in IBD. *Inflammatory Bowel Diseases*, *20*(1), 166–175. https://doi.org/10.1097/MIB.0b013e3182a69dca

Steinbrecher, K. a, Harmel-Laws, E., Sitcheran, R., & Baldwin, A. S. (2008). Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. *Journal of Immunology (Baltimore, Md.: 1950)*, *180*(4), 2588–2599. https://doi.org/180/4/2588 [pii]

Suenaert, P., Bulteel, V., Lemmens, L., Noman, M., Geypens, B., Van Assche, G., … Rutgeerts, P. (2002). Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. *The American Journal of Gastroenterology*, *97*(8), 2000–2004. https://doi.org/10.1111/j.1572-0241.2002.05914.x

Sugihara, T., Kobori, A., Imaeda, H., Tsujikawa, T., Amagase, K., Takeuchi, K., … Andoh, A. (2010). The increased mucosal mRNA expressions of complement C3 and interleukin-17 in inflammatory bowel disease. *Clinical and Experimental Immunology*, *160*(3), 386–393. https://doi.org/10.1111/j.1365-2249.2010.04093.x

Sugimoto, K., Ogawa, A., Mizoguchi, E., Shimomura, Y., Andoh, A., Bhan, A. K., … Mizoguchi, A. (2008). IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *The Journal of Clinical Investigation*, *118*(2), 534–44. https://doi.org/10.1172/JCI33194

Sun, L., Wang, H., Wang, Z., He, S., Chen, S., Liao, D., … Wang, X. (2012). Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell*, *148*(1–2), 213–227. https://doi.org/10.1016/j.cell.2011.11.031

Swan, N. C., Geoghegan, J. G., O'Donoghue, D. P., Hyland, J. M., & Sheahan, K. (1998). Fulminant colitis in inflammatory bowel disease: detailed pathologic and clinical analysis. *Diseases of the Colon and Rectum*, *41*(12), 1511–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9860331

Tada, K., Okazaki, T., Sakon, S., Kobarai, T., Kurosawa, K., Yamaoka, S., … Nakano, H. (2001). Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. *The Journal of Biological Chemistry*, *276*(39), 36530–4. https://doi.org/10.1074/jbc.M104837200

Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., … Akira, S. (1999). Limb and skin abnormalities in mice lacking IKKalpha. *Science*, *284*(5412), 313–316. https://doi.org/10.1126/science.284.5412.313

Takiuchi, T., Nakagawa, T., Tamiya, H., Fujita, H., Sasaki, Y., Saeki, Y., … Iwai, K. (2014). Suppression of LUBAC-mediated linear ubiquitination by a specific interaction between LUBAC and the deubiquitinases CYLD and OTULIN. *Genes to Cells*, *19*(3), 254–272. https://doi.org/10.1111/gtc.12128

Tanaka, T., Grusby, M. J., & Kaisho, T. (2007). PDLIM2-mediated termination of transcription factor NF-κB activation by intranuclear sequestration and degradation of the p65 subunit. *Nature Immunology*, *8*(6), 584–591. https://doi.org/10.1038/ni1464

Tartaglia, L. A., Ayres, T. M., Wong, G. H. W., & Goeddel, D. V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell*, *74*(5), 845–853. https://doi.org/10.1016/0092-8674(93)90464-2

Tavares, R. M., Turer, E. E., Liu, C. L., Advincula, R., Scapini, P., Rhee, L., … Ma, A. (2010). The Ubiquitin Modifying Enzyme A20 Restricts B Cell Survival and Prevents Autoimmunity. *Immunity*, *33*(2), 181–191. https://doi.org/10.1016/j.immuni.2010.07.017

Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., … Meier, P. (2011). The Ripoptosome, a Signaling Platform that Assembles in Response to Genotoxic Stress and Loss of IAPs. *Molecular Cell*, *43*(3), 432–448. https://doi.org/10.1016/j.molcel.2011.06.006

Toedter, G., Li, K., Sague, S., Ma, K., Marano, C., MacOritto, M., … Baribaud, F. (2012). Genes associated with intestinal permeability in ulcerative colitis: Changes in expression following infliximab therapy. *Inflammatory Bowel Diseases*, *18*(8), 1399–1410. https://doi.org/10.1002/ibd.22853

Tokunaga, F., Nakagawa, T., Nakahara, M., Saeki, Y., Taniguchi, M., Sakata, S., … Iwai, K. (2011). SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex. *Nature*, *471*(7340), 633–636. https://doi.org/10.1038/nature09815

Tokunaga, F., Nishimasu, H., Ishitani, R., Goto, E., Noguchi, T., Mio, K., … Nureki, O. (2012). Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-κB regulation. *The EMBO Journal*, *31*(19), 3856–3870. https://doi.org/10.1038/emboj.2012.241

Tokunaga, F., Sakata, S., Saeki, Y., Satomi, Y., Kirisako, T., Kamei, K., … Iwai, K. (2009). Involvement of linear polyubiquitylation of NEMO in NF-κB activation. *Nature Cell Biology*, *11*(2), 123–132. https://doi.org/10.1038/ncb1821

Trompouki, E., Hatzivassiliou, E., Tsichritzis, T., Farmer, H., Ashworth, A., & Mosialos, G. (2003). CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature*, *424*(6950), 793–6. https://doi.org/10.1038/nature01803

Tumanov, A. V., Koroleva, E. P., Guo, X., Wang, Y., Kruglov, A., Nedospasov, S., & Fu, Y. X. (2011). Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. *Cell Host and Microbe*, *10*(1), 44–53. https://doi.org/10.1016/j.chom.2011.06.002

Udalova, I. a, Mott, R., Field, D., & Kwiatkowski, D. (2002). Quantitative prediction of NF-kappa B DNA-protein interactions. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(12), 8167–72. https://doi.org/10.1073/pnas.102674699

Uhlig, H. H., McKenzie, B. S., Hue, S., Thompson, C., Joyce-Shaikh, B., Stepankova, R., … Powrie, F. (2006). Differential Activity of IL-12 and IL-23 in Mucosal and Systemic Innate Immune Pathology. *Immunity*, *25*(2), 309–318. https://doi.org/10.1016/j.immuni.2006.05.017

Upton, J. W., Kaiser, W. J., & Mocarski, E. S. (2012). DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. *Cell Host and Microbe*, *11*(3), 290–297. https://doi.org/10.1016/j.chom.2012.01.016

Uslu, R., & Bonavida, B. (1996). Involvement of the mitochondrion respiratory chain in the synergy achieved by treatment of human ovarian carcinoma cell lines with both tumor necrosis factor-α and cis-diamminedichloroplatinum. *Cancer*, *77*(4), 725–732. https://doi.org/10.1002/(SICI)1097-0142(19960215)77:4<725::AID-CNCR19>3.0.CO;2-2

Vainer, B., Nielsen, O. H., Hendel, J., Horn, T., & Kirman, I. (2000). Colonic expression and synthesis of interleukin 13 and interleukin 15 in inflammatory bowel disease. *Cytokine*, *12*(10), 1531–6. https://doi.org/10.1006/cyto.2000.0744

Vallabhapurapu, S., Matsuzawa, A., Zhang, W., Tseng, P.-H., Keats, J. J., Wang, H., … Karin, M. (2008). Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-κB signaling. *Nature Immunology*, *9*(12), 1364–1370. https://doi.org/10.1038/ni.1678

Van den Brande, J. M. H., Koehler, T. C., Zelinkova, Z., Bennink, R. J., te Velde, A. A., ten Cate, F. J. W., … Hommes, D. W. (2007). Prediction of antitumour necrosis factor clinical efficacy by real-time visualisation of apoptosis in patients with Crohn's disease. *Gut*, *56*(4), 509–517. https://doi.org/10.1136/gut.2006.105379

van Raam, B. J., Ehrnhoefer, D. E., Hayden, M. R., & Salvesen, G. S. (2013). Intrinsic cleavage of receptor-interacting protein kinase-1 by caspase-6. *Cell Death and Differentiation*, *20*(1), 86–96. https://doi.org/10.1038/cdd.2012.98

Vandenabeele, P., Declercq, W., Van Herreweghe, F., & Vanden Berghe, T. (2010). The Role of the Kinases RIP1 and RIP3 in TNF-Induced Necrosis. *Science Signaling*, *3*(115), re4-re4. https://doi.org/10.1126/scisignal.3115re4

Varfolomeev, E., Goncharov, T., Fedorova, A. V, Dynek, J. N., Zobel, K., Deshayes, K., … Vucic, D. (2008). c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. *J Biol Chem*, *283*(36), 24295–24299. https://doi.org/10.1074/jbc.C800128200

Varshavsky, A. (2017). The Ubiquitin System, Autophagy, and Regulated Protein Degradation. *Annual Review of Biochemistry*, *86*(1), 123–128. https://doi.org/10.1146/annurev-biochem-061516-044859

Vercammen, D., Beyaert, R., Denecker, G., Goossens, V., Van Loo, G., Declercq, W., … Vandenabeele, P. (1998). Inhibition of Caspases Increases the Sensitivity of L929 Cells to Necrosis Mediated by Tumor Necrosis Factor. *The Journal of Experimental Medicine*, *187*(9), 1477–1485. https://doi.org/10.1084/jem.187.9.1477

Vereecke, L., Beyaert, R., & van Loo, G. (2009). The ubiquitin-editing enzyme A20 (TNFAIP3) is a central regulator of immunopathology. *Trends in Immunology*, *30*(8), 383–391. https://doi.org/10.1016/j.it.2009.05.007

Vereecke, L., Sze, M., Guire, C. M., Rogiers, B., Chu, Y., Schmidt-Supprian, M., … van Loo, G. (2010). Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor–induced toxicity and experimental colitis. *The Journal of Experimental Medicine*, *207*(7), 1513–1523. https://doi.org/10.1084/jem.20092474

Vereecke, L., Vieira-Silva, S., Billiet, T., van Es, J. H., Mc Guire, C., Slowicka, K., … van Loo, G. (2014). A20 controls intestinal homeostasis through cell-specific activities. *Nature Communications*, *5*, 5103. https://doi.org/10.1038/ncomms6103

Verhelst, K., Carpentier, I., Kreike, M., Meloni, L., Verstrepen, L., Kensche, T., … Beyaert, R. (2012). A20 inhibits LUBAC-mediated NF-κB activation by binding linear polyubiquitin chains via its zinc finger 7. *The EMBO Journal*, *31*(19), 3845–3855. https://doi.org/10.1038/emboj.2012.240

Vlantis, K., Wullaert, A., Sasaki, Y., Schmidt-Supprian, M., Rajewsky, K., Roskams, T., & Pasparakis, M. (2011). Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice. *Journal of Clinical Investigation*, *121*(7), 2781–2793. https://doi.org/10.1172/JCI45349

Vos, A. C. W., Wildenberg, M. E., Duijvestein, M., Verhaar, A. P., van den Brink, G. R., & Hommes, D. W. (2011). Anti–Tumor Necrosis Factor-α Antibodies Induce Regulatory Macrophages in an Fc Region-Dependent Manner. *Gastroenterology*, *140*(1), 221–230.e3. https://doi.org/10.1053/j.gastro.2010.10.008

Wagner, S. A., Satpathy, S., Beli, P., & Choudhary, C. (2016). SPATA2 links CYLD to the TNF-α receptor signaling complex and modulates the receptor signaling outcomes. *The EMBO Journal*, *35*(17), 1–17. https://doi.org/10.15252/embj.201694300

Wallach, D., Kang, T.-B., Dillon, C. P., & Green, D. R. (2016). Programmed necrosis in inflammation: Toward identification of the effector molecules. *Science*, *352*(6281), aaf2154-aaf2154. https://doi.org/10.1126/science.aaf2154

Wang, H., Sun, L., Su, L., Rizo, J., Liu, L., Wang, L. F., … Wang, X. (2014). Mixed Lineage Kinase Domain-like Protein MLKL Causes Necrotic Membrane Disruption upon Phosphorylation by RIP3. *Molecular Cell*, *54*(1), 133–146. https://doi.org/10.1016/j.molcel.2014.03.003

Wang, J., Anders, R. a, Wang, Y., Turner, J. R., Abraham, C., Pfeffer, K., & Fu, Y.-X. (2005). The critical role of LIGHT in promoting intestinal inflammation and Crohn's disease. *Journal of Immunology (Baltimore, Md.: 1950)*, *174*(31), 8173–8182. https://doi.org/10.4049/jimmunol.174.12.8173

Wang, K., Baldassano, R., Zhang, H., Qu, H. Q., Imielinski, M., Kugathasan, S., … Hakonarson, H. (2010). Comparative genetic analysis of inflammatory bowel disease and type 1 diabetes implicates multiple loci with opposite effects. *Human Molecular Genetics*, *19*(10), 2059–2067. https://doi.org/10.1093/hmg/ddq078

Wang, L., Du, F., Wang, X., Orenstein, J., Chan, F. K., Lenardo, M., … Al., E. (2008). TNF-α Induces Two Distinct Caspase-8 Activation Pathways. *Cell*, *133*(4), 693–703. https://doi.org/10.1016/j.cell.2008.03.036

Wang, S.-L., Wang, Z.-R., & Yang, C.-Q. (2012). Meta-analysis of broad-spectrum antibiotic therapy in patients with active inflammatory bowel disease. *Experimental and Therapeutic Medicine*, *4*(6), 1051–1056. https://doi.org/10.3892/etm.2012.718

Wang, X., Jiang, W., Yan, Y., Gong, T., Han, J., Tian, Z., & Zhou, R. (2014). RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway. *Nature Immunology*, *15*(12), 1126–1133. https://doi.org/10.1038/ni.3015

Ware, C. F., VanArsdale, T. L., Crowe, P. D., & Browning, J. L. (1995). The ligands and receptors of the lymphotoxin system. *Current Topics in Microbiology and Immunology*, *198*, 175–218. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7774281

Wehkamp, J., Fellermann, K., Herrlinger, K. R., Baxmann, S., Schmidt, K., Schwind, B., … Stange, E. F. (2002). Human beta-defensin 2 but not beta-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. *European Journal of Gastroenterology & Hepatology*, *14*(7), 745–752. https://doi.org/10.1097/00042737-200207000-00006

Wehkamp, J., Harder, J., Weichenthal, M., Mueller, O., Herrlinger, K. R., Fellermann, K., … Stange, E. F. (2003). Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. *Inflammatory Bowel Diseases*, *9*(4), 215–23. https://doi.org/10.1097/00054725-200307000-00001

Wehkamp, J., Salzman, N. H., Porter, E., Nuding, S., Weichenthal, M., Petras, R. E., … Bevins, C. L. (2005). Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(50), 18129–34. https://doi.org/10.1073/pnas.0505256102

Wehkamp, J., Schwind, B., Herrlinger, K. R., Baxmann, S., Schmidt, K., Duchrow, M., … Fellermann, K. (2002). Innate immunity and colonic inflammation: enhanced expression of epithelial alpha-defensins. *Digestive Diseases and Sciences*, *47*(6), 1349–55. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12064812

Weinlich, R., & Green, D. R. (2014). The Two Faces of Receptor Interacting Protein Kinase-1. *Molecular Cell*, *56*(4), 469–480. https://doi.org/10.1016/j.molcel.2014.11.001

Wertz, I. E., & Dixit, V. M. (2010). Signaling to NF-kappaB: regulation by ubiquitination. *Cold Spring Harbor Perspectives in Biology*. https://doi.org/10.1101/cshperspect.a003350

Wertz, I. E., Newton, K., Seshasayee, D., Kusam, S., Lam, C., Zhang, J., … Dixit, V. M. (2015). Phosphorylation and linear ubiquitin direct A20 inhibition of inflammation. *Nature*, *528*(7582), 370–375. https://doi.org/10.1038/nature16165

Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., … Dixit, V. M. (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. *Nature*, *430*(7000), 694–699. https://doi.org/10.1038/nature02794

Wilkinson, K. D., Urban, M. K., & Haas, A. L. (1980). Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *Journal of Biological Chemistry*, *255*(16), 7529–7532. https://doi.org/6249803

Won, M., Park, K. a, Byun, H. S., Sohn, K.-C., Kim, Y.-R., Jeon, J., … Hur, G. M. (2010). Novel anti-apoptotic mechanism of A20 through targeting ASK1 to suppress TNF-induced JNK activation. *Cell Death and Differentiation*, *17*(12), 1830–1841. https://doi.org/10.1038/cdd.2010.47

Wu, X.-N., Yang, Z.-H., Wang, X.-K., Zhang, Y., Wan, H., Song, Y., … Han, J. (2014). Distinct roles of RIP1–RIP3 hetero- and RIP3–RIP3 homo-interaction in mediating necroptosis. *Cell Death and Differentiation*, *21*(11), 1709–1720. https://doi.org/10.1038/cdd.2014.77

Wullaert, A., Bonnet, M. C., & Pasparakis, M. (2010). NF- κ B in the regulation of epithelial homeostasis and inflammation. *Nature Publishing Group*, *21*(1), 146–158. https://doi.org/10.1038/cr.2010.175

Xanthoulea, S., Pasparakis, M., Kousteni, S., Brakebusch, C., Wallach, D., Bauer, J., … Kollias, G. (2004). Tumor Necrosis Factor (TNF) Receptor Shedding Controls Thresholds of Innate Immune Activation That Balance Opposing TNF Functions in Infectious and Inflammatory Diseases. *The Journal of Experimental Medicine*, *200*(3), 367–376. https://doi.org/10.1084/jem.20040435

Xu, X.-R., Liu, C.-Q., Feng, B.-S., & Liu, Z.-J. (2014). Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease. *World Journal of Gastroenterology : WJG*, *20*(12), 3255–3264. https://doi.org/10.3748/wjg.v20.i12.3255

Yamaguchi, N., & Yamaguchi, N. (2015). The seventh zinc finger motif of A20 is required for the suppression of TNF-α-induced apoptosis. *FEBS Letters*, *589*(12), 1369–1375. https://doi.org/10.1016/j.febslet.2015.04.022

Yamamoto, M., Yoshizaki, K., Kishimoto, T., & Ito, H. (2000). IL-6 Is Required for the Development of Th1 Cell-Mediated Murine Colitis. *The Journal of Immunology*, *164*(9), 4878–4882. https://doi.org/10.4049/jimmunol.164.9.4878

Yamazaki, K., McGovern, D., Ragoussis, J., Paolucci, M., Butler, H., Jewell, D., … Nakamura, Y. (2005). Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. *Human Molecular Genetics*, *14*(22), 3499–3506. https://doi.org/10.1093/hmg/ddi379

Yang, X. O., Chang, S. H., Park, H., Nurieva, R., Shah, B., Acero, L., … Dong, C. (2008). Regulation of inflammatory responses by IL-17F. *The Journal of Experimental Medicine*, *205*(5), 1063–1075. https://doi.org/10.1084/jem.20071978

Yau, R., & Rape, M. (2016). The increasing complexity of the ubiquitin code. *Nature Cell Biology*, *18*(6), 579–586. https://doi.org/10.1038/ncb3358

Yen, D., Cheung, J., Scheerens, H., Poulet, F., McClanahan, T., Mckenzie, B., … Rennick, D. (2006). IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *Journal of Clinical Investigation*, *116*(5), 1310–1316. https://doi.org/10.1172/JCI21404

Yin, L., Wu, L., Wesche, H., Arthur, C. D., White, J. M., Goeddel, D. V, & Schreiber, R. D. (2001). Defective lymphotoxin-beta receptor-induced NF-kappaB transcriptional activity in NIK-deficient mice. *Science (New York, N.Y.)*, *291*(5511), 2162–5. https://doi.org/10.1126/science.1058453

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology*, *9*(1), 47–59. https://doi.org/10.1038/nrm2308

Yuan, W. C., Lee, Y. R., Lin, S. Y., Chang, L. Y., Tan, Y. P., Hung, C. C., … Chen, R. H. (2014). K33-Linked Polyubiquitination of Coronin 7 by Cul3-KLHL20 Ubiquitin E3 Ligase Regulates Protein Trafficking. *Molecular Cell*, *54*(4), 586–600. https://doi.org/10.1016/j.molcel.2014.03.035

Zarnegar, B. J., Wang, Y., Mahoney, D. J., Dempsey, P. W., Cheung, H. H., He, J., … Cheng, G. (2008). Noncanonical NF-κB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. *Nature Immunology*, *9*(12), 1371–1378. https://doi.org/10.1038/ni.1676

Zeissig, S. (2004). Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumour necrosis factor antibody treatment. *Gut*, *53*(9), 1295–1302. https://doi.org/10.1136/gut.2003.036632

Zeissig, S., Burgel, N., Gunzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., … Schulzke, J.-D. (2007). Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut*, *56*(1), 61–72. https://doi.org/10.1136/gut.2006.094375

Zenewicz, L. A., Yancopoulos, G. D., Valenzuela, D. M., Murphy, A. J., Stevens, S., & Flavell, R. A. (2008). Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity*, *29*(6), 947–57. https://doi.org/10.1016/j.immuni.2008.11.003

Zhang, D.-W., Shao, J., Lin, J., Zhang, N., Lu, B.-J., Lin, S.-C., … Han, J. (2009). RIP3, an Energy Metabolism Regulator That Switches TNF-Induced Cell Death from Apoptosis to Necrosis. *Science*, *325*(5938), 332–336. https://doi.org/10.1126/science.1172308

Zhang, J., Stirling, B., Temmerman, S. T., Ma, C. A., Fuss, I. J., Derry, J. M. J., & Jain, A. (2006). Impaired regulation of NF-κB and increased susceptibility to colitis-associated tumorigenesis in CYLD-deficient mice. *Journal of Clinical Investigation*, *116*(11), 3042–3049. https://doi.org/10.1172/JCI28746

Zhang, L., Blackwell, K., Workman, L. M., Chen, S., Pope, M. R., Janz, S., & Habelhah, H. (2015). RIP1 Cleavage in the Kinase Domain Regulates TRAIL-Induced NF-κB Activation and Lymphoma Survival. *Molecular and Cellular Biology*, *35*(19), 3324–3338. https://doi.org/10.1128/mcb.00692-15

Zhang, Y., Su, S. S., Zhao, S., Yang, Z., Zhong, C.-Q., Chen, X., … Han, J. (2017). RIP1 autophosphorylation is promoted by mitochondrial ROS and is essential for RIP3 recruitment into necrosome. *Nature Communications*, *8*, 14329. https://doi.org/10.1038/ncomms14329

Zhao, J., Jitkaew, S., Cai, Z., Choksi, S., Li, Q., Luo, J., & Liu, Z.-G. (2012). Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proceedings of the National Academy of Sciences*, *109*(14), 5322–5327. https://doi.org/10.1073/pnas.1200012109

Zheng, C. F., & Huang, Y. (2011). [Expression of zinc finger protein A20 in pediatric inflammatory bowel disease]. *Zhonghua Er Ke Za Zhi. Chinese Journal of Pediatrics*, *49*, 261–265.

Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., … Ouyang, W. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine*, *14*(3), 282–289. https://doi.org/10.1038/nm1720

Zhong, H., May, M. J., Jimi, E., & Ghosh, S. (2002). The phosphorylation status of nuclear NF-κB determines its association with CBP/p300 or HDAC-1. *Molecular Cell*, *9*(3), 625–636. https://doi.org/10.1016/S1097-2765(02)00477-X

Zhu, G., Wu, C. J., Zhao, Y., & Ashwell, J. D. (2007). Optineurin Negatively Regulates TNFα- Induced NF-κB Activation by Competing with NEMO for Ubiquitinated RIP. *Current Biology*, *17*(16), 1438–1443. https://doi.org/10.1016/j.cub.2007.07.041

Chapter 8:
ABBREVIATIONS

c-FLIP	Cellular FLICE-like inhibitory protein long isoform
C8	Caspase-8
cC8	Cleaved caspase-8
CD	Crohn's Disease
CHX	Cyclohexamide
cIAP	Cellular inhibitor of apoptosis protein
CYLD	Cylindromatosis
DC	Dendritic cell
DISC	Death-inducing signaling complex
DSS	Dextran sodium sulphate
DUB	Deubiquitinase
FADD	Fas-associated death domain-containing protein
GI	Gastointestinal
GWAS	Genome-wide association study
HOIL	Haem-oxidized IRP2 ubiquitin ligase-1
HOIP	HOIL-1 interacting protein, also known as E3 ubiquitin-protein ligase RNF31
HVEM	Herpes-virus entry mediator
i.p.	Intra-peritoneal
i.v.	Retro-orbital
IBD	Inflammatory bowel diesase
IEC	Intestinal epithelial cell
IFN	Interferon
IκB	Inhibitor of κB
IKK	IκB kinase
IP	Immunoprecipitation
JNK	JUN N-terminal kinase
LIGHT	Lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells
LPS	Lipopolysaccharide
LTβ	Lymphotoxin β
LTβR	Lymphotoxin β receptor
LUBAC	Linear ubiquitin chain assembly complex
MAPK	Mitogen-activated kinase
Mø	Macrophages
Nec-1	Necrostatin-1
Nec1s	Necrostatin-1s
NF-κB	Nuclear factor-κB
NIK	NF-κB-inducing kinase
NK	Natural killer
O/N	Over night
OTU	Ovarian tumor
OTULIN	OTU DUB with linear linkage specificity
RIPK	Receptor-interacting serine/threonine-protein kinase
ROS	Reactive oxygen species
RT	Room temperature

SHARPIN	SHANK-associated RH domain-interacting protein
SNPs	Single-nucleotide polymorphisms
TAB	TAK1-binding protein
TAK1	Transforming growth factor-β activated kinase 1
TJ	Tight junctions
TNF	Tumor necrosis α
TNFAIP3	TNFα-induced protein 3
TNFR	TNFα receptor
TNFRSF	Tumor necrosis factor receptor super family
TNFSF	Tumor necrosis factor super family
TRAD	TNFR1-associated death domain protein
TRAF	TNFR-associated factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Ub	Ubiquitin
UC	Ulcerative Colitis
ZnF	Zinc finger