The role of necroptosis in inflammatory bowel diseases (IBD)

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ABSTRACT

Inflammatory bowel diseases (IBD) are a group of complex inflammatory diseases of the gastrointestinal tract, the two main forms of which being Crohn's disease (CD) and ulcerative colitis (UC). Mechanisms involved in disruption of the intestinal epithelial barrier, microbiota symbiosis and immunological tolerance are posited to contribute to IBD. Genetic and functional studies have recently implicated cell death as a potential pathological mechanism in IBD. The predominant view that apoptosis, a physiologically essential cell death program, is the dominant form of cell death in IBD is now challenged with the recent discovery of regulated necrosis, including necroptosis. 'Patchy necrosis' has been previously observed in the ileum and colon of patients with CD (Dourmashkin et al., 1983). However, whether necrotic cell death is causative in IBD pathogenesis has yet to be clarified. The main objective of the project was to investigate necroptosis and apoptosis in preclinical animal models of IBD, to determine the contribution of cell death to colitis induction and severity and to identify potential therapeutic targets for IBD. Using *Tbx21*$^{-/-}$/*Rag2*$^{-/-}$ TRUC (*Tr*ansferable *UC*) mice, we demonstrate that both apoptosis and necrosis were induced in the inflamed colon but that necrosis was associated with disease severity. We also studied a chemically induced model of colitis, in which cIAP2-deficient mice were infected with murine chronic norovirus (MNV) prior to treatment with dextran sulfate sodium (DSS). We demonstrate that MNV infection exacerbates DSS-mediated injury in cIAP2 deficient mice by modulating Paneth cell homeostasis and cell death. Our results suggest that cIAP2 may play a dual role in autophagy and protection against ER stress-induced cell death. The second aim of the project was to explore regulatory mechanisms controlling the expression of cell death genes in humans. We identified a number of significant eSNPs, including in *MLKL*, the executioner of necroptosis. Towards understanding this eSNP mechanism, we characterize the transcriptional requirement of the *Mlkl* gene and found it to be induced by type I and II interferons. These results shed light on the occurrence of necroptosis in the context of intestinal inflammation, opening new avenues to further the investigation of the links between this cell death modality and IBD.

RÉSUMÉ

Les maladies inflammatoires de l'intestin (MII) forment un ensemble de syndromes complexes du système gastro-intestinale, regroupant deux formes principales: la maladie de Crohn's (CD) et la colite ulcérative (UC). La perturbation de la barrière épithéliale intestinale, la symbiose avec la microflore ainsi que la tolérance immunologique sont des mécanismes importants qui participent à la pathologie des MII. Des études génétiques ont récemment impliqué la mort cellulaire comme mécanisme potentiel qui pourrait également souligner la pathologie des MII. Le dogme impliquant l'apoptose, qui est une mort cellulaire programmée et physiologique, comme type de mort cellulaire principal dans les MII est maintenant remis en cause par la découverte d'un nouveau type mort cellulaire nommé la necroptose (necrose programmee). La présence de "plaques nécrotiques" dans la muqueuse du colon de patients atteints de CD suggère la présence de ce mécanisme dans les MII. Cependant, que la necroptose soit une cause ou une conséquence dans la pathologie des MII reste a être démontré. Afin de dans discerner la contribution relative de l'apoptose et de la nécroptose dans l'induction et la sévérité des MII, l'objectif principal de ce projet consiste à détecter et analyser ces deux types de mort cellulaire dans des modèles murins de MII. L'utilisation de souris *Tbx21*$^{-/-}$/*Rag2*$^{-/-}$ TRUC (*Tr*ansferable *UC*) nous a permis non seulement de démontrer que l'apoptose et la nécroptose étaient induites dans le colon inflammé, mais que la necroptose est directement associée avec la sévérité de la maladie. Nous avons également étudié un autre modèle animal de colite, où des souris *Birc3*$^{-/-}$ (déficientes en cIAP2) infectées avec norovirus murin (MNV) étaient traitées avec du dextran sulfate sodium (DSS). Nous avons démontré que l'infection avec MNV sensibilise les souris à la pathologie induite par le DSS dans les souris *Birc3*$^{-/-}$ en influençant l'homéostase et la mort des cellules de Paneth dans l'iléum. Nos résultats suggèrent que cIAP2 pourrait jouer un rôle dans l'autophagie ainsi que dans la protection contre la mort cellulaire due au stress du réticulum endoplasmique (RE). Le deuxième objectif de ce projet consistait à étudier les mécanismes de régulations qui gouvernent l'expression des gènes de mort cellulaires chez les humains. Nous avons identifié un nombre de loci de caractères quantitatifs associés à l'expression de gènes (eQTLs). Parmi ces gènes, l'expression de *MLKL*, une protéine centrale dans le processus de nécroptose, a été démontré comme étant régulée

par des mutations ponctuelles. Nous décrivons également des caractéristiques de transcription du gène MLKL, notamment qu'il peut être induit par les interférons de type I et de type II. Ces résultats illuminent donc la contribution de la nécroptose dans l'inflammation intestinale, ouvrant d'autres voies de recherche qui permettront de potentiellement connecter cette mort cellulaire et les MII.

CONTRIBUTION OF AUTHORS

The research conducted and presented in this thesis is entirely my own. Claudia Champagne, Maryse Dagenais, Jeremy Dupaul-Chicoine, Anna Kinio and Yifei (Phoebe) Zhong assisted with mouse dissections and sample collection. Alexander Skeldon also aided with gavaging procedures. Dr. David Langlais furnished us with RNA-seq and ChIP-seq data, as well as BMDM cDNA samples. Dr. Maya Saleh designed and supervised the work. This thesis was written by me with correction by Dr. Maya Saleh.

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ABBREVIATIONS

AMP	Antimicrobial peptide
ARE	AU rich element
CD	Crohn's disease
cDC	Conventional dendritic cell
ChIP-seq	Chromatin immunoprecipitation-sequencing
CMV	Cytomegalovirus
CR	Colonization resistance
CYLD	Cylindromatosis
DAI	DNA-dependent activator of IFN-regulatory factors
DISC	Death-inducing signaling complex
DSS	Dextran sulphate sodium
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EHEC	Entero-haemorragic Escherichia coli
EPEC	Entero-pathogenic Escherichia coli
ER	Endoplasmic reticulum
FADD	Fas-Associated protein with Death Domain
FLIP	FLICE-like inhibitory protein
GALT	Gut-associated lymphoid tissue
GF	Germ-free
GWAS	Genome-wide association study
IAP	Inhibitor of apoptosis protein
IBD	Inflammatory bowel diseases
IECs	Intestinal epithelial cells
IFN	Interferon
IKK	Inhibitor of nuclear factor kappa-B kinase
IL-10	Interleukin-10
IL-10RA	Interleukin-10 receptor A
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-22	Interleukin-22
ILC	Innate lymphoid cell
IRGM	Immunity-related GTPase family M
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin assembly complex
MCMV	Murine cytomegalovirus
MDP	Muramyl dipeptide
MLKL	Mixed-linked kinase-like protein

MNV	Murine norovirus
NEMO	NF-ϰB essential modulator
NF-kB	Nuclear factor kappa-B
NK	cell Natural killer cell
NKT	cell Natural killer T cell
pDC	Plasmacytoid dendritic cell
RBC	Red blood cell
REGIIIβ/γ	Regenerating islet-derived protein 3 beta/gamma
RNA-seq	RNA-sequencing
RHIM	RIP homotypic interaction motif
ROS	Reactive oxygen species
SFB	Segmented filamentous bacteria
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRADD	TNFRSF1A-associated via death domain
Treg	Regulatory T cell
TRIF	TIR domain-containing adaptor-inducing interferon beta
TRUC	*Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ Ulcerative colitis
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TWEAK	NF-related weak inducer of apoptosis
UC	Ulcerative colitis
UPR	Unfolded protein response
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis protein

LITERATURE REVIEW

Intestinal homeostasis

Intestinal architecture

The gut constitutes the largest boundary against the external environment, with an area of about 300 m^2. Following the stomach, the intestine is divided in two main segments, namely the small and large intestine. The small intestine begins at the pylorus and ends at the ileocaecal valve, which is an entry point into the large intestine. It is divided in three main segments, with the duodenum closest to the stomach, followed by the jejunum and then the ileum. The large intestine starts at the caecum, followed by the ascending colon, the transverse colon, the distal colon and the rectum, terminating at the anus. Finger-like projections, known as villi, characterize the surface of the small intestine, which protrude from the intestinal mucosa into the lumen and increase the surface area of digestively active epithelium; in contrast, villi are absent form the caecum and the colon, resulting in a flat surface. In all parts of the intestine, the surface epithelium is continuously renewed by immature cells arising from invaginations known as the crypts of Lieberkühn, where multipotent stem cells give rise to several different types of mature intestinal epithelial cells (IECs) (Mowat & Agace, 2014). Proliferating progenitor cells located at the bottom of the crypts migrate along the crypt-villus axis and undergo terminal differentiation into two main groups, the absorptive and secretory lineages. The enterocytes are absorptive IECs and are the most abundant cell type in the intestinal epithelium (van der Flier & Clevers, 2009). The physical nature of the intestinal epithelium is in part provided by the presence of tight junctions between enterocytes, preventing leakage of intestinal content into the underlying tissues. The convolution of the luminal surface of enterocytes into microvilli allows for enhanced absorption of nutrients form the intestinal lumen (Mowat & Agace, 2014). Progenitor cells also differentiate into secretory cells of the intestine, namely Paneth cells, goblet cells and enteroendocrine cells. Paneth cells specialize in the secretion of antimicrobial peptides, such as α-defensins, lysozymes and phospholipase A, which are key microbicidal effectors in the control of bacterial, fungal and viral infection (Ostaff, Stange, & Wehkamp, 2013). Paneth cells are found in the crypts of the small

intestine. Goblet cells, on the other hand, are increasingly abundant in the colon, and participate in intestinal barrier defences by the secretion of glycoproteins called mucins. These are the principal components of mucus, a gel-like matrix that lines the intestinal mucosa, providing protection against physical and chemical insults (Birchenough, Johansson, Gustafsson, Bergstrom, & Hansson, 2015) Finally, the last secretory cell type present in the gastro-intestinal tract is that of enteroendocrine cells, which coordinate gut functions by the secretion of gut-specific hormones (Yen & Wright, 2006). Together, absorptive and secretory cells of the gut represent the building blocks of the intestinal epithelial barrier (Figure 1).

The intestinal immune landscape

The bulk of intestinal immune responses take place in the mucosa, which comprises the epithelium, the underlying lamina propria and the muscularis mucosa. The lamina propria consists of a loosely packed connective tissue that forms the scaffolding for the villi, as well as containing the blood supply, lymph drainage and nervous supply for the mucosa. More importantly, it contains many cells of the innate and adaptive immune systems. The organized structures of the gut-associated lymphoid tissue (GALT) and the draining lymph nodes are the principal locations for priming adaptive immune cell responses in the intestine. Macroscopically visible GALT structures that have been well studied are intestinal Peyer's patches, particularly concentrated in the distal ileum (Brandtzaeg, 2010). Peyer's patches are composed of numerous B cell lymphoid follicles, flanked by smaller T cell areas. GALT-associated structures in the large intestine include caecal patches around the ileocaecal valve and colonic patches throughout the colon and rectum (Mowat & Agace, 2014). Access of antigen from the lumen across the epithelium to underlying immune cells requires specialized transport mechanisms that vary depending on the region of the intestine. In the small intestine, microbial particles are taken up by microfold cells (M cells) in the follicle-associated epithelium of the Peyer's patches and isolated lymphoid follicles (ILFs) to be transported to dendritic cells (DCs) in the adjacent subepithelial dome region (Mabbott, Donaldson, Ohno, Williams, & Mahajan, 2013). Similar mechanisms may occur in ILFs of the colon and caecal patches, which were recently suggested to be the main source of IgA-producing plasma cells in the colon

(Masahata et al., 2014). By contrast, the Peyer's patches seem to be the main source of resident IgA plasmablasts in the small intestine (Masahata et al., 2014).

Whereas the epithelium primarily contains T cells, the lamina propria harbours many cell populations of both the adaptive and innate immune systems, namely B cells, T cells, dendritic cells (DCs) and macrophages. Lamina propria $CD4^+$ and $CD8^+$ T cells represent the main adaptive effectors of the intestine, and are thought to derive from conventional T cells primed in regional lymphoid structures. Accordingly, most lamina propria resident $CD4^+$ and $CD8^+$ T cells are found to display an effector memory phenotype (Sathaliyawala et al., 2013). The $CD4^+$ compartment is highly diverse, with pro-inflammatory Th_1, Th_2 and Th_{17} subsets, as well as $Foxp3^+$ regulatory T cells (T_{regs}). IL-17-producing Th_{17} cells heavily populate the lamina propria of the small intestine. These effector cells play a key role in host defence and autoimmune disease by producing the pro-inflammatory cytokines IL-17 and IL-22 (Littman & Rudensky, 2010). T_{regs} function in maintaining tolerance in the gut by modulating DC activation, by negative regulation of effector T cells and by the production of anti-inflammatory effectors, namely transforming growth factor β (TGF-β) and/or IL-10 (Josefowicz, Lu, & Rudensky, 2012). Inverse correlation between Th_{17} and T_{regs} cell numbers along the length of the intestine in mice has been reported, with Th_{17} cell numbers progressively decreasing from the duodenum to the colon while T_{regs} numbers increasing in the colon (Denning et al., 2011). Conversely, in humans, higher proportions of IL-17-producing $CD4^+$ T cells have been reported in the lamina propria of the colon and ileum compared to the jejunum (Sathaliyawala et al., 2013). By contrast, the frequencies of Th_1 and Th_2 $CD4^+$ T cells do not seem to vary significantly along the length of the human intestine (Wolff et al., 2012). Unlike most healthy tissues, normal intestinal lamina propria contains large numbers of B cell-derived plasma cells, the density of which is highest in the proximal and distal parts of the intestinal tract (Brandtzaeg & Johansen, 2005). Whereas IgM-producing B cell-derived plasma cells are scarce, IgA-producing plasma cells predominate throughout the intestine (Brandtzaeg & Johansen, 2005). IgA plays a critical role in mucosal defences. Following antigen presentation and B cell differentiation and activation, secreted IgA (sIgA) dimers bind to commensal bacteria and soluble antigens, thereby protecting the surface epithelium while shaping the composition

and function of the gut microbiota (Fagarasan, Kawamoto, Kanagawa, & Suzuki, 2010). IgA secretion has been shown to be profoundly dependent on the presence of the microbiota, as well as the associated polymeric Ig receptor (pIgR), enabling the transport of IgA into the lumen (Brandtzaeg & Johansen, 2005). Recently described innate cell types, termed innate lymphoid cells (ILCs), are believed to have important roles in intestinal immunity, inflammation and GALT development (Vonarbourg et al., 2010). Among the non-lymphoid innate effector cells that play a prominent role in gut immunity, mononuclear phagocytes, namely macrophages and dendritic cells (DCs), are implicated in uptake and presentation of antigens in the intestine. These innate cells, as well as various other lymphoid and myeloid cell types, are equipped with a set of receptors, termed pattern recognition receptors (PRRs), that detect imminent dangers such as microbial invasion, environmental or endogenous noxious substances, and elicit protective responses to contain and eliminate these harmful triggers while providing the host with resistance mechanisms to tolerate damage and restore normalcy. PRRs can be classified into five main classes that include Toll-like receptors (TLRs), nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)- like receptors (RLRs), absent-in-melanoma (AIM)-like receptors (ALRs) and C-type lectins (CTLs) (Barbe, Douglas, & Saleh, 2014). Macrophages are the most abundant leukocytes in healthy intestinal lamina propria. They have many functions in intestinal homeostasis, including phagocytosis of microorganisms and cellular debris, as well as production of pro-inflammatory mediators that drive cytokine and chemokine production and epithelial cell renewal (Mowat & Agace, 2014). They are also an important source of IL-10, which prevents inflammation by blocking pro-inflammatory responses to stimuli such as TLR ligation (Ueda et al., 2010), but also provide the survival and function of local T_{regs} in the mucosa (Murai et al., 2009). Intestinal conventional and plasmacytoid DCs (cDCs and pDCs) are critical in relaying the immune response to adaptive immunity. Both DC subtypes are recruited to the intestinal mucosa in response to inflammation or virus infection (Smit et al., 2011). Whereas conventional DCs were shown to mobilize to neighbouring lymph nodes upon antigen exposure, pDCs do not migrate, but rather drive the mobilization of cDCs in the lymph in response to TLR7 and/or TLR8 ligands (Yrlid et al., 2006). Plasmacytoid DCs

have also been involved in the induction of tolerance, with protective roles in models of small intestinal inflammation (Mizuno et al., 2012).

The microbiota

The human gut harbours more than 100 trillion microbes referred to as the gut microbiota (Wlodarska, Kostic, & Xavier, 2015). The gut microbiota plays a paramount role in health and disease in humans, the mains benefit of which include metabolic, trophic, and immunologic functions (Hooper & Macpherson, 2010). Germ-free (GF) mice are severely compromised in terms of immune function and lymphoid organ development, and it is now clear that individual members of the commensal microbiota can influence the regional specialization of the immune system along the length of the intestine (Figure 2) (Min & Rhee, 2015).

A close relationship exists between the presence of the microbiota and gut-specific B cell responses (Figure 3). Indeed, germ-free mice display markedly decreased IgA-producing cells in the intestine (Fagarasan et al., 2010). Colonization of mice with segmented filamentous bacteria (SFB) drives enhanced IgA production and increases intestinal effector $CD4^+$ T cell numbers, especially those producing IL-17 (Talham, Jiang, Bos, & Cebra, 1999). Generation of Th_{17} cells requires the presentation of SFB antigens by intestinal DCs (Ivanov et al., 2009). In addition, SFB were recently shown to promote IgA and IL-17 production by driving the development of isolated lymphoid follicles and germinal centres in PPs (Lecuyer et al., 2014). Palm and Zoete *et al*. exploited the tendency of IgA to target particularly pathogenic bacteria to identify a collection of colitogenic bacteria in human IBD that are heavily coated by IgA (Palm et al., 2014). Introduction of these colitogenic populations in GF mice rendered these mice more susceptible to chemically-induced colitis (Palm et al., 2014). A new study reports important changes in fecal IgA levels in WT mice, which were vertically transmissible and correlated with susceptibility to chemically induced colitis (Moon et al., 2015). A specific bacterial genus, *Sutterella*, caused the degradation of the secretory component of IgA and reduced levels of IgA resulted in increased mouse susceptibility to colitis (Moon

et al., 2015). Together, these recent findings provide further insight in the IgA-microbiota crosstalk in the gut (Figure 3).

Intestinal homeostasis in the gut is achieved, in part, by the balance between pro- and anti-inflammatory signals in response to the intestinal microflora. In concert with microbial populations preferentially eliciting pro-inflammatory responses, some commensals are able to dampen the inflammatory response, thereby promoting tolerance. *Bacteroides fragilis,* for example, produces polysaccharide A that was shown to restore the Th_1/Th_2 balance by inducing T_{regs}, which protected GF mice from colitis (Mazmanian, Round, & Kasper, 2008). Honda and colleagues have shown that anaerobic *Clostridium* species can induce colonic T_{regs} in mice, resulting in a TGF-β environment in the colon which protects from colitis and allergy (Atarashi et al., 2013). Short-chain fatty acids (SCFAs), such as butyrate, acetate and propionate, are produced by colonic bacteria as fermentation end products of non-digestible carbohydrates and can influence immune function in this part of the intestine. Indeed, SCFAs produced by bacteria enhanced the numbers and function of peripheral T_{regs} cells in the colon (Furusawa et al., 2013).

The role of the microbiota in ILC development has been controversial. The ILC3 sub-type was suggested to directly influence intestinal homeostasis and host defence through the production of IL-22, which can induce the production of the C-type lectin antimicrobial peptides REGenerating islet-derived protein 3β (REGIIIβ) and REGIIIγ production by IECs (Sanos et al., 2009; Vaishnava et al., 2011). Tryptophan metabolism by *Lactobacillus* species was shown to be another mechanism underpinning microbiota-driven IL-22 production by Th_{17} cells in the ileum (Zelante et al., 2013). Another group, however, suggested that the microbiota suppressed IL-22 production by ILC3 cells (Sawa et al., 2011). Conversely, it was recently shown that the microbiota promotes intestinal homeostasis through a cross talk between macrophages and ILC3. Il-1β produced by macrophages stimulated ILC3-derived GM-CSF production, which drove macrophages and DCs to produce regulatory molecules such as IL-10 and retinoic acid (Mortha et al., 2014).

The normal microbiota shapes the local immune landscape, but also crowds the intestine to prevent the colonization of opportunistic pathogens by competing for nutrients, through a mechanism termed colonization resistance (CR). Indeed, colonization

of various pathogens such as *Salmonella typhimurium, Shigella flexneri, Pseudomonas aeruginosa* and *Vibrio cholera* were previously shown to be exacerbated by antibiotic treatment in mice, showing the critical role of the sole presence of commensals in the gut in preventing colonization (Bohnhoff, Drake, & Miller, 1954; Hentges & Freter, 1962; Lawley et al., 2009). Recent studies suggest that pathogen elimination by crowding is most effective with commensals that are metabolically related to the pathogen. *E. coli*, but not *Bacteroides* species, were shown to effectively outcompete *Citrobacter rodentium*, a metabolically related pathogen used to mimic enteropathogenic (EPEC) and enterohaemorragic (EHEC) *E. coli* infections in mice (Kamada et al., 2012). Similar observations were reported in mice harbouring a limited microbiota, which were increasingly colonized by *Salmonella typhimurium* (Endt et al., 2010). Thus, the regulation of intestinal immune cells by commensal bacteria has profound effects that are not limited to immune homeostasis, but also include the regulation of the balance between beneficial and potentially pathogenic commensals (referred to as pathobionts) within the microbiota, and resistance to intestinal pathogens and pathology.

Intestinal epithelium turnover

The intestinal epithelium has a remarkable capacity to self-renew, within only 4 to 5 days (van der Flier & Clevers, 2009). Undifferentiated stem cells proliferate in the crypt region, forming a pool of partially differentiated transit amplifying cells. These intestinal epithelial progenitor cells subsequently arrest cell division to migrate upwards along the crypt-villus axis, presumably driven by the constant generation of new cells in the crypt area (Yen & Wright, 2006). After 3 days, terminally differentiated cells reach the top of the villi from where they are shed. An initial study had suggested that IEC slough off the epithelium through anoikis, a specialized form of apoptosis driven by detachment from the extracellular matrix (Gilmore, 2005). Indeed, active caspase-3 staining indicated that shed cells were undergoing apoptosis (Gilmore, 2005). Transgenic mouse studies showed that TNF-induced cell shedding could be blocked when caspase activation was inhibited (Marchiando et al., 2011). Conversely, mice deficient in caspase-3 did not show any impairment in intestinal epithelium turnover, suggesting that apoptosis is dispensable for gut homeostasis (Brinkman et al., 2011; Duprez et al., 2011). In line with this

observation, a recent study has shown that IECs at the top of the villi did not undergo caspase-3 activation. Instead, sloughing occurs mechanically due to crowding and apoptosis occurred after and not prior to cell detachment (Eisenhoffer et al., 2012). Even though apoptosis seems to be dispensable for the structural integrity of the gut, compelling evidence suggest that deregulated or excessive apoptosis can lead to severe gut pathology.

Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are a group of complex inflammatory diseases of the intestine, characterized by chronic inflammation and lymphoid hyperplasia, ulcerative lesions, submucosal edema and fibrosis in affected areas (Neuman, 2007). IBD is believed to be associated with an increased "Westernized lifestyle" in industrialized nations, including increased hygiene, use of antibiotics, a high fat diet and other environmental factors that converge on changes in the microbiota (Bernstein, 2006). Consistent with a major role of the environment in IBD, the incidence and prevalence of IBD have progressively increased not only in developed but also in developing countries in the last ten years (Bernstein, 1999; Kaser, Zeissig, & Blumberg, 2010). IBD affects approximately 1.5 million Americans and 2.2 million individuals in Europe (Cosnes, Gower-Rousseau, Seksik, & Cortot, 2011). In 2012, approximately 233,000 Canadians were living with IBD, corresponding to a prevalence of 0.67%, with about 10,200 incident cases every year (Rocchi et al., 2012). Despite advances in the field, there are still no known cures for IBD, and patients are prescribed anti-inflammatory and immunosuppressive medication, antibiotics and in severe cases surgery to manage the disease (Rutgeerts, Vermeire, & Van Assche, 2009; Van Assche, Vermeire, & Rutgeerts, 2009). Anti-tumor necrosis factor (TNF) antibody therapy, a widely used treatment for IBD, has been shown to alleviate symptoms and restore mucosal barrier integrity (Colombel et al., 2010). However, a fraction of IBD patients do not respond to anti-TNF treatment (Hanauer et al., 2006). *Atreya et al*. investigated the basis of this non-responsiveness using molecular imaging, and showed that expression of membrane-

bound TNF on myeloid cells in the gut dictated responsiveness to anti-TNF therapy (Atreya et al., 2014).

Depending on the nature and site of inflammation, IBD comprehends two main phenotypes: Crohn's disease (CD) and ulcerative colitis (UC) (Figure 4). CD affects various segments of the GI tract, but the terminal ileum is most commonly affected. Whereas inflammation in CD is patchy, UC spreads in continuity from the rectum, and frequently involves the periappendiceal region (Both, Torp-Pedersen, Kreiner, Hendriksen, & Binder, 1983; Sanders, 1998). The depth of inflammation also differs in both diseases; in UC, inflammation is superficial and confined to the colonic mucosa, whereas it is transmural in CD, leading to granuloma formation and fibrosis (Neuman & Nanau, 2012). The balance between pro- and anti-inflammatory cytokines is essential for gut homeostasis, and its deregulation is linked to IBD with a Th_1-dominated response in CD versus a Th_2 response in UC (Muzes, Molnar, Tulassay, & Sipos, 2012).

IBD is multifactorial and results from the interaction between complex genetic predisposition and environmental factors that converge on the gut microbiota (Leone, Chang, & Devkota, 2013). A three-step scenario leading to IBD onset has been proposed: an intestinal microorganism with pathogenic characteristics (a pathogen or a pathobiont) penetrates the epithelial barrier and provokes an inflammatory response. This acute inflammation is tissue-destructive and can lead to a vicious cycle of microbiota translocation, chronic inflammation and tissue ulceration (Sewell, Marks, & Segal, 2009).

The complex genetics of IBD

The genetic component of IBD has been thoroughly investigated in the last decade. The prevalence of IBD varies amongst populations, and a positive family history is exhibited amongst first-degree relatives in 6 to 22% of affected patients (Monsen, Bernell, Johansson, & Hellers, 1991). Twin studies and segregation analysis suggest that monozygotic twins had higher concordance rates of IBD compared to dizygotic twins. Moreover, the concordance rate of CD in monozygotic twins was estimated at 30-35% compared to 10-15% in UC, suggesting that CD may have a stronger genetic component compared to UC (Ahmad, Marshall, & Jewell, 2006; Spehlmann et al., 2008). A recent genome-wide association studies (GWAS) meta-analysis has identified 163 loci that are

associated with IBD risk in the Caucasian population (Jostins et al., 2012). Although these risk loci only explain a fraction of heritability, they have begun to shed light on potential pathogenic mechanisms in IBD. Indeed, genetic evidence has shown that the impaired recognition and killing of commensal bacteria might contribute to IBD development, as has been suggested by the fact that numerous IBD associated genes regulate host-microbial interactions. NOD2, an intracellular sensor of bacterial peptidoglycan, was the first identified susceptibility factor for IBD, and thereafter numerous pathways that interact with NOD2 signalling were also implicated (Ogura et al., 2001). Indeed, NOD2 signalling is involved in processes such as autophagy, endoplasmic reticulum (ER) stress, Th_{17} differentiation, as well as in MDP-independent pathways, such as single stranded RNA (ssRNA)-induced type I IFN responses (Figure 5) (Barbe et al., 2014). These functions are impaired in cells from patients with the CD-associated NOD2 mutation 3020insC (Hugot et al., 2001; Ogura et al., 2001). Although the exact role of NOD2 deficiency in IBD still remains unclear, multiple hypotheses have been suggested. Saleh *et al*. list potential pathways in which NOD2 deficiency could cause IBD, namely by modulating adaptive immunity through lymphoid tissue development, by signalling in the myeloid compartment that indirectly contributes to the maintenance of the microbiota, or by acting via antimicrobial peptide-producing Paneth cells (Saleh & Trinchieri, 2011).

Mutations in the autophagy regulatory genes autophagy related gene 16-like 1 (ATG16L1) and immunity related GTPase family (IRGM) are also linked to CD (Hampe et al., 2007), and defects in autophagy were associated with impaired bacterial killing (Cooney et al., 2010; Homer, Richmond, Rebert, Achkar, & McDonald, 2010). Interestingly, NOD2 and autophagy proteins were shown to regulate the function of Paneth cells (Figure 6) (Homer et al., 2010). Indeed, previous studies have reported the diminished expression of Paneth cell α-defensin expression in CD patients harbouring the 3020insC NOD2 mutation (Ogura et al., 2003; Wehkamp et al., 2004). Concordantly, *Nod2*$^{-/-}$ mice displayed impaired Paneth cell function, leading to overt ileal bacterial colonization (Petnicki-Ocwieja et al., 2009). Similarly, ATG16L1 hypomorphic mice ($ATG16L1^{HM}$) mice showed Paneth cell granule packaging defects, mirroring what was seen in CD patients harbouring the T300A variant in ATG16L1 (Cadwell et al., 2008). It

is thus possible that impaired NOD2 or autophagy function might result in the accumulation of intestinal commensal bacteria that have the capacity to locally invade the intestinal mucosa and trigger an abnormal inflammatory response.

Genetic variants underlying IBD can vary in frequency depending on the cohort ethnicity, raising the possibility that some such variants may have emerged in the context of differential genetic pressure across populations. Indeed, NOD2 and ATG16L1 polymorphisms are observed in Caucasian patients, but not in Japanese, Korean and Chinese patients with IBD (Gazouli et al., 2010). One of the first genome-wide studies for IBD was conducted in a Japanese cohort in 2005, where a strong correlation was established between polymorphisms in the tumor necrosis alpha super family 15 (TNFSF15) gene and Crohn's disease (Yamazaki et al., 2005). TNFSF15 encodes a TNF-related factor named TL1A, a cytokine required for T cell co-stimulation (Meylan, Richard, & Siegel, 2011). Since then, four GWAS were conducted Japanese populations, and a recent meta-analysis has shown that NFKBIL1 and CD14 were not identified as UC susceptibility genes in Caucasian populations (Arimura et al., 2014). Hallmark genetic variations of the human leukocyte antigen (HLA) region were also reported to differ between European and Japanese IBD patients (Fernando et al., 2008). For example, two mutations in the HLA DR beta 1 gene (HLA-DRB1), HLA-DRB1*1502 and HLA-DRB1*0103, have shown consistent association with UC (Ahmad et al., 2003). The HLA-DRB1*0103 allele was shown to be strongly associated to Caucasian UC, but not in Japanese UC (Arimura et al., 2014). Conversely, the HLA-DRB1*1502 haplotype was highly prevalent in Japanese UC patients (20-25%) (Matsumura et al., 2008), but only rarely frequent in European populations (<1%) (Ahmad et al., 2006).

Currently, GWAS are typically powered to characterize frequent variants (>1% frequency) and do not include the contribution of rare variants (<1% frequency). Exome sequencing has been used to identify rare variants, such as those in IL-10 receptor A (IL-10RA) in early-onset IBD (Glocker et al., 2009). Early functional studies attempting to determine causality have largely focused on coding variants, whereas the vast majority of polymorphisms linked to IBD are in non-coding regions. Such non-coding SNPs can be associated with qualitative or quantitative changes in gene expression. Furthermore, intergenic changes may affect transcription factor binding sequences, locus accessibility,

translational efficiency and trans-regulators such as noncoding RNAs and microRNAs (miRNAs). Important variants called expression quantitative trait loci (eQTLs) that influence the expression of genes have been detected for approximately half of the IBD risk regions, indicating that allele-specific gene expression changes might contribute to disease risk (Khor, Gardet, & Xavier, 2011).

Viruses and the pathogenesis of IBD

While the role of bacteria as an etiological factor in triggering relapse has been well studied, the role of viruses in the pathogenesis of IBD still remains elusive. The dominant theory suggests that certain infections that occur during childhood may predispose to IBD onset later in life (Bosca-Watts et al., 2015). For example, early case-control studies showed that the incidence of CD was higher in children that had been born in the three months following measles outbreaks (Wakefield et al., 1993). These findings stimulated the execution of observational studies, based on the relationship between early viral infections and the development of IBD. During the same time period, parotiditis virus infection was also proposed as a pathogenic agent in IBD (Montgomery, Morris, Pounder, & Wakefield, 1999). However, reports addressing perinatal measles and mumps infection and co-infection in IBD did not generate the minimum data necessary to establish a causal biological relationship, leaving the implication of both viruses in the pathogenesis of IBD uncertain. Some viruses, however, have been proposed to modulate inflammatory diseases, namely Cytomegalovirus (CMV) (Waters & Ahmad, 2011) and Epstein-Barr virus (EBV) (Yanai, Shimizu, Nagasaki, Mitani, & Okita, 1999). While the causal involvement of viral infections in IBD still remains unclear, the potential for viral infection to trigger loss of tolerance to bacteria in a susceptible host has recently been shown (Cadwell et al., 2010). ATG16L1^{HM} mice infected with murine norovirus displayed abnormal Paneth cell function and morphology similar to that seen in CD patients homozygous for the risk allele of ATG16L1 (Cadwell et al., 2010). Noroviruses are encapsidated positive-sense RNA viruses responsible for the majority of epidemic nonbacterial gastroenteritis in humans (Goto et al., 2009). While the human strain causes disease, murine norovirus (MNV) infection has been shown to be asymptomatic in immunosufficient mice (Thackray et al., 2007) and lethal for mice genetically deficient in

type I IFN signalling (Karst, Wobus, Lay, Davidson, & Virgin, 2003). MNV infection of ATG16L1^{HM} mice displayed exacerbated DSS colitis compared to DSS treatment alone or compared to wild-type mice (Cadwell et al., 2010), which demonstrates how a genetic factor and an environmental agent could contribute to the pathogenesis of CD (Figure 7).

TNFR1 signalling

TNF is a pleiotropic, pro-inflammatory cytokine whose function is implicated in a wide range of inflammatory, infectious, malignant and autoimmune conditions. TNF is produced in response to infection to confer immunity to the host. While the effects of TNF during infection are beneficial, tight regulation of TNF production is required to protect the host from its detrimental activities (Apostolaki, Armaka, Victoratos, & Kollias, 2010). It is now well established that TNF exerts pathological effects in IBD, specifically impacting the intestinal barrier, which is supported by the positive effects of anti-TNF therapy in restoring barrier integrity in IBD patients (Zeissig et al., 2004). Various studies utilizing mouse models of intestinal disease driven by excessive TNF production have solidified this notion. For example, deletion of the adenylate-uridylate-rich elements (ARE) in the 3'-UTR of the TNF gene in mice (TNF$^{\Delta ARE}$ mice) results in TNF mRNA stabilization and a severe spontaneous intestinal inflammation (Kontoyiannis, Pasparakis, Pizarro, Cominelli, & Kollias, 1999). Genetic ablation of *TNFR1* in TNF$^{\Delta ARE}$ mice abrogated the colitis phenotype, underlying the importance of TNF signalling in orchestrating the early inflammatory response leading to disease (Kontoyiannis et al., 1999).

It remains unclear, however, how TNF exerts pathology in IBD. TNF signalling results in either inflammation and cell survival or cell death (Figure 8). Upon engagement and trimerization of the TNF receptor, TNF induces the assembly of complex I, a membrane-bound complex which consists of the TNF receptor-associated protein with a death domain (TRADD) and the serine-threonine kinase RIP1. Subsequent ubiquitination of RIP1 by various E3 ubiquitin ligases, namely the cellular inhibitor of apoptosis proteins (cIAP)1 and cIAP2 and the linear ubiquitination assembly complex (LUBAC), leads to the recruitment of the inhibitor of nuclear factor kappa-B (NF-κB) kinase (IKK)

complex to complex I and induction of the canonical NF-κB survival pathway (Hacker & Karin, 2006; Pateras, Giaginis, Tsigris, Patsouris, & Theocharis, 2014). While RIP1 polyubiquitination promotes survival, RIP1 deubiquitination, by the inhibition of E3 ubiquitin ligases or by the action of deubiquitinases such as A20 or cylindromatosis (CYLD), promotes destabilization of complex I into an alternative cytosolic complex termed complex IIa (Flusberg & Sorger, 2015). This death inducing signalling complex (DISC) promotes cell death by apoptosis, and is composed of procaspase-8, Fas-associated protein with death-domain (FADD), TRADD and the long isoform of FLICE-like inhibitory protein (FLIP) to trigger canonical death receptor-induced apoptosis (Lavrik, Golks, & Krammer, 2005). In this context, caspase-8 cleaves caspase-3 and caspase-7, which subsequently leads to the classical morphological manifestations of apoptotic cell death, namely membrane blebbing, cellular shrinkage, nuclear condensation and DNA fragmentation (Lalaoui, Lindqvist, Sandow, & Ekert, 2015). The cell death capacity of TNFR1 complex II through apoptosis was recently complemented by the recent discovery of a novel programmed cell death modality, termed necroptosis, or regulated necrosis (Holler et al., 2000). In this model, RIP1 deubiquitination leads to heterodimerization through the RIP homotypic interaction motif (RHIM) with its associated kinase, RIP3, leading to the formation of complex IIb, or the necrosome (Micheau & Tschopp, 2003). Under steady state conditions, studies have shown that RIP1-RIP3 interaction is controlled by the proteolytic activity of caspase-8, which cleaves RIP1 and RIP3, blocking necrosome formation (He et al., 2009). Indeed, necroptosis was shown to proceed in a context devoid of active caspase-8, either chemically or by the action of endogenous inhibitors, such as the short isoform of FLIP ($FLIP_s$) (Holler et al., 2000). Similar to caspase-8, FLIP contains two death effector domains, allowing its association with the DISC, subsequently blocking caspase-8 mediated apoptosis (Scaffidi, Schmitz, Krammer, & Peter, 1999). Additionally, caspase-8 was shown to cleave CYLD, a RIP1 deubiquitinase, which promotes survival (O'Donnell et al., 2011). When caspase-8 is inhibited, RIP1 and RIP3 undergo trans-autophosphorylation and assemble into cytosolic amyloid structures composed of filaments containing RIP3-RIP3 homodimers and RIP1-RIP3 heterodimers (J. Li et al., 2012). RIP3 then phosphorylates mixed lineage kinase-domain like protein (MLKL),

followed by MLKL homo-trimerization and translocation to the plasma membrane (Murphy et al., 2013; Zhao et al., 2012). The MLKL oligomer subsequently triggers membrane permeabilization by mechanisms involving its association with ion channels (Cai et al., 2014) or by direct pore formation via interaction with membrane phospholipids (Dondelinger et al., 2014). A role for the mitochondria in necroptosis was proposed by Wang *et al*., suggesting that MLKL caused mitochondrial fragmentation via mitochondrial phosphatase PGAM5, resulting in increased reactive oxygen species (ROS) and increased intracellular calcium (Wang, Jiang, Chen, Du, & Wang, 2012). Conversely, a later study demonstrated the dispensable role for mitochondria, as cells devoid of the respiring organelle could undergo necroptosis (Tait et al., 2013).

Cell death in inflammatory diseases

Despite the initial belief that cell death is a consequence of inflammation, increasing evidence suggests that cell death can precede, trigger or even amplify the inflammatory response (Figure 9). The following recapitulates recent findings linking cell death by apoptosis or necroptosis to inflammation, with a focus on intestinal disease.

Apoptosis in intestinal disease

As previously mentioned, TNFR complex I mediates survival through activation of the NF-κB pathway. Impairment in complex I formation shifts the TNF-induced response towards cell death. Even though apoptosis seems to be dispensable for the structural integrity of the gut at normalcy, increasing evidence suggests that impairment in the survival pathway leading to overt cell death can lead to pathology in the gut. IEC-specific deletion of NF-κB essential modulator (NEMO) in mice ($NEMO^{IEC\text{-}KO}$) results in spontaneous inflammation in the colon, in which intestinal barrier integrity is compromised due to increased IEC apoptosis. The pathology in these mice was ameliorated by genetically ablating *Tnfr1* (Nenci et al., 2007). Similarly, IEC-specific deletion of IKK1 and IKK2 ($IKK1/2^{IEC\text{-}KO}$) and TAK1 ($TAK1^{IEC\text{-}KO}$) resulted in increased susceptibility to spontaneous colitis (Kajino-Sakamoto et al., 2008; Nenci et al., 2007). $TAK1^{IEC\text{-}KO}$ mice died one day after birth, and histological examination of the intestine revealed increased apoptosis (Kajino-Sakamoto et al., 2008). The anti-apoptotic role of

cFLIP in regulating caspase-8 activity was emphasized in mice in which cFLIP was specifically deleted in IECs ($cFLIP^{IEC\text{-}KO}$). Interestingly, these mice were embryonically lethal. A tamoxifen-inducible model of IEC-specific deletion of cFLIP ($cFLIP^{iIEC\text{-}KO}$) mice displayed increased caspase-3 and caspase-8 activity in the intestine (Wittkopf et al., 2013). Therefore, it seems that modulation of apoptosis in IECs is critical in maintaining intestinal homeostasis during development and adulthood.

Necroptosis: an inflammatory cell death antagonized by apoptosis

The physiological relevance of necroptosis was initially investigated in embryonic development in reports showing that mice deficient in *Caspase-8*, *Fadd* or *Flip* died in utero at embryonic day 10.5 (E. E. Varfolomeev et al., 1998; Yeh et al., 1998; Yeh et al., 2000). Interestingly, these mice shared common pathological features, and all converged towards excessive necroptosis. Interestingly, caspase-8-deficient mice genetically ablated for *Rip3* were viable (Kaiser et al., 2011; Oberst et al., 2011), and *Rip1* deficiency in FADD knock out mice also restored viability at birth (Zhang et al., 2011). These studies thus highlight a crosstalk between apoptosis and necroptosis, where necroptotic cell death is induced when the apoptotic machinery is impaired. Moreover, this crosstalk was shown to be crucial for both embryonic development and throughout adulthood (Piao et al., 2012; Weinlich et al., 2013). Thus far, in most *in vivo* experimental models, sensitization to necroptosis was achieved by blocking apoptosis by genetically ablating the FADD-caspase-8 signalling pathway.

Interestingly, cell type-specific deletion of various apoptotic effectors in mice has shed light on the role of necroptosis in the onset of inflammation. For instance, keratinocyte-specific deletion of *caspase-8* and *Fadd* in mice resulted in spontaneous inflammation of the skin, which was RIP3 dependent (Weinlich et al., 2013). Similarly, in a mouse model of double-stranded RNA-induced retinal degeneration, RIP3-dependent cell death of retinal pigment epithelial cells triggered inflammation (Murakami et al., 2014). MLKL- and RIP3-driven necroptosis were shown to worsen inflammation and tissue injury in a mouse model of acute pancreatitis and sepsis (Duprez et al., 2011; Wu et al., 2013).

Necroptosis in IBD

Two recent studies however have demonstrated that necroptosis of IECs can lead to intestinal inflammation with features that resemble those seen in IBD (Gunther et al., 2011; Welz et al., 2011). In these studies, deletion of *caspase-8* and *Fadd* specifically in IECs ($FADD^{IEC\text{-}KO}$, $Casp8^{IEC\text{-}KO}$) resulted in mice with spontaneous TNF-driven intestinal inflammation that was RIP1- and RIP3-dependent. Dying cells in the gut of $FADD^{IEC\text{-}KO}$ mice were devoid of active capsase-3, and exhibited cellular and organelle swelling, impaired plasma membrane and absence of chromatin condensation, all hallmarks of necrotic cell death (Welz et al., 2011). Additionally, $FADD^{IEC\text{-}KO}$ and $Casp8^{IEC\text{-}KO}$ mice suffered a robust decrease in Paneth cells, which is concomitant with the constitutive expression of RIP3 in this cell type in humans and mice (Gunther et al., 2011). Furthermore, crossing $FADD^{IEC\text{-}KO}$ mice with mice deficient in *Rip3*, *Cyld* or *Tnf* rescued the colitis phenotype, suggesting that the disease resulted from overt necroptosis downstream of TNF signalling (Welz et al., 2011). Similarly, $Casp8^{IEC\text{-}KO}$ mice were very susceptible to TNF-induced intestinal tissue injury, which was preventable by the administration of Necrostatin-1, a RIP1 kinase inhibitor (Gunther et al., 2011). Interestingly, in contrast to $FADD^{IEC\text{-}KO}$, $Casp8^{IEC\text{-}KO}$ did not develop colitis, suggesting differential roles for FADD and caspase-8 in the colonic epithelium or differential environmental factors in different animal facilities (Gunther et al., 2011). Collectively, these studies suggest a pathological role for necroptosis in conditional models of IBD, where IEC necrotic death causes a breach in barrier integrity and impaired AMP production, allowing microbiota translocation and chronic inflammation. In line with these findings, evidence from human studies has equally suggested the presence of necrotic cell death in the intestine of patients with IBD. Indeed, necrotic cell death features were observed in the intestinal epithelium of CD patients, namely cellular swelling, large vacuolar structures and hollow nuclei (Dourmashkin et al., 1983). In inflamed ileal sections of CD patients, decreased Paneth cell numbers correlated with increased necrotic cell death in the crypts, suggesting that Paneth cell death by necroptosis is a feature of CD (Gunther et al., 2011). A recent report has shown that children with IBD had elevated expression of RIP3 and MLKL in the colon and ileum, which was potentially linked to inflammation (Pierdomenico et al., 2014).

GOALS OF THIS STUDY

Apoptosis, an immunologically silent (and often tolerogenic) form of cell death, has long been considered as an essential process for normal embryonic development and adult tissue homeostasis. A novel function of apoptosis signalling has recently been discovered that furthers supports its function in tissue tolerance: the antagonism of a pathogenic inflammatory cell death modality referred as necroptosis (E. E. Varfolomeev et al., 1998; Yeh et al., 1998). In the gut, mice with impaired extrinsic apoptosis in their intestinal epithelium through an IEC-specific deletion of *caspase-8* or *Fadd* display spontaneous ileitis and colitis, respectively, inferring a pathological role for necroptosis in this conditional model of IBD (Gunther et al., 2011; Welz et al., 2011). However, whether necrotic cell death is causative in IBD where the apoptotic machinery is intact was not clear.

To address the role of necroptosis in IBD pathogenesis, we proposed the following research aims: i) to detect and investigate the contribution of necroptosis and apoptosis in preclinical animal models of IBD, ii) to interrogate the influence of viral infection on cell death modalities in the gut in these models, and iii) to identify expression SNPs in cell death genes and examine their association with IBD in humans. We used the TRUC mouse model to explore necroptosis induction in a spontaneous TNF-driven model of intestinal inflammation, and cIAP2-deficient mice treated with DSS, as an established DSS colitis model. Through the investigation of cell death modalities and effector mechanisms in these models, our primary objective was to identify potential therapeutic targets for IBD.

Materials and methods

Animal strains

Wild-type (WT; C57Bl/6), cIAP2$^{-/-}$(*Birc3*$^{-/-}$), *Rag1*$^{-/-}$ mice on a C57Bl/6J background, TRUC (*Tbx21*$^{-/-}$*Rag2*$^{-/-}$), *Rag2*$^{-/-}$ mice on a Balb/c background were bred and maintained at the McGill University Life Science Complex animal Facility. WT and cIAP2$^{-/-}$ mice were used at over 6 weeks of age. TRUC mice and age-matched *Rag1*$^{-/-}$ mice were used at 6, 10 and 14 weeks of age. All animal experiments were performed under guidelines of the animal ethics committee of McGill University.

Cell culture

HT29 cells (colon adenocarcinoma, ATCC® HTB-38™) were maintained in 5% CO2 at 37 ^{oC} in complete McCoy's 5A modified medium (Hyclone) supplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine and 100 μg ml^{-1} penicillin/streptomycin. To obtain cell lysates, cells were seeded in 6 well plates at 0.6 x 10^6 cells per well overnight in Opti-MEM (Life technologies), and pre-treated with a Smac mimetic (BV6; Genentech), a pan caspase inhibitor (Z-VAD-fmk; Invivogen), and Necrostatin-1s (Nec-1s; Calbiochem) for 30 minutes, then stimulated with hTNF-α (Peprotech) for 6 hours. Cells were lysed in the well using 150 μl of Laemmli sample buffer. Samples were then boiled at 95^{oC} for 5 minutes and vortexed for 30 seconds. To obtain the MLKL oligomer (Figure 12C), cells were lysed in Laemmli sample buffer devoid of a reducing agent (without SDS), and samples were processed accordingly.

Immunoblotting

For mouse colon and ileum protein lysates, cells were lysed in B150 (20 mM Tris-HCl (pH 8.0), 150 mM KCl, 10% glycerol, 5 mM $MgCl_2$, and 0.1% NP-40) supplemented with a protease inhibitor tablet (Roche). Lysates were then complemented with Laemmli sample buffer, boiled for 5 minutes and analyzed by SDS-polyacrilamide gel. Membranes were blocked 1h at room temperature using 5% milk in TBS 0.01% Tween. Primary antibodies for human HT29 cell lysates included: human cIAP1 (R&D; AF8181), human

cIAP2 (R&D; AF8171), active caspase-3 (Cell signalling; 9661), RIP1 (BD; 610549), MLKL (Abnova, clone 3B2; H00197259-M02) and Ser358 phospho-MLKL (Abcam; 187091). Primary antibodies for mouse intestinal tissue included: RIP1 (BD; 610549), MLKL (Abcam; 172876), RIP3 (ProSci; 2283), FADD (Santa Cruz; 6036), CYLD (Invitrogen; 437700), and cFLIP (Santa Cruz; 8347), cleaved caspase-3 (Cell Signalling; 9661L), cleaved caspase-8 (Cell Signalling; 429S) and lysozyme P (Santa Cruz; 27958). Primary antibody incubation was done overnight at 4ºC in 5% milk TBS 0.01% Tween and 10 mM sodium azide (NaN_3). Membranes were then washed 3 times in TBS 0.01% Tween, followed by secondary antibody incubation for 1 hour at room temperature. Following secondary antibody incubation, membranes were washed 5 times 5 minutes with TBS 0.01% Tween. Depending on the sensitivity of the primary antibody, membranes were developed using enhanced chemiluminescence reagent (ECL, Amersham), ECL+ (Amersham) or West Femto (Pierce).

Norovirus infection and DSS colitis

Male *Birc3*$^{-/-}$ mice that were over 6 weeks of age were infected by oral gavage with CR6 murine norovirus (MNV) (Washington University), at 10^5 PFU per mice by oral gavage in 100 µl of PBS, controls were gavaged with 100 µl of PBS. Fecal pellets were collected every other day to monitor viremia. Viremia was not measured in this thesis but future work will quantify viral content by quantitative plaque assay as previously described (Cadwell et al. 2010) or RT-qPCR using the following primers: Forward: 5'-CACGCCACCGATCTGTTCTG-3', Reverse: 5'-GCGCTGCGCCATCACTC-3'. After 7 days, *Birc3*$^{-/-}$ mice were given 2.5% DSS in drinking water for 6 days (days 0 – 5), then placed on regular water for 3 days (Days 6 – 8). Mice were monitored daily for body weight loss. Mice were sacrificed on day 8 for tissue collection. Histological colitis scoring was measured by quantifying areas of healthy, damaged and eroded areas of the colon. Mucosal lymphoid aggregates, located above the lamina propria, were counted in the region adjacent to the ano-rectal junction in the colon, as previously reported (Cadwell et al., 2010).

Immunohistochemistry

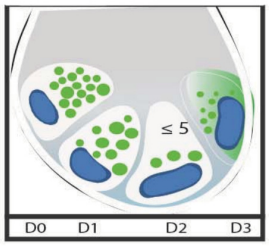
Colonic and ileal tissue were fixed in 10% formalin for 24 hours. Paraffin-embedded and haematoxylin and eosin histology slides were generated by the GCRC Histology Facility. Unstained slides were deparaffinized using the following sequence of treatments: twice in Xylene for 5 minutes, 5 minutes in Xylene 50:50 99% EtOH, 5 minutes in EtOH 99%, 5 minutes in 95% EtOH, 5 minutes in 70% EtOH, 5 minutes in 50% EtOH and 5 minutes in dH_20. Antigen retrieval was done by boiling the rehydrated slides in citrate buffer (0.1M citric acid, 0.1M sodium citrate) for 20 minutes, and then left to cool at room temperature for 1 hour. Permeabilization was performed using 0.25% Triton X-100 in PBS for 20 minutes at room temperature. Cell viability was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Life technologies), according to the manufacturer's protocol. Slides were then blocked in FBS 10% and 3% BSA in PBS for 30 minutes at 37ºC. Primary antibody staining then followed, using the following antibodies: cleaved caspase-3 (R&D; MAB835), e-cadherin (Abcam; 15148), Lysosyme P (Santa Cruz; 27958), MLKL (Abcam; 172876), CD-31 (Abcam; 28364), in 3% BSA in PBS for 2 hours at 37ºC. Both primary antibodies were used at 1:300. Slides were then washed 3 times in 0.05% Tween in PBS. Secondary antibody step consisted of Hoeschst staining (1:5000), Alexa Fluor anti-Rabbit 594 (1:500) and Alexa Fluor anti-mouse 488 (1:500) secondary antibodies for 30 minutes at 37ºC. Samples were then washed 3 times in 0.05% Tween in PBS, once in PBS alone and once in dH_20. Slides were mounted using mounting media (Glycerol 50:50, glycine buffer: 0.2M glycine, 0.3M NaCL, NaN_3, pH 8.6). Slides were visualized on a Zeiss Axiovert 200M Fully Automated Inverted Microscope, and pictures were taken using a Zeiss Axiocam Monochrome (HR) camera.

RNA extraction and mRNA quantification

HT29 cells were stimulated with increasing concentrations IFN-α (Sigma; SRP4594) and IFN-γ (R&D; 285-IF101) for 16h. Total RNA was extracted from cells with Trizol reagent (Invitrogen; 48190011), followed by isopropanol precipitation. Reverse-transcription was done with 2 μg total RNA to complementary DNA (cDNA) by using random hexamers and the M-MLV reverse transcriptase (Invitrogen; 28025-013)

according to the manufacturer's protocol. qPCR with reverse transcription was performed using iTaq SYBR green supermix (Bio-Rad; 172-5852).

Paneth cell analysis



Ileal sections were stained for lysozyme and TUNEL by immunofluorescence as described above. Paneth cells were then graded according to previous reports: D0 (Healthy), D1 (Abnormal), D2 (Depleted) and D3 (Diffuse) (Cadwell et al. 2007; Image adapted from Adolph et al. 2013). Paneth cell death and total cell death were quantified as numbers of $TUNEL^{+}lysozyme^{+}$ cells and total $TUNEL^{+}$ cells per field, respectively. Paneth cell numbers were quantified as the number of $Lysozyme^{+}$ cells per well-oriented crypt.

Statistical analysis

Data are represented as mean ± standard error or standard deviation as indicated. Two-tailed student t-test was used to evaluate significant differences amongst two groups. A p-value of <0.05 was considered statistically significant. Two-way Anova test was used for survival analyses. Statistical analysis and graphs were generated using Prism version 5.0c (GraphPad Software).

RESULTS

TRUC mice display increased necrosome formation in the colon

Mice deficient in T-bet and Rag2, referred to as *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ ulcerative colitis (TRUC) mice, are a model of TNF-induced spontaneous intestinal inflammation that closely resembles human UC (Garrett et al., 2007). T-bet is a T-box transcription factor expressed in T cells, B cells, NK cells, NKT cells and dendritic cells (DCs), and is involved in the regulation of numerous cytokines, chemokines and chemokine receptors. Loss of T-bet induces deregulated TNF production by lamina propria myeloid dendritic cells (Garrett et al., 2007). The overproduction of TNF by DCs is especially deleterious in the absence of regulatory T cells in the *Rag2*$^{-/-}$ background. High levels of mucosal TNF drive epithelial cell death leading to ulceration of the epithelium and a leaky barrier. These mice are protected from colitis when genetically ablated for *Tnfr1*, confirming the key role of TNF in the pathology of this model (Garrett et al., 2007). Moreover, anti-TNF antibody administration alleviated the colitis in TRUC mice and decreased cell death (Garrett et al., 2007). TRUC mice harbour a colitogenic microbiota, as proven by a transferable disease in cross-fostering experiments, as well as rescue of the colitis phenotype when treating the mice with antibiotics (Garrett et al., 2007).

To decipher the nature of cell death in the colon of TRUC mice, we performed histological examination of colon sections of 14-week-old TRUC mice. We observed increased IEC hyperplasia and colonic epithelium tissue damage compared to age-matched Balb/c control mice (Figure 10). Garrett *et al*. reported increased cell death in the colon of TRUC mice, as indicated by increased TUNEL staining (Garrett et al., 2007). Because TUNEL staining does not discriminate between apoptosis and necroptosis, an active caspase-3 (CC3)/ TUNEL co-stain was performed. Increased early and late apoptotic cell death was observed in TRUC mice, as shown by increased CC3+ TUNEL- and CC3+ TUNEL+ cells, respectively (Figure 10). Moreover, TUNEL-only staining in the colonic epithelium of TRUC mice, interpreted as caspase-independent necrotic cell death, was increased compared to controls of the same age (Figure 10).

We sought to assess the protein level of expression of various cell death effectors in the gut of these mice. Among the necrosome components that were assessed, RIP3 and

MLKL levels were upregulated in the colon of TRUC mice compared to age-matched *Rag1*$^{-/-}$ controls (Figure 11). Even though this increase in expression might be a consequence of increased inflammation, increased expression of RIP3 and MLKL are indicative of increased sensitivity towards necroptosis (Gunther et al., 2011). Collectively, these results suggest that cell death by necroptosis and necrosome formation are increased in the colon of TRUC mice compared to control animals.

Cellular inhibitor of apoptosis protein (cIAP)1 and 2 are protective against necroptosis

Cell death by necroptosis downstream of TNFR1 was shown to occur when pro-survival effectors, such as the cIAPs, as well as pro-apoptotic effectors, particularly caspase-8, were blocked, allowing formation of the necrosome (Murphy et al., 2013). We sought to reproduce what was shown in the literature to confirm the cellular requirements favouring necroptosis. HT29 cells are colon adenocarcinoma cells that are widely used in the field to characterize mechanisms of necroptosis (Dondelinger et al., 2014; Remijsen et al., 2014). As previously mentioned, chemical induction of necroptosis downstream of TNFR1 requires inhibition of the cIAPs (Vince et al., 2007). Stimulation of HT29 cells with TNF induced cIAP2 expression (Figure 12A), which is consistent with previous observations (Mitsiades et al., 2002). On the other hand, TNF treatment led to cIAP1 downregulation (Figure 12A). cIAP1 degradation downstream of TNF has also been observed by others, as TNFR2-induced activation of the non-canonical NF-κB pathway leads to degradation of cIAP1 by TNF-like weak inducer of apoptosis (TWEAK) protein (E. Varfolomeev et al., 2007). To fully inhibit cIAP expression, we used an IAP antagonist, also referred to as a SMAC mimetic (BV6), and observed rapid degradation of both cIAP1 and cIAP2 (Figure 12A). TNFR1 engagement in the absence of cIAPs leads to complex IIa formation, which subsequently promotes caspase-3 cleavage by caspase-8. By using the pan-caspase inhibitor Z-VAD.fmk, caspase activity was blocked, as shown by absence of the active caspase-3 p20 fragment on immunoblot (Figure 12B). Phosphorylation and homo-oligomerization of MLKL at the plasma membrane is the last reported step of necroptosis (Cai et al., 2014). Accordingly, trimerization of phosphorylated MLKL was detected during TNFR1-induced necroptosis in HT29 cells, which was rescued by blocking RIP1 kinase activity using Nec-1 (Figure 12C). Together,

these *in vitro* results recapitulate the reported role of cIAPs in protecting against cell death by apoptosis and necroptosis. We concluded that mice genetically devoid of cIAPs would be an adequate *in vivo* platform in our characterization of cell death modalities during inflammatory disease of the intestine.

Norovirus infection exacerbates DSS-induced colitis in cIAP2-deficient mice

It was recently reported that ATG16L1^{HM} mice infected with MNV displayed worsened DSS-induced colitis compared to uninfected controls (Cadwell et al., 2010). Interestingly, ATG16L1^{HM} mice infected with MNV displayed abnormalities in granule packaging and unique patterns of gene expression in Paneth cells, which are known to have elevated expression of RIP3 (Gunther et al., 2011). It is thus plausible that MNV infection promotes disease exacerbation by stimulating alternative and more pathogenic types of cell death in IECs, namely RIP3-dependent necroptosis. Indeed, many viruses, including both poxviruses and herpesviruses, inhibit caspase-8 activity (Mocarski, Upton, & Kaiser, 2012) and therefore have the potential to unleash this alternate death pathway. Necroptosis is thought to have emerged as a product of evolution that acts as a *bona fide* autonomous host defence pathway subjected to a specific viral countermeasure (Mocarski, Guo, & Kaiser, 2015). This is confirmed by the fact that some viral pathogens developed the ability to block necroptosis. For example, Murine cytomegalovirus (MCMV) encodes a viral inhibitor of RIP activation (vIRA), which contains an amino-terminal RHIM motif able to block signal transduction leading to cell death and cytokine production (Upton, Kaiser, & Mocarski, 2010, 2012). Homotypic RHIM-RHIM motif binding enables various interactions between RIP3 and other proteins, such as the viral nucleic acid sensor DNA-induced activator of interferon (DAI), TIR-domain-containing adapter-inducing interferon β (TRIF) and RIP1, which ultimately culminate in the execution of necroptosis (Upton et al., 2010, 2012). Interestingly, our laboratory has shown that influenza infection of cIAP2-deficient mice displayed increased necrotic cell death in the lungs that was abrogated by the genetic deletion of RIP3 (Rodrigue-Gervais et al., 2014). Necroptosis was triggered due to suppression of apoptosis by host expression of c-FLIP$_S$ in the lung. The observation that lung inflammatory pathology was independent of differences in viral loads suggests that necroptosis is a detrimental

bystander consequence of influenza infection. In light of these reports, we hypothesized that MNV infection, in a mechanism similar to that exerted by influenza, could trigger a switch in cell death modalities towards necroptosis in the intestine of genetically predisposed mice, causing pathology.

The cIAPs inhibit both apoptosis and necroptosis by promoting RIP1 kinase activity and pathological responses to death or microbial ligands. Our laboratory has shown that cIAP2 is required to protect against influenza A-mediated pulmonary necrosis (Rodrigue-Gervais et al., 2014). Notably, other IAPs, including cIAP1 and XIAP do not compensate for cIAP2 loss in conferring protection to the lung (Rodrigue-Gervais et al., 2014). Moreover, Dagenais *et al*. have recently demonstrated the role of cIAP2 as a critical regulator of intestinal homeostasis, as cIAP2-deficient mice are more susceptible to DSS-induced colitis compared to WT mice (Dagenais et al., 2015). We therefore hypothesized that MNV infection could tip the cell death balance towards necroptosis in DSS treated *Birc3*$^{-/-}$ mice and exacerbate colitis. In order to investigate this hypothesis, we infected *Birc3*$^{-/-}$ mice with the persistent MNV CR6 strain at day 0, and on day 6 post-infection, subjected the mice to acute DSS-induced tissue injury and colitis. 2.5% DSS in the drinking water was administered for 5 days, followed by 3 days of regular drinking water (Figure 13A). DSS-treated *Birc3*$^{-/-}$ mice infected with MNV.CR6 displayed exacerbated colitis compared to MNV.CR6-only or DSS-only controls, as reflected in reduced colon length at necropsy on day 12 (Figure 13B, C). Consistently, MNV.CR6-infected *Birc3*$^{-/-}$ mice showed increased DSS-induced tissue damage, with increased damaged and fully eroded areas in the colon (Figure 13D, E). MNV.CR6-infected DSS-treated mice also displayed increased colonic lymphoid aggregates compared to uninfected controls, similar to what was seen with MNV.CR6-infected ATG16L1^{HM} mice treated with DSS (Figure 13F) (Cadwell et al., 2010). Collectively, these results demonstrate that MNV.CR6 infection exacerbates DSS-induced colitis in cIAP2-deficient mice.

CR6 infection modulates cell death machinery expression in the gut

We sought to examine the mechanisms by which MNV infection could modulate disease severity. Western blot analysis of colon homogenates showed increased protein

expression of lysozyme P in CR6-infected DSS-treated mice compared to DSS- or CR6-only cohorts (Figure 14A). Increased lysozyme P expression in the colon, a protein highly expressed by Paneth cells, has been shown to correlate with disease severity (Fahlgren, Hammarstrom, Danielsson, & Hammarstrom, 2003). Interestingly, CR6 infection alone in *Birc3*$^{-/-}$ mice induced increased levels of active caspase-3 compared to uninfected controls (Figure 14A), suggesting a role for apoptosis in CR6-mediated disease exacerbation. To investigate the cell death modalities occurring in DSS-induced colitis in the context of CR6 infection, colon sections were labelled by immunofluorescence with active caspase-3 antibodies and TUNEL. This analysis revealed the presence of both apoptosis and caspase-3-independent cell death in all treatment groups (Figure 14B). CR6 infection did not influence a shift in cell death programs, shown by similar proportions of apoptotic (TUNEL^{+} Caspase-3^{+}) and necrotic (TUNEL^{-} Caspase-3^{-}) cell death (Figure 14C). Data we have recently generated, however, suggests that cell death occurs more prominently in *Birc3*$^{-/-}$ mice at earlier time points during the time course of DSS treatment (Dagenais et al., 2015). Indeed, caspase-3 activation and total cell death levels in the colon were increased in *Birc3*$^{-/-}$ mice compared to WT controls on days 3 and 5 post-DSS and these differences were less apparent at endpoint, as intestinal cell death at later time points is compensated for by engagement of IL-18-mediated tissue repair pathway (Allen et al., 2010; Dagenais et al., 2015; Dupaul-Chicoine et al., 2010). This could underlie the similar cell death burdens seen between infected and uninfected DSS-treated cIAP2-deficient mice (Figure 14C), suggesting that necropsy on days 3 and 5 following DSS is required to adequately depict cell death modalities in the colon during CR6 + DSS colitis.

The observation that ATG16L1^{HM} mice harboured defective Paneth cells linked functional autophagy to Paneth cell homeostasis. Adolph *et al*. showed that disruption of autophagy function or the unfolded protein response (UPR) in IECs leads to their compensatory engagement, and that defects in both mechanisms resulted in spontaneous transmural ileitis (Adolph et al., 2013). Genetically altering UPR function in IECs by Xbp1 deletion lead to increased ER stress, which subsequently lead to Paneth cell abnormalities and spontaneous enteritis, which was worsened on an IEC-specific Atg16l1-deficient background (Adolph et al., 2013). The ileal phenotype in these mice

was similar to what was seen in MNV.CR6-infected ATG16L1^{HM} mice, suggesting that MNV.CR6 could act as an environmental source of ER stress that impacts autophagy and Paneth cell function. We therefore hypothesized that MNV.CR6 infection in cIAP2-deficient mice could disrupt Paneth cell homeostasis in a similar fashion, thereby causing inflammation. Immunoblot analysis of distal ileal tissue shows that MNV.CR6 infection alone caused a mild decrease in Paneth cell-associated lysozyme P protein expression (Figure 15). Moreover, decreased lysozyme P expression levels was accompanied by increased active caspase-3 and caspase-8 expression, suggesting that MNV.CR6 infection in *Birc3*$^{-/-}$ mice could promote Paneth cell death by apoptosis (Figure 15). Additionally, elevated levels of the short isoform of c-FLIP$_S$, an NF-κB-inducible inhibitor of caspase-8, suggests that both apoptotic and necroptotic cell death could contribute to the pathogenesis of MNV.CR6 in the ileum (Figure 8). Together, these results suggest that MNV.CR6-mediated Paneth cell death could play a role in the exacerbation of DSS colitis in cIAP2 deficient mice.

CR6 infection in Birc3$^{-/-}$ *mice induces Paneth cell abnormalities and cell death*

Lysozyme P protein downregulation caused by MNV.CR6 infection in *Birc3*$^{-/-}$ mice in the ileum suggested that Paneth cell integrity could be affected (Figure 15). We therefore sought to examine the morphology, health and functionality of these secretory cells in fixed ileal tissue sections. Paneth cells were detected using lysozyme P labelling by immunofluorescence in ileal sections, and graded according to previous reports (Figure 16C) (Adolph et al., 2013; Cadwell et al., 2010). Untreated and DSS-treated uninfected *Birc3*$^{-/-}$ mice displayed >80% normal (D0) Paneth cells, whereas MNV.CR6-infected untreated and DSS-treated *Birc3*$^{-/-}$ mice displayed <50% normal Paneth cells, confirming the hypothesis that MNV.CR6 infection impacts Paneth cell health in *Birc3*$^{-/-}$ mice (Figure 16A, B). Moreover, DSS treatment in MNV.CR6 infected mice worsened Paneth cell abnormalities, as shown by <40% healthy and >20% diffuse (D3) Paneth cells (Figure 16B). These results thus consolidate the hypothesis that MNV.CR6 infection impairs Paneth cell homeostasis in *Birc3*$^{-/-}$ mice.

In light of the observed MNV.CR6-induced increased death effector protein expression in the ileum (Figure 15), together with MNV.CR6-induced Paneth cell

abnormalities, we sought to investigate the cell death programs present in the ileum. MNV.CR6 infection in *Birc3*$^{-/-}$ mice caused increased cell death in the ileum, as shown by increased TUNEL staining compared to untreated controls (Figure 17C). Interestingly, Paneth cell death was observed in MNV.CR6-infected mice, and DSS treatment augmented the number of TUNEL^{+} Lysozyme^{+} cells (Figure 17A, D). MNV.CR6 infection was sufficient to significantly reduce the number of lysozyme^{+} cells per crypt compared to uninfected *Birc3*$^{-/-}$ mice (Figure 17B). Collectively, these data suggest that MNV.CR6 infection in cIAP2-deficient mice promotes Paneth cell abnormalities and cell death.

Role of MLKL in colitis

The last reported executioner of necroptosis, MLKL, has been shown to be an indispensable component of the necrosome (Wu et al., 2013). Even though the function of MLKL in cell death is now well established, alternative roles still remain to be unravelled. Since MLKL protein expression is induced in the inflamed colon (Figure 10), we decided to examine the expression and localisation of MLKL during colitis. Colon section labelling by immunofluorescence shows that shed IECs in 16-week-old TRUC mice have increased MLKL expression compared to Balb/c control of the same age (Figure 18A). This finding suggested that overt TNF levels in TRUC mice colitis burdens the colon with excessive cell death, that is either apoptotic (Figure 10B) or necroptotic in nature (Figure 18A), and could contribute to TNF-driven pathology.

We sought to investigate the expression pattern of MLKL in another mouse model of colitis, namely *Birc3*$^{-/-}$ mice on acute 2.5% DSS regimen. Histological analysis of colon sections revealed that MLKL expression co-localizes with the endothelial cell marker CD31 (Figure 18B). It has been shown that during DSS-induced colitis, pathogenic angiogenesis occurs to promote lymphocyte and monocyte trafficking to the site of inflammation (Tolstanova et al., 2011). Angiogenic cytokines produced at the site of inflammation promotes endothelial cell activation, leading to increased vascular permeability, and degradation of the underlying basement membrane. Endothelial cell proliferation and directional migration then ensues, guided by local extracellular matrix (ECM) remodelling to allow novel sprouting blood vessels to form (Chidlow, Shukla,

Grisham, & Kevil, 2007). Endothelial cell apoptosis has been shown to play a potent role in vessel remodelling. Indeed, inhibition of apoptosis blocked *in vitro* vascular morphogenesis (Segura et al., 2002), and negatively charged membranes of apoptotic endothelial cells promoted sprouting of adjacent vessels by causing localized plasma membrane hyperpolarization *in vivo* (Weihua, Tsan, Schroit, & Fidler, 2005). It is therefore appealing to hypothesize that endothelial cell death by other cell death modalities, namely MLKL-driven necroptosis, could promote pathogenic angiogenesis during disease. Accordingly, a recent study reports lung endothelial cell necroptosis induced by red blood cell (RBC) transfusion, which increased susceptibility to lung inflammation (Qing et al., 2014).

To conclude our investigation on the role of cell death in IBD, we sought to characterize genetic polymorphisms that modulate the expression of certain genes by searching for regulatory eQTLs. We collaborated with Dr. Michel Georges (Université de Liège) to interrogate 52 genes in cell death and innate immunity pathways. Using Illumina DNA microarray and cDNA from 9 primary cell types from 350 normal Caucasian individuals, strong eQTLs were identified based on a threshold of significance set at a p-value $<10^{-5}$. Among the primary cell types that were examined, CD4+ T cells, CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD15+ myelomonocytic cells displayed a strong eQTL for MLKL (Figure 19A). Because of the critical role of MLKL as a decisive pro-necrotic effector and the strong eQTL in this gene, we focused on defining the regulatory mechanisms governing its expression. In collaboration with Dr. Phillipe Gros (McGill University), we analyzed Chip-Seq, RNA-seq and ImmGen datasets and determined that *Mlkl* expression is induced by type I & II IFN and LPS (data not shown). Validation RT-qPCR analysis of cDNA extracted from WT B6 bone marrow-derived macrophages (BMDMs) stimulated with IFN-γ for 3h or 21h was performed, and *Mlkl* mRNA expression increased 4- to 5- fold upon IFN-γ treatment (Figure 19B). Increased MLKL gene expression was also assessed in human HT29 cells treated with IFN-α or IFN-γ for 16h, where MLKL mRNA expression increased 3-fold upon IFN-α treatment and increased 2-fold following IFN-γ stimulation (Figure 19C). Together, these results demonstrate that MLKL gene expression is inducible by type I and type II IFN.

DISCUSSION

In this work, we attempted to address the role of cell death modalities in the pathogenesis of IBD primarily by using animal models of the disease. Our objectives were to 1) develop methods to discriminate between apoptosis and necroptosis in the intestine, 2) to monitor cell death induction during disease progression, 3) to assess whether environmental triggers, such as viral infection, modulate the nature and extent of cell death in the gut promoting pathogenesis, and 4) to define expression SNP in cell death genes and their association with IBD in humans. Our results reveal a correlation between cell death induction and the severity of IBD-like symptoms in two mouse models and identify eSNPs in a number of human genes encoding cell death effectors. The implications of these findings and the future directions arising from this work are discussed below.

Necrosome formation and cell death in TRUC colitis

TRUC mice are a spontaneous TNF-driven mouse model of colitis that closely resembles human UC (Garrett et al., 2007). 14-week-old TRUC mice showed increased hyperplasia and damage to the colonic epithelium compared to age-matched Balb/c control mice (Figure 10). Garrett *et al*. reported increased cell death in the colon of TRUC mice, as indicated by increased TUNEL staining. Our findings corroborate these results, as we have demonstrated the presence of TUNEL+ cells in the colon of TRUC mice compared to controls (Figure 10). In an attempt to discriminate between apoptotic and necrotic cell death, co-labelling with active caspase-3 showed the presence of both apoptotic and necrotic cell death in the colon of TRUC mice. Interestingly, the proportion of $TUNEL^{+}Caspase\text{-}3^{-}$ cells was higher in advanced disease compared to early disease, suggesting that caspase-independent necrotic cell death is linked to disease severity in this model.

Increased necrosome protein expression *in vivo,* namely MLKL and RIP3, was shown to be indicative of increased sensitivity towards necroptosis (Gunther et al., 2011). We report that MLKL expression is robustly induced in the colon of 6- and 10-week-old

TRUC mice compared to *Rag1*$^{-/-}$ controls, accompanied with a mild induction in RIP3 and FADD expression, suggesting increased necrosome formation. This is concordant with a recent report showing increased MLKL and RIP3 expression in the inflamed colon and ileum of children with IBD (Pierdomenico et al., 2014). The short isoform of FLIP ($FLIP_s$) blocks apoptosis by acting as a dominant negative inhibitor of caspase-8, thus promoting RIP3-dependent necrosis, whereas FLIP long ($FLIP_L$) blocks both apoptosis and necroptosis (Oberst et al., 2011). Surprisingly, $FLIP_s$ expression was completely suppressed in the colon of TRUC mice compared to that of age-matched *Rag1*$^{-/-}$ mice (Figure 11). Together, these results suggest that the cell death burden present in the colon of TRUC mice comprehends both necroptosis and apoptosis. However, the discrepancy of whether TNF-α-mediated necroptosis acts as a driver or a consequence of disease still remains elusive in this model.

Virus-plus-gene interaction during DSS colitis

The cIAPs are important regulators of innate immunity. They are essential for cytokine and chemokine production following NOD1 and NOD2 stimulation (Bertrand et al., 2009), mediate the activation of MAP kinases pathways downstream of Toll-like receptor (TLR)4 (Bertrand et al., 2009; Tseng et al., 2010), and are required for efficient activation of the inflammasome (Labbe, McIntire, Doiron, Leblanc, & Saleh, 2011). Besides their role in immunity, cIAPs are prominent arbiters of cell death downstream of various death receptors and PRRs. In line with previous reports (Feoktistova et al., 2011), we show that chemical inhibition of cIAPs *in vitro* promotes TNF-α-induced apoptosis in HT29 cells (Figure 12). Further inhibition of caspase activity was required to induce phospho-MLKL trimerization and necroptosis, which was rescued by chemically inhibiting RIP1 kinase activity with Nec-1.

In vivo, whereas cIAP1 (*Birc2*$^{-/-}$)- or cIAP2 (*Birc3*$^{-/-}$)-deficient mice are viable, deletion of both genes results in embryonic lethality, which is delayed to shortly after birth through co-deletion of *Tnfr1*, *Ripk1* or *Ripk3* (Moulin et al., 2012). As cIAP1 and cIAP2 are the main IAPs involved in the negative regulation of the necrosome (also referred as ripoptosome) (Feoktistova et al., 2011), we hypothesized that mice deficient

in cIAP1 or cIAP2 would be increasingly susceptible to cell death-driven pathology. We demonstrate here that *Birc3*$^{-/-}$ mice are more susceptible to DSS-induced colitis compared to WT mice (Figure 13). Increased caspase-3-dependent and –independent cell death modalities were measured in the colon of DSS-treated mice, agreeing with our hypothesis that cIAP deficiency *in vivo* promotes exuberant cell death signalling. Our recent data suggest that apoptosis is the main pathological cell death program involved in DSS-induced colitis in *Birc3*$^{-/-}$ mice, as the colitis phenotype was alleviated by chemically inhibiting caspase activity *in vivo* (Dagenais et al., 2015) Indeed, active caspase-3 was robustly induced in the colon of DSS-treated *Birc3*$^{-/-}$ mice (Figure 14). This is in line with a previous report that demonstrated that mice genetically ablated of the pro-apoptotic inducer PUMA were resistant to DSS colitis (Qiu et al., 2011). Collectively, our results show that cIAP2-deficient mice treated with DSS are burdened with pathogenic cell death in the colon that is mainly apoptotic in nature.

Previous studies have shown that certain viral pathogens, such as herpes virus and murine cytomegalovirus (MCMV), have the ability to sabotage the host cell death machinery and block apoptosis, in which necroptosis acts as a "back-up" cell death pathway (Mocarski et al., 2012). This is of particular relevance in the context of IBD, as environmental factors such as viral infections can have a profound impact on disease. For instance, higher prevalence of Epstein-Barr virus (EBV) was detected in intestinal tissue from patients with IBD with exacerbated disease compared to patients in remission (Dimitroulia, Pitiriga, Piperaki, Spanakis, & Tsakris, 2013).

A recent report has shown that virus-plus-gene interactions in CR6 murine norovirus (MNV.CR6)-infected ATG16L1^{HM} mice caused exacerbation of DSS-induced colitis compared to uninfected controls (Cadwell et al., 2010). We therefore considered the possibility that MNV infection in a mouse model of intestinal colitis could modulate disease by shifting the intestinal cell death burden towards necroptosis. Surprisingly, *Birc3*$^{-/-}$ mice infected with MNV.CR6 were more susceptible to DSS-induced colitis compared to uninfected DSS-treated controls (Figure 13). Others have shown that MV.CR6 infection alone in immunocompetent mice is asymptomatic (Tomov et al., 2013). Similarly, *Birc3*$^{-/-}$ mice infected with MNV.CR6 did not show signs of disease (data not shown). MNV.CR6 infection alone, however, was sufficient to induce active

caspase-3 in the colon of *Birc3*$^{-/-}$ mice, suggesting that MNV.CR6 promotes apoptosis in the colon of *Birc3*$^{-/-}$ mice. Notably, there were no significant differences in apoptotic or necrotic colonic cell deaths in MNV.CR6-infected and uninfected DSS-treated mice, possibly due to the delayed time point of analysis (Dagenais et al., 2015). Collectively, we demonstrate that cIAP2-deficient mice display increased susceptibility to DSS-induced colitis when infected with MNV.CR6.

MNV.CR6 disrupts ileal homeostasis by affecting Paneth cell function in Birc3$^{-/-}$ *mice*

The discovery of ATG16L1 as a genetic risk factor in CD has unravelled the critical role of autophagy in CD pathogenesis (Hampe et al., 2007; Jostins et al., 2012). CD patients homozygous for the ATG16L1 risk allele displayed Paneth cell granule abnormalities, similar to those observed in mice with hypomorphic ATG16L1 activity (Cadwell et al., 2008). Autophagy is thus required for Paneth cell homeostasis.

Paneth cell abnormalities were exacerbated in ATG16L1^{HM} mice infected with MNV.CR6 compared to uninfected controls, suggesting that MNV.CR6 infection negatively modulates Paneth cell function in an autophagy-deplete system (Cadwell et al., 2010). We observe a similar phenotype, where cIAP2 deficient mice infected with MNV.CR6 display abnormal Paneth cell morphologies compared to uninfected controls (Figure 16). Therefore, it is possible that cIAP2 participates in autophagy-related processes. Accordingly, a recent publication demonstrates that XIAP and cIAP1 induce autophagosome formation by upregulating Beclin 1 expression via activation of the NF-kB pathway (Lin et al., 2015). *In vitro* pharmacological inhibition of XIAP reduced autophagic activity in HeLa cells (Lin et al., 2015). It is therefore possible that cIAP2 maintains Paneth cell homeostasis by directly stabilizing autophagy through induction of Beclin-1.

Strikingly, the Paneth cell phenotype observed in MNV.CR6-infected ATG16L1^{HM} mice and *Birc3*$^{-/-}$ mice closely resembled those in mice specifically deleted in IECs for both Atg16l1 and Xbp1 (Atg16l1/Xbp1$^{\Delta IEC}$ mice), a protein involved in the unfolded protein response (UPR) (Adolph et al., 2013). The UPR is a well-conserved stress response among mammals and metazoans, where stress induced by misfolded proteins sensing in

the endoplasmic reticulum (ER) leads to a set of death programs when the cells fail to successfully adapt to ER stress or restore homeostasis (Hiramatsu, Chiang, Kurt, Sigurdson, & Lin, 2015). When autophagy is functional, UPR-driven activation of NF-kB is inhibited by compensatory activation of autophagy, where autophagosome formation blocks inositol requiring enzyme 1 α (IRE1α)-mediated NF-kB activation (Adolph et al., 2013). In Atg16l1/Xbp1$^{\Delta IEC}$ mice, Xbp1 deletion causes constitutive UPR activation in IECs, leading to overt ER stress, which signals towards unregulated NF-kB activation, ultimately leading to inflammation, cell death and disease (Adolph et al., 2013). In summary, impaired autophagy in IECs increasingly sensitizes the intestinal epithelium to environmental triggers that further challenge the UPR pathway. This suggests that ER stress is a stimulus that can disrupt Paneth cell homeostasis when the autophagosome is impaired, which correlates with augmented ER stress in Paneth cells of CD patients harbouring the ATG16L1^{T300A} variant (Deuring et al., 2014). Respectfully, we hypothesize that MNV.CR6 infection and DSS treatment act as environmental triggers that overwhelm Paneth cells with ER stress, leading to abnormalities and disease when autophagy is impaired by cIAP2 deficiency.

In the ileum of *Birc3*$^{-/-}$ mice, MNV.CR6 infection alone strongly induced the activity of apoptotic caspases (Figure 15). Moreover, increased levels of Paneth cell death occurred when DSS-treated cIAP2-deficient mice were previously infected with MNV.CR6 (Figure 16). These results support our hypothesis of an exaggerated ER stress-mediated response in MNV.CR6-infected DSS-treated *Birc3*$^{-/-}$ mice that leads to aberrant pathogenic cell death in the ileum. Accordingly, ER stress-driven cell death by apoptosis was shown to be an early event during disease in Atg16l1/Xbp1$^{\Delta IEC}$ mice (Adolph et al., 2013). A group have recently shown that loss of an associated IAP, named X-linked inhibitor of apoptosis protein (XIAP), resulted in increased sensitivity to ER stress-induced cell death (Hiramatsu et al., 2014). Therefore, aside from its potential role in autophagy, cIAP2 may also act to protect against cell death downstream or ER stress-related inflammatory signals. Interestingly, CR6 infection alone was sufficient to reduce the number of Paneth cells per crypt to half, while DSS-only treated and untreated controls harboured similar levels (Figure 17B). This could be explained by the increased potential of DSS to elicit intestinal survival and repair signals (Chassaing, Aitken,

Malleshappa, & Vijay-Kumar, 2014), whereas MNV.CR6 was shown to mainly signal through type I IFN signalling pathway (Kernbauer, Ding, & Cadwell, 2014).

Collectively, we suggest that cIAP2 is required for autophagosome stabilization and Paneth cell homeostasis, and is protective of cell death downstream or ER stress-associated stimuli.

Regulation of MLKL expression in colitis

MLKL is indispensable in the execution of necroptosis (Wu et al., 2013). It has been shown to be upregulated in the inflamed colon and ileum of CD patients (Pierdomenico et al., 2014), which mirrors what we report in the colon of TRUC mice (Figure 10). Shedding IECs highly express MLKL, suggesting that overt TNF signalling can promote IEC necroptosis in the gut of TRUC mice (Figure 18A). Interestingly, we report that MLKL expression in the colon co-localizes with the endothelial cell marker CD-31 in *Birc3*$^{-/-}$ mice treated with 2.5% DSS (Figure 18B). During DSS-induced colitis, pathogenic angiogenesis occurs to promote lymphocyte and monocyte trafficking to the site of inflammation (Tolstanova et al., 2011). Angiogenic cytokines produced at the site of inflammation promotes endothelial cell activation, leading to increased vascular permeability, and degradation of the underlying basement membrane. Endothelial cell proliferation and directional migration then ensues, guided by local extracellular matrix (ECM) remodelling to allow novel sprouting blood vessels to form (Chidlow et al., 2007). Endothelial cell apoptosis has been shown to play a potent role in vessel remodelling. Indeed, inhibition of apoptosis appeared to block *in vitro* vascular morphogenesis (Segura et al., 2002), and negatively charged membranes of apoptotic endothelial cells were suggested to promote sprouting of adjacent vessels by causing localized plasma membrane hyperpolarization *in vivo* (Weihua et al., 2005). It is therefore appealing to stipulate that endothelial cell death by other cell death modalities, namely MLKL-driven necroptosis, could drive pathogenic angiogenesis during disease. Mice deficient for Mlkl (*Mlkl*$^{-/-}$ mice) would therefore display reduced angiogenesis formation in the gut during chemically induced colitis, for example, and this would verify the requirement of MLKL in pathogenic angiogenesis. One could also chemically inhibit

MLKL function *in vivo* with necrosulfonamide (NSA) by intraperitoneal injection prior to chemically induced colitis.

To broaden our investigation on the regulation of MLKL gene expression, we sought to identify genetic polymorphisms that had a tangible effect on MLKL gene regulation. Over the past decade, GWAS have been paramount in identifying numerous loci related to complex diseases. Recent GWAS of IBD identified 163 genomic regions associated with CD or UC (Jostins et al., 2012). With GWAS-identified risk loci spanning immense genomic regions, one strategy in aiding the characterization of causative genes is to identify eQTL that strongly correlate with the disease. Not only can eQTL mapping identify unsuspected candidates, it also increases the power of GWAS analyses (L. Li et al., 2013). Here, we report a strong eQTL in *MLKL* in various cell types, suggesting that mutations in MLKL regulatory elements might potentially influence its expression in disease (Figure 19A). Whether these mutations actually induce or repress gene expression requires further investigation (Westra et al., 2015). Modulation of MLKL expression across cell types therefore shapes the sensitivity to necroptotic cell death in a cell type specific fashion.

We report that interferon signalling promote MLKL gene expression in mice and humans *in vitro*. Type I IFN are massively produced in response to viral and other microbial infections, and play a pivotal role in innate defences (Gonzales-van Horn & Farrar, 2015). Necroptosis downstream of type I IFN signalling has been shown to block intracellular pathogen dissemination in macrophages such as in *S. typhimurium* infection (Robinson et al., 2012). Cell death and inflammation were attenuated when mice were genetically ablated for *Ifnar*$^{-/-}$ (Robinson et al., 2012). This mechanism was shown to be dependent on the transcription factors IFN-stimulated gene factor 3 (ISGF3), STAT1/2 and IRF9 (McComb et al., 2014). Thus type I IFN signalling might confer anti-viral or anti-bacterial replication by inducing MLKL-driven necroptosis.

CONCLUSIONS AND FUTURE PERSPECTIVES

Necroptosis has been detected in various models of intestinal inflammatory diseases, however its precise role in the pathogenesis of such disorders is still not clear. Our experiments in TRUC mice have shown that the necroptotic burden in the colon correlates with disease severity, suggesting that necrotic cell death-derived signals in the colon participate in the inflammatory milieu of this model. The causative nature of this cell death modality, however, still remains elusive. To clarify this discrepancy, deletion of genes indispensable to the execution of necroptosis, namely RIP3 or MLKL, on a *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ background would reveal whether necrotic cell death acts as a trigger to spontaneous TNF-driven colitis in TRUC mice. It seems more likely, however, that necroptosis acts as an amplifier of inflammation during intestinal disease. To determine whether necroptosis is a *bona fide* driver of inflammation, we plan to chemically induce colitis with DSS in mice genetically devoid of *Mlkl* or *Rip3*, in an attempt to alleviate the colitis phenotype in these mice compared to treated WT mice. We also plan to infect *Mlkl*$^{-/-}$ and *Rip3*$^{-/-}$ mice with *C. rodentium,* to fully address the involvement of necroptosis during chemical- and bacterial-induced intestinal disease. To unequivocally confirm that caspase-independent necrotic cell death occurs in the gut of mouse models of intestinal inflammation, we plan to co-stain with a yet-to-be generated antibody that targets murine phosphorylated MLKL with caspase-3 and TUNEL by immunofluorescence in colon tissue. With this assay, we hope to clearly identify necrotic cells in this model, and utilize this technique to interrogate the contribution of necroptosis in other models of inflammation.

Through our findings in *Birc3*$^{-/-}$ mice, we consolidate the notion of cIAP2 as a guardian of cell survival. We show that cIAP2 deficiency in mice increases disease susceptibility to DSS colitis. Furthermore, we suggest an unanticipated dual role for cIAP2 in both autophagy, critical for Paneth cell function and homeostasis, and protection against ER stress-driven cell death. In order to interrogate the role of cIAP2 in autophagy, we plan to quantify and assess autophagy function in Paneth cells of cIAP2-deficient mice orally infected with MNV.CR6 compared to infected WT mice via western blot and immunofluorescence labelling of autophagy marker p62 in ileal tissue. Cytosolic

accumulation of p62 was shown to correlate with defective autophagy, and p62 accumulation at the bottom of ileal crypts in infected *Birc3*$^{-/-}$ mice would further suggest a positive role for cIAP2 in Paneth cell homeostasis. Autophagy is highly active in cells that generate and secrete great amounts of protein. Thus, to broaden our investigation of the role of cIAP2 in autophagy, we plan to examine the functionality of another highly secretory cell type in the gut, namely Goblet cells. Impaired mucus production in cIAP2 due to defective autophagy in mice could possibly be an additional mechanism by which *Birc3*$^{-/-}$ mice are more susceptible to DSS colitis. Once we strengthen the link between cIAP2 and autophagy, we plan to assess ER stress levels in the ileum of MNV-infected WT and *Birc3*$^{-/-}$ mice treated with DSS by probing for GRP78, a sensor of misfolded proteins that accumulates during ER stress. If autophagy is hypofunctional in Paneth cells of *Birc3*$^{-/-}$ mice, ER stress-driven IRE1α-mediated activation of NF-ϰB cannot be dampened by autophagosome assembly, leading to inflammation and cell death. We plan to further characterize the inflammatory milieu in the ileum during MNV-mediated DSS-induced disease exacerbation in cIAP2-deficient mice by doing a panel of ELISAs for pro- and anti-inflammatory cytokines (i.e. IL-6, TNF, IL-17, IL-10, TGF-β) of ileal organ culture as well as RT-qPCR of RNA extracted from ileal tissue. We also aim to investigate the cell death programs that govern Paneth cell death downstream of ER stress signalling by co-staining for caspase-3, TUNEL and phospho-MLKL, in order to clearly phenotype the fashion by which these AMP-producing cells succumb.

Finally, we show eQTLs associated with the differential expression of MLKL in healthy individuals. In order to correlate these polymorphisms with disease susceptibility, we plan to align these results with known IBD-risk loci from CD and UC patients. Additionally, to fully explore the areas of interest involved in the genetic regulation of cell death genes, we also plan to map for eQTLs located outside of the IBD-risk genomic regions, in an attempt to fully encompass the body of eQTL effects that influence susceptibility to cell death.

With this research, we hope to build a more comprehensive picture of the role of cell death in intestinal disease, and advance our understanding in dissecting the mechanisms underlying cell death programs, namely apoptosis and necroptosis, which will potentially drive the development of targeted clinical therapies.

REFERENCES

Adolph, T. E., Tomczak, M. F., Niederreiter, L., Ko, H. J., Bock, J., Martinez-Naves, E., . . . Blumberg, R. S. (2013). Paneth cells as a site of origin for intestinal inflammation. *Nature, 503*(7475), 272-276. doi: 10.1038/nature12599

Ahmad, T., Armuzzi, A., Neville, M., Bunce, M., Ling, K. L., Welsh, K. I., . . . Jewell, D. P. (2003). The contribution of human leucocyte antigen complex genes to disease phenotype in ulcerative colitis. *Tissue Antigens, 62*(6), 527-535.

Ahmad, T., Marshall, S. E., & Jewell, D. (2006). Genetics of inflammatory bowel disease: the role of the HLA complex. *World J Gastroenterol, 12*(23), 3628-3635.

Allen, I. C., TeKippe, E. M., Woodford, R. M., Uronis, J. M., Holl, E. K., Rogers, A. B., . . . Ting, J. P. (2010). The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med, 207*(5), 1045-1056. doi: 10.1084/jem.20100050

Apostolaki, M., Armaka, M., Victoratos, P., & Kollias, G. (2010). Cellular mechanisms of TNF function in models of inflammation and autoimmunity. *Curr Dir Autoimmun, 11*, 1-26. doi: 10.1159/000289195

Arimura, Y., Isshiki, H., Onodera, K., Nagaishi, K., Yamashita, K., Sonoda, T., . . . Shinomura, Y. (2014). Characteristics of Japanese inflammatory bowel disease susceptibility loci. *J Gastroenterol, 49*(8), 1217-1230. doi: 10.1007/s00535-013-0866-2

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., . . . Honda, K. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature, 500*(7461), 232-236. doi: 10.1038/nature12331

Atreya, R., Neumann, H., Neufert, C., Waldner, M. J., Billmeier, U., Zopf, Y., . . . Neurath, M. F. (2014). In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat Med, 20*(3), 313-318. doi: 10.1038/nm.3462

Barbe, F., Douglas, T., & Saleh, M. (2014). Advances in Nod-like receptors (NLR) biology. *Cytokine Growth Factor Rev, 25*(6), 681-697. doi: 10.1016/j.cytogfr.2014.07.001

Bernstein, C. N. (1999). Cancer surveillance in inflammatory bowel disease. *Curr Gastroenterol Rep, 1*(6), 496-504.

Bernstein, C. N. (2006). Natural history and management of flat and polypoid dysplasia in inflammatory bowel disease. *Gastroenterol Clin North Am, 35*(3), 573-579. doi: 10.1016/j.gtc.2006.07.010

Bertrand, M. J., Doiron, K., Labbe, K., Korneluk, R. G., Barker, P. A., & Saleh, M. (2009). Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. *Immunity, 30*(6), 789-801. doi: 10.1016/j.immuni.2009.04.011

Birchenough, G. M., Johansson, M. E., Gustafsson, J. K., Bergstrom, J. H., & Hansson, G. C. (2015). New developments in goblet cell mucus secretion and function. *Mucosal Immunol*. doi: 10.1038/mi.2015.32

Bohnhoff, M., Drake, B. L., & Miller, C. P. (1954). Effect of streptomycin on susceptibility of intestinal tract to experimental Salmonella infection. *Proc Soc Exp Biol Med, 86*(1), 132-137.

Bosca-Watts, M. M., Tosca, J., Anton, R., Mora, M., Minguez, M., & Mora, F. (2015). Pathogenesis of Crohn's disease: Bug or no bug. *World J Gastrointest Pathophysiol, 6*(1), 1-12. doi: 10.4291/wjgp.v6.i1.1

Both, H., Torp-Pedersen, K., Kreiner, S., Hendriksen, C., & Binder, V. (1983). Clinical appearance at diagnosis of ulcerative colitis and Crohn's disease in a regional patient group. *Scand J Gastroenterol, 18*(7), 987-991.

Brandtzaeg, P. (2010). Function of mucosa-associated lymphoid tissue in antibody formation. *Immunol Invest, 39*(4-5), 303-355. doi: 10.3109/08820131003680369

Brandtzaeg, P., & Johansen, F. E. (2005). Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunol Rev, 206*, 32-63. doi: 10.1111/j.0105-2896.2005.00283.x

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 Dis, 2*, e220. doi: 10.1038/cddis.2011.101

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

Cadwell, K., Patel, K. K., Maloney, N. S., Liu, T. C., Ng, A. C., Storer, C. E., . . . Virgin, H. W. (2010). Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell, 141*(7), 1135-1145. doi: 10.1016/j.cell.2010.05.009

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. *Nat Cell Biol, 16*(1), 55-65. doi: 10.1038/ncb2883

Chassaing, B., Aitken, J. D., Malleshappa, M., & Vijay-Kumar, M. (2014). Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol, 104*, Unit 15 25. doi: 10.1002/0471142735.im1525s104

Chidlow, J. H., Jr., Shukla, D., Grisham, M. B., & Kevil, C. G. (2007). Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. *Am J Physiol Gastrointest Liver Physiol, 293*(1), G5-G18. doi: 10.1152/ajpgi.00107.2007

Colombel, J. F., Sandborn, W. J., Reinisch, W., Mantzaris, G. J., Kornbluth, A., Rachmilewitz, D., . . . Group, S. S. (2010). Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med, 362*(15), 1383-1395. doi: 10.1056/NEJMoa0904492

Cooney, R., Baker, J., Brain, O., Danis, B., Pichulik, T., Allan, P., . . . Simmons, A. (2010). NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med, 16*(1), 90-97. doi: 10.1038/nm.2069

Cosnes, J., Gower-Rousseau, C., Seksik, P., & Cortot, A. (2011). Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology, 140*(6), 1785-1794. doi: 10.1053/j.gastro.2011.01.055

Dagenais, M., Dupaul-Chicoine, J., Champagne, C., Skeldon, A., Morizot, A., & Saleh, M. (2015). A critical role for cellular inhibitor of protein 2 (cIAP2) in colitis-associated colorectal cancer and intestinal homeostasis mediated by the inflammasome and survival pathways. *Mucosal Immunol*. doi: 10.1038/mi.2015.46

Denning, T. L., Norris, B. A., Medina-Contreras, O., Manicassamy, S., Geem, D., Madan, R., . . . Pulendran, B. (2011). Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. *J Immunol, 187*(2), 733-747. doi: 10.4049/jimmunol.1002701

Deuring, J. J., Fuhler, G. M., Konstantinov, S. R., Peppelenbosch, M. P., Kuipers, E. J., de Haar, C., & van der Woude, C. J. (2014). Genomic ATG16L1 risk allele-restricted Paneth cell ER stress in quiescent Crohn's disease. *Gut, 63*(7), 1081-1091. doi: 10.1136/gutjnl-2012-303527

Dimitroulia, E., Pitiriga, V. C., Piperaki, E. T., Spanakis, N. E., & Tsakris, A. (2013). Inflammatory bowel disease exacerbation associated with Epstein-Barr virus infection. *Dis Colon Rectum, 56*(3), 322-327. doi: 10.1097/DCR.0b013e31827cd02c

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 Rep, 7*(4), 971-981. doi: 10.1016/j.celrep.2014.04.026

Dourmashkin, R. R., Davies, H., Wells, C., Shah, D., Price, A., O'Morain, C., & Levi, J. (1983). Epithelial patchy necrosis in Crohn's disease. *Hum Pathol, 14*(7), 643-648.

Dupaul-Chicoine, J., Yeretssian, G., Doiron, K., Bergstrom, K. S., McIntire, C. R., LeBlanc, P. M., . . . Saleh, M. (2010). Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity, 32*(3), 367-378. doi: 10.1016/j.immuni.2010.02.012

Duprez, L., Takahashi, N., Van Hauwermeiren, F., Vandendriessche, B., Goossens, V., Vanden Berghe, T., . . . Vandenabeele, P. (2011). RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. *Immunity, 35*(6), 908-918. doi: 10.1016/j.immuni.2011.09.020

Eisenhoffer, G. T., Loftus, P. D., Yoshigi, M., Otsuna, H., Chien, C. B., Morcos, P. A., & Rosenblatt, J. (2012). Crowding induces live cell extrusion to maintain homeostatic cell numbers in epithelia. *Nature, 484*(7395), 546-549. doi: 10.1038/nature10999

Endt, K., Stecher, B., Chaffron, S., Slack, E., Tchitchek, N., Benecke, A., . . . Hardt, W. D. (2010). The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal Salmonella diarrhea. *PLoS Pathog, 6*(9), e1001097. doi: 10.1371/journal.ppat.1001097

Fagarasan, S., Kawamoto, S., Kanagawa, O., & Suzuki, K. (2010). Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. *Annu Rev Immunol, 28*, 243-273. doi: 10.1146/annurev-immunol-030409-101314

Fahlgren, A., Hammarstrom, S., Danielsson, A., & Hammarstrom, M. L. (2003). Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin Exp Immunol, 131*(1), 90-101.

Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D. P., Langlais, C., Hupe, M., . . . Leverkus, M. (2011). cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell, 43*(3), 449-463. doi: 10.1016/j.molcel.2011.06.011

Fernando, M. M., Stevens, C. R., Walsh, E. C., De Jager, P. L., Goyette, P., Plenge, R. M., . . . Rioux, J. D. (2008). Defining the role of the MHC in autoimmunity: a review and pooled analysis. *PLoS Genet, 4*(4), e1000024. doi: 10.1371/journal.pgen.1000024

Flusberg, D. A., & Sorger, P. K. (2015). Surviving apoptosis: life-death signaling in single cells. *Trends Cell Biol*. doi: 10.1016/j.tcb.2015.03.003

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., . . . Ohno, H. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature, 504*(7480), 446-450. doi: 10.1038/nature12721

Garrett, W. S., Lord, G. M., Punit, S., Lugo-Villarino, G., Mazmanian, S. K., Ito, S., . . . Glimcher, L. H. (2007). Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell, 131*(1), 33-45. doi: 10.1016/j.cell.2007.08.017

Gazouli, M., Pachoula, I., Panayotou, I., Mantzaris, G., Chrousos, G., Anagnou, N. P., & Roma-Giannikou, E. (2010). NOD2/CARD15, ATG16L1 and IL23R gene polymorphisms and childhood-onset of Crohn's disease. *World J Gastroenterol, 16*(14), 1753-1758.

Gilmore, A. P. (2005). Anoikis. *Cell Death Differ, 12 Suppl 2*, 1473-1477. doi: 10.1038/sj.cdd.4401723

Glocker, E. O., Kotlarz, D., Boztug, K., Gertz, E. M., Schaffer, A. A., Noyan, F., . . . Klein, C. (2009). Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med, 361*(21), 2033-2045. doi: 10.1056/NEJMoa0907206

Gonzales-van Horn, S. R., & Farrar, J. D. (2015). Interferon at the crossroads of allergy and viral infections. *J Leukoc Biol*. doi: 10.1189/jlb.3RU0315-099R

Goto, K., Hayashimoto, N., Yasuda, M., Ishida, T., Kameda, S., Takakura, A., & Itoh, T. (2009). Molecular detection of murine norovirus from experimentally and spontaneously infected mice. *Exp Anim, 58*(2), 135-140.

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

Hacker, H., & Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. *Sci STKE, 2006*(357), re13. doi: 10.1126/stke.3572006re13

Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., . . . Schreiber, S. (2007). A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet, 39*(2), 207-211. doi: 10.1038/ng1954

Hanauer, S. B., Sandborn, W. J., Rutgeerts, P., Fedorak, R. N., Lukas, M., MacIntosh, D., . . . Pollack, P. (2006). Human anti-tumor necrosis factor monoclonal antibody

(adalimumab) in Crohn's disease: the CLASSIC-I trial. *Gastroenterology, 130*(2), 323-333; quiz 591. doi: 10.1053/j.gastro.2005.11.030

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-alpha. *Cell, 137*(6), 1100-1111. doi: 10.1016/j.cell.2009.05.021

Hentges, D. J., & Freter, R. (1962). In vivo and in vitro antagonism of intestinal bacteria against Shigella flexneri. I. Correlation between various tests. *J Infect Dis, 110*, 30-37.

Hiramatsu, N., Chiang, W. C., Kurt, T. D., Sigurdson, C. J., & Lin, J. H. (2015). Multiple Mechanisms of Unfolded Protein Response-Induced Cell Death. *Am J Pathol*. doi: 10.1016/j.ajpath.2015.03.009

Hiramatsu, N., Messah, C., Han, J., LaVail, M. M., Kaufman, R. J., & Lin, J. H. (2014). Translational and posttranslational regulation of XIAP by eIF2alpha and ATF4 promotes ER stress-induced cell death during the unfolded protein response. *Mol Biol Cell, 25*(9), 1411-1420. doi: 10.1091/mbc.E13-11-0664

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. *Nat Immunol, 1*(6), 489-495. doi: 10.1038/82732

Homer, C. R., Richmond, A. L., Rebert, N. A., Achkar, J. P., & McDonald, C. (2010). ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology, 139*(5), 1630-1641, 1641 e1631-1632. doi: 10.1053/j.gastro.2010.07.006

Hooper, L. V., & Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol, 10*(3), 159-169. doi: 10.1038/nri2710

Hugot, J. P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, 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. doi: 10.1038/35079107

Ivanov, II, Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., . . . Littman, D. R. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell, 139*(3), 485-498. doi: 10.1016/j.cell.2009.09.033

Josefowicz, S. Z., Lu, L. F., & Rudensky, A. Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol, 30*, 531-564. doi: 10.1146/annurev.immunol.25.022106.141623

Jostins, L., Ripke, S., Weersma, R. K., Duerr, R. H., McGovern, D. P., Hui, K. Y., . . . Cho, J. H. (2012). Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature, 491*(7422), 119-124. doi: 10.1038/nature11582

Kaiser, W. J., Upton, J. W., Long, A. B., Livingston-Rosanoff, D., Daley-Bauer, L. P., Hakem, R., . . . Mocarski, E. S. (2011). RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature, 471*(7338), 368-372. doi: 10.1038/nature09857

Kajino-Sakamoto, R., Inagaki, M., Lippert, E., Akira, S., Robine, S., Matsumoto, K., . . . Ninomiya-Tsuji, J. (2008). Enterocyte-derived TAK1 signaling prevents

epithelium apoptosis and the development of ileitis and colitis. *J Immunol, 181*(2), 1143-1152.
Kamada, N., Kim, Y. G., Sham, H. P., Vallance, B. A., Puente, J. L., Martens, E. C., & Nunez, G. (2012). Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. *Science, 336*(6086), 1325-1329. doi: 10.1126/science.1222195
Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin, H. W. t. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science, 299*(5612), 1575-1578. doi: 10.1126/science.1077905
Kaser, A., Zeissig, S., & Blumberg, R. S. (2010). Inflammatory bowel disease. *Annu Rev Immunol, 28*, 573-621. doi: 10.1146/annurev-immunol-030409-101225
Kernbauer, E., Ding, Y., & Cadwell, K. (2014). An enteric virus can replace the beneficial function of commensal bacteria. *Nature, 516*(7529), 94-98. doi: 10.1038/nature13960
Khor, B., Gardet, A., & Xavier, R. J. (2011). Genetics and pathogenesis of inflammatory bowel disease. *Nature, 474*(7351), 307-317. doi: 10.1038/nature10209
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*(3), 387-398.
Labbe, K., McIntire, C. R., Doiron, K., Leblanc, P. M., & Saleh, M. (2011). Cellular inhibitors of apoptosis proteins cIAP1 and cIAP2 are required for efficient caspase-1 activation by the inflammasome. *Immunity, 35*(6), 897-907. doi: 10.1016/j.immuni.2011.10.016
Lalaoui, N., Lindqvist, L. M., Sandow, J. J., & Ekert, P. G. (2015). The molecular relationships between apoptosis, autophagy and necroptosis. *Semin Cell Dev Biol*. doi: 10.1016/j.semcdb.2015.02.003
Lavrik, I., Golks, A., & Krammer, P. H. (2005). Death receptor signaling. *J Cell Sci, 118*(Pt 2), 265-267. doi: 10.1242/jcs.01610
Lawley, T. D., Clare, S., Walker, A. W., Goulding, D., Stabler, R. A., Croucher, N., . . . Dougan, G. (2009). Antibiotic treatment of clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun, 77*(9), 3661-3669. doi: 10.1128/IAI.00558-09
Lecuyer, E., Rakotobe, S., Lengline-Garnier, H., Lebreton, C., Picard, M., Juste, C., . . . Gaboriau-Routhiau, V. (2014). Segmented filamentous bacterium uses secondary and tertiary lymphoid tissues to induce gut IgA and specific T helper 17 cell responses. *Immunity, 40*(4), 608-620. doi: 10.1016/j.immuni.2014.03.009
Leone, V., Chang, E. B., & Devkota, S. (2013). Diet, microbes, and host genetics: the perfect storm in inflammatory bowel diseases. *J Gastroenterol, 48*(3), 315-321. doi: 10.1007/s00535-013-0777-2
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. doi: 10.1016/j.cell.2012.06.019

Li, L., Kabesch, M., Bouzigon, E., Demenais, F., Farrall, M., Moffatt, M. F., . . . Liang, L. (2013). Using eQTL weights to improve power for genome-wide association studies: a genetic study of childhood asthma. *Front Genet, 4*, 103. doi: 10.3389/fgene.2013.00103

Lin, F., Ghislat, G., Luo, S., Renna, M., Siddiqi, F., & Rubinsztein, D. C. (2015). XIAP and cIAP1 amplifications induce Beclin 1-dependent autophagy through NFkappaB activation. *Hum Mol Genet, 24*(10), 2899-2913. doi: 10.1093/hmg/ddv052

Littman, D. R., & Rudensky, A. Y. (2010). Th17 and regulatory T cells in mediating and restraining inflammation. *Cell, 140*(6), 845-858. doi: 10.1016/j.cell.2010.02.021

Mabbott, N. A., Donaldson, D. S., Ohno, H., Williams, I. R., & Mahajan, A. (2013). Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol, 6*(4), 666-677. doi: 10.1038/mi.2013.30

Marchiando, A. M., Shen, L., Graham, W. V., Edelblum, K. L., Duckworth, C. A., Guan, Y., . . . Watson, A. J. (2011). The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding. *Gastroenterology, 140*(4), 1208-1218 e1201-1202. doi: 10.1053/j.gastro.2011.01.004

Masahata, K., Umemoto, E., Kayama, H., Kotani, M., Nakamura, S., Kurakawa, T., . . . Takeda, K. (2014). Generation of colonic IgA-secreting cells in the caecal patch. *Nat Commun, 5*, 3704. doi: 10.1038/ncomms4704

Matsumura, Y., Kinouchi, Y., Nomura, E., Negoro, K., Kakuta, Y., Endo, K., . . . Shimosegawa, T. (2008). HLA-DRB1 alleles influence clinical phenotypes in Japanese patients with ulcerative colitis. *Tissue Antigens, 71*(5), 447-452. doi: 10.1111/j.1399-0039.2008.01031.x

Mazmanian, S. K., Round, J. L., & Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature, 453*(7195), 620-625. doi: 10.1038/nature07008

McComb, S., Cessford, E., Alturki, N. A., Joseph, J., Shutinoski, B., Startek, J. B., . . . Sad, S. (2014). Type-I interferon signaling through ISGF3 complex is required for sustained Rip3 activation and necroptosis in macrophages. *Proc Natl Acad Sci U S A, 111*(31), E3206-3213. doi: 10.1073/pnas.1407068111

Meylan, F., Richard, A. C., & Siegel, R. M. (2011). TL1A and DR3, a TNF family ligand-receptor pair that promotes lymphocyte costimulation, mucosal hyperplasia, and autoimmune inflammation. *Immunol Rev, 244*(1), 188-196. doi: 10.1111/j.1600-065X.2011.01068.x

Micheau, O., & Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell, 114*(2), 181-190.

Min, Y. W., & Rhee, P. L. (2015). The Role of Microbiota on the Gut Immunology. *Clin Ther, 37*(5), 968-975. doi: 10.1016/j.clinthera.2015.03.009

Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., . . . Anderson, K. C. (2002). Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. *Oncogene, 21*(37), 5673-5683. doi: 10.1038/sj.onc.1205664

Mizuno, S., Kanai, T., Mikami, Y., Sujino, T., Ono, Y., Hayashi, A., . . . Hibi, T. (2012). CCR9+ plasmacytoid dendritic cells in the small intestine suppress development of intestinal inflammation in mice. *Immunol Lett, 146*(1-2), 64-69. doi: 10.1016/j.imlet.2012.05.001

Mocarski, E. S., Guo, H., & Kaiser, W. J. (2015). Necroptosis: The Trojan horse in cell autonomous antiviral host defense. *Virology, 479-480C*, 160-166. doi: 10.1016/j.virol.2015.03.016

Mocarski, E. S., Upton, J. W., & Kaiser, W. J. (2012). Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. *Nat Rev Immunol, 12*(2), 79-88. doi: 10.1038/nri3131

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), 46-54 e42; quiz e30. doi: 10.1053/j.gastro.2011.10.001

Monsen, U., Bernell, O., Johansson, C., & Hellers, G. (1991). Prevalence of inflammatory bowel disease among relatives of patients with Crohn's disease. *Scand J Gastroenterol, 26*(3), 302-306.

Montgomery, S. M., Morris, D. L., Pounder, R. E., & Wakefield, A. J. (1999). Paramyxovirus infections in childhood and subsequent inflammatory bowel disease. *Gastroenterology, 116*(4), 796-803.

Moon, C., Baldridge, M. T., Wallace, M. A., Burnham, C. A., Virgin, H. W., & Stappenbeck, T. S. (2015). Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. *Nature, 521*(7550), 90-93. doi: 10.1038/nature14139

Mortha, A., Chudnovskiy, A., Hashimoto, D., Bogunovic, M., Spencer, S. P., Belkaid, Y., & Merad, M. (2014). Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science, 343*(6178), 1249288. doi: 10.1126/science.1249288

Moulin, M., Anderton, H., Voss, A. K., Thomas, T., Wong, W. W., Bankovacki, A., . . . Vaux, D. L. (2012). IAPs limit activation of RIP kinases by TNF receptor 1 during development. *EMBO J, 31*(7), 1679-1691. doi: 10.1038/emboj.2012.18

Mowat, A. M., & Agace, W. W. (2014). Regional specialization within the intestinal immune system. *Nat Rev Immunol, 14*(10), 667-685. doi: 10.1038/nri3738

Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C. L., Cheroutre, H., & Kronenberg, M. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol, 10*(11), 1178-1184. doi: 10.1038/ni.1791

Murakami, Y., Matsumoto, H., Roh, M., Giani, A., Kataoka, K., Morizane, Y., . . . Vavvas, D. G. (2014). Programmed necrosis, not apoptosis, is a key mediator of cell loss and DAMP-mediated inflammation in dsRNA-induced retinal degeneration. *Cell Death Differ, 21*(2), 270-277. doi: 10.1038/cdd.2013.109

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. doi: 10.1016/j.immuni.2013.06.018

Muzes, G., Molnar, B., Tulassay, Z., & Sipos, F. (2012). Changes of the cytokine profile in inflammatory bowel diseases. *World J Gastroenterol, 18*(41), 5848-5861. doi: 10.3748/wjg.v18.i41.5848

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. doi: 10.1038/nature05698

Neuman, M. G. (2007). Immune dysfunction in inflammatory bowel disease. *Transl Res, 149*(4), 173-186. doi: 10.1016/j.trsl.2006.11.009

Neuman, M. G., & Nanau, R. M. (2012). Inflammatory bowel disease: role of diet, microbiota, life style. *Transl Res, 160*(1), 29-44. doi: 10.1016/j.trsl.2011.09.001

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. *Nat Cell Biol, 13*(12), 1437-1442. doi: 10.1038/ncb2362

Oberst, A., Dillon, C. P., Weinlich, R., McCormick, L. L., Fitzgerald, P., Pop, C., . . . Green, D. R. (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature, 471*(7338), 363-367. doi: 10.1038/nature09852

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. doi: 10.1038/35079114

Ogura, Y., Lala, S., Xin, W., Smith, E., Dowds, T. A., Chen, F. F., . . . Nunez, G. (2003). Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut, 52*(11), 1591-1597.

Ostaff, M. J., Stange, E. F., & Wehkamp, J. (2013). Antimicrobial peptides and gut microbiota in homeostasis and pathology. *EMBO Mol Med, 5*(10), 1465-1483. doi: 10.1002/emmm.201201773

Palm, N. W., de Zoete, M. R., Cullen, T. W., Barry, N. A., Stefanowski, J., Hao, L., . . . Flavell, R. A. (2014). Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell, 158*(5), 1000-1010. doi: 10.1016/j.cell.2014.08.006

Pateras, I., Giaginis, C., Tsigris, C., Patsouris, E., & Theocharis, S. (2014). NF-kappaB signaling at the crossroads of inflammation and atherogenesis: searching for new therapeutic links. *Expert Opin Ther Targets, 18*(9), 1089-1101. doi: 10.1517/14728222.2014.938051

Petnicki-Ocwieja, T., Hrncir, T., Liu, Y. J., Biswas, A., Hudcovic, T., Tlaskalova-Hogenova, H., & Kobayashi, K. S. (2009). Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci U S A, 106*(37), 15813-15818. doi: 10.1073/pnas.0907722106

Piao, X., Komazawa-Sakon, S., Nishina, T., Koike, M., Piao, J. H., Ehlken, H., . . . Nakano, H. (2012). c-FLIP maintains tissue homeostasis by preventing apoptosis and programmed necrosis. *Sci Signal, 5*(255), ra93. doi: 10.1126/scisignal.2003558

Pierdomenico, M., Negroni, A., Stronati, L., Vitali, R., Prete, E., Bertin, J., . . . Cucchiara, S. (2014). Necroptosis is active in children with inflammatory bowel disease and contributes to heighten intestinal inflammation. *Am J Gastroenterol, 109*(2), 279-287. doi: 10.1038/ajg.2013.403

Qing, D. Y., Conegliano, D., Shashaty, M. G., Seo, J., Reilly, J. P., Worthen, G. S., . . . Mangalmurti, N. S. (2014). Red blood cells induce necroptosis of lung endothelial cells and increase susceptibility to lung inflammation. *Am J Respir Crit Care Med, 190*(11), 1243-1254. doi: 10.1164/rccm.201406-1095OC

Qiu, W., Wu, B., Wang, X., Buchanan, M. E., Regueiro, M. D., Hartman, D. J., . . . Zhang, L. (2011). PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. *J Clin Invest, 121*(5), 1722-1732. doi: 10.1172/JCI42917

Remijsen, Q., Goossens, V., Grootjans, S., Van den Haute, C., Vanlangenakker, N., Dondelinger, Y., . . . Vandenabeele, P. (2014). Depletion of RIPK3 or MLKL blocks TNF-driven necroptosis and switches towards a delayed RIPK1 kinase-dependent apoptosis. *Cell Death Dis, 5*, e1004. doi: 10.1038/cddis.2013.531

Robinson, N., McComb, S., Mulligan, R., Dudani, R., Krishnan, L., & Sad, S. (2012). Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. *Nat Immunol, 13*(10), 954-962. doi: 10.1038/ni.2397

Rocchi, A., Benchimol, E. I., Bernstein, C. N., Bitton, A., Feagan, B., Panaccione, R., . . . Ghosh, S. (2012). Inflammatory bowel disease: a Canadian burden of illness review. *Can J Gastroenterol, 26*(11), 811-817.

Rodrigue-Gervais, I. G., Labbe, K., Dagenais, M., Dupaul-Chicoine, J., Champagne, C., Morizot, A., . . . Saleh, M. (2014). Cellular inhibitor of apoptosis protein cIAP2 protects against pulmonary tissue necrosis during influenza virus infection to promote host survival. *Cell Host Microbe, 15*(1), 23-35. doi: 10.1016/j.chom.2013.12.003

Rutgeerts, P., Vermeire, S., & Van Assche, G. (2009). Biological therapies for inflammatory bowel diseases. *Gastroenterology, 136*(4), 1182-1197. doi: 10.1053/j.gastro.2009.02.001

Saleh, M., & Trinchieri, G. (2011). Innate immune mechanisms of colitis and colitis-associated colorectal cancer. *Nat Rev Immunol, 11*(1), 9-20. doi: 10.1038/nri2891

Sanders, D. S. (1998). The differential diagnosis of Crohn's disease and ulcerative colitis. *Baillieres Clin Gastroenterol, 12*(1), 19-33.

Sanos, S. L., Bui, V. L., Mortha, A., Oberle, K., Heners, C., Johner, C., & Diefenbach, A. (2009). RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol, 10*(1), 83-91. doi: 10.1038/ni.1684

Sathaliyawala, T., Kubota, M., Yudanin, N., Turner, D., Camp, P., Thome, J. J., . . . Farber, D. L. (2013). Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity, 38*(1), 187-197. doi: 10.1016/j.immuni.2012.09.020

Sawa, S., Lochner, M., Satoh-Takayama, N., Dulauroy, S., Berard, M., Kleinschek, M., . . . Eberl, G. (2011). RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol, 12*(4), 320-326. doi: 10.1038/ni.2002

Scaffidi, C., Schmitz, I., Krammer, P. H., & Peter, M. E. (1999). The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem, 274*(3), 1541-1548.

Segura, I., Serrano, A., De Buitrago, G. G., Gonzalez, M. A., Abad, J. L., Claveria, C., . . . Riese, H. H. (2002). Inhibition of programmed cell death impairs in vitro vascular-like structure formation and reduces in vivo angiogenesis. *FASEB J, 16*(8), 833-841. doi: 10.1096/fj.01-0819com

Sewell, G. W., Marks, D. J., & Segal, A. W. (2009). The immunopathogenesis of Crohn's disease: a three-stage model. *Curr Opin Immunol, 21*(5), 506-513. doi: 10.1016/j.coi.2009.06.003

Smit, J. J., Bol-Schoenmakers, M., Hassing, I., Fiechter, D., Boon, L., Bleumink, R., & Pieters, R. H. (2011). The role of intestinal dendritic cells subsets in the establishment of food allergy. *Clin Exp Allergy, 41*(6), 890-898. doi: 10.1111/j.1365-2222.2011.03738.x

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. *Inflamm Bowel Dis, 14*(7), 968-976. doi: 10.1002/ibd.20380

Tait, S. W., Oberst, A., Quarato, G., Milasta, S., Haller, M., Wang, R., . . . Green, D. R. (2013). Widespread mitochondrial depletion via mitophagy does not compromise necroptosis. *Cell Rep, 5*(4), 878-885. doi: 10.1016/j.celrep.2013.10.034

Talham, G. L., Jiang, H. Q., Bos, N. A., & Cebra, J. J. (1999). Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immun, 67*(4), 1992-2000.

Thackray, L. B., Wobus, C. E., Chachu, K. A., Liu, B., Alegre, E. R., Henderson, K. S., . . . Virgin, H. W. t. (2007). Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. *J Virol, 81*(19), 10460-10473. doi: 10.1128/JVI.00783-07

Tolstanova, G., Deng, X., Khomenko, T., Garg, P., Paunovic, B., Chen, L., . . . Sandor, Z. (2011). Role of anti-angiogenic factor endostatin in the pathogenesis of experimental ulcerative colitis. *Life Sci, 88*(1-2), 74-81. doi: 10.1016/j.lfs.2010.10.026

Tomov, V. T., Osborne, L. C., Dolfi, D. V., Sonnenberg, G. F., Monticelli, L. A., Mansfield, K., . . . Wherry, E. J. (2013). Persistent enteric murine norovirus infection is associated with functionally suboptimal virus-specific CD8 T cell responses. *J Virol, 87*(12), 7015-7031. doi: 10.1128/JVI.03389-12

Tseng, P. H., Matsuzawa, A., Zhang, W., Mino, T., Vignali, D. A., & Karin, M. (2010). Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. *Nat Immunol, 11*(1), 70-75. doi: 10.1038/ni.1819

Ueda, Y., Kayama, H., Jeon, S. G., Kusu, T., Isaka, Y., Rakugi, H., . . . Takeda, K. (2010). Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. *Int Immunol, 22*(12), 953-962. doi: 10.1093/intimm/dxq449

Upton, J. W., Kaiser, W. J., & Mocarski, E. S. (2010). Virus inhibition of RIP3-dependent necrosis. *Cell Host Microbe, 7*(4), 302-313. doi: 10.1016/j.chom.2010.03.006

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 Microbe, 11*(3), 290-297. doi: 10.1016/j.chom.2012.01.016

Vaishnava, S., Yamamoto, M., Severson, K. M., Ruhn, K. A., Yu, X., Koren, O., . . . Hooper, L. V. (2011). The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science, 334*(6053), 255-258. doi: 10.1126/science.1209791

Van Assche, G., Vermeire, S., & Rutgeerts, P. (2009). Infliximab therapy for patients with inflammatory bowel disease: 10 years on. *Eur J Pharmacol, 623 Suppl 1*, S17-25. doi: 10.1016/j.ejphar.2009.10.025

van der Flier, L. G., & Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol, 71*, 241-260. doi: 10.1146/annurev.physiol.010908.163145

Varfolomeev, E., Blankenship, J. W., Wayson, S. M., Fedorova, A. V., Kayagaki, N., Garg, P., . . . Vucic, D. (2007). IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell, 131*(4), 669-681. doi: 10.1016/j.cell.2007.10.030

Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., . . . Wallach, D. (1998). Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity, 9*(2), 267-276.

Vince, J. E., Wong, W. W., Khan, N., Feltham, R., Chau, D., Ahmed, A. U., . . . Silke, J. (2007). IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell, 131*(4), 682-693. doi: 10.1016/j.cell.2007.10.037

Vonarbourg, C., Mortha, A., Bui, V. L., Hernandez, P. P., Kiss, E. A., Hoyler, T., . . . Diefenbach, A. (2010). Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity, 33*(5), 736-751. doi: 10.1016/j.immuni.2010.10.017

Wakefield, A. J., Pittilo, R. M., Sim, R., Cosby, S. L., Stephenson, J. R., Dhillon, A. P., & Pounder, R. E. (1993). Evidence of persistent measles virus infection in Crohn's disease. *J Med Virol, 39*(4), 345-353.

Wallach, D., Kovalenko, A., & Kang, T. B. (2011). 'Necrosome'-induced inflammation: must cells die for it? *Trends Immunol, 32*(11), 505-509. doi: 10.1016/j.it.2011.07.004

Wang, Z., Jiang, H., Chen, S., Du, F., & Wang, X. (2012). The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell, 148*(1-2), 228-243. doi: 10.1016/j.cell.2011.11.030

Waters, O., & Ahmad, T. (2011). Opportunistic infections and vaccinations in IBD patients. *J Crohns Colitis, 5*(3), 263; author reply 264. doi: 10.1016/j.crohns.2011.02.006

Wehkamp, J., Harder, J., Weichenthal, M., Schwab, M., Schaffeler, E., Schlee, M., . . . Stange, E. F. (2004). NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut, 53*(11), 1658-1664. doi: 10.1136/gut.2003.032805

Weihua, Z., Tsan, R., Schroit, A. J., & Fidler, I. J. (2005). Apoptotic cells initiate endothelial cell sprouting via electrostatic signaling. *Cancer Res, 65*(24), 11529-11535. doi: 10.1158/0008-5472.CAN-05-2718

Weinlich, R., Oberst, A., Dillon, C. P., Janke, L. J., Milasta, S., Lukens, J. R., . . . Green, D. R. (2013). Protective roles for caspase-8 and cFLIP in adult homeostasis. *Cell Rep, 5*(2), 340-348. doi: 10.1016/j.celrep.2013.08.045

Welz, P. S., Wullaert, A., Vlantis, K., Kondylis, V., Fernandez-Majada, V., Ermolaeva, M., . . . Pasparakis, M. (2011). FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. *Nature, 477*(7364), 330-334. doi: 10.1038/nature10273

Westra, H. J., Arends, D., Esko, T., Peters, M. J., Schurmann, C., Schramm, K., . . . Franke, L. (2015). Cell Specific eQTL Analysis without Sorting Cells. *PLoS Genet, 11*(5), e1005223. doi: 10.1371/journal.pgen.1005223

Wittkopf, N., Gunther, C., Martini, E., He, G., Amann, K., He, Y. W., . . . Becker, C. (2013). Cellular FLICE-like inhibitory protein secures intestinal epithelial cell survival and immune homeostasis by regulating caspase-8. *Gastroenterology, 145*(6), 1369-1379. doi: 10.1053/j.gastro.2013.08.059

Wlodarska, M., Kostic, A. D., & Xavier, R. J. (2015). An Integrative View of Microbiome-Host Interactions in Inflammatory Bowel Diseases. *Cell Host Microbe, 17*(5), 577-591. doi: 10.1016/j.chom.2015.04.008

Wolff, M. J., Leung, J. M., Davenport, M., Poles, M. A., Cho, I., & Loke, P. (2012). TH17, TH22 and Treg cells are enriched in the healthy human cecum. *PLoS One, 7*(7), e41373. doi: 10.1371/journal.pone.0041373

Wu, J., Huang, Z., Ren, J., Zhang, Z., He, P., Li, Y., . . . Han, J. (2013). Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. *Cell Res, 23*(8), 994-1006. doi: 10.1038/cr.2013.91

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. *Hum Mol Genet, 14*(22), 3499-3506. doi: 10.1093/hmg/ddi379

Yanai, H., Shimizu, N., Nagasaki, S., Mitani, N., & Okita, K. (1999). Epstein-Barr virus infection of the colon with inflammatory bowel disease. *Am J Gastroenterol, 94*(6), 1582-1586. doi: 10.1111/j.1572-0241.1999.01148.x

Yeh, W. C., de la Pompa, J. L., McCurrach, M. E., Shu, H. B., Elia, A. J., Shahinian, A., . . . Mak, T. W. (1998). FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science, 279*(5358), 1954-1958.

Yeh, W. C., Itie, A., Elia, A. J., Ng, M., Shu, H. B., Wakeham, A., . . . Mak, T. W. (2000). Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity, 12*(6), 633-642.

Yen, T. H., & Wright, N. A. (2006). The gastrointestinal tract stem cell niche. *Stem Cell Rev, 2*(3), 203-212. doi: 10.1007/s12015-006-0048-1

Yrlid, U., Milling, S. W., Miller, J. L., Cartland, S., Jenkins, C. D., & MacPherson, G. G. (2006). Regulation of intestinal dendritic cell migration and activation by plasmacytoid dendritic cells, TNF-alpha and type 1 IFNs after feeding a TLR7/8 ligand. *J Immunol, 176*(9), 5205-5212.

Zeissig, S., Bojarski, C., Buergel, N., Mankertz, J., Zeitz, M., Fromm, M., & Schulzke, J. D. (2004). Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumour necrosis factor alpha antibody treatment. *Gut, 53*(9), 1295-1302. doi: 10.1136/gut.2003.036632

Zelante, T., Iannitti, R. G., Cunha, C., De Luca, A., Giovannini, G., Pieraccini, G., . . . Romani, L. (2013). Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity, 39*(2), 372-385. doi: 10.1016/j.immuni.2013.08.003

Zhang, H., Zhou, X., McQuade, T., Li, J., Chan, F. K., & Zhang, J. (2011). Functional complementation between FADD and RIP1 in embryos and lymphocytes. *Nature, 471*(7338), 373-376. doi: 10.1038/nature09878

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. *Proc Natl Acad Sci U S A, 109*(14), 5322-5327. doi: 10.1073/pnas.1200012109

FIGURE LEGENDS

Figure 10: Enhanced colonic pathology and cell death in TRUC mice

Tbx21$^{-/-}$*Rag2*$^{-/-}$ mice and Balb/c control mice colon sections for histopathological scoring and cell death quantification. *A*. Schematic representation of colon section with associated hematoxylin & eosin stain of TRUC and Balb/c mice that were 14 weeks of age. Numbered sections (1,2) match the sections used to score disease. *B*. Immunofluoresence labelling of active caspase-3 (red) and TUNEL (green) in colon sections of TRUC mice with mild and severe disease progression. ***, $p < 0.001$

Figure 11: Increased necrosome formation in the colon of TRUC mice

Tbx21$^{-/-}$*Rag2*$^{-/-}$ and *Rag1*$^{-/-}$ colon lysates from mice that were 6 and 10 weeks of age for each genotype. Western blots of colonic tissue directly adjacent to the anal verge were used. *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ mice were bred on a Balb/c background, and *Rag1*$^{-/-}$ control mice were bred on a C57BL/6 background.

Figure 12: cIAP2 is required for protection against TNFR1-mediated cell death

Whole cell lysates (WCLs) generated from stimulated HT29 cells assayed by western blot. *A*. HT29 cells were pre-treated with BV6 (20μM) for 30 minutes, and were then stimulated with TNFα (15 ng/ml) for 1 hour. TNFα-only treated cells were not pre-treated with BV6. *B*. HT29 cells were pre-treated with BV6 (20μM) and/or Z.VAD.fmk (20μM) for 30 minutes, and were then stimulated with TNFα (15 ng/ml) for 1 hour. *C*. HT29 cells were pre-treated with BV6 (20μM), Z.VAD.fmk (20μM) and Nec-1 (15μM) for 30 minutes, and were then stimulated with TNFα (15 ng/ml) for 3 hours.

Figure 13: MNV infection increases disease susceptibility in DSS-treated *Birc3*$^{-/-}$ mice

A. *Birc3*-/- males between 8 and 10 weeks of age were orally gavaged with MNV.CR6 (10^5 PFU/mice) at D0. At D6, infected and uninfected *Birc3*-/- mice were put on a 2.5% DSS regimen until D10, which was switched to regular water up to D12. Mice were sacrificed at D13 post infection. *B*. Colons from MNV-infected and noninfected DSS-treated mice were dissected at day 8 of the DSS regimen (D13 p.i.) and measured from the anal verge to the cecocolic junction. *, $p < 0.05$; ***, $p < 0.001$. *C*. Representative gross images of colons from MNV.CR6-infected and uninfected DSS-treated *Birc3*-/- mice at day 8 of the DSS regimen (D13 p.i.). *D, E, F*. Hematoxylin & eosin stain of colon sections from infected and uninfected DSS-treated *Birc3*$^{-/-}$ mice, with associated quantifications for histopathological scoring of colon pathology (E) and mucosal lymphoid aggregates (F). *, $p < 0.05$; **, $p < 0.01$; Data represent mean ± standard error (error bars) from two independent experiments.

Figure 14: MNV.CR6 infection does not shift cell death programs in the colon during DSS colitis in *Birc3*$^{-/-}$ mice

Birc3-/- males between 8 and 10 weeks of age were orally gavaged with MNV.CR6 (10^5 PFUs per mice) at D0. At D6, infected and uninfected *Birc3*-/- mice were put on a 2.5% DSS regimen until D10, which was switched to regular water up to D12. Mice were sacrificed at D13 post infection. *A*. Western blot analysis of colonic tissue directly adjacent to the anorectal junction in MNV.CR6-infected and uninfected DSS-treated *Birc3*-/- mice at day 8 of the DSS regimen (D13 p.i.). *B*,*C*. Immunofluoresence labelling of active caspase-3 (red) and TUNEL (green) in colon sections of MNV.CR6-infected and uninfected DSS-treated *Birc3*-/- mice at day 8 of the DSS regimen (D13 p.i.) (B), with associated quantifications for single positive (Caspase-3+TUNEL-, Caspase-3-TUNEL+) or double positive (Caspase-3+TUNEL+) cell death events. *, $p < 0.05$; **, $p < 0.01$. Data represent mean ± standard error (error bars) from one experiment.

Figure 15: MNV.CR6 infection promotes caspase-3 and caspase-8 activity in the ileum

Birc3-/- males between 8 and 10 weeks of age were orally gavaged with MNV.CR6 (10^5 PFUs per mice) at D0. At D6, infected and uninfected *Birc3*-/- mice were put on a 2.5% DSS regimen until D10, which was switched to regular water up to D12. Mice were sacrificed at D13 post infection. Western blot analysis of distal ileal tissue directly adjacent to the cecoileal junction in MNV.CR6-infected and uninfected DSS-treated *Birc3*-/- mice at day 8 of the DSS regimen (D13 p.i.).

Figure 16: MNV.CR6 infection triggers Paneth cell abnormalities in *Birc3*$^{-/-}$ mice

Birc3$^{-/-}$ males between 8 and 10 weeks of age were orally gavaged with MNV.CR6 (10^5 PFUs per mice) at D0. At D6, infected and uninfected *Birc3*-/- mice were put on a 2.5% DSS regimen until D10, which was switched to regular water up to D12. Mice were sacrificed at D13 post infection (UT, n= 5; DSS, n=5; CR6, n=3; CR6 + DSS, n=4). *A*. Immunofluorescence labelling of lysozyme (magenta) and nuclei (blue) in fixed ileal tissue. B. Percentage of Paneth cells displaying each of the four types of lysozyme distribution patterns in MNV-infected and uninfected DSS-treated *Birc3*$^{-/-}$ mice (n > 1700 cells from 3-5 mice/conditions). **, $p < 0.01$; ***, $p < 0.001$. *C*. Four types of lysozyme distribution patterns observed in Paneth cells: normal (D0), disordered (D1), depleted (D2), diffuse (D3). Dotted lines denote single cells.

Figure 17: MNV-mediated disease exacerbation correlates with increased Paneth cell death

Birc3$^{-/-}$ males between 8 and 10 weeks of age were orally gavaged with MNV.CR6 (10^5 PFUs per mice) at D0. At D6, infected and uninfected *Birc3*-/- mice were put on a 2.5% DSS regimen until D10, which was switched to regular water up to D12. Mice were sacrificed at D13 post infection. *A*. Immunofluorescence labelling of lysozyme (green), TUNEL (magenta) and nuclei (blue) of ileum tissue. *B*. Average number of lysozyme+ cells per well oriented crypt. C. Average number of Lysozyme+TUNEL+ cell per field (n= 6 fields/mice). D. Average number of TUNEL+ cell per field (n= 6 fields/mice). *, *p*

< 0.05; **, $p < 0.01$. Data represent mean ± standard error (error bars) from two independent experiments.

Figure 18: MLKL expression during disease

A. Histological colon sections of *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ and Balb/*c* mice that were 16 weeks of age. Immunofluorescence labelling of MLKL (red), e-cadherin (green) and nuclei (blue). *B*. Histological colon sections of *Birc3*$^{-/-}$ male mice that were 8 weeks of age treated with 2.5% DSS for 7 day and sacrificed at day 8 post-DSS. Immunofluorescence labelling of MLKL (red), CD-31 (white) and nuclei (blue).

Figure 19: Expression variants and regulatory elements for MLKL

A. Identification of expression quantitative trait loci (eQTLs) in apoptosis and necroptosis genes. Illumina DNA microarray and cDNA from 9 primary cell types were generated from 350 normal Caucasian individuals, strong eQTLs were identified based on a threshold of significance set at a *p*-value $<10^{-5}$. CD4+ T cells, CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD15+ myelomonocytic cells were purified from peripheral blood. Transverse colon biopsies were obtained from the same individuals. The chromosomal (CHR) location and position (SNP) of the top eSNP are shown. Multiple probes for one gene correlates with multiple splice variants for the gene (FAS, MLKL). B. Mlkl mRNA quantification by RT-qPCR of cDNA generated from BMDMs seeded triplicates, stimulated with IFN-γ (400 UI/ml) for 3 and 21 hours. C. MLKL mRNA quantification by RT-qPCR of cDNA generated form HT29 cells seeded in triplicates, stimulated with IFN-α and IFN-γ for 16 hours at indicated concentrations.

FIGURES

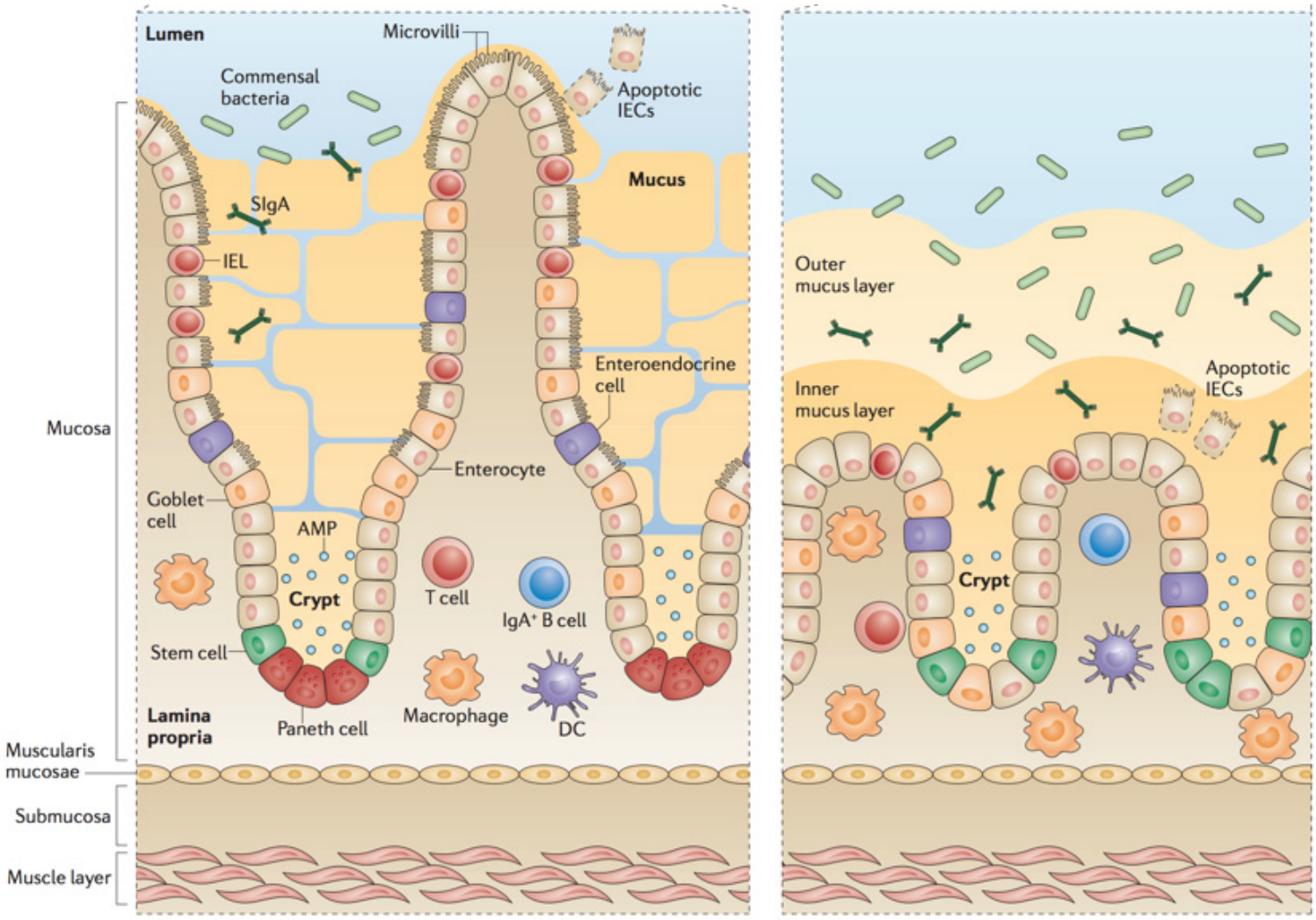


Figure 1: Intestinal mucosal architecture
Mowat, A.M., and Agace W.A. (2014). Regional specialization within the intestinal epithelium. *Nat Rev Imm* 14, 667-685

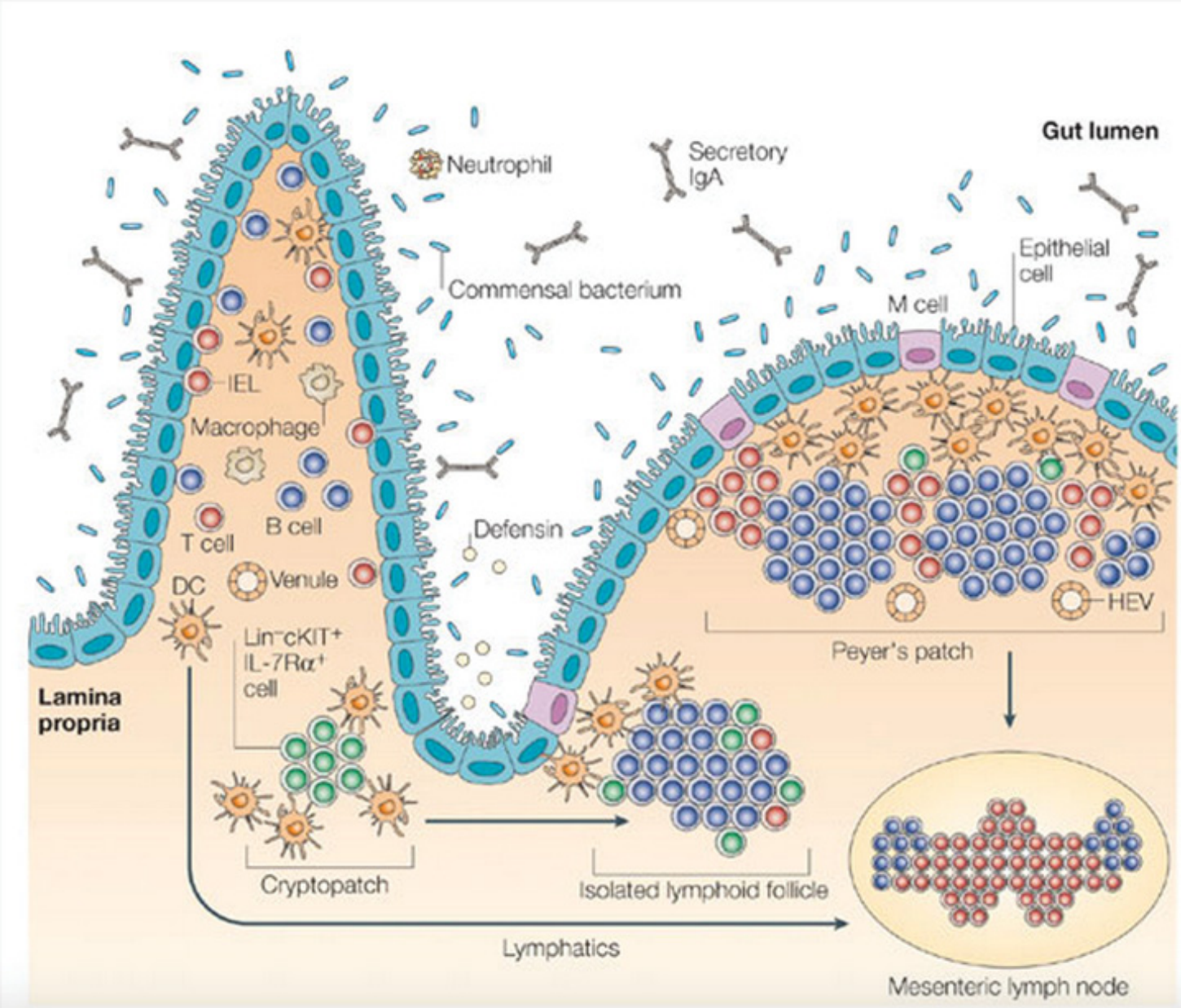


Figure 2: Gut associated lymphoid tissue

Eberl, G. (2005). Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? *Nat Rev Imm* 5, 413-420

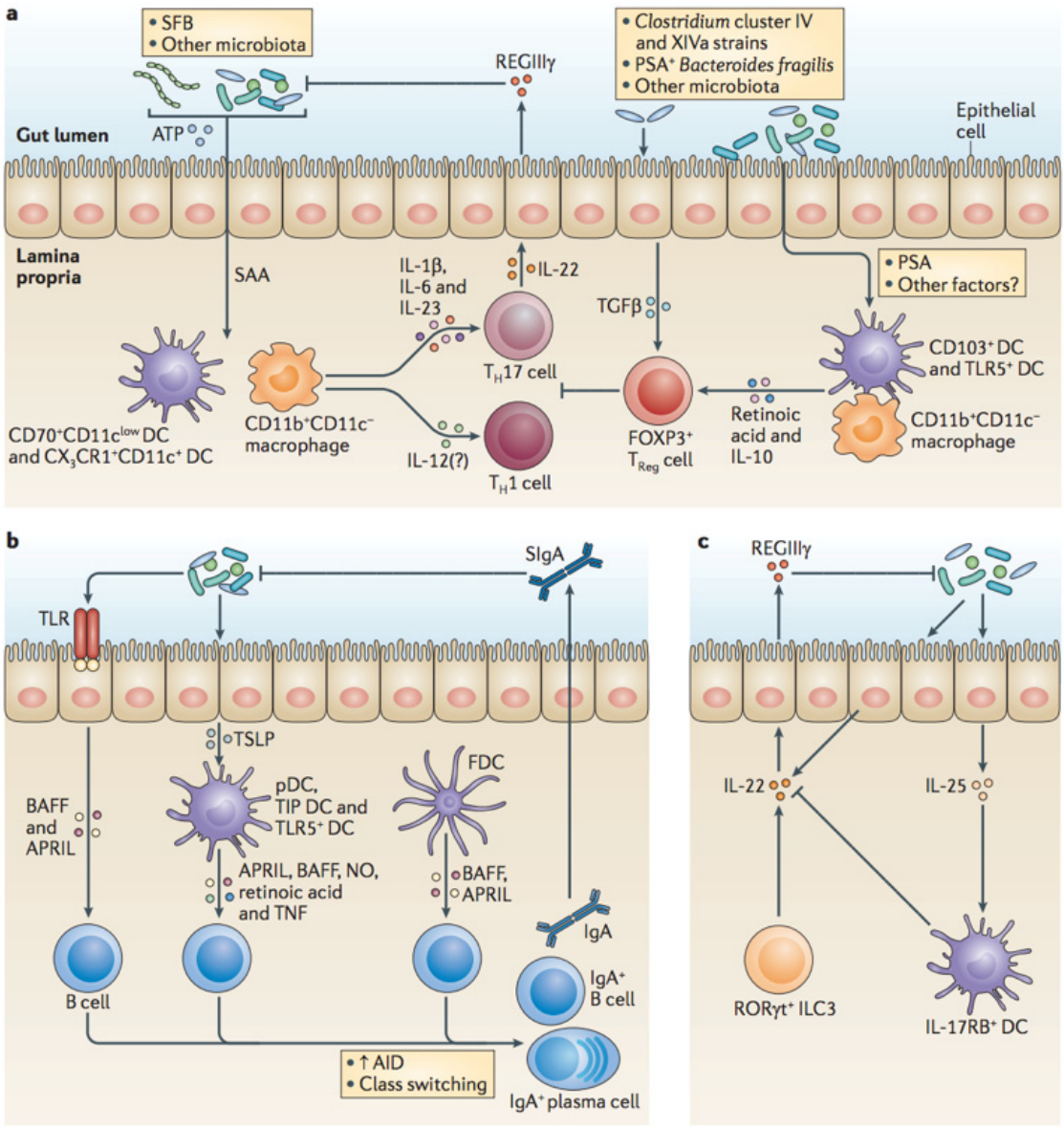


Figure 3: Microbiota-driven shaping of the intestinal immune system
Kamada N., Sang-Uk S., Chen G.Y., and Nùñez G. (2013). Role of the gut microbiota in immunity and inflammatory diseases. *Nat Rev Imm* 13, 321-335

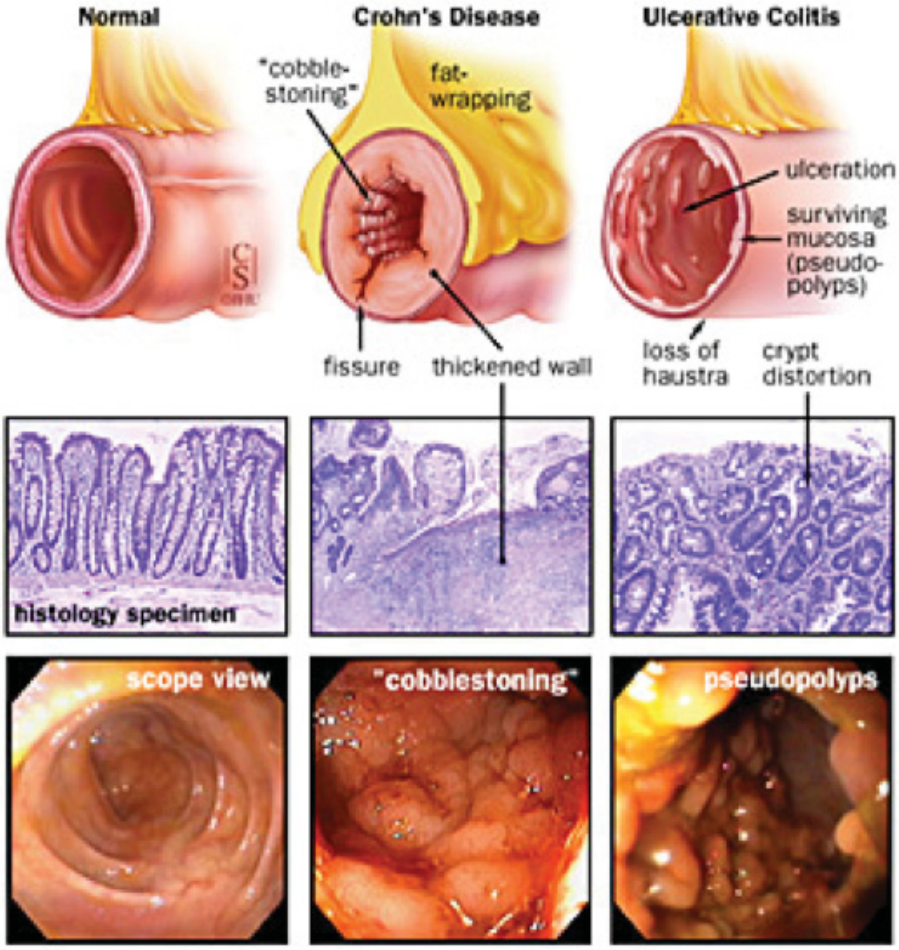


Figure 4: Mucosal pathology in Crohn's disease and ulcerative colitis
Johns Hopkins Medicine: Gastroenterology & Hepatology

URL:https://gi.jhsps.org/GDL_Disease.aspx?CurrentUDV=31&GDL_Disease_ID=2A4995B2-DFA5-4954-B770-F1F5BAFED033&GDL_DC_ID=D03119D7-57A3-4890-A717-CF1E7426C8BA

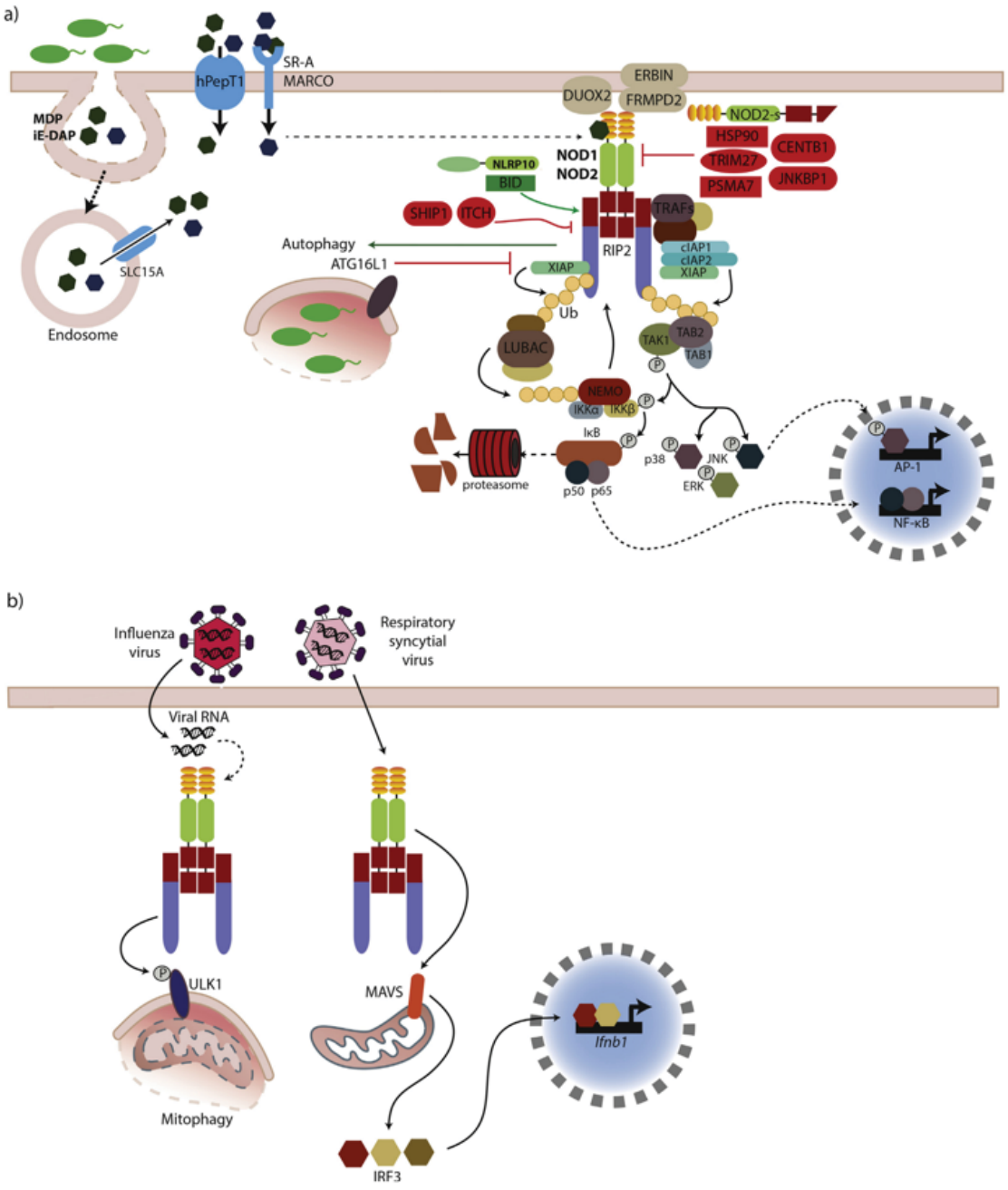


Figure 5: Activation of NOD1 and NOD2 pathways
Barbé, F., Douglas. T.M. and Saleh, M. (2014). Advances in Nod-like receptors (NLR) biology. *Cytokine & Growth Factor Reviews* 25, 681-697

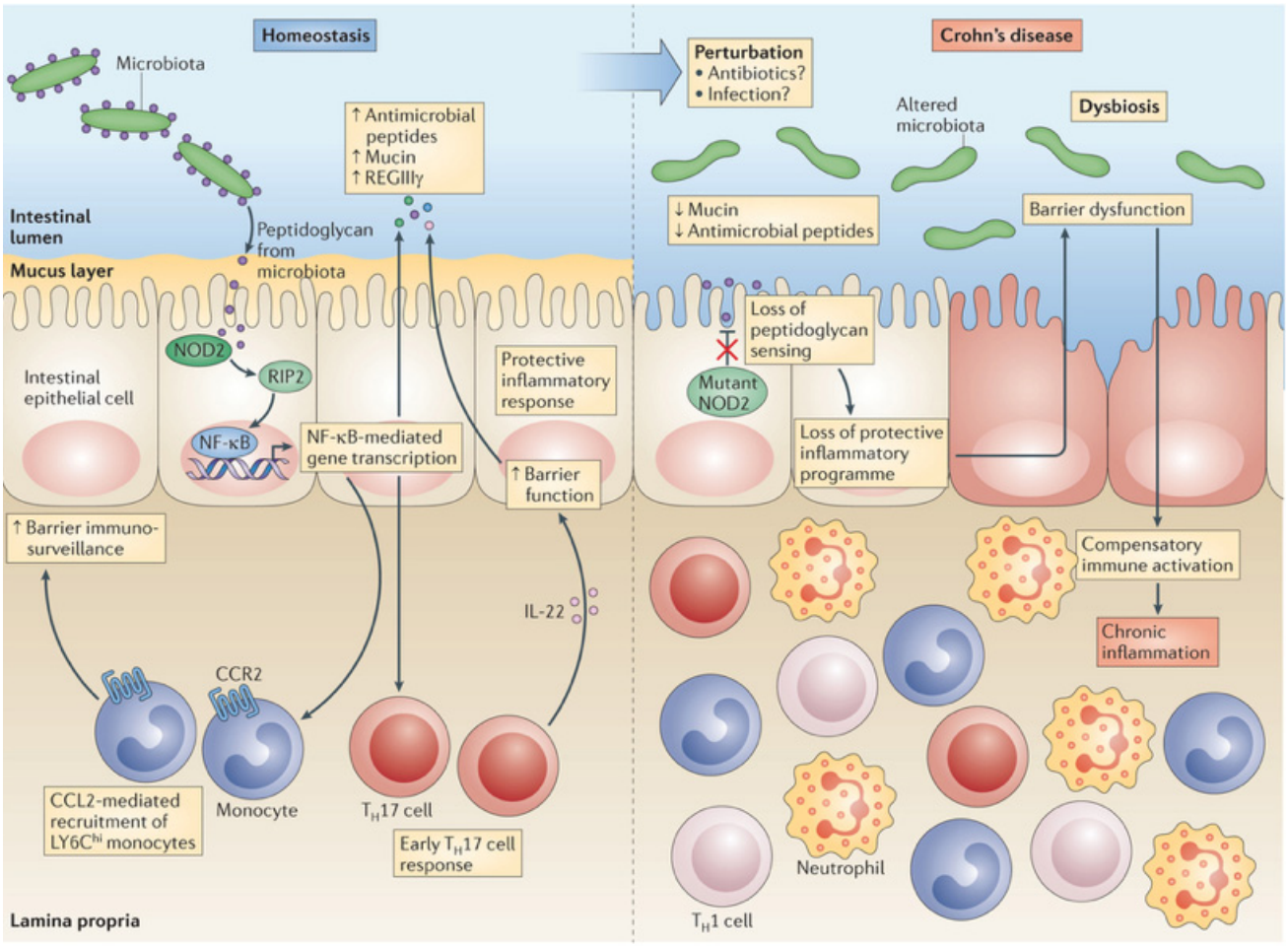


Figure 6: NOD2 in the pathogenesis of Crohn's disease

Philpott D.J., Sorbara M.T., Robertson S.J., Croitoru K. and Girardin S.E. (2014). NOD proteins: regulators of inflammation in health and disease. *Nat Rev Imm* 14, 9-23

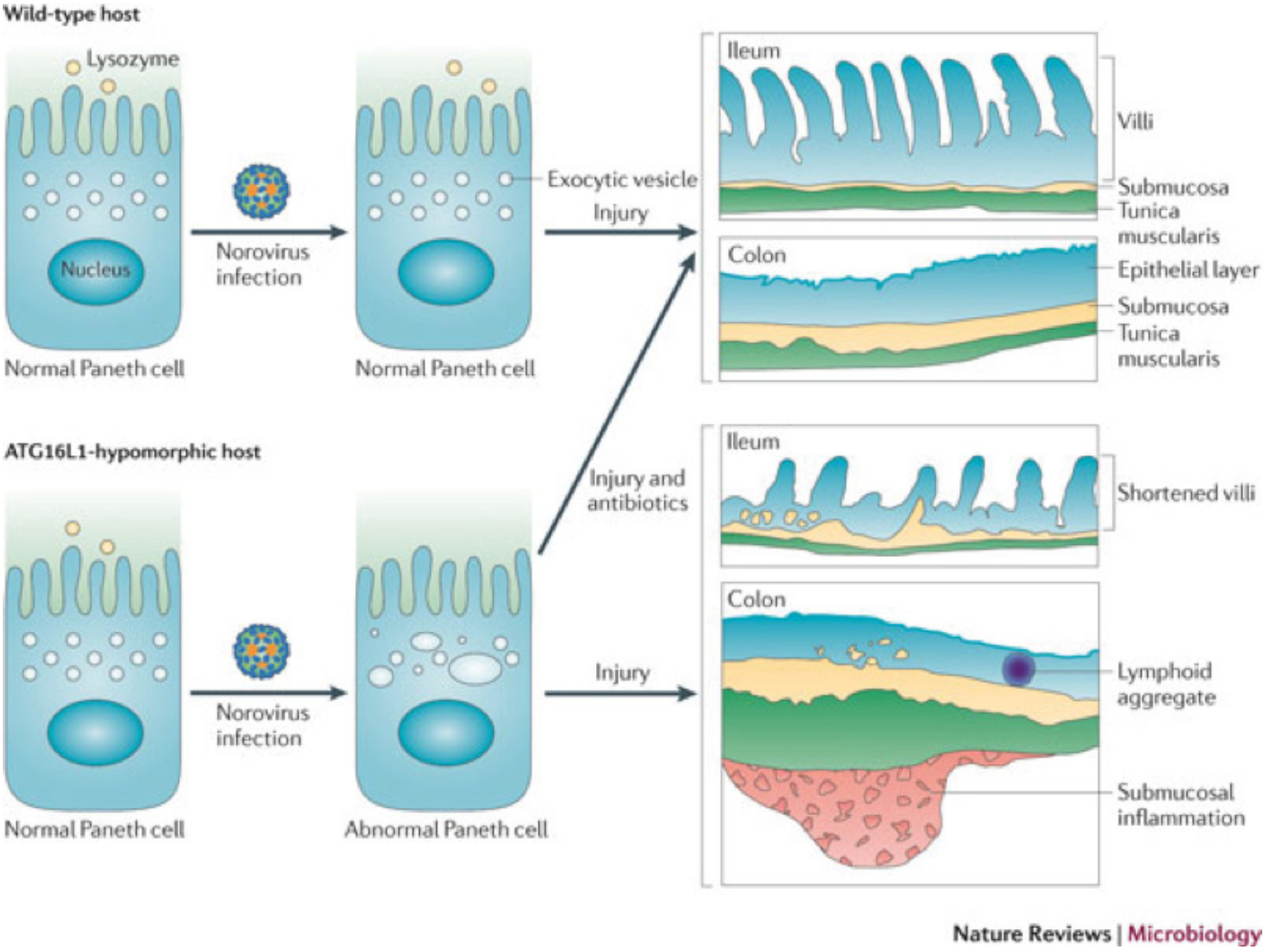


Figure 7: Virus-genome interaction in the onset of IBD

Foxman E.F., and Iwasaki A. (2011). Genome-virome interactions: examining the role of common viral infections in complex disease. *Nat Rev Micr* 9, 254-264

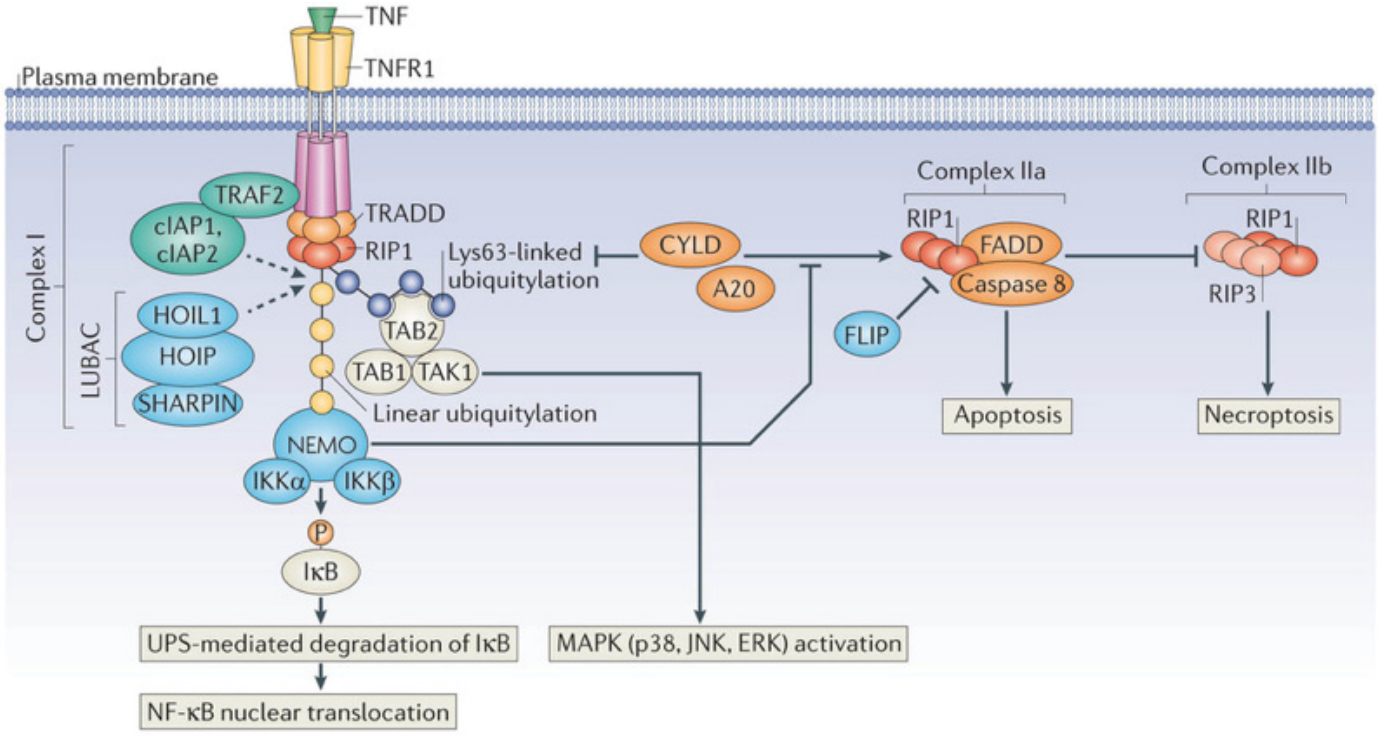


Figure 8: TNFR1-induced cell death complexes

Ofengeim D., and Yuan J. (2013). Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nat Rev Mol Cell Bio* 14,727–736

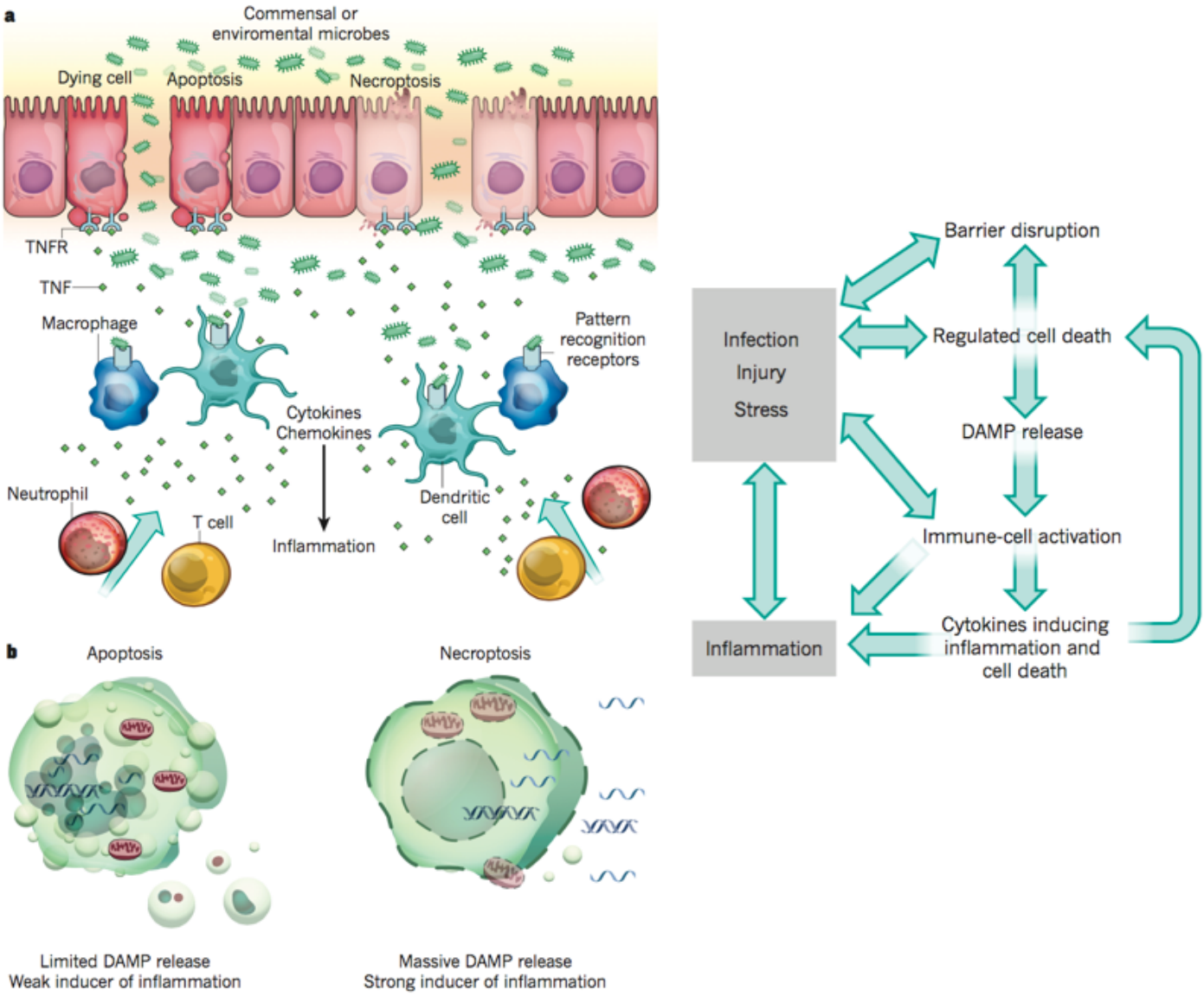
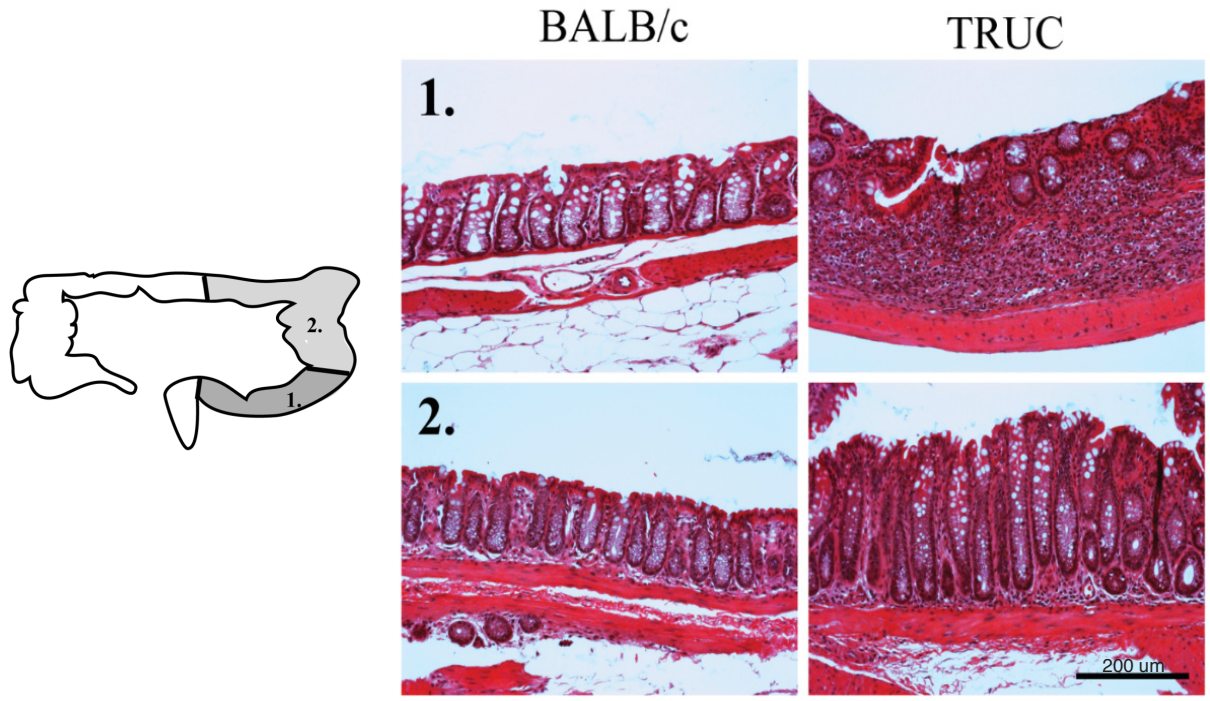
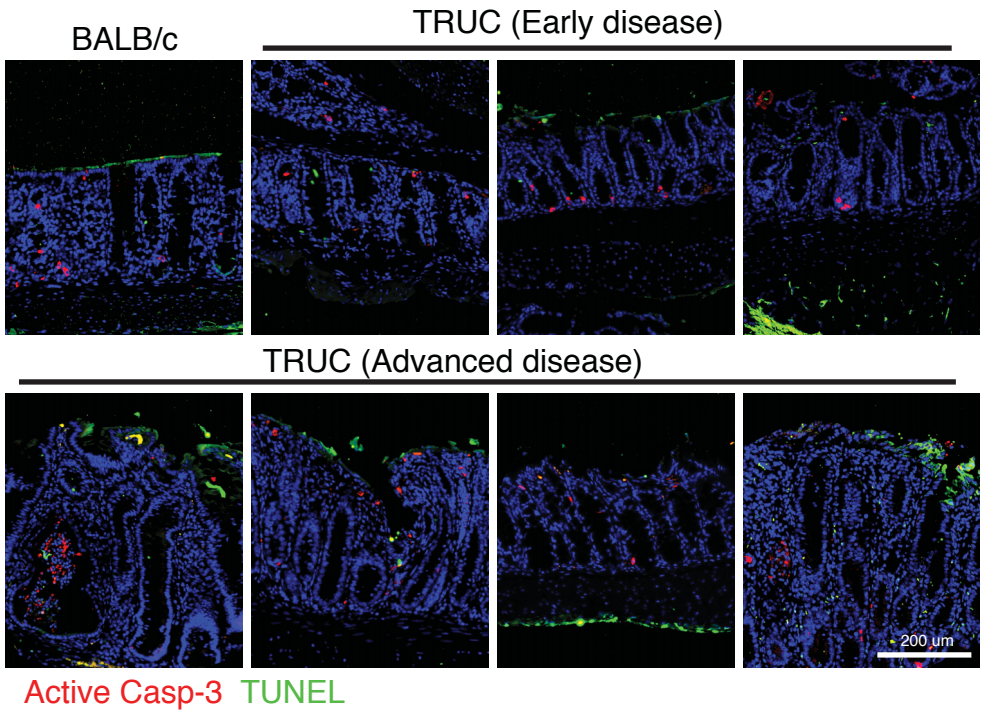


Figure 9: Cell death in disease

Pasparakis M., and Vandenabeele P. (2015). Necroptosis and its role in inflammation. *Nature* 517, 311-320





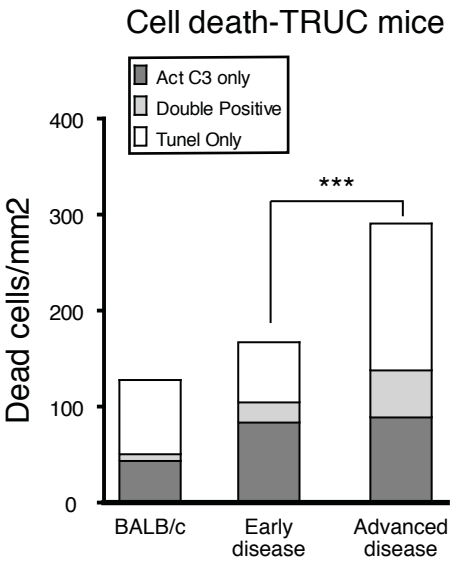


Figure 10: Enhanced colonic pathology and cell death in TRUC mice

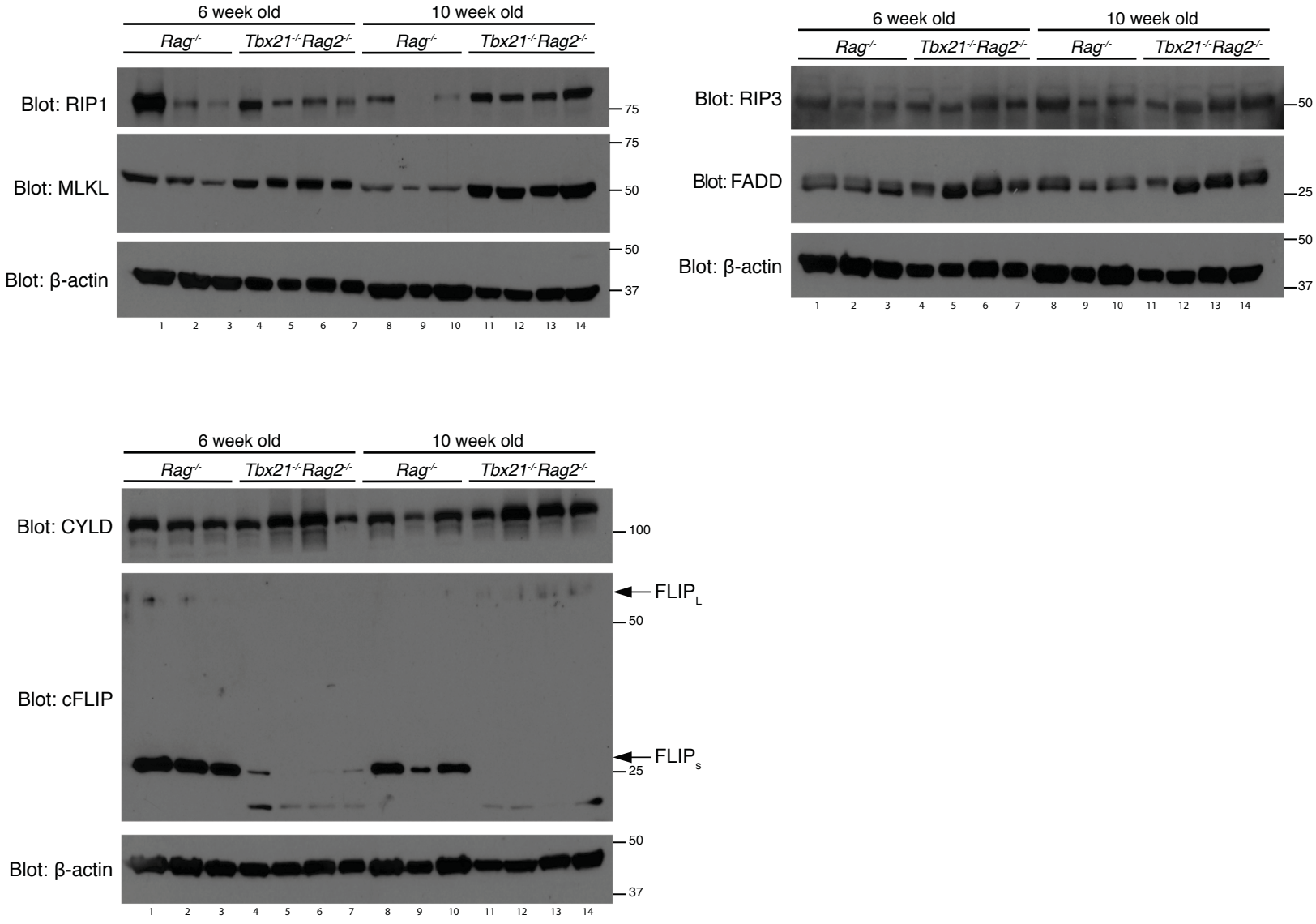


Figure 11: Increased necrosome formation in the colon of TRUC mice

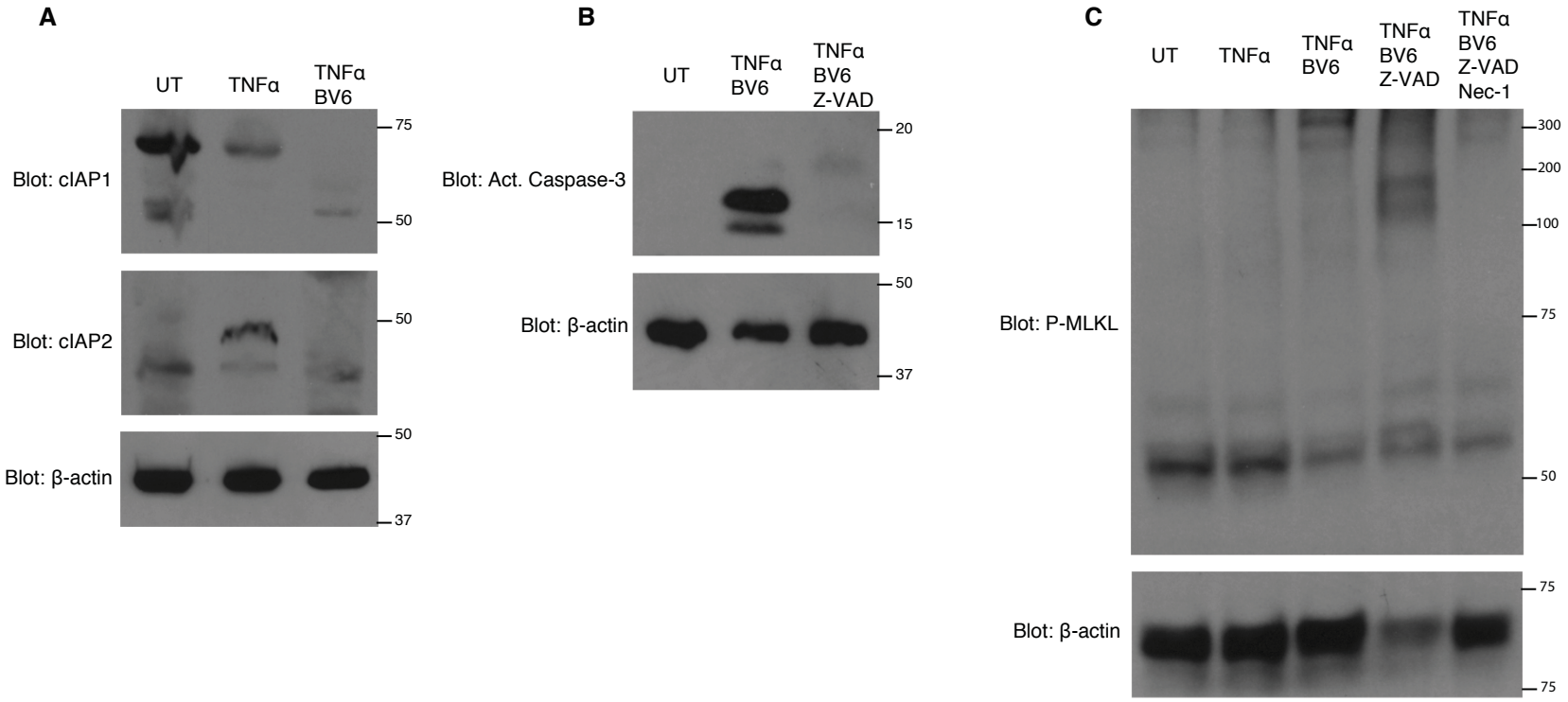


Figure 12: cIAP2 is required for protection against TNFR1-mediated cell death

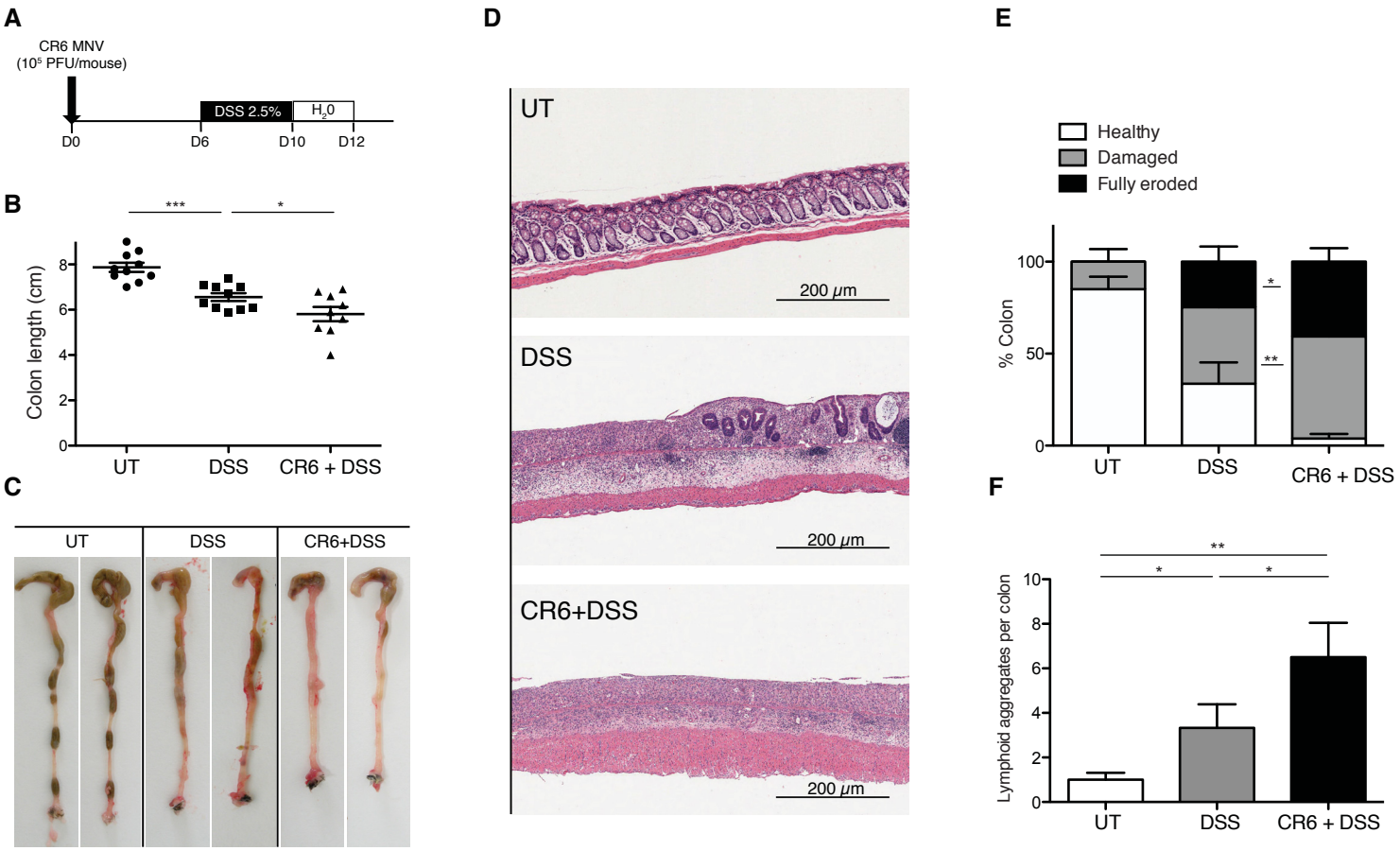
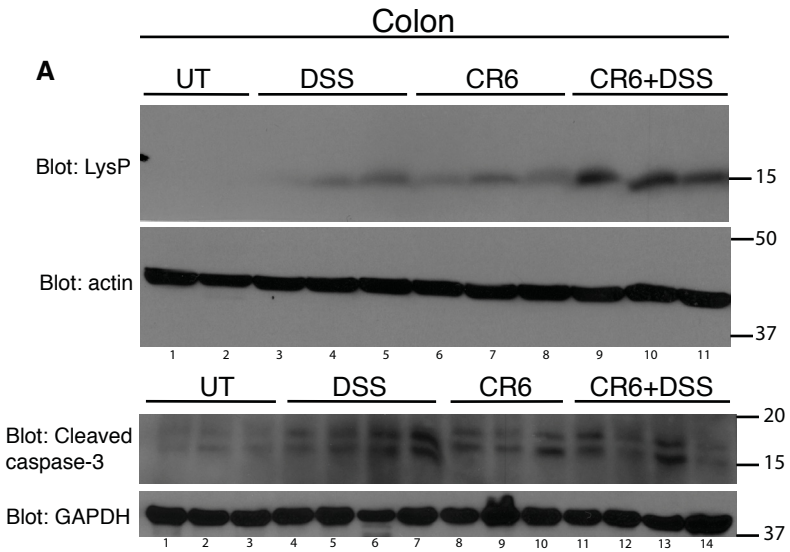
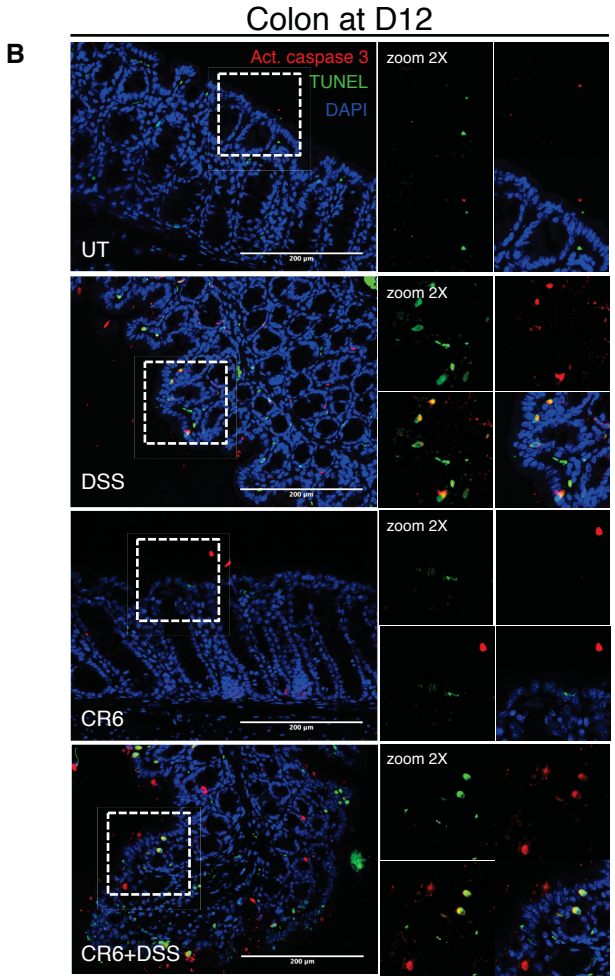


Figure 13: MNV infection increases disease susceptibility in DSS-treated *Birc3*$^{-/-}$ mice





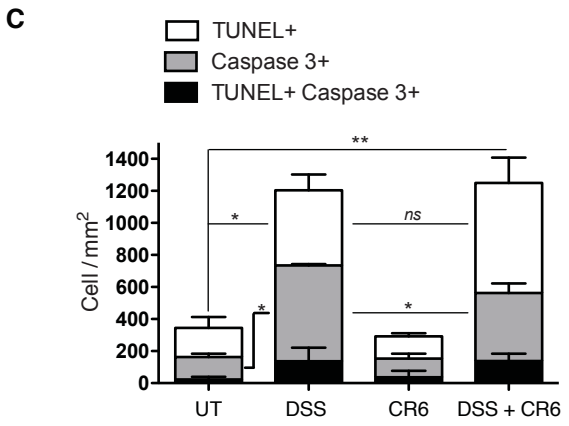


Figure 14: MNV.CR6 infection does not shift cell death programs in the colon during DSS colitis in *Birc3*$^{-/-}$ mice

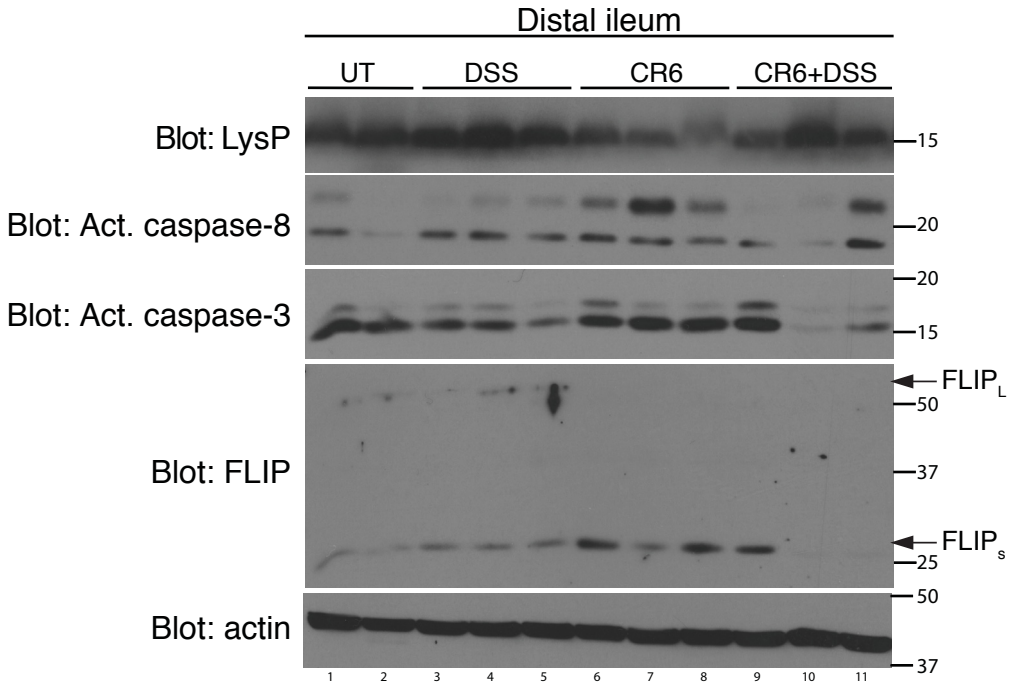


Figure 15: MNV.CR6 infection promotes caspase-3 and caspase-8 activity in the ileum

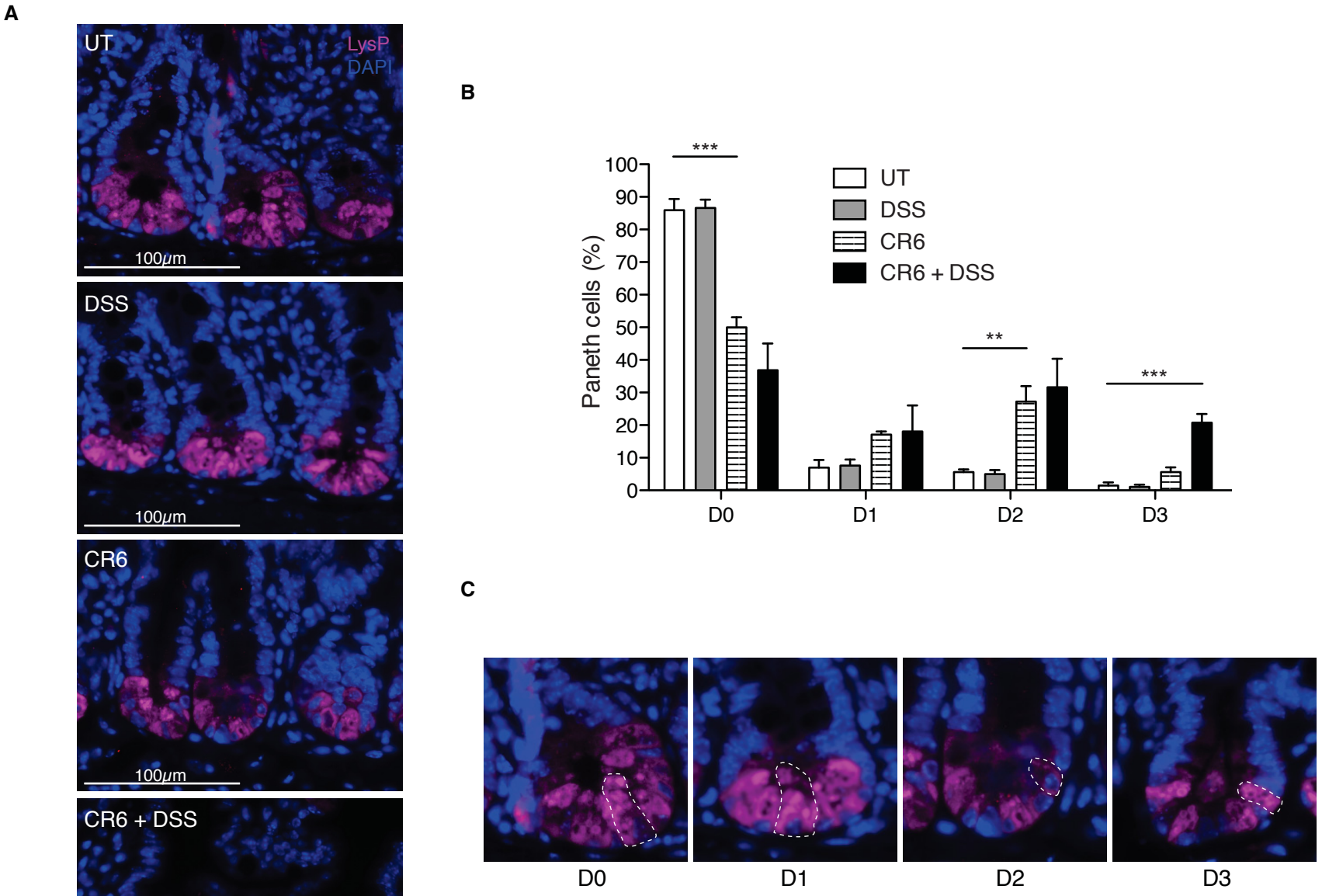
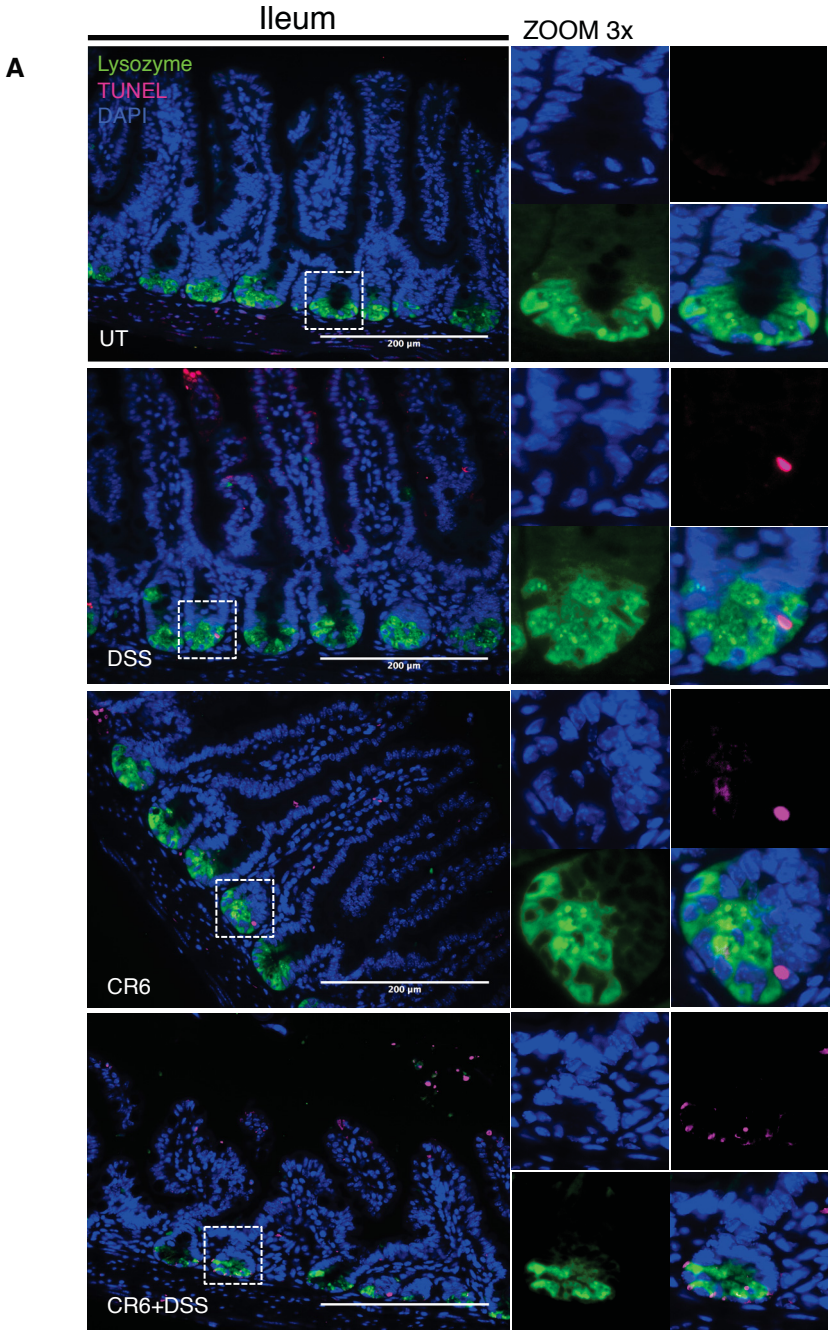


Figure 16: MNV.CR6 infection triggers Paneth cell abnormalities in *Birc3*$^{-/-}$ mice



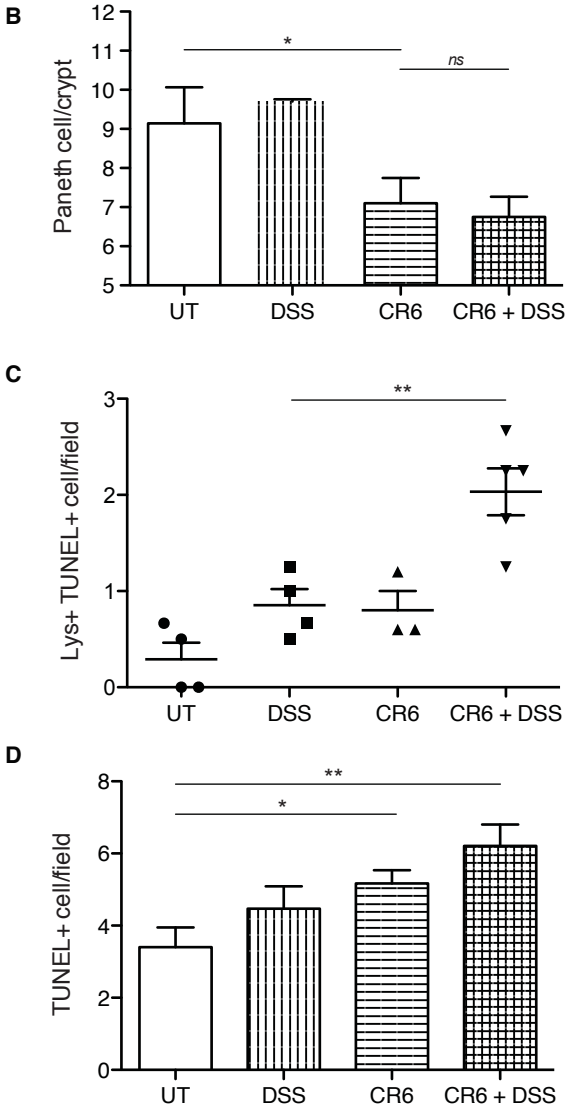


Figure 17: MNV-mediated disease exacerbation correlates with increased Paneth cell death

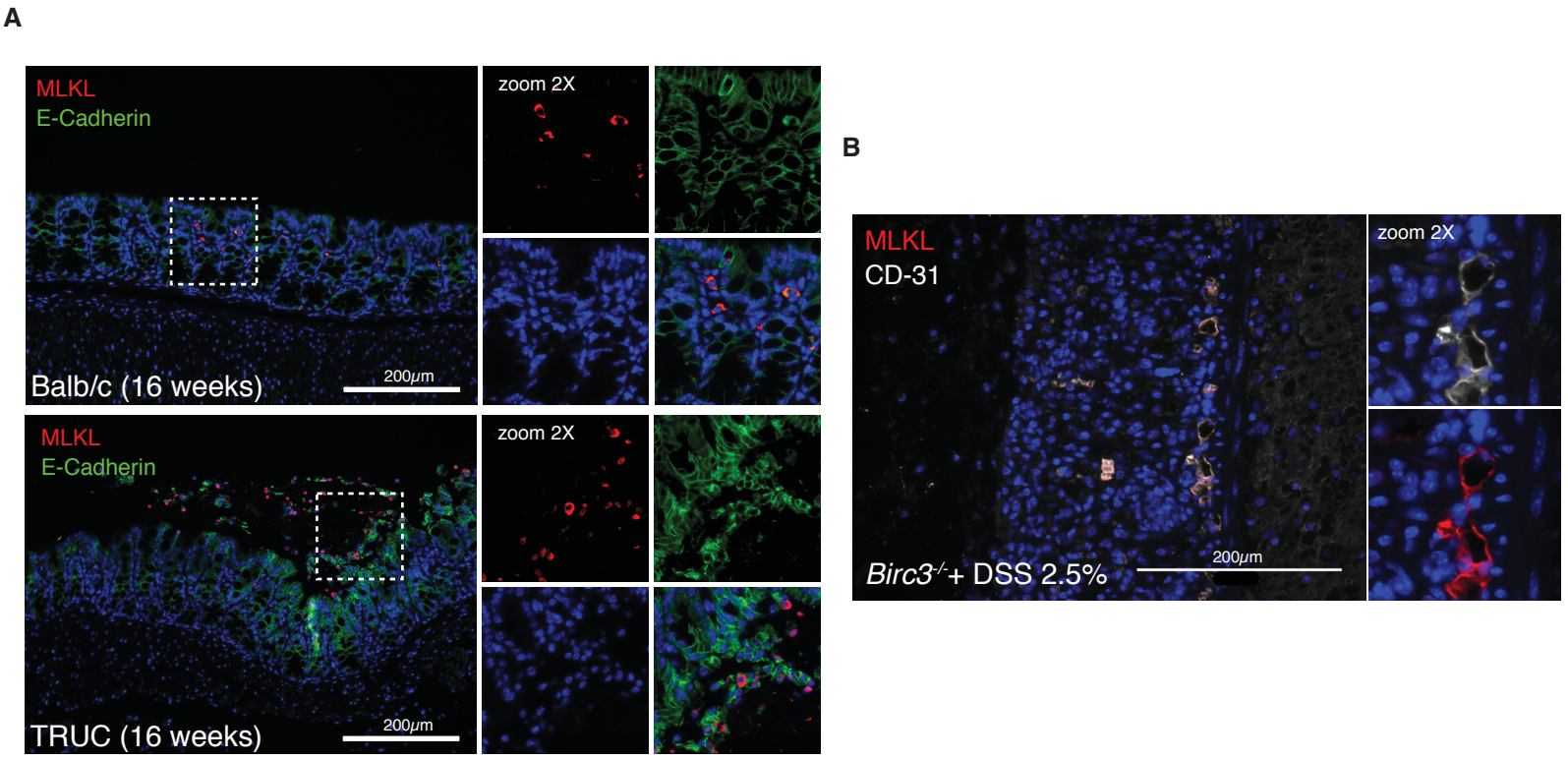
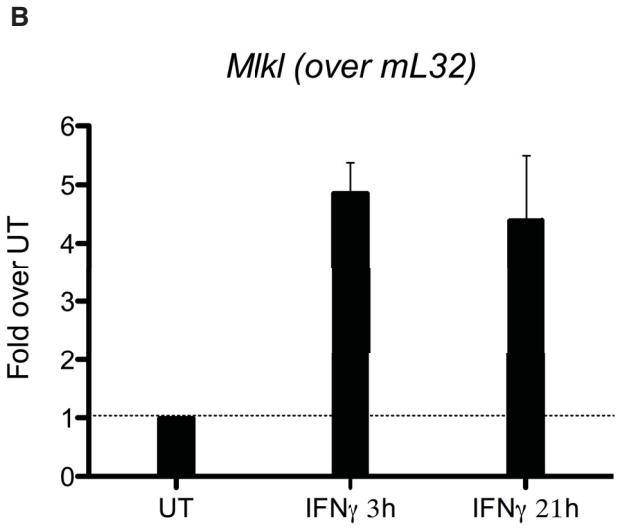


Figure 18: MLKL expression during disease

A

CD4+	ProbeID	From	CHR	SNP	BP	A1	p-value
FAS	3420026	90775097	10	rs2234978	90771829	T	5.61E-08
FAS	460438	90775175	10	rs2234978	90771829	T	1.91E-10
MLKL	4560541	74705809	16	rs9932977	74735148	G	8.10E-24
MLKL	5810022	74712848	16	rs9932977	74735148	G	3.33E-07
MLKL	5810022	74716549	16	rs9932977	74735148	G	3.58E-07
RBCK1	2510523	411499	20	rs6139109	391025	C	7.18E-15
CFLAR	1240593	202028943	2	rs1594	202025621	A	1.55E-07
CD8+							
TNFRSF14	6520725	2495103	1	rs734999	2513216	T	9.73E-10
TRADD	2650706	67188445	16	rs1053612	67423939	G	9.34E-06
MLKL	4560541	74705809	16	rs9932977	74735148	G	9.60E-19
MLKL	5810022	74712848	16	rs28579687	74746403	G	1.19E-05
MLKL	5810022	74716549	16	rs28579687	74746403	G	7.26E-06
RBCK1	2510523	411499	20	rs6139109	391025	C	7.44E-12
ITCH	7560538	33099121	20	rs6088512	33095891	G	1.96E-08
CFLAR	1240593	202028943	2	rs1594	202025621	A	3.66E-06
RIPK2	4150520	90802376	8	rs42490	90778513	G	9.86E-10
CD19+							
FADD	1300674	70053214	11	rs875108	70004695	G	1.45E-05
MLKL	4560541	74705809	16	rs9932977	74735148	G	1.01E-07
RBCK1	2510523	411499	20	rs6139109	391025	C	1.24E-08
CFLAR	1240593	202028943	2	rs1594	202025621	A	3.20E-05
CD14+							
RIPK3	360142	24805314	14	rs12891732	24799843	C	1.92E-05
TNFSF12	1090747	7460967	17	rs34790908	7451110	T	2.36E-10
CD15+							
MLKL	4560541	74705809	16	rs9932977	74735148	G	1.87E-09
TNFRSF10B	2600463	22877811	8	rs11784599	22932122	A	9.88E-13
Colon							
FAS	3420026	90775097	10	rs2031613	90766924	C	1.53E-09
FAS	460438	90775175	10	rs2031613	90766924	C	2.65E-05
RIPK3	360142	24805314	14	rs3212251	24809185	C	1.30E-05
ITCH	7560538	33099121	20	rs1205340	32923871	A	4.58E-05



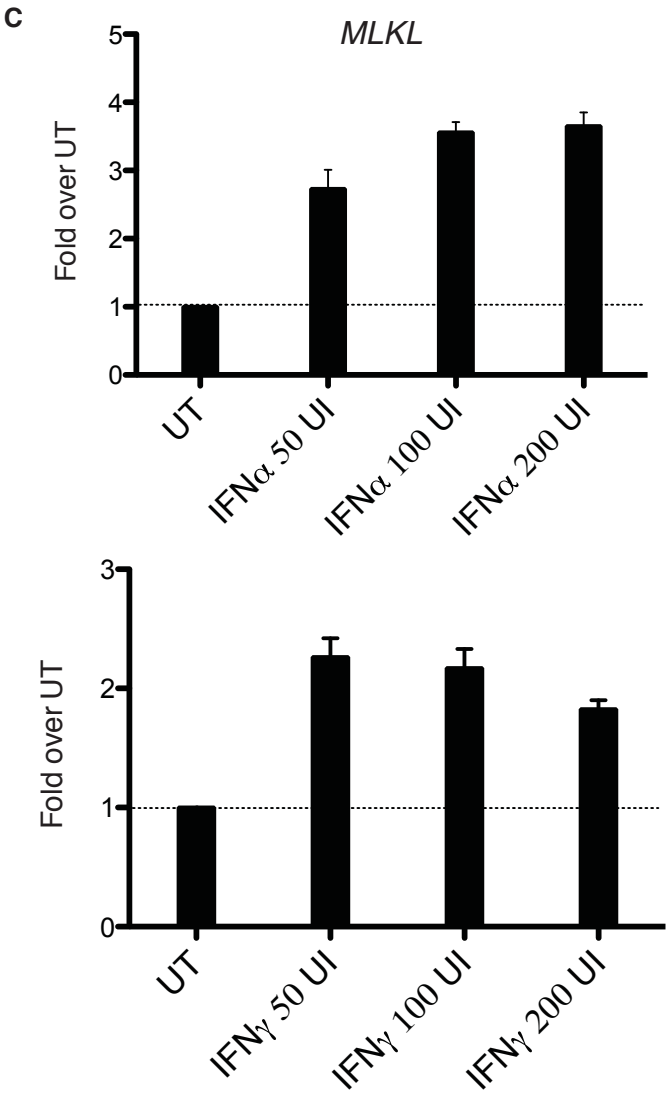


Figure 19: Expression variants and regulatory elements for MLKL