**Technische Universität München**
**Fakultät für Medizin**

# Dissecting aberrant host-microbiota interactions in a model of TNF-driven intestinal inflammation

**Adam Wahida**

Vollständiger Abdruck der von der Fakultät für Medizin der
Technischen Universität München zur Erlangung eines

**Doctor of Philosophy (Ph.D.)**

genehmigten Dissertation.

**Vorsitz:** Prof. Dr. Stefan Lichtenthaler

**Betreuer*in:** Prof. Dr. Florian Bassermann

**Prüfer*innen der Dissertation:**

1. Prof. Dr. Philipp J. Jost
2. Prof. Dr. Roland M. Schmid
3. Prof. Dr. Sebastian Zeissig

Die Dissertation wurde am 21.02.2023 bei der Fakultät für Medizin der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 15.03.2023 angenommen.

*Meinen Eltern*

## TABLE OF CONTENTS

## PUBLISHED WORK

**Parts of this thesis have been previously published in peer reviewed journals and/or presented at international conferences.**

### PEER REVIEWED PUBLICATION

**A Wahida***, M Müller*, A Hiergeist, B Popper, K Steiger, C Branca, M Tschurtschenthaler, T Engleitner, S Donakonda, J De Coninck, R Öllinger, MK Pfautsch, N Müller, M Silva, S Usluer, E Thiele Orberg, JP Böttcher, N Pfarr, M Anton, JB Slotta-Huspenina, AG Nerlich, T Madl, M Basic, A Bleich, G Berx, J Ruland, PA Knolle, R Rad, TE Adolph, P Vandenabeele, H Kanegane, A Gessner, PJ Jost#, M Yabal#† (*equal contribution, #co-senior authors, † corresponding author)

*XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells*

**SCIENCE IMMUNOLOGY (2021)** (PMID: 34739338)

### CONFERENCE ABSTRACT

**A Wahida**, K Steiger, B Popper, T Engleitner, R Öllinger, M Ehrenschwender, R Rad, H Kanegane, M Yabal, PJ Jost†

*Molecular Characterization of the TNF-RIPK3-axis in Paneth cell Dysfunction and Dysbiosis as drivers of Inflammatory Bowel Disease in XIAP-deficiency* **(selected as a Talk)**

**11th EUROPEAN WORKSHOP ON CELL DEATH (EWCD) 2018**

## GLOSSARY

| | |
|---|---|
| AMP | Antimicrobial peptide |
| CASP8 | Caspase 8 |
| CD | Crohn's disease |
| CED | *Chronisch entzündliche Darmerkrankungen* |
| DC | Dendritic Cell |
| DSS | Dextran sulfate sodium |
| GI | Gastrointestinal tract |
| GSEA | Gene set enrichment analysis |
| GWAS | Genome-wide association study |
| IBD | Inflammatory bowel disease |
| IECs | Intestinal epithelial cells |
| IHC | Immunohistochemistry |
| MLKL | Mixed Lineage Kinase Like |
| NGS | Next generation sequencing |
| NLRP6 | NOD-like receptor family pyrin domain containing 6 |
| NOD2 | Nucl.-binding oligomerization domain-containing protein 2 |
| PAS | Periodic acid–Schiff |
| PC | Paneth Cell |
| PCR | Polymerase chain reaction |
| RIPK1 | Receptor interacting serine/threonine kinase 1 |
| RIPK2 | Receptor interacting serine/threonine kinase 3 |
| RIPK3 | Receptor interacting serine/threonine kinase 3 |
| SNP | Single nucleotide polymorphism |
| SPF | Specific pathogen-free |

| | |
|---|---|
| TEM | Transmission electron microscopy |
| Th | T helper cells |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TNFR1 | Tumor necrosis factor receptor 1 |
| TNFR2 | Tumor necrosis factor receptor 2 |
| UAE1 | *Ulex europaeus* lectin |
| UC | Ulcerative colitis |
| XIAP | X-linked inhibitor of apoptosis protein |
| XLP-2 | X-linked lymphoproliferative syndrome type 2 |

## ZUSAMMENFASSUNG

Die intestinale Homöostase beruht maßgeblich auf der symbiotischen Interaktion von Wirt und Mikrobiom. Ein Verlust dieser Symbiose (kann auch als Dysbiose bezeichnet werden) kann eine Immun-Dysregulation auslösen und beeinflusst die Suszeptibilität für chronisch entzündliche Darmerkrankungen (CED), welche ein komplexes Erkrankungsspektrum darstellen, verursacht durch eine genetische Veranlagung sowie äußeren Umwelteinflüssen. Mutationen im Gen *X-linked inhibitor of apoptosis protein* (XIAP) verursachen das seltene X-chromosomale lymphoproliferative Syndrom Typ 2 (XLP2), welches häufig mit CED in Erscheinung tritt. Die genaue Rolle von XIAP in entzündlichen Darmerkrankungen ist jedoch weitestgehend unerforscht. Im Rahmen dieser Dissertation wurde anhand einer Reihe von genetischen Mausmodellen gezeigt, dass XIAP eine mukosalen Entzündung im Dünndarm verhindert, welche durch beide Tumor-Nekrose-Faktor (TNF)-Rezeptoren 1 sowie 2 ausgelöst wird. Dementsprechend ist XIAP erforderlich, um die Integrität des Darmepithels und insbesondere die Funktion der Paneth-Zellen zu erhalten. Ein Fehlen von XIAP im Sinne des XLP2 Syndroms, wirkt darüber hinaus auch auf die durch den Toll-like-Rezeptor 5 (TLR5) vermittelten Immunfunktionen aus, welche unter anderem durch mikrobielle Einflüsse im Darm gesteuert werden. Diese Dissertation bietet einen Verständnisrahmen für jene Krankheiten, die durch einen genetisch-induzierten XIAP-Mangel gekennzeichnet sind, und verdeutlicht die essenzielle Rolle der Interaktion von Wirt und Mikrobiom in der TNF-induzierten intestinalen Inflammation von CED.

## ABSTRACT

Intestinal homeostasis relies on the symbiotic nature of host-microbe interactions. Loss of symbiosis (referred to as dysbiosis) can trigger immune dysregulation and is considered to shape the susceptibility to inflammatory bowel diseases (IBD), a complex trait influenced by genetic predisposition and environmental cues. The X-linked lymphoproliferative syndrome type 2 (XLP2), a rare condition, is caused by mutations in the X-linked inhibitor of apoptosis protein (XIAP). This syndrome is often linked to Inflammatory Bowel Disease (IBD) and variants of XIAP have been found to be connected to pediatric IBD. However, the role of XIAP in intestinal inflammation is largely unexplored. By using a series of genetic mouse models, this thesis elucidates how XIAP restricts mucosal inflammation in the small intestine which is driven by tumor necrosis factor (TNF) receptor signaling. XIAP is required to maintain intestinal epithelial integrity and specifically Paneth cell function. Moreover, XIAP-restricted TNF signaling impacts toll-like receptor 5 (TLR5)-mediated immune functions which are controlled by gut microbial cues. This dissertation provides a framework for understanding diseases that are characterized by genetically induced XIAP deficiency and highlight a critical role for host-microbe interactions in TNF receptor-driven mucosal inflammation.

# I. INTRODUCTION

Inflammatory Bowel Disease (IBD) is the term used for a clinical condition describing a set of chronic and relapsing inflammatory diseases affecting the gastrointestinal tract (**Maloy and Powrie 2011**). These can present in two major forms – Crohn's disease (CD) and ulcerative colitis (UC) (**Roda et al. 2020; Kobayashi et al. 2020**). From a heuristic point of view, CD and UC are diagnosed by combining clinical, histologic, endoscopic, and radiologic examinations (**Roda et al. 2020; Kobayashi et al. 2020**). One of the key differences herein, used to discriminate UC from CD, is the location and extent of inflammation along the gastrointestinal (GI) tract. While UC spreads proximally from the rectum but is generally limited to the colon, CD can discontinuously affect virtually any part of the GI tract, but mostly manifests in the terminal ileum (**Figure 1**) (**Roda et al. 2020; Kobayashi et al. 2020**).

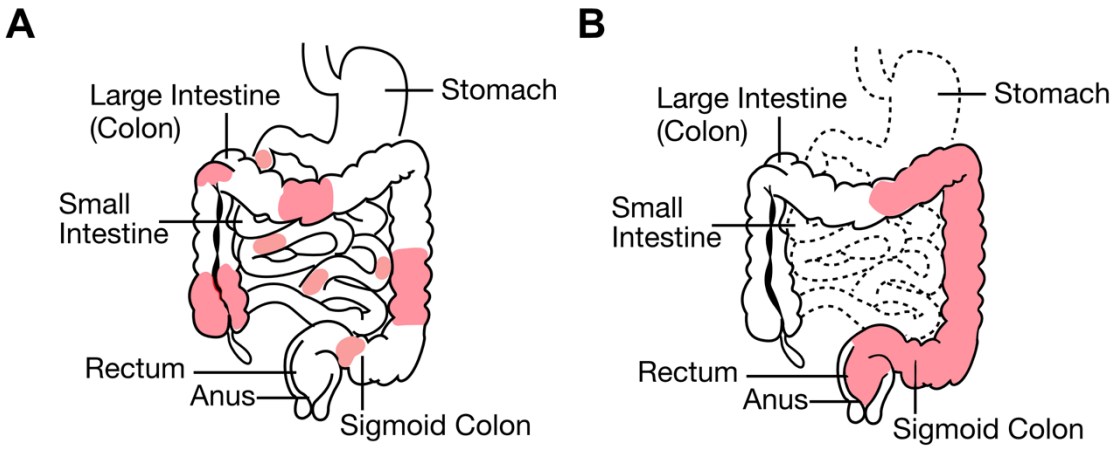


**Figure 1. Anatomical regions affected by IBD**. Schematic depiction of affected parts in salmon red in the context of CD (A) or Ulcerative colitis (B). Adapted from open-source figure: https://commons.wikimedia.org/wiki/File:Patterns_of_Crohn%27s_Disease.svg

From a macroscopic perspective, in UC, large-scale ulcers span along the affected colonic section. For one, these superficial ulcers impair the physiological roles of the

intestine and can be the source of precancerous lesions developing over the course of the disease (**Kakiuchi et al. 2020**). At the same time, mucosal lesions in CD exist but are more often found in the vicinity of Peyer's patches, which can be considered lymphoid tissue located within the mucosal layers of the small intestine (**de Saussure and Soravia 2005**). Histologically these ulcers are the macroscopic correlate of chronically inflamed tissue, elucidating the molecular reasons for which are the subject of this dissertation. One key histological hallmark of CD is the occurrence of macrophage aggregates which often form noncaseating granulomas (**Yi Li et al. 2015**). In contrast, UC is characterized by micro-abscesses composed of neutrophils in the mucosa and crypts.

Importantly, also, differences in adaptive immune cell infiltration exist. While in CD, the inflammatory component is amplified by T helper cells type 1 (Th1) with interferon and IL-2 dominated milieu, UC is characterized by a strong involvement of Th2 cells (**Graham and Xavier 2020**). Clearly, they represent the clinical challenge and reason for complication and ensuing surgical approaches. The ultimate long-term consequence of chronically inflamed mucosa is the development of fibrosis, fistulas, and malignant transformation, representing a pressing need to better understand the underlying molecular underpinnings.

Clinically, treatment modalities for IBD vary depending on the type, extent, and severity of the disease and are comprehensively discussed in (**Roda et al. 2020; Kobayashi et al. 2020**). And while the onset and course of IBD are determined by a combination of genetic, host-specific, and microbial factors, the exact sequence of events leading to IBD remains elusive. While Crohn's Disease (CD) and Ulcerative

Colitis (UC) are distinct from a clinical perspective, it remains unclear whether they also differ pathologically or if they fall within a single range. Therefore, genetic studies, such as genomic fine-mapping efforts in the context of genome-wide association studies (**GWAS**), are of critical importance. More recent studies have expanded our view on the genetic basis of IBD, adding layers of complexity such as transcriptomic, microbiomic, or metabolic and contributed significantly to a more comprehensive understanding of the pathogenesis of IBD (**Schirmer et al. 2019; Graham and Xavier 2020**).

## Mucosa and mucosal inflammation

### *Mucosal Anatomy*

By definition, the gastrointestinal tract spans from the oral cavity to the anus and is covered by varying kinds of epithelial cell layers. Next to mechanical protection, the intestinal epithelium takes over most tasks specific to the gastrointestinal tract ranging from nutrient and water resorption to metabolic and hormonal functions. The intestinal epithelium plays a crucial role in innate immunity by actively shielding the host through various mechanisms that defend the mucosal layer (**Allaire et al. 2018**). The primary line of defense against harmful factors in the lumen is provided by the intestinal epithelial cells (IECs), and only when these defenses are breached do professional immune cells become activated. This is because the IECs act as the initial line of defense. The epithelium prepares and communicates with immune cells to enhance an effective immune response or an inflammatory response. The intestinal epithelium consists of various cell types such as enterocytes, goblet cells, neuroendocrine cells, tuft cells, Paneth cells, and M cells, all subject to further subclassification.

The various epithelial cell types work together to preserve the stability of the intestine and support the host's defense mechanisms, utilizing their unique functions. In conclusion, mucosal immunity has to be able to tolerate the luminal microbiota and not react to their products while still safeguarding the intestinal mucosa from potentially dangerous dietary antigens and invading pathogens.

### *Anatomy of the crypt*

Next to stratified intestinal non-keratinized squamous epithelium covering the oral cavity, the upper two-thirds of the esophagus, as well as the rectum, the majority of the functional gastrointestinal tract, is covered by glandular epithelium (**van der Flier and Clevers 2009; Sato et al. 2009; Beumer and Clevers 2021**). These glands consist of intestinal crypt, small, tube-like structures that are found in the lining of the small intestine and colon. The crypts and villi are covered with specialized epithelial cells, which are responsible for secreting mucus and other substances that help to protect and lubricate the intestinal lining.

Crypts are important because they are the site of stem cell proliferation and differentiation, which helps to maintain the intestinal lining (**van der Flier and Clevers 2009; Sato et al. 2009; Beumer and Clevers 2021**). When the intestinal lining is damaged, stem cells in the crypts are activated to divide and differentiate into new epithelial cells, which helps to repair the damage and maintain the integrity of the intestinal lining. Crypts also play a role in the immune system, as they contain immune cells called Paneth cells, which produce antimicrobial proteins that help to protect the intestine from infection.

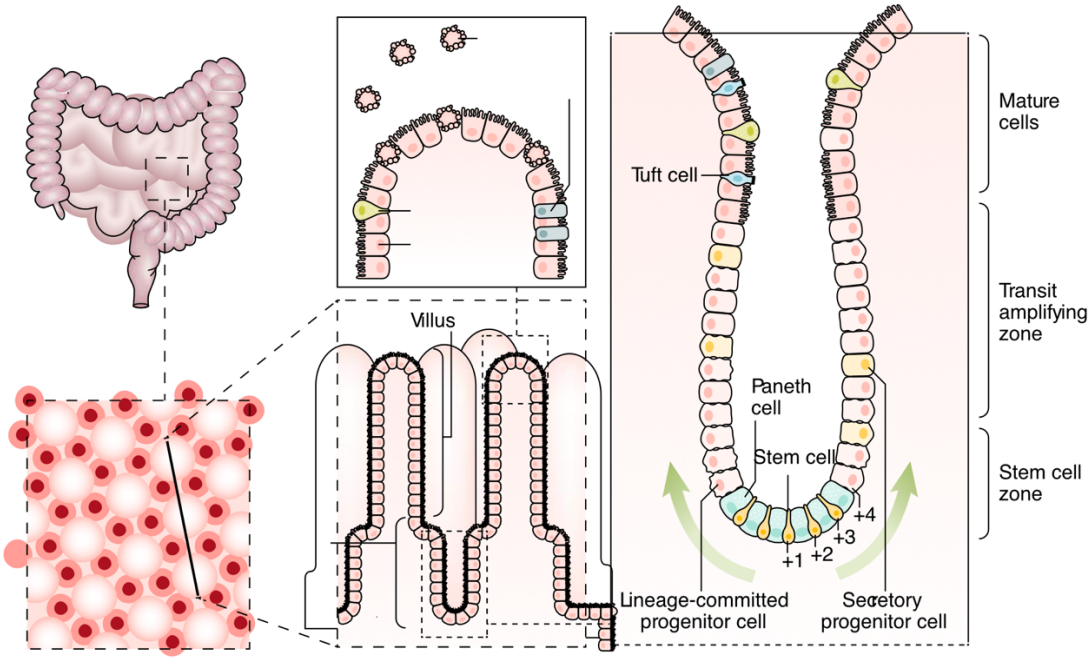


**Figure 2. Mucosal architecture of the villus crypt unit.** The micro-histological depiction of the mucosal architecture throughout the intestinal tract, especially the small intestinal tract as depicted. Crypt-villus units line the entire small intestinal lumen and are rejuvenated by a constant pool of self-replicating stem cells in a Paneth cell niche. Adapted from Gehart and Clevers 2019, *Nat Rev. Gastroenterology and Hepatology*

***Epithelial cell types with functions in immunity***

As discussed, a variety of specialized epithelial cell decorates the intestinal cell lining, thus including the intestinal crypts. Among these, so-called Paneth cells are of particular interest. Paneth Cells, originally coined as „*Paneth-Körnerzellen*“ by the Austrian Physiologist Joseph Paneth (1857 – 1890), are merocrine cells, which are hallmarked by the presence of eosinophile granules and reside at the basis of small intestinal crypts (crypts of Lieberkühn) but can occasionally also be found in the stomach or rectal mucosa. The function of Paneth cells can be divided into two main clades; first and foremost, these cells act similarly to immune cells secreting anti-bacterial/microorganismal proteins such as lysozyme, several kinds of peptidases, lactoferrins, or alpha-defensins. Secondly, Paneth cells play a vital role in maintaining a viable intestinal stem cell pool by providing these ISCs with critical growth factors and other ligands. A unique expression signature of these cells also qualifies them as sensors for qualitative and quantitative aspects of bacterial or other microorganismal presence in the lumen of the gut.

Notably, Paneth cells express high levels of Nucleotide-binding oligomerization domain-containing protein 2 (NOD2), whose importance in the context of IBD will be of special interest throughout this dissertation (**Damgaard et al. 2012**). NOD2 is a pathogen recognition receptor (PRR) and is involved in the immune response to bacteria. As a member of the NOD-like receptor family, variants in *NOD2*, have been linked to an increased risk of developing Crohn's disease, a type of inflammatory bowel disease (IBD). These mutations can cause NOD2 to be overactive or underactive, leading to an abnormal immune response in the digestive tract and the development

of IBD or Blau’s Syndrome. While NOD2 mutations are not the only cause of Crohn's disease, they are thought to play a role in the development of the condition in some individuals. Clearly, more mechanistic insights are required to decipher how NOD2 variants cause hyperactivation of the immune system or immunodeficiencies.

## Considerations on the Origins of Inflammatory Bowel Disease (IBD): genetic and non-genetic basis

As mentioned earlier, CD and UC are summarized and grouped together under the term IBD. Symptomatic commonalities include diarrhea, and blood discharge from the intestine, occasionally accompanied by abdominal pain. Clinically the disease occurs in flares which is largely due to a cycling dysregulation of mucosal immunity triggered by and shaping aberrant microbiota signatures, which molecular underpinnings are the subject of this dissertation. IBD typically presents between the second and third decade, although theoretically, it can begin at any age (**GBD 2017 Inflammatory Bowel Disease Collaborators 2020**). Epidemiologically prevalence is increasing in virtually all age groups, including within pediatric or geriatric populations, arguing for strong non-genetic but habitual factors shaping the onset of disease. Among these metabolic and diet-linked aspects of Western life are suspected to play a significant role, although the exact etiopathology remains to be fully elucidated (**Adolph et al. 2022**). Importantly, genetic tools are increasingly used to elucidate etiopathological aspects or to ascertain diagnoses in clinically suspicious cases (**Zeissig et al. 2015**).

Inflammatory Bowel Disease (IBD) is driven by both genetic and environmental factors, resulting in mucosal inflammation and erosion. It is linked to an imbalanced commensal microbiome that leads to a recurring cycle of increasing and decreasing pathological inflammation. While IBD was once considered to primarily affect Western countries, its occurrence is growing in the Eastern Hemisphere, following the spread of industrialization and westernization in these regions.

**The intestinal microbiome and inflammatory bowel disease (IBD)**

The term microbiota encompasses microorganisms such as viruses (e.g. bacteriophages), bacteria, archaea, and fungi, which colonize various niches within the (human) body as a shared habitat (**Manichanh et al. 2012; Wahida, Ritter, and Horz 2016; Wahida, Tang, and Barr 2021**). The relationship of the individual organism to the host can be symbiotic, commensal, or under certain conditions, pathogenic. In the human body, various niches are colonized by microbiota; the gastrointestinal tract contains the largest number of microorganisms. By definition, the microbiome comprises the totality of all microbial genomes and functions of a given habitat. Numerically, the microbiota of the human intestine consists of up to 1014 bacterial species, most of which can be assigned to four bacterial phyla: *Actinobacteria*, *Bacteriodetes*, *Proteobacteria*, and *Firmicutes.* The composition of the intestinal flora can vary considerably even between related individuals. For example, an analysis of the intestinal microbiome of a monozygotic twin pair only a match of 17 %. In contrast, the core functions performed by the intestinal flora as an "organ", such as the breakdown of long-chain carbohydrates, are almost universally detectable in healthy individuals. Metagenomic studies have shown that the diversity of microbial species in the gut can serve as an indicator of the host's health. In contrast, disturbances in microbiota composition are now associated with diseases of various forms. These include gastrointestinal infections, malignancies, and rheumatological, metabolic, and neurological diseases.

Pathological changes in microbiota diversity are also referred to as dysbiosis. Thanks to the possibilities of modern metagenomic methods, such as high-

performance gene sequencing and rapid advances in bioinformatics, our knowledge of the human microbiome has multiplied. Data on the gut microbiome are largely based on DNA obtained from stool samples and only to a small extent on biopsies. The clinical applicability of these methods is currently still limited. One reason for this is the high variability of results between different sequencing centers. Causative to the development of IBD is an inappropriate immunological response to commensal microbiota in individuals who are carriers of genetic susceptibilities (**Strigli et al. 2021**).

Dysbiosis was shown to be a feature of inflammatory bowel diseases. Differences in the microbiota composition of patients with Crohn's disease or ulcerative colitis, on the one hand, and healthy individuals, on the other hand, have been demonstrated. This is generally reflected in a globally reduced biodiversity. As representatives of this bacterial phylum, some *Clostridia* species, such as *Faecalibacterium prausnitzii* or members of the *Lachnospiraceae*, produce short-chain fatty acids, such as butyrate, which has anti-inflammatory properties and is also an important source of energy for epithelial cells of the colon as well as potent signaling properties on various cells in the gut. In contrast, patients with Crohn's disease, especially with the involvement of the terminal ileum, experience an increase in *Enterobacteriaceae* (phylum: *Proteobacteria*). Thus, Escherichia coli can be detected more frequently in the area of granulomas or fistulas in patients with terminal Crohn's ileitis. A shift in the ratio of Firmicutes to Bacteroidetes is associated with a predisposition to various diseases. However, since this ratio can vary widely even between healthy individuals, its clinical relevance is debatable. (**Kurilshikov et al. 2021**).

One of the early assumptions was that, since so many genetic susceptibilities were found in genes encoding pattern recognition receptors, such as NOD2, deregulated sensing of microorganisms was the reason behind the occurrence of aberrant microbial architectures. Single nucleotide polymorphisms (SNPs) in various genes important to immunity were therefore scrutinized regarding their potential effect in affecting the microbiome and thereby representing a genetic liability in the context of IBD. One of the foremost studies testing this hypothesis was conducted at the Flavell lab and investigated the role of NLRP6 and its potential ability to alter the overall microbiome structure. Indeed, it was found that Nlrp6-knockout mice were more susceptible to developing intestinal inflammation in a DSS model and this was attributed to their inability to mount proper Nlrp6-mediated signaling cues.

Clearly, this established a conceptual framework for how impaired innate signaling represented the common genetic basis for microbiome-driven inflation in the gut. While this and subsequent studies provided an attractive and orthogonal model for intestinal inflammation in general, one important procedural detail was left unnoticed. In general, these studies were largely based on gene-targeted mice comparing these to wildtype controls. Inbreeding of strains to generate the genotypes of interest without the consideration of using heterozygous mice as breeders to generate both wildtype animals, as well as homozygous knockout mice, led to the accumulation of strain-specific maternally inherited microbiome signatures that overruled potential genotype-driven patterns. The use case of NLRP6 as a candidate relays shaping the microbiome and thereby the subsequent susceptibility to inflammatory triggers was reevaluated in various hygienic/geographic contexts and

using a rigorous breeding strategy with sufficient wildtype controls involved in the generation of heterozygous animals (**Mamantopoulos et al. 2017**).

Unexpectedly, a detailed analysis of all 16S results of this study revealed that deletion of *Nlrp6* in mice bore no effect on the propensity to develop exaggerated inflammatory responses and by virtue of these to the microbiome overall. This seminal work led to a reconsideration of what it actually meant when observing altered microbiome changes were observed in different transgenic murine models (**Wullaert, Lamkanfi, and McCoy 2018; Elinav et al. 2018**). Mouse husbandry and maternal inheritance and the more or less spontaneous occurrence of intestinal inflammation in conjuncture with altered microbiome patterns thus became one of the mainstays in controlling for potential experimental biases. Nevertheless, the hypothesis that impaired sensing of the microbiome as illustrated by the occurrence of genetic variants in such genes or pathways still remains a valid path forward to disentangle aberrant host-microbiota interactions.

The notion that NOD2 and IL23R are one of the three most frequent genes affected in IBD, argues for this line of research, but mouse models fail to recapitulate the human situation in its full. One of the reasons thereof is that mice are bred and held in controlled environments minimizing the potential impact of pathogens or alternative exponents which might lead to variability in experimental series. This has been countered by the elegant so-called “rewilding” experiment (**Lin et al. 2020; Yeung et al. 2020**). In the absence of spontaneous microbiome alterations or inflammatory cues in conventional/SPF housing conditions, various mice lacking or

carrying genes thought to play a critical role in IBD (*i.e., Nod2, Atg16l1*) were housed in an outside enclosure with exposure to a vastly diverse microbial specter.

From a scientific point of view, one major question which needs to be addressed is, whether dysbiosis represents the cause or alternatively the consequence of the disease (**Sommer et al. 2017**). One could even reformulate this question to ask; are the host's genetics the determinants which shape the architecture of the microbiome (**Weissbrod et al. 2018**)? In healthy subjects, this question was addressed by the Elinav group in 2018 (**Rothschild et al. 2018**). Here using a large-scale sequencing effort, no considerable effect size was detected. Earlier studies have tried to address the same question in patient collectives affected by IBD but were unable to identify a clear genotypic basis for microbiota signatures, albeit identifying genetic variants associated with subtle changes on the species-level (**Jostins et al. 2012**).

Taken together, GWAS efforts have landscaped a large collection of genetic susceptibilities predisposing to IBD and converging with the development of microbial dysbiosis, associated with a reduced diversity within the microbiome and a marked loss of commensals in particular *Firmicutes*.

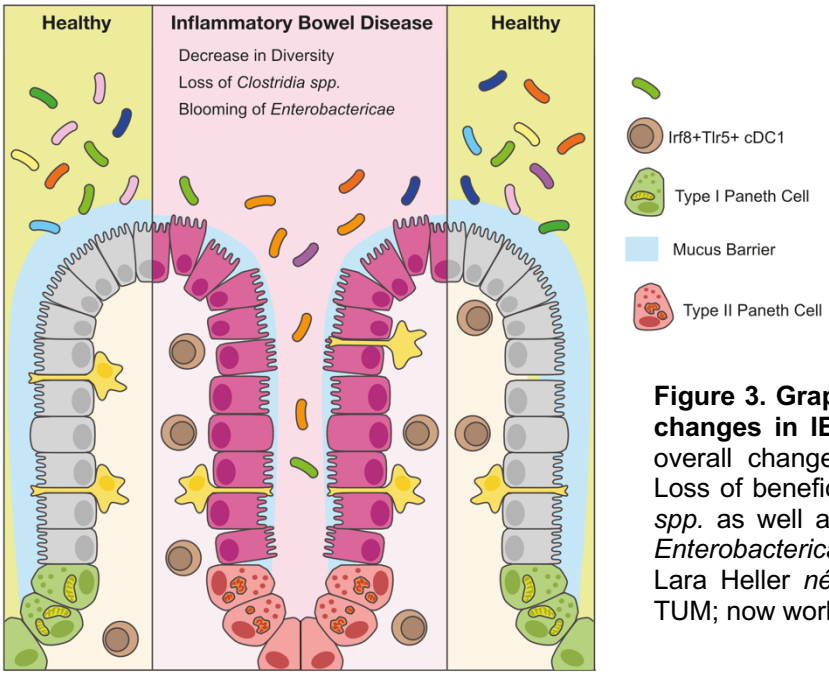


**Figure 3. Graphical summary of microbiome changes in IBD.** Disease model summarizing overall changes in microbial patterns in IBD: Loss of beneficial symbionts such as *Clostridia spp.* as well as blooming of several clades of *Enterobactericae*. Adapted and conceived by Lara Heller *née* Hartjes (formerly AG Ruland, TUM; now working at CeGaT, Tübingen)

## Genetics of IBD

Subsequent to the deciphering of the draft human genome in 2001 (**Venter et al. 2001**), large-scale efforts began rapidly trying to map the genetic heterogeneity of human populations. The aim was to functionally assign genotype-phenotype relationships, especially in the context of complex trait disorders, thereby identifying both triggers and therapeutics to these conditions. To date, IBD has been linked to over 200 gene loci, but identifying causative genes or variants within these loci requires more than just sequencing. GWAS stands for "*genome-wide association study*". This is a high throughput method using genomics to identify genetic variants associated with a particular (complex) trait or disease.

These studies employ genomic data from a vast number of individuals, trying to identify specific single nucleotide polymorphisms (SNPs) or other genetic variations which are statistically more common in individuals with a particular trait or disease compared to control individuals lacking the trait or being disease free. Subsequently these genetic variations or so-called "associated variants," can provide hints regarding the underlying pathological mechanisms implicated in the trait or disease.

Over the past 20 years, GWAS has been a valuable tool in identifying genetic factors involved in various diseases, such as cardiovascular disease, diabetes, cancer, and mental illness, as well as non-disease traits such as height, weight, and hair color. While GWAS has been effective in identifying IBD risk loci, identifying the causative genes and variants within these loci requires further investigation. Trans-ancestry studies of IBD have shown that NOD2 is more commonly associated with

IBD in European populations, while TNFSF15 is more prevalent in East Asian populations. The search for rare causal variants by exome sequencing can be limited by the need for large cohort sizes, but there are ways to overcome this challenge. Populations with a founder effect or genetic bottleneck, such as Ashkenazi Jews, can provide a richer source of rare alleles and their associated phenotypes. Exome sequencing studies of Ashkenazi Jews compared to non-Ashkenazi Jewish Europeans have revealed new coding variants in GWAS loci and new genes contributing to the higher incidence of CD in Ashkenazi Jews. Another solution is to conduct genetic studies in diverse ethnic populations, which may uncover the heterogeneity in genetic risk factors and suggest the existence of distinct disease subtypes.

Exome sequencing has revealed multiple separate genetic variations in genes that represent a range of allelic variations, which are associated with a spectrum of outcomes ranging from disease risk to disease protection. For example, exome sequencing identified risk and protective Risk and protection variants were identified that form allelic series in multiple genes, including CARD9, IL23R, and RNF. These results highlight the significance of innate microbiome detection pathways, cytokine networks, and barrier function in determining the risk of IBD. Further functional studies are needed to elucidate the mechanistic basis of risk and protection. These efforts are crucial for creating treatment plans that either suppress mechanisms related to disease risk or imitate mechanisms that provide protection.

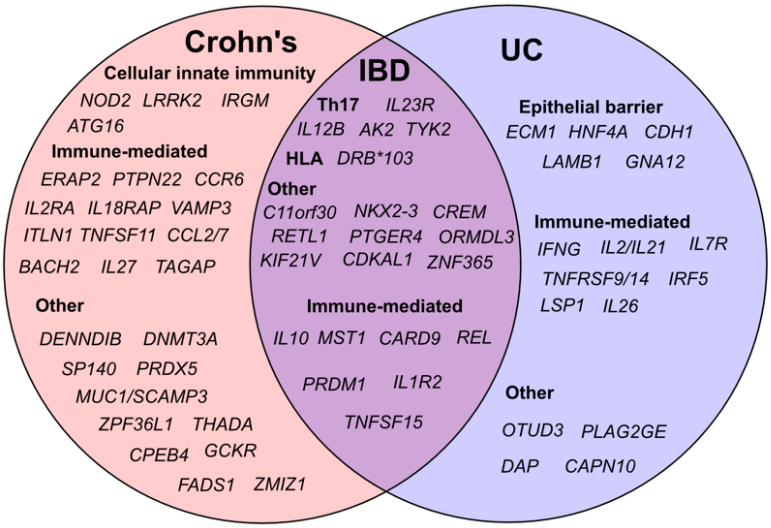


**Figure 4**. **Venn diagram with genes identified through GWAS studies**. GWAS genes where polymorphisms and variants have been associated to development of CD (salmon red), UC (mauve) or IBD in general (overlap). Genes are clustered according to their biological function e.g., immune related. Adapted from: https://www.ibdresearch.co.uk/what-is-inflammatory-bowel-disease-ibd/identifying-the-ibd-genes/

Although IBD is heritable, common susceptibility variants explain less than 20% of IBD incidence in pediatric and adult-onset IBD (**Jostins et al. 2012; Cutler et al. 2015**). Remarkably, both adult- and pediatric IBD share the same spectrum of over 200 susceptibility variants, implying that non-genetic factors are involved in triggering the pediatric disease. Genetic loci associated with disease progression are enriched in pathways active in mononuclear phagocytes, T–cell exhaustion, and antigen presentation. An exception is monogenic primary immunodeficiencies that have a higher disease penetrance, with pediatric very-early-onset (VEO-IBD), as observed in patients with mutations in IL-10 or Il-10-receptor deficiency (**Glocker et al. 2009; Zhu et al. 2017**).

Yet, despite these strong genetic drivers, not all primary immunodeficiency patients succumb to IBD. Turning to sporadic IBD in adult patients, one of the most frequently affected genes is *ATG16L1*, which in mice, even though homozygously affected, does not show spontaneous inflammation even if Paneth cells are impaired (**Cadwell et al. 2008**), arguing for (1) differences in rodent models of disease (2) Paneth cell dysfunction alone is not sufficient to trigger disease – a concept which will be important later on this dissertation.

However, a subsequent story was able to show that a second axis, if impaired, could jointly with a loss of *Atgl16l1* deficiency trigger intestinal inflammation (**Adolph et al. 2013**). Biologically in essence is a member of the autophagy-related protein family, which is critically involved in the process of autophagy. Autophagy plays a role in getting rid of damaged or redundant cellular elements, especially during stressful periods. Lastly, these fine mapping efforts also yield knowledge about yet

understudied aspects of IBD susceptibility beyond the regulation of inflammation. Variants directly affecting epithelial integrity, such as epithelial polarity (*TTC7A*) have been associated with very early-onset IBD (**Bigorgne et al. 2014**). This gene is understood to maintain epithelial apicobasal polarity by acting as a critical component of the PI4KIIIα complex. Moreover, other examples exist, such as genes essential to epithelial adhesion (*EPCAM*) (**Lei et al. 2012**), which when affected can lead to congenital Tufting enteropathy or congenital sodium diarrhea.

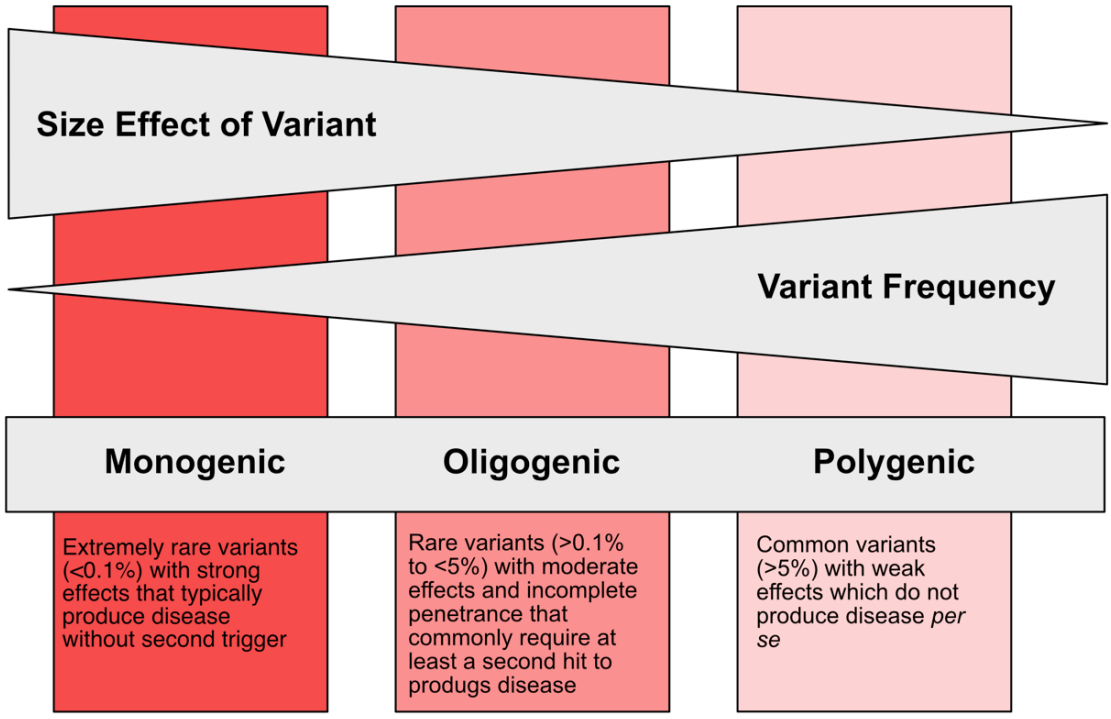


**Figure 5. Anticorrelation of effect size and prevalence of disease associated genetic variants.** Graphical summary of how low frequent variants which are associated to monogenic disease and present with a large effect size i.e. penetrance. On the contrary, complex genetic trait disorders such as sporadic IBD present with variants with an inherent low effect size but high prevalence. Adapted from Giudicessi et al. (**Giudicessi and Ackerman 2013**)

Beyond epithelial polarity, other important aspects of intestinal physiology such as electrolyte transport mechanism are important genetic sites of susceptibility. For instance, the familial diarrhea syndrome is caused by GUCY2C polymorphisms (**Mishra et al. 2021**), and the gain of function variants controlling the secretion of NaCl and water. GUCY2C encodes an epithelial receptor for endogenous ligands uroguanylin and guanylin, which can activate the cyclic guanosine monophosphate (cGMP) production and by virtue of this activation of CFTR via activation of protein kinase GII. Taken together, these findings broaden our understanding of new genes that control metabolic processes such as electrolyte balance, epithelial orientation, and barrier function.

**Primary Immunodeficiencies and (very)-early onset (VEO-)IBD as a tool to dissect genotype-phenotype interactions**

I elaborated on the fact that IBD progresses through a multifactorial stepwise process driven by complex interactions of a series of polygenetic predispositions and environmental factors. Interestingly, IBD is frequently diagnosed in the context of monogenic diseases, especially primary immunodeficiencies (**Pazmandi et al. 2019**). Next generation sequencing (NGS) of genomes has revolutionized the identification of genetic variants for monogenic disorders. So far, over 350 primary immune disorders have been discovered and connected to various molecular causes, of which approximately a third are related to gastrointestinal symptoms.

Recently, a growing number of these rare diseases have been found to frequently present as IBD, or solely as IBD. Early identification of these conditions allows for the adaptation of treatments, which has a direct impact on the course of IBD. One of the genes initially associated with extremely early-onset Inflammatory Bowel Disease (IBD) was IL10RA. Individuals with severe hypomorphic alleles in this gene have reduced IL-10 signaling, leading to a breakdown in tolerance. IL-10R signaling in macrophages is critical for limiting intestinal inflammation. Similarly, GWAS studies have found noncoding SNPs near IL10 and weaker signaling in the IL10RA locus to be associated with adult IBD. Other examples include lymphoproliferative disorders related to a lack of cell death regulatory genes such as caspase 8 (CASP8) and XIAP, or genes involved in regulating T cells, such as FOXP3 or CTLA4, which often cause intestinal inflammation. On the other hand, mutations associated with immunodeficiencies have shed light on intestinal mucosal immunity. In patients with a mutation in MALT1 that impairs NF-κB signaling, defective selection

of the T-cell receptor repertoire was observed. In addition, intestinal symptoms occur in congenital immunodeficiencies such as chronic granulomatous disease, which is linked to mutations in CYBB. Potential signs of the disease include an early onset, reliance on intravenous nutrition, poor response to typical immunosuppressive treatment, a family history of close relatives marrying, heightened vulnerability to infections, specific histopathological observations, and blood test results that are atypical for traditional Inflammatory Bowel Disease (IBD).

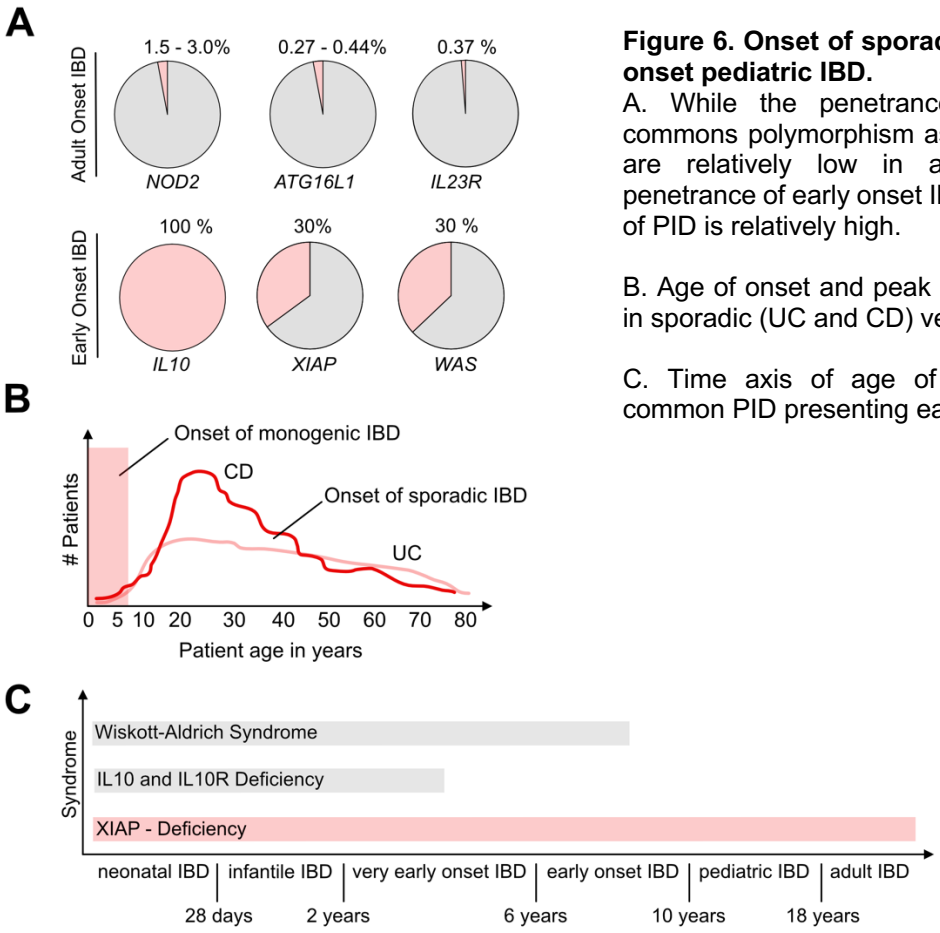


**Figure 6. Onset of sporadic versus early onset pediatric IBD.**
A. While the penetrance of the most commons polymorphism associated to IBD are relatively low in adult-onset IBD, penetrance of early onset IBD in the context of PID is relatively high.

B. Age of onset and peak incidence of IBD in sporadic (UC and CD) versus PID

C. Time axis of age of onset in three common PID presenting early onset IBD

Taking these factors into account, one could propose a diagnostic route to uncover PID-dependent IBD (**Tegtmeyer et al. 2017**). Hyperinflammatory states associated with cytokine imbalances, and Immunodeficiencies related to impaired antimicrobial response. Immunodeficiencies can lead to an unhealthy relationship with the

microbiome, thus providing a possible explanation for the intestinal Localization of these pathologies.

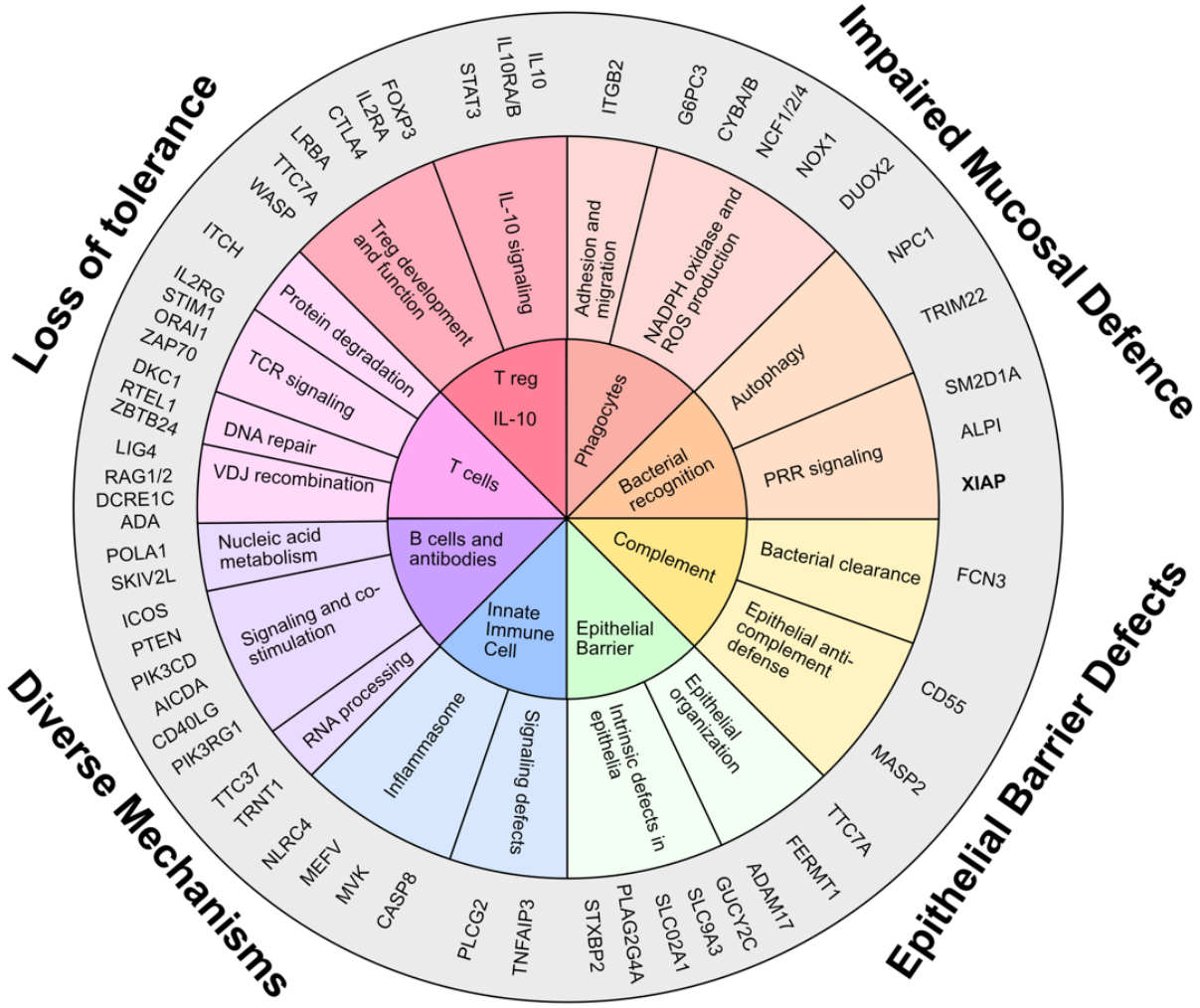


**Figure 7.Gene Clusters of PID associated with early onset IBD.** Summary of all genes associated to IBD in Primary immunodeficiencies, clustered according to gene function. Adapted from (**Pazmandi et al. 2019**)

## XIAP deficiency at the intersection of aberrant TNF signaling and cell death in intestinal inflammation

The intestinal epithelium has a very high cell replacement rate, and this process is strictly controlled (**Fuchs and Steller 2015**). The transit-amplifying progenitor cells in the intestinal epithelium produce approximately 300 cells per crypt on a daily basis. Controlled cell death and shedding at the top surface maintains the overall cell count in balance. An increase in the rate of death of intestinal epithelial cells (IECs) leads to extensive epithelial erosion, which is a hallmark of several intestinal disorders such as inflammatory bowel disease and infectious colitis. Growing evidence suggests that necroptosis, autophagy, and pyroptosis play vital roles as forms of programmed cell death in the intestine, in addition to apoptosis. The way in which cells die has an impact on the tissue repair responses and, as a result, determines the potential for developing intestinal fibrosis and colorectal cancer in the long term. A harmful cycle of intestinal barrier disruption, abnormal cell death, and resulting inflammation is the central issue in chronic inflammatory and infectious gastrointestinal conditions. (**Patankar and Becker 2020**).

### *TNF Signaling*

Tumor necrosis factor (TNF) is a critical cytokine in pathological inflammation, underscored by the fact that biologicals that can neutralize TNF are among the most successful therapeutics for autoimmune and chronic inflammatory diseases (**van Loo and Bertrand 2022**). More importantly, in physiology, TNF plays a pivotal role in mediating inflammation to enforce homeostasis in various contexts. By instructing inflammatory gene expression programs or, this has become clear in recent years, by triggering cell death (**Manolis Pasparakis and Vandenabeele 2015)**, TNF can fine-tune and amplify inflammatory cues. And while cell death downstream of TNF has clear physiological roles, aberrant activation thereof is the subject of intense research. This dissertation will focus on one scenario in which TNF-dependent death is relevant due to a deficiency of the X-linked inhibitor of apoptosis protein XIAP (encoded by *BIRC4*).

TNF (tumor necrosis factor) signaling is a complex process that involves the activation of TNF receptors, the production of downstream signaling molecules, and the regulation of various cellular processes. TNF is a cytokine secreted by mainly innate immune cells, playing a key role in the immune system's response to infection, inflammation, and tissue damage. There are two types of TNF binding receptors, TNF-alpha receptor 1 (TNFR1) and TNF-alpha receptor 2 (TNFR2). When TNF binds to these receptors, it activates signaling pathways that lead to various cellular responses, including inflammation, cell death, and changes in gene expression. TNF signaling can be either pro-inflammatory or anti-inflammatory, depending on the context in which it occurs.

Nevertheless, prolonged inflammation can also lead to tissue damage or chronic disease (**van Loo and Bertrand 2022**). TNF signaling is regulated by various signaling molecules, including protein kinases and transcription factors, which control the activation and expression of downstream targets. TNF signaling involves various physiological and pathological processes, including immune responses, inflammation, cancer, and autoimmune diseases. Dysregulation of TNF signaling has been linked to various diseases, including rheumatoid arthritis, Crohn's disease, and cancer. Understanding TNF signaling may help to develop new therapies for these and other diseases. In recent years, it has become clear that TNF triggers inflammatory responses not only directly by inducing inflammatory gene expression, but also indirectly by triggering cell death, which triggers inflammatory immune responses and disease development. Therefore, cell death inhibitors are emerging as novel therapies for TNF-dependent inflammatory diseases.

### *X-linked lymphoproliferative syndrome type 2 (XIAP deficiency) and as an ultra-rare cause of mendelian IBD*

XIAP is best known as a checkpoint of non-immunogenic apoptosis. However, mutations in the gene encoding for XIAP, *BIRC4*, are the mendelian cause of the immunodeficiency termed X-linked lymphoproliferative syndrome type 2 (XLP2). Original reports describing young boys with a fatal early onset lymphoproliferative syndrome, histologically hallmarked by lymphohistiocytosis, a marked hyperactivation by various T-cell and dendritic cell subsets, were published in 1975 by Purtilo *et al*. (**Purtilo et al. 1975**) In 2006, seminal work revealed the underlying genetic cause of XLP2 syndrome, i.e. mutations in BIRC4 the gene coding for XIAP (**Rigaud et al., 2006**). About 30% of these XLP2 patients succumb to a form of IBD that is frequently resistant to therapy and associated with a lethal outcome in roughly 10% of all patients (**Speckmann et al., 2013**). In addition, XIAP mutations were identified in 4% of pediatric very-early-onset (VEO) IBD male patients (**Zeissig et al., 2015**). Recently our group implicated XIAP in restricting RIPK3-dependent inflammatory cell death (**Yabal et al. 2014**). However, the role of TNFR2 mediating inflammatory cell death *in vivo* and thus pathology in XLP2 disease remained unclear.

***XIAP and cell death – molecular biology***

XIAP directs cell survival through various regulated cell death pathways and directs a spectrum of inflammatory signaling events. Initially classified as a protein that binds to caspases, XIAP was initially believed to primarily prevent apoptosis caused by both internal and external triggers. On the other hand, XIAP prevents cell death caused by TNF and receptor-interacting protein 3 (RIPK3) by regulating the ubiquitylation of RIPK1 and inhibiting the death of inflammatory cells. Identifying individuals with hereditary mutations in XIAP, known as XLP-2 syndrome, emphasized the significance of XIAP in regulating inflammation. XIAP promotes the proinflammatory signaling of NOD2 by enhancing the ubiquitination of RIPK2 within the NOD2 signaling complex, leading to the activation of NF-κB and MAPK and the production of cytokines and chemokines that cause inflammation. In general, XIAP plays a crucial role in regulating various cell death and inflammatory processes, making it a promising target for drugs in treating tumors and inflammatory diseases. (**Damgaard et al. 2012; Yabal et al. 2014; Jost and Vucic 2020**).

***Cell death in IBD – general aspects and TNF therapy***

A crucial series of genes not originally identified in GWAS encode proteins that regulate inflammatory cell death, also known as regulated necrosis, or necroptosis. The importance of this pathway in intestinal homeostasis was first shown in murine studies of Caspase-8 (**Günther et al., 2011**). Epithelial deletion of Caspase-8 resulted in severe ileal inflammation characterized by Paneth cell death and increased immune cell infiltration and as well as marked elevated expression of RIPK3. Cell death was induced by TNF-mediated activation of RIPK1. Importantly, the central role of TNF, RIPK1 and RIPK3 in mediating intestinal epithelial cell death was also demonstrated in the murine model of ATG16L1 deficiency, an IBD susceptibility gene discussed above (**Matsuzawa-Ishimoto et al., 2017**). Furthermore, deleting epithelial *Ripk1* resulted in an increased TNF-dependent apoptotic cell death (**Takahashi et al. 2014**). Supporting these murine findings, in the subsequent, mutations in *RIPK1* and *CASP8* have been identified in patients with severe immune deficiencies and IBD (**Cuchet-Lourenço et al. 2018; Lehle et al. 2019; Yue Li et al. 2019**). However, in addition to promoting cell death, RIPK3 was also shown to promote epithelial tissue repair in the murine DSS-colitis model (**Moriwaki et al. 2014**). Thus, there remain open questions on the precise role of these proteins in both disease initiation and progression, especially in non-mendelian IBD (**Matsuzawa-Ishimoto et al. 2017**). *This paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

## Open questions and aim of this study

As broadly discussed, one important point in IBD research is linking genetic susceptibility with non-genetic factors triggering the disease. The second main question is establishing a clear sequence of events leading to chronic inflammation in the gut. Lastly, specific questions related to XIAP deficiency, in particular, will be of special interest to this dissertation. The first deals with establishing the exact roles of TNF-receptors 1 and 2 and their pleiotropic role in different cell types in the intestinal tract. The second question largely relates to the question of varying penetrance in IBD. While the penetrance of developing IBD-like symptoms in XLP-2 syndrome is much higher compared to sporadic IBD, 30 % versus < 1 %, there is still room for elucidating non-genetic factors triggering pathology. In the absence of large and diverse human specimens to work with, the main model that was used throughout this project is a murine model of XIAP-deficiency, the *Xiap*$^{-/-}$ mouse generated almost two decades ago (**Olayioye et al. 2005**). This mouse constitutes a “full-body” knockout model, therefore mimicking the clinical situation in patients carrying truncating and therefore deleterious variants in *BIRC4*.

Additionally, we leveraged a series of gene-targeted murine models to dissect the role of both TNF receptors in the pathogenesis of intestinal inflammation. Next to this main workhorse, a small number of patient biopsies served as the basis for omic analyses, supporting the hypotheses and data generated *a priori* in a murine setting.

## II. RESULTS

### Intestinal inflammation in *Xiap*$^{-/-}$ mice varies in husbandries with varying hygienic contexts

Although a murine model of the XIAP-deficiency syndrome (XLP-2 disease) had been developed before the discovery that genetic lesions in *BIRC4* are the genetic cause of this primary immunodeficiency (**Rigaud et al. 2006**), the intestinal phenotype of these mice was never studied in depth (**Olayioye et al. 2005**). To address this question, the intestinal histological phenotypes of wildtype and *Xiap*$^{-/-}$ were assessed. Using the "*Swiss Roll*" technique (**Moolenbeek and Ruitenberg 1981**), a longitudinal examination of the entire intestinal tract becomes possible.

Next to basic staining methods allowing for the assessment of basic histopathological findings, intestinal tissue embedded with this technique also allows for epitope-specific immunohistochemistry in relation to the anatomical/spatial expression.

The first assessment of the small intestines of a series of mice of both genotypes and sexes and of 8 – 14 weeks old, revealed that, overall, there was no overt phenotype in the sense of massive intestinal inflammation such as accumulation of immune cell infiltrates, erosion of the mucosa with tissue architecture destruction or the occurrence of fissures/fistulas and fibrosis, reminiscent of the human histopathological correlate. On the contrary, overall tissue architecture seemed intact, with a regular lining of crypt-villus structure along the duodenal-jejunal-ileal tract. In

fact, section-specific examination and comparison revealed that in comparison to wildtype controls, the duodenum and jejunum did not show any differences when examined in a blinded manner.

However, a closer examination of the terminal ileum, an anatomical site especially vulnerable to the development of IBD-like phenotypes, revealed that in *Xiap*$^{-/-}$ mice intestinal villi showed some signs of apical congestion (**Figure 8A-B**). A distortion of the upper two-thirds of the villus characterized this. This was hallmarked by a dislocation of the basal layer of the epithelial cell lining with basal membrane bridges connecting the epithelium with the lamina propria. Although fixing and dehydration would not confirm or exclude the exact pathogenic origin of this, rodent pathology classifies this finding as a mild subepithelial edematous swelling. This finding is observed during transient intestinal infections or sublethal radiation and could indicate intestinal inflammation in the context of genetic susceptibility. This yields the necessity of several control experiments and considerations which will be described in the subsequent sections.

Before doing so, an in-depth histological examination was performed. First, and in a plausible manner regarding the intestinal phenotype, hyperproliferation as a possible consequence of low-grade inflammation was observed. For instance, a BRDU proliferation measurement showed a significant increase in positive nuclei in single-housed mice which were knockouts for XIAP compared to wildtype controls (**Figure 9A-B**). Additionally, Beta-catenin nuclearization was observed, indicative of elevated Wnt signaling in the context of hyperproliferation (**Figure 9A-B**).

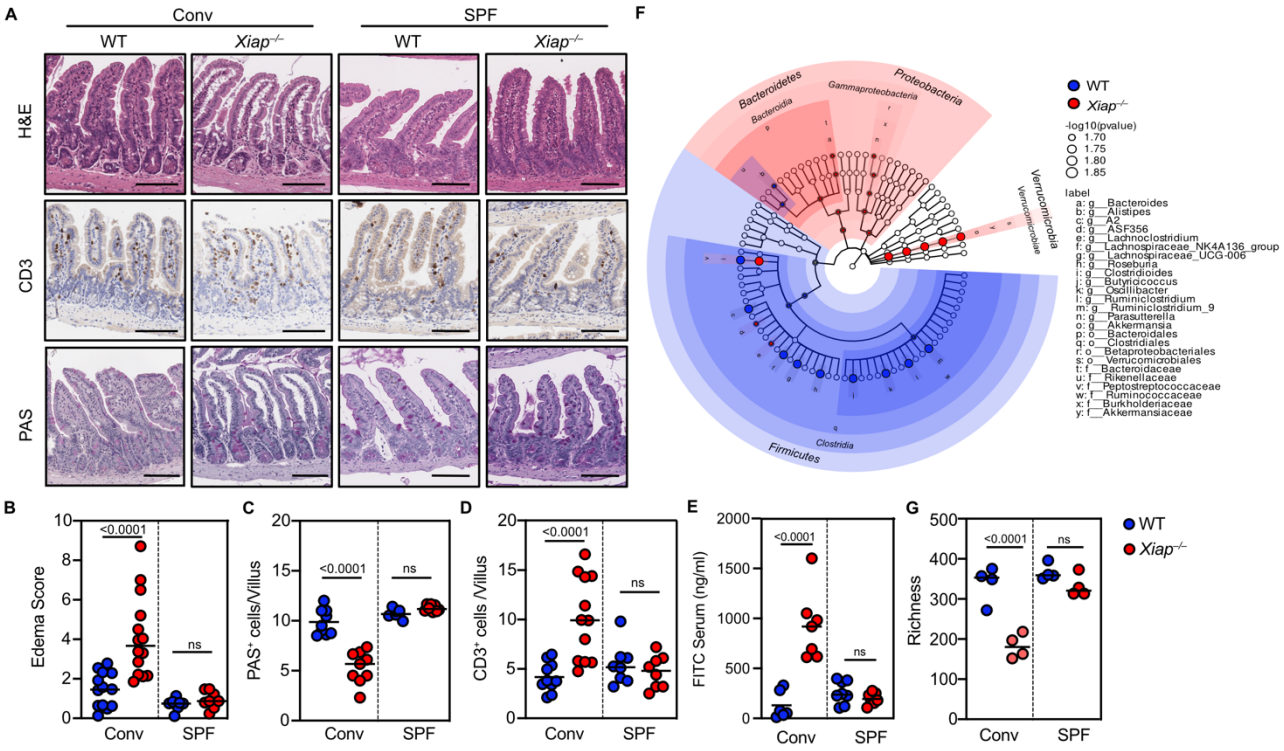


**Figure 8**. **Ileitis in *XIAP-deficient* mice is dependent on the hygienic context**.

A. Representative pictures (scale bars are at 200 µm) of ileal microsections stained for Hematoxylin and eosin, CD3 via IHC and PAS reaction.
B. Quantification of edema score of HE sections of wildtype and *Xiap*$^{-/-}$ animals from the conventional housing (Conv), or SPF-housing (SPF)
C. Number of goblet cells were counted for 20 representative villi of wildtype and *Xiap*$^{-/-}$ animals from the conventional housing (Conv), or SPF-housing (SPF) and averaged per mouse
D. Number of CD3+ T-cells were counted for 20 representative villi of wildtype and *Xiap*$^{-/-}$ animals from the conventional housing (Conv), or SPF-housing (SPF) and averaged per mouse
E. Serum concentration of FITC-dextran as a proxy for intestinal permeability in wildtype and *Xiap*$^{-/-}$ mice from the conventional or SPF-housing context.
F. Cladogram of differentially abundant bacterial taxa between wildtype and *Xiap*$^{-/-}$ mice. Significantly altered species and families or taxa (alpha < 0.05) are color-coded according to corresponding sample groups. Nonsignificant taxa are represented in translucent circles.
G. Bacterial diversity (Richness) based on 16S sequencing of wildtype and *Xiap*$^{-/-}$ mice from conventional and SPF-housing.

All data were analyzed by one-way ANOVA (P < 0.0001) and in figure are reported P values from post hoc analysis with Holm-Šidák's correction. In all figure panels, each dot represents a mouse.

*Figure and Legend were modified and taken from "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Furthermore, in accordance with the hyperproliferative and inflammatory character of the subepithelial edema, CD3+ cells were quantified as a marker for T-cell infiltration (**Figure 9D**) and PAS reaction (**Figure 9C**), which stains mucin-positive cells such as goblet cells and whose abundance can serve as differentiation marker and therefore proliferative turnover rate. Both an increased infiltration of T cells as measured by CD3, the T cell receptor as a pan T cell marker, and decreased number of PAS+ cells, which constitute the goblet cell population, were indicative of low-grade inflammation.

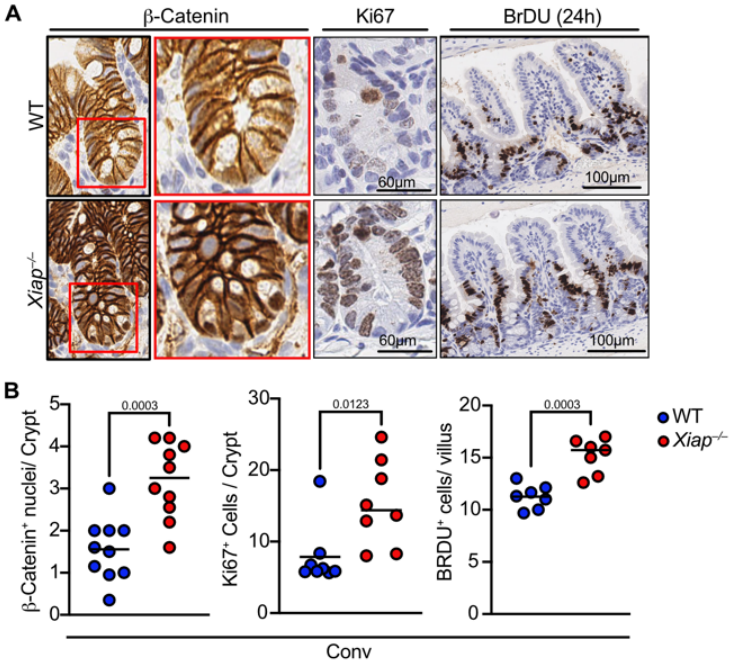


**Figure 9. Hyperproliferative effect on inflamed tissue of XIAP-deficient mice.**

A. Representative sections of beta-Catenin, Ki67, and BrDU in ileal tissue microsections from wildtype and *Xiap*$^{-/-}$ mice from the conventional housing facility. BrDU staining 24h after injection.
B. Quantification of A using average count in 20 representative crypts or villi averaged per mouse.

All data were analyzed by Student's t-Test and p-values are reported in figure.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

A general consideration, also in conjuncture with the absence of observed phenotypes in the same mouse lines in different mouse husbandries, was that the phenotype could be mouse line and/or housing specific rather than genotype driven. Indeed, mouse husbandries differ regarding their hygiene status, and mouse inbreeding introduces breeding artifacts and accumulation of polymorphisms potentially masking or amplifying the underlying genetic lesion in question. In our case, mice were housed in a conventional facility, not fulfilling the requirements to receive SPF (specific pathogen-free) status, usually standard for mouse experiments. Among other microorganisms, the mouse husbandry harbored various *Helicobacter* species, a bacterium known to induce low-grade inflammation in genetically susceptible animals (**Chichlowski et al. 2008**) (**Figure 10B**). Thus, to challenge this hypothesis, *Xiap*$^{-/-}$ mice were rederived, recrossed with wildtype animals, and kept in an alternative mouse husbandry, located not in Munich but in Italy (Calco, LC).

Surprisingly, mice that were housed in an SPF facility did not show signs of intestinal inflammation as observed in the original cohort bred in Munich (**Figure 9A**). Clearly, this finding suggested that either a breeding artifact (and) or an external factor triggered the onset of intestinal inflammation in XIAP-deficiency. The first reason would be a clearly disappointing finding and signify a clear mismatch between murine and human pathophysiology. The latter would on the contrary support the notion of a two-hit model in IBD-like clinics in the context of XLP-2 disease, with environmental factors triggering disease in a genetically susceptible host.

Next, a functional test, corroborating the consequences of the histological phenotype, we performed a barrier test to investigate whether the inflammation would

affect intestinal permeability (**Figure 9E**). To this end, we employed the FITC-dextran permeability assay, where a large molecular dextran molecule is coupled to a fluorophore (FITC) and orally gavaged to mice. Subsequent to an “incubation time” of four hours, upon which the permeability of the intestinal barrier will more or less let these molecules trespass into the bloodstream. The concentration in the serum of mice is then measured using an automated plate reader able to detect fluorescence. This experiment revealed that in line with the histological findings indeed the barrier function of XIAP-deficient mice was impaired and that the leakiness, as measured by the FITC-dextran permeability assay, was significantly heightened.

Clearly, a hygienic context drove a functionally relevant loss of mucosal integrity, raising the question of how the microbiome composition in our mice was constituted. Therefore, we set out to map out microbial diversity and variance in our different mouse lines. To investigate the microbiome, most stable to habitual or other external factors, we chose the cecal microbiome, reported to reflect long-term changes in the intestinal flora landscape. We collected cecal content from hour mice and collaborated with Prof. Dr. Dr. André Gessner and Dr. Andreas Hiergeist (both at the Department of Microbiology, University of Regensburg) who aided us in performing ribosomal 16S sequencing of our samples. This method allows for a phylogenetic-based characterization of the full breadth of bacterial diversity in a specific sample and is a routinely used method in microbiota research. After 16S-rRNA sequencing several parameters regarding overall diversity and the specific abundance of individual clades, families, or even species can be performed. This experiment consisted of wildtype animals compared to XIAP-deficient animals of whom we compared the microbiome

of several animals in several batches to exclude any cage effects possibly influencing the microbiome signatures independent of any genotypic influence (**Figure 9F**).

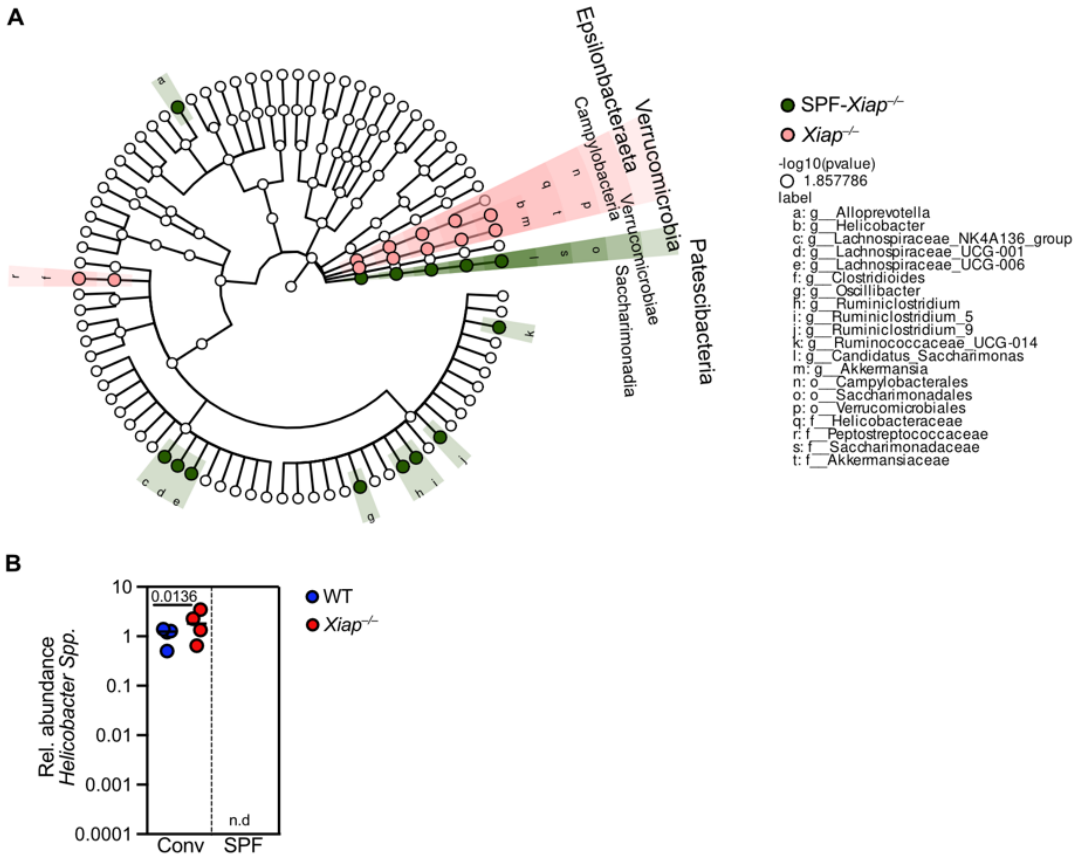


**Figure 10 Hyperproliferative effect on inflamed tissue of XIAP-deficient mice.**

A. Cladogram of differentially abundant bacterial taxa between conventionally- housed and SPF-housed *Xiap*$^{-/-}$ mice. Significantly altered lineages (α, 0.05) are color-coded according to corresponding sample groups. Non-significant taxa are represented in white.
B. Relative abundance of *Helicobacter spp*. in wildtype or Xiap-deficient mice from conventional versus SPF housing. N.d. not determined.

Data are reported as dot plots, where every dot represents an animal and were analyzed by one-way ANOVA (correction $p<0.0001$). p values from post hoc analysis with Šidák's correction are reported in figure.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Detailed analysis showed a distinct bacterial composition of conventionally-housed *Xiap*$^{-/-}$ mice compared to wildtype mice. In addition, conventionally-housed *Xiap*$^{-/-}$ mice showed a loss of bacterial richness and an extensive reduction in the *Firmicutes* phylum (particularly various members of *Clostridium* Clusters IV and XIVa within the class *Clostridia*), which was not observed in SPF-housed *Xiap*$^{-/-}$ mice (**Figure 9F**). To establish gut microbial differences between these two facilities (irrespective of the genotype), we performed an in-depth analysis of the 16S sequencing and identified various members from the *Helicobacter* genus, that were existent only in conventionally-housed wildtype and *Xiap*$^{-/-}$ mice but not SPF-housed mice. The amplicon sequence variants that mapped to the *Helicobacter* genus showed a 98% sequence identity with *Helicobacter rodentium* (alignment against the NCBI reference RNA sequence database) (**Chichlowski et al. 2008**). Taken together, we observed that conventionally-housed *Xiap*$^{-/-}$ mice spontaneously displayed signs of intestinal inflammation and epithelial dysfunction, which was associated with a clear gut microbiota signature (*i.e.*, loss of diversity and specifically *Clostridia* when compared to wildtype mice, and the presence of *Helicobacter spp.*).

**Co-housing experiments test the transmissibility of microbial signatures as well as the possibility of an infectious agent triggering inflammation**

Since inflammation was clearly correlated with exposure to the hygienic context of the conventional housing facility, one control experiment was required to exclude the presence of an infectious agent triggering disease in genetically susceptible animals. Obviously, one possible explanation of the onset of inflammation could be an infectious agent that caused inflammation and would therefore be transmissible to wildtype animals housed in the conventional husbandry. Next to testing this hypothesis, the transmissibility of potentially protective factors present in the wildtype line could be investigated. To test these hypotheses, mice of either sex or genotype were housed together just after weaning enabling the possibility for a cross-genotype exposure to strain-specific microbiota. Mice possess the intrinsic behavior of coprophagy (**Klaasen et al. 1990**), which allow for a swift and efficient dispersal of microbiota species present in stool pellets to be transmitted across animals present in one cage. This behavioral trait was exploited to check whether an infectious agent was present in XIAP-deficient mice or secondly whether protective bacterial strains were absent, thereby triggering disease in a genotype-dependent manner.

In analogy to the initial analysis of *Xiap*-deficient mice, first, an overall histological examination was performed (**Figure 11**). To our surprise, hematoxylin and eosin staining revealed that the mild ileitis was entirely abrogated in knockout animals, although they were housed in the facility that actually triggered the disease. The fact that single-housed mice (mice being housed only with littermates of the same genotype) were affected by intestinal inflammation, but as soon as these mice were housed with wildtype mice, were “cured”, strongly argued against the involvement of

an infectious agent of microorganismal nature triggering inflammation. Moreover, the data suggested that some kind of transmission of several protective microbiota strains happened during the cohousing experiment. Since mice were cohoused over the duration of four weeks, ample opportunity for microbial exchange was given. Supporting the data obtained by Hematoxylin and eosin staining, goblet cell numbers were also restored to wildtype levels, as indicated by the quantification of PAS-positive cells (**Figure 11B**) along the small intestinal parts that were quantified. Also, the infiltration of CD3-positive T-cells (**Figure 11C**) into the mucosal layers of the ileum was diminished and not distinguishable from wildtype levels. Thus, clearly, cohousing abrogated inflammation in *Xiap*-deficient hosts, which prompted us to conclude that the microbiome signatures observed in single-housed mice – and that were associated with inflammation in the conventional facility – were the drivers of inflammation but possessed a recessive nature.

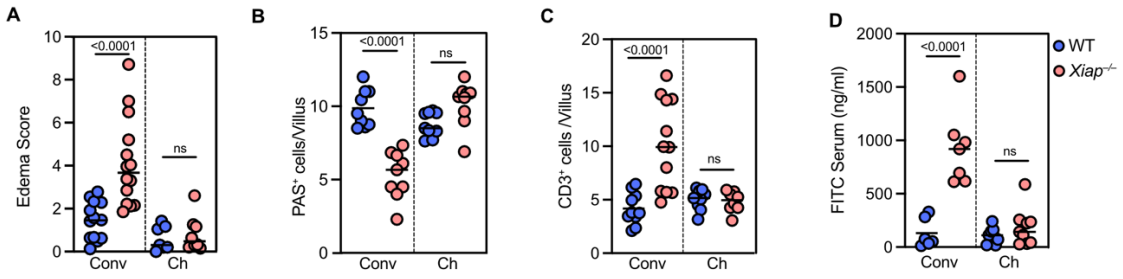


**Figure 11. Cohousing rescues ileitis in XIAP-deficient mice.**

A. Quantification of edema as "Edema score" of wildtype and *Xiap*$^{-/-}$ in the conventional facility (Conv) and cohoused wildtype and Xiap$^{-/-}$ mice (chWT and *chXiap*$^{-/-}$) in the same housing facility.
B. Goblet cell count of wildtype and *Xiap*$^{-/-}$ in the conventional facility (Conv) and cohoused wildtype and Xiap$^{-/-}$ mice (chWT and *chXiap*$^{-/-}$) in the same housing facility.
C. Quantification CD3$^{+}$ cells of wildtype and *Xiap*$^{-/-}$ in the conventional facility (Conv) and cohoused wildtype and Xiap$^{-/-}$ mice (chWT and *chXiap*$^{-/-}$) in the same housing facility.
D. Serum concentration of FITC-dextran as a proxy for intestinal permeability of wildtype and *Xiap*$^{-/-}$ in the conventional facility (Conv) and cohoused wildtype and Xiap$^{-/-}$ mice (chWT and *chXiap*$^{-/-}$) in the same housing facility.

All data were analyzed by a one-way ANOVA ($P < 0.0001$) and in figure are reported P values from post hoc analysis with Holm-Šidák's correction.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

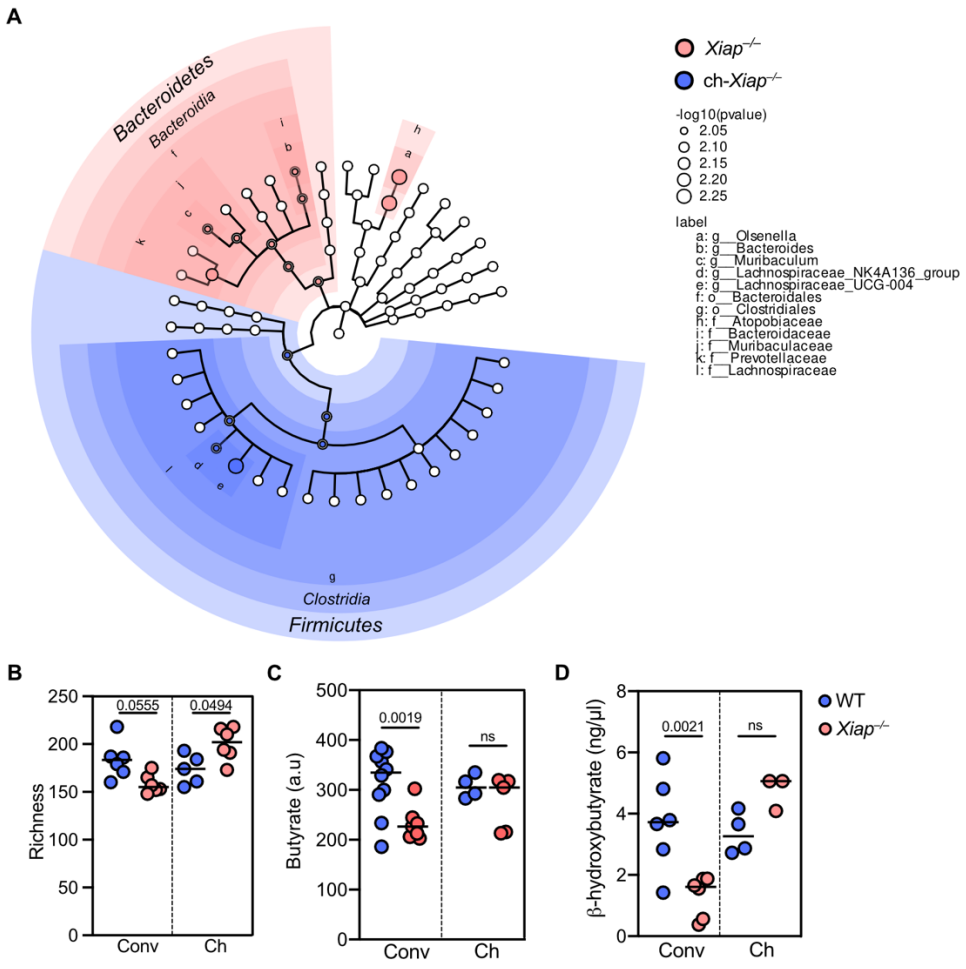


**Figure 12. Restoring Clostridia abundance prevents intestinal inflammation in *Xiap*-deficient mice.**

A. Cladogram with significantly altered lineages (alpha < 0.01) which are color-coded according to corresponding sample groups.
B. Bacterial diversity based on 16S sequencing of *Xiap*$^{-/-}$ and *chXiap*$^{-/-}$ mice from conventional housing. Statistical significance of richness was determined by one-way ANOVA and in the figure are reported P values from post hoc analysis with Holm-Holm-Šidák's correction.
C. Butyrate concentrations in cecal content from all the experimental groups. One-way ANOVA analyzed data and in the figure are reported P values from post hoc analysis with Holm-Šidák's correction.
D. beta-hydroxybutyrate concentrations in cecal content from all the experimental groups.

One-way ANOVA analyzed data and in the figure are reported P values from post hoc analysis with Holm-Šidák's correction.

*Figure and Legend were modified and taken from "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Next, we investigated whether indeed the “curative” exchange that had proceeded between wildtype and knockout animals concerned the species which were a priori found to be depleted in knockout animals, or for that matter enriched but with a still unknown pathobiological value. Thus, and in analogy to previous approaches to mapping the intestinal microbiome of our mice, we sampled the cecal content of cohoused mice of several groups consisting of both sexes, several cages, and several animals of both genotypes and proceeded to profile the ribosomal diversity of the microbiome by 16S sequencing (**Figure 12**). Surprisingly almost no bacterial clades were significantly differentially abundant in both genotypes when housed together. Moreover, we noted that co-housing with reversed loss of diversity in *Xiap*$^{-/-}$ mice. These data indicated that the wildtype microbiota corrects intestinal pathology in *Xiap*$^{-/-}$ mice and argued against a pathogen as a cause of inflammation in *Xiap*$^{-/-}$ mice. Importantly this effect was transient underscoring the inability of the XIAP-deficient host to sustain a symbiotic relationship with its microbiome (**Figure 13A-B**).

Given that members of the Clostridia class, particularly *Roseburia* species, *Faecalibacterium*, and other *Lachnospiraceae*, are significant producers of butyrate, a powerful anti-inflammatory short-chain fatty acid, we theorized that the decline in butyrate levels would result from a reduction in the abundance of Clostridia. Unbiased metabolite analysis through nuclear magnetic resonance (NMR) and colorimetric analysis of fecal matter revealed a decrease in both butyrate and beta-hydroxybutyrate, a byproduct of butyrate, in *Xiap*$^{-/-}$ mice (**Figure 12C-D**). Given that butyrate regulates gene expression by suppressing histone deacetylases, we conducted transcriptomic analysis of the small intestine ileal crypts from conventionally

housed animals to determine the impact of co-housing on host epithelial stem cells and PCs.

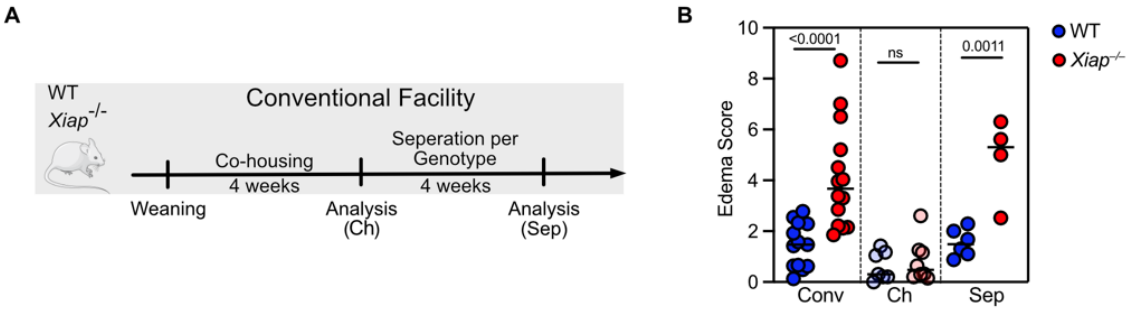


**Figure 13. Separation experiment.**

A. Schematic of experimental setup specifying housing conditions, treatment timepoint, and duration as well as the time point of analysis of cohousing of wildtype and *Xiap*$^{-/-}$ mice from conventional housing facility.
B. Edema score of WT, *Xiap*$^{-/-}$, chWT, and *chXiap*$^{-/-}$ mice and separated (sep) wildtype and *Xiap*$^{-/-}$

Data are reported as dot plots, where every dot represents an animal, and were analyzed by one-way ANOVA (correction $p<0.0001$). *P* values from post hoc analysis with Šidák's correction are reported in the figure.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

**Epithelial defects induced by TNF trigger intestinal inflammation in *Xiap*-deficiency**

The previous findings suggested that the way the host reacts to changes in the microbiome – or vice versa – was encrypted by host-dependent factors, most importantly genetics. With a clear presence of a microorganism – triangulation of the differences between SPF and conventionally housed animals revealed a clear hint towards *Helicobacter spp*. – the question was how the genetically susceptible host reacted to the presence of such “pathobionts”. The fact that *Helicobacter spp*. might represent a pathobiont and trigger disease in XIAP deficiency was suggested by elegant work from our collaborator Prof. Sebastian Zeißig (**Strigli et al. 2021**) and reviewed in depth by Azabdaftari *et al.* (**Azabdaftari and Uhlig 2021**).

Therefore, to understand the signaling cues underlying the misguided response of the XIAP-deficient host, an unbiased survey of RNA transcripts was performed using RNA sequencing. The tissue that was chosen to undergo bulk RNA-seq consisted of purified intestinal crypts, the microanatomic site of inflammation harboring Paneth cells. Crypt isolation was performed as previously described (**Sato et al. 2009**), and subsequently, whole transcriptomic analysis was performed using polyA-specific SCRB-Seq as described in (**Bagnoli et al. 2018**) with the help of Dr. Rupert Öllinger (library prep) and Thomas Engleitner (bioinformatic analysis) both from the group of Prof. Roland Rad, as well as Dr. Sainitin Donakonda (bioinformatic analysis) from the group of Prof. Percy A. Knolle.

For the analysis and reflecting the initial hypothesis, four groups were included each consisting of 4 – 5 mice. The experiment involved four groups of mice, consisting of two pairs of wildtype and *Xiap*$^{-/-}$ mice. In two of the groups, the mice were co-housed regardless of their genotype, while in the other two groups, mice were housed according to their genotype. Initial unsupervised clustering analysis of the differentially expressed genes revealed two distinct groups, closely matching the pathological segregation observed through H&E staining and scoring.

This result was surprising and suggested that the clustering of genes was able to reflect the underlying pathohistological differences seen in the gut rolls. While one group consisted of single-housed *Xiap*$^{-/-}$ mice exhibiting signs of inflammation, the other group was composed of both co-housed *Xiap*$^{-/-}$ and wildtype mice, as well as single-housed wildtype mice. These data suggest that the changes detected by RNA sequencing could serve as a pathological signature reflecting the underlying causes of the disrupted host-mediated responses leading to intestinal inflammation, potentially providing insights into the relationship between the changes in gene expression and the onset of ileitis. A thorough analysis of biological pathways revealed increased transcriptional activity in pathways related to oxidative and endoplasmic reticulum (ER) stress, as well as lipid metabolism. Additionally, we detected a loss of expression of AMPs as well as mucins (*i.e.*, *Muc13*, *Reg3g*, *Reg3b*, and *Reg4*) underscoring the concept of Paneth cell together with goblet cell dysfunction in single housed *Xiap*$^{-/-}$ mice, all while being absent in cohoused knockout mice. Given the impact of the intestinal microbiota on inflammation in *Xiap*-deficient mice, our subsequent transcriptional analysis focused on the expression patterns of toll-like receptors.

We specifically analyzed TLR5 and observed that the absence of XIAP had a potent effect on suppressing TLR5 expression. This highlights the potential role of TLR5 in the development of inflammation in *Xiap*-deficient mice. We next created a transcriptomic gene expression signature for TLR5 through gene-set enrichment analysis (GSEA), using data from a stimulation experiment in which wildtype, PC-enriched intestinal organoids were exposed to flagellin (GSE117772). This allowed us to identify genes that were differentially expressed in response to TLR5 activation. The results showed that compared to wildtype or co-housed *Xiap*-deficient mice, flagellin-stimulated TLR5 signaling was absent in the epithelial organoids of *Xiap*-deficient mice. In addition to the impaired TLR5 response, we observed that the TNF signaling pathway was highly upregulated. This suggests that TNF signaling could play a decisive role in the development of inflammation in *Xiap*$^{-/-}$ mice. Based on these observations, we hypothesized that TNF-mediated signaling is a crucial factor in the dysfunction of epithelial cells and the development of inflammation in *Xiap*-deficient mice.

Importantly to test this hypothesis we measured transcript levels of Tnf and its receptors and found that both were significantly upregulated in XIAP-deficient animals who showed signs of intestinal inflammation (**Figure 17**). Secondly, we crossed *Xiap*-deficient mice to *Tnf*-deficient mice to check whether genetic loss of *Tnf* was sufficient to abrogate inflammation and reverse the phenotypes observed in *Xiap*$^{-/-}$ mice (**Figure 18**).

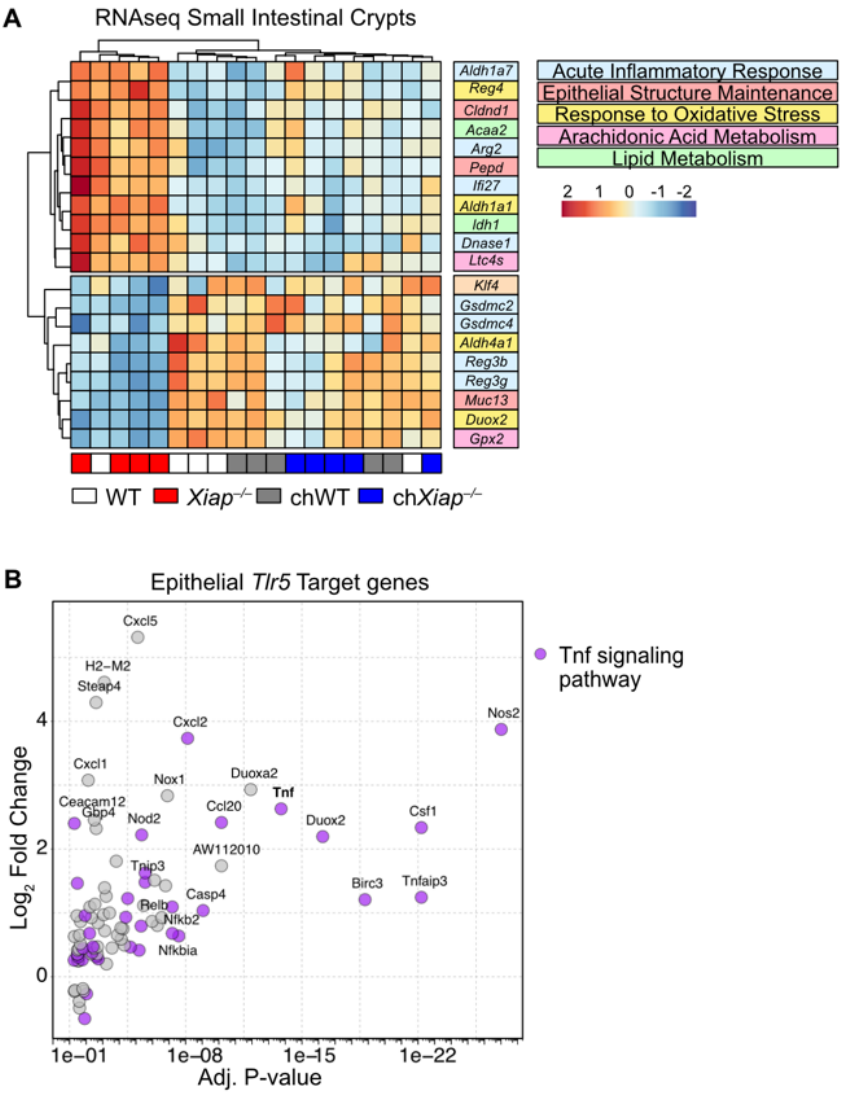


**Figure 14. Transcriptomic analysis of XIAP deficient versus wildtype tissue.**

A. Heatmap of selected deregulated genes from RNA-seq of SI crypts from WT, *Xiap*$^{-/-}$, chWT, and *ch Xiap*$^{-/-}$ mice.
B. Dot plot of epithelial *Tlr5* target genes and TNF signaling genes highlighted in mauve, based on GSE117772.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Next, we utilized CRISPR-Cas9-mediated sgRNA to delete either Tlr5 or the adaptor protein Myd88 in the organoids and assessed their ability to respond to flagellin stimulation. This allowed us to examine the role of these genes in the development of inflammation in *Xiap*-deficient mice. When either *Tlr5* or *Myd88* was absent, both wildtype and *Xiap*-deficient organoids were unable to induce TNF expression in response to flagellin stimulation. This indicates that these genes play a crucial role in activating TNF signaling in response to flagellin stimulation and provides further insight into the mechanisms underlying inflammation in *Xiap*-deficient mice. Based on these findings, we propose that the dysregulation of TLR5 signaling in *Xiap*-deficient mice leads to the dysregulation of TNF signaling, which in turn drives the development of inflammation in these mice.

We observed that TNF signaling was the primary pathway activated by TLR5 stimulation. This highlights the critical role of TNF signaling in the response to TLR5 stimulation and provides further evidence of its involvement in the development of inflammation in *Xiap*-deficient mice. This was a noteworthy discovery, as TNF is known to play a key role in the development of IBD and TLR5 is predominantly expressed by PCs, which have been suggested to be the primary site of origin for IBD. This observation further underscores the importance of TNF and TLR5 signaling in the development of IBD and provides a potential target for therapeutic intervention. Therefore, we evaluated the ability of wildtype and *Xiap*-deficient intestinal epithelial organoids to react to the cognate TLR5-ligand flagellin as well as TNF. This analysis aimed to determine the role of TLR5 and TNF in the development of inflammation in *Xiap*-deficient mice.

When exposed to flagellin, the expression levels of TNF mRNA did not differ significantly between wildtype and *Xiap*-deficient organoids. However, when stimulated with recombinant TNF, the expression of TNF in *Xiap*-deficient organoids was found to be higher compared to wildtype organoids. This suggests that the lack of XIAP leads to increased sensitivity to TNF stimulation, potentially contributing to the development of inflammation.

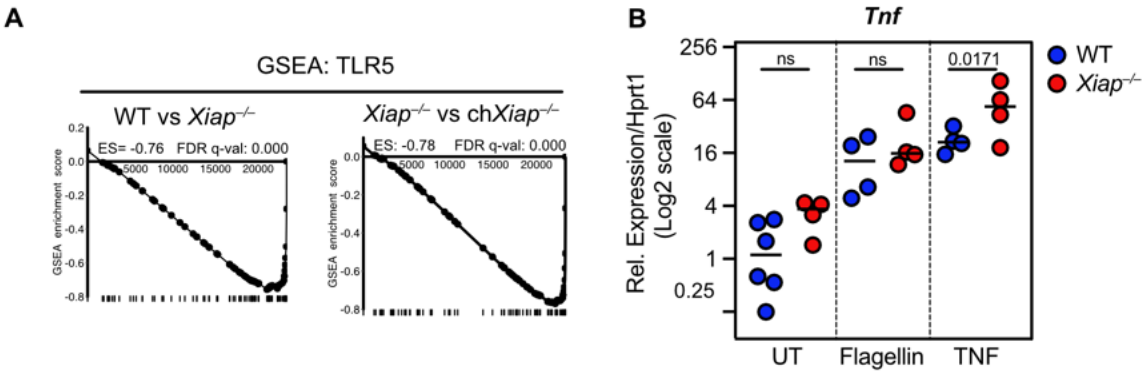


**Figure 15. TLR5 signaling is abrogated in XIAP-deficient mice**

A. *Tlr5* GSEA was based on GSE117772, comparing gene expression of crypts from wildtype versus *Xiap*$^{-/-}$
B. Quantification of *Tnf* mRNA levels via qPCR in flagellin- and TNF-stimulated wildtype and *Xiap*$^{-/-}$ SI organoids

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

We utilized the primary epithelial organoid system to further explore whether, beyond immune-derived TNF, the intrinsic production of TNF in epithelial cells could cause epithelial cell damage.

We stimulated both wildtype or *Xiap*-KO organoids with either flagellin or, alternatively TNF and carried out a morphometric survey to study the differentiation capacity of epithelial cells. This analysis aimed to provide insights into how the stimulation affected the differentiation of these cells. In response to stimulation with the TLR5 ligand flagellin, *Xiap*-deficient organoids demonstrated a higher occurrence of decaying cystic organoids, suggesting a decrease in cellular growth as well as differentiation. This result was not present in wildtype or organoid cultures that lacked Tlr5 or Myd88. Organoids lacking both *Xiap* and *Tlr5* or *Myd88* remained susceptible to TNF stimulation and showed a decrease in the number of differentiated enteroids. The findings show that upon stimulation of TLR5 by flagellin, the intrinsic production of TNF has a harmful impact on epithelial cells lacking XIAP.

Finally, to explore the mechanism by which the rise in butyrate following co-housing of both genotypes alleviated inflammation in knockout mice, we treated both organoids from both genotypes with TNF in the presence and also absence of butyrate and conducted a morphometric survey. The addition of butyrate to TNF-stimulated wildtype organoids did not result in any noticeable alterations in epithelial differentiation when comparing these to the stimulated cultures with TNF only.

In *Xiap*-knockout organoids, the presence of both TNF and butyrate resulted in a significant decrease in the frequency of cystic organoids compared to those treated with TNF alone. Together, these results suggest that one of the ways in which the increased presence of *Clostridial* species in *Xiap*$^{-/-}$ mice led to diminished inflammation was by mitigating the harmful effects of TNF on *Xiap*$^{-/-}$intestinal cells.

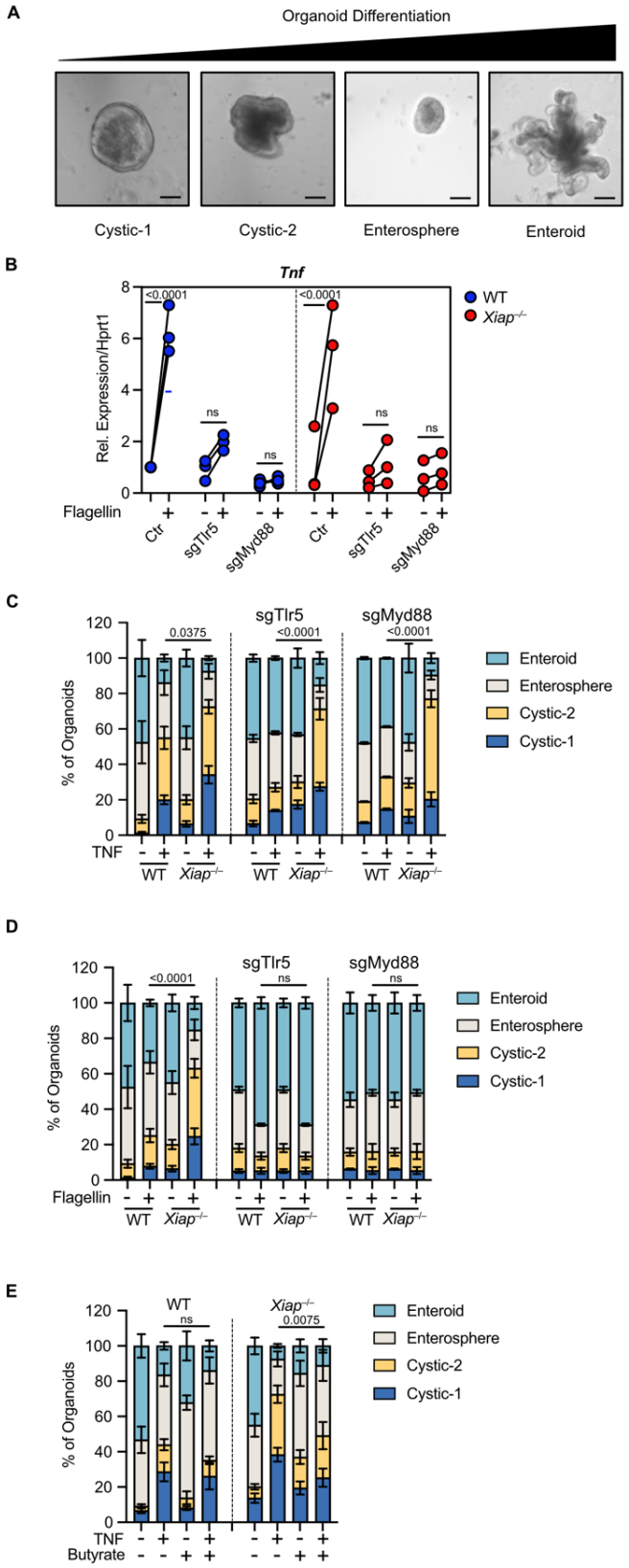


**Figure 16. TNF-dependent toxicity in *Xiap*$^{-/-}$ epithelium is induced by TLR5 signaling.**

A. Representative bright-field microscopy images of the morphological classification of SI organoid differentiation (×20 magnification; scale bars, 100 µm).
B. Quantification of Tnf mRNA levels in flagellin- and TNF-stimulated WT and *Xiap*$^{-/-}$ SI organoids not transduced or transduced with CRISPR-Cas9 and sgTLR5 and sgMyD88
C. to E. Morphometric analysis of organoids treated as reported (flagellin = 500 ng/ml; TNF = 20 ng/ml; butyrate = 1 mM).

Data were generated by at least three independent experiments, each including two to three domes, with 100 to 200 organoids per dome, and analyzed by an experimenter blind to genotype and treatment. Statistical analysis was performed using a chi-square test, with exact P values reported in the figures.

*Figure and Legend were modified and taken from "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth*

## Both TNF receptors drive ileal inflammation in *Xiap*-deficiency and dysbiosis

Since TNF was identified as the main driver of epithelial damage in XIAP-deficient mice, the next obvious question was through which receptor this detrimental effect was mediated. Especially within the context of XIAP deficiency TNF receptors act differently than normally expected in wildtype cells. For one, XIAP deficiency significantly elevates the propensity for cells (especially TLR expressing) to die in an inflammatory (necroptotic) manner, but also the downstream effects (as elaborated in the introduction) significantly differ from the wildtype situation. To further investigate the importance of both TNF receptors in the context of XIAP deficiency, we crossed our mice to gene-targeted mice lacking either receptor (*Tnfrsf1a* or *Tnfrsf1b*). The desired genotypes consisting of double-knockout animals were then subjected to the same series of experimental procedures to investigate their intestinal phenotype (**Figure 19**).

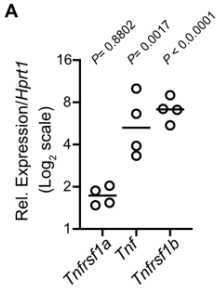


**Figure 17. TNF and its receptors are upregulated in XIAP-deficiency.**

A. Quantification of the depicted genes from ileal tissues from *Xiap*$^{-/-}$ mice, from the conventional facility, relative to their wildtype counterparts. Each dot represents an animal.

Data were analyzed by one-way ANOVA ($p<0.0001$) and in figure are reported p values from post hoc analysis with Holm-Šidák's correction.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

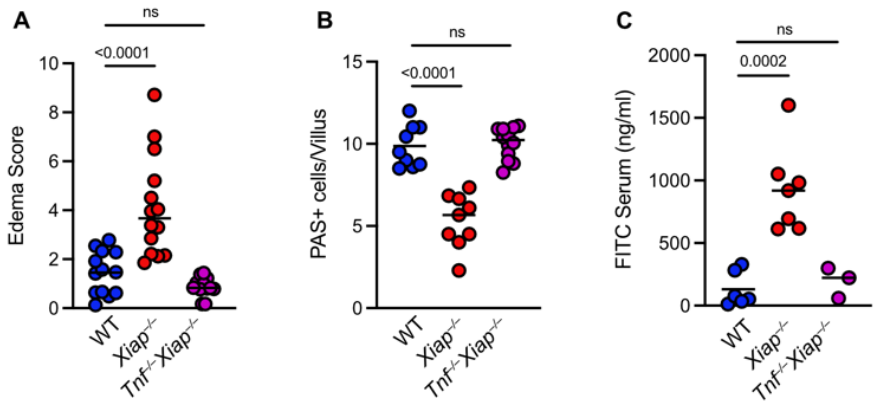


**Figure 18. TNF drives intestinal inflammation in *Xiap*-deficient mice**

(A-C) Edema score (A) and Goblet cell count (B), and FITC-dextran assay (C) of epithelial integrity of WT, Xiap$^{-/-}$, and Tnf$^{-/-}$Xiap$^{-/-}$ mice from conventional housing.

Data are reported as dot plots, where every dot represents an animal and were analyzed by one-way ANOVA (correction p<0.0001 for Edema and PAS stain, p =0.0002 for FITC). p values from post hoc analysis with Šidák's correction (PAS stain) or Dunnett's correction (Edema score, FITC-dextran concentration) are reported in figure.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

First, the overall microanatomical architecture was examined, and to our surprise, the apical villous edema, hallmarking the low-grade inflammation in *Xiap*$^{-/-}$ animals was completely absent in both *Tnfr1*$^{-/-}$/ *Xiap*$^{-/-}$ as well as *Tnfr2*$^{-/-}$/*Xiap*$^{-/-}$ animals. Additionally, the phenotype reversal was also true for the aberrant T-cell infiltration (marked by CD3$^{+}$ cells) and goblet cell count in the case of both receptors (**Figure 19**).

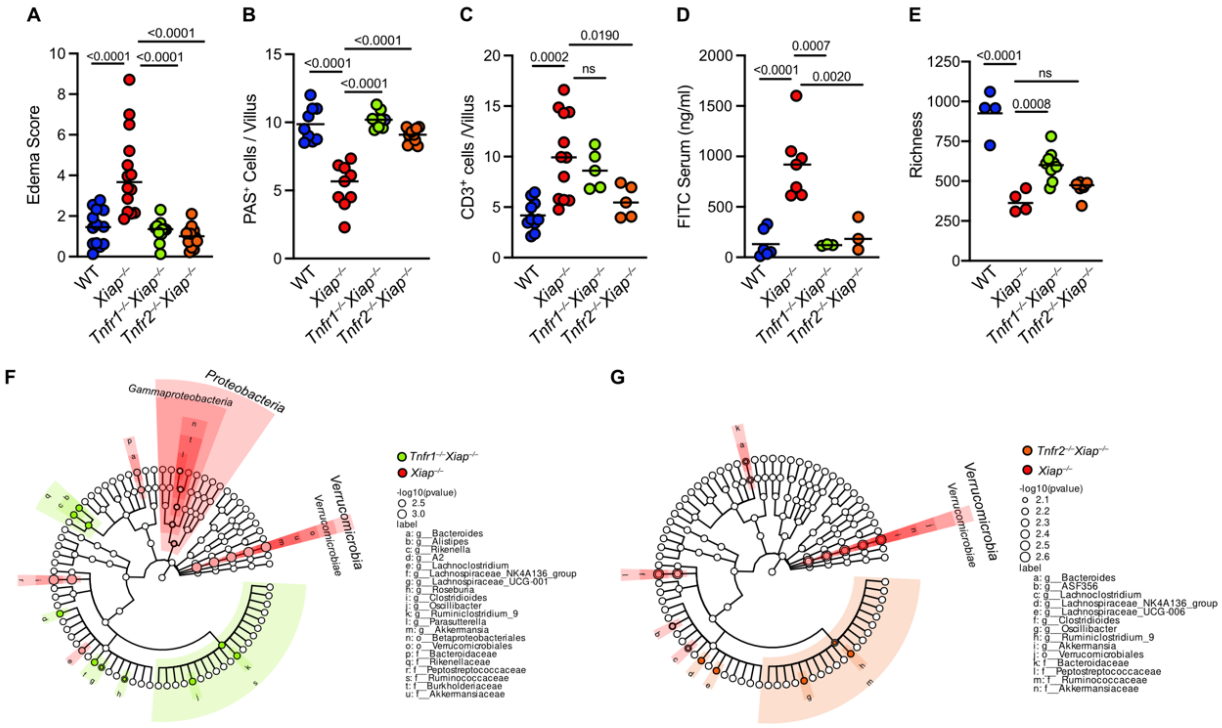


**Figure 19. TNF drives intestinal inflammation in Xiap-deficient mice**

A. Edema score of WT, *Xiap*$^{-/-}$, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$, and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice
B. goblet cell count of WT, *Xiap*$^{-/-}$, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$, and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice
C. Quantification of CD3+ cells per villus of WT, *Xiap*$^{-/-}$, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$, and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice
D. Serum concentration of FITC-dextran of of WT, *Xiap*$^{-/-}$, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$, and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice
E. Bacterial diversity (Richness) of WT, *Xiap*$^{-/-}$, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$, and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice
F. and cladogram of differentially abundant bacterial taxa based on 16S sequencing of *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$
G. and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice from conventional housing. In the cladogram, significantly altered lineages (alpha < 0.01) are color-coded according to corresponding sample groups.

Data in (A) to (E) are reported as dot blot, where each dot represents one mouse. Data in (A) to (E) were analyzed by one- way ANOVA ($P < 0.0001$) and in figure are reported P values from post hoc analysis with Holm-Šidák's correction.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

## The epithelial compartment is rescued by the deletion of TNFR1

So far, the data clearly established a pathogenic role for TNF and both of its receptors TNFR1 and TNFR2. Upstream thereof is the engagement of TLR5 which efficiently induces the expression of TNF in small intestinal organoids, leading to their demise in *Xiap*-deficient tissue. This argued for the presence of an epithelial cell type able to sense flagellin by TLR5 and then engage either TNFR1 or 2, depending on their cell type-specific expression. Thus, this warranted the presence of an epithelial cell type mediating this effect. A TLR-wide expression survey indicated that Paneth cells expressed TLR5 by using a reporter mouse system (**Price et al. 2018**). To test the hypothesis of whether Paneth cells were at the origin of intestinal inflammation, a finding that would put XIAP deficiency in line with a range of other genetic causes of inflammatory bowel disease triggered by Paneth cell loss/dysfunction, we proceeded by morphologically examining their abundance. To do so, we first stained tissue obtained from wildtype and *Xiap*$^{-/-}$ mice for a lectin expressed on Paneth cells, UAE1, and imaged its distribution across small intestinal crypts. Examination revealed a significant volume decrease of UEA1-positive cells and especially vesicles only in Paneth cells originating in *Xiap*-deficient mice, while no change could be detected in wildtype Paneth cells. These data were the first indication that in *Xiap*-deficiency Paneth Cells are unable to appropriately respond to microbial triggers.

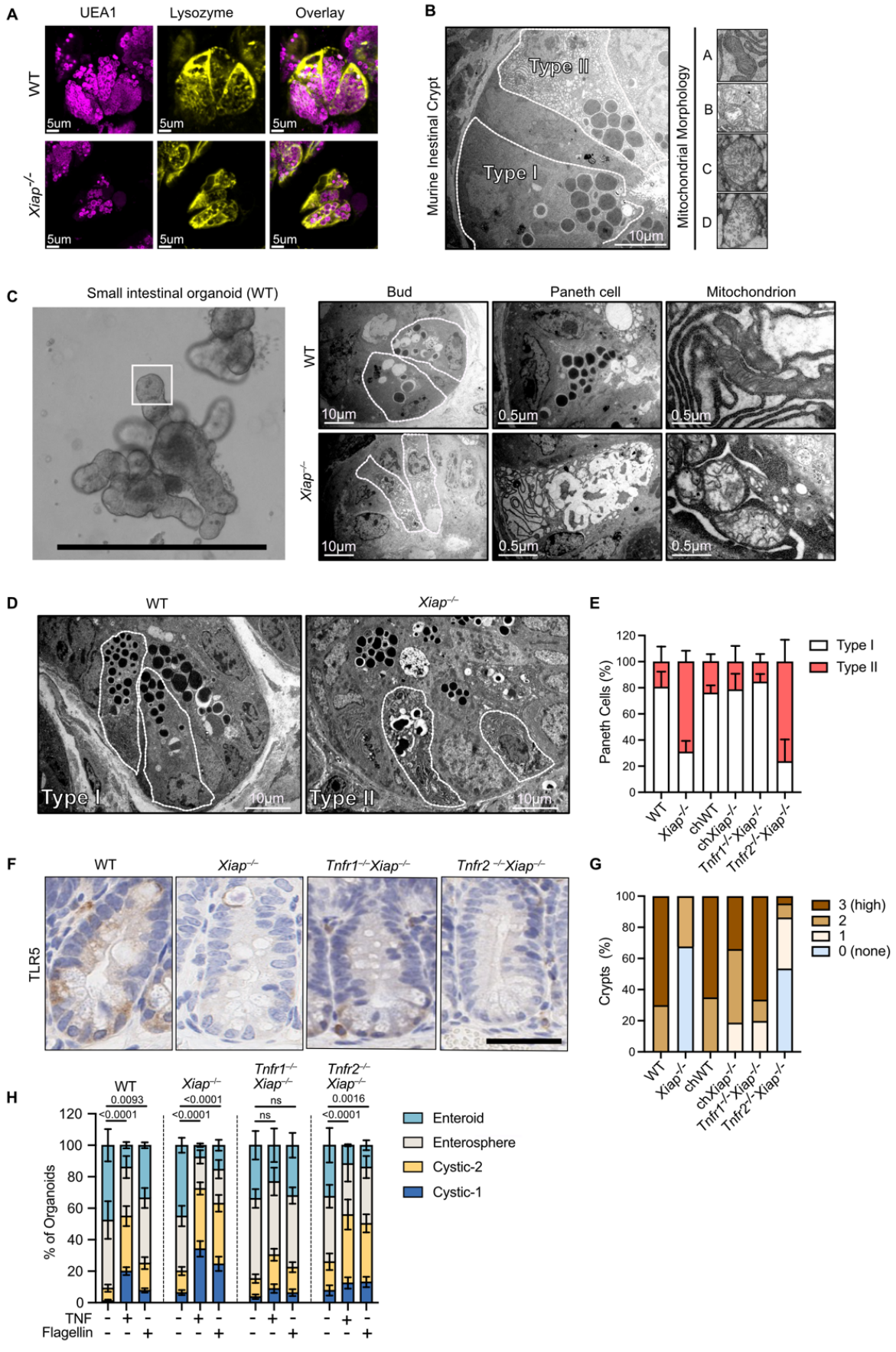
A
UEA1
Lysozyme
Overlay
WT
Xiap-/-
5um
B
Murine Inestinal Crypt
Type II
Type I
10μm
Mitochondrial Morphology
A
B
C
D
C
Small intestinal organoid (WT)
Bud
Paneth cell
Mitochondrion
WT
Xiap-/-
10μm
0.5μm
D
WT
Xiap-/-
Type I
Type II
10μm
E
Paneth Cells (%)
Type I
Type II
WT
Xiap-/-
chWT
chXiap-/-
Tnfr1-/-Xiap-/-
Tnfr2-/-Xiap-/-
F
WT
Xiap-/-
Tnfr1-/-Xiap-/-
Tnfr2-/-Xiap-/-
TLR5
G
Crypts (%)
3 (high)
2
1
0 (none)
H
WT
Xiap-/-
Tnfr1-/- Xiap-/-
Tnfr2-/- Xiap-/-
0.0093
<0.0001
<0.0001
ns
ns
0.0016
<0.0001
% of Organoids
TNF
Flagellin
Enteroid
Enterosphere
Cystic-2
Cystic-1

**Figure 20. TNFR1 signaling impairs TLR5 PC function in *Xiap*-deficient mice**

A. Representative images of Paneth cells stained with UEA1 (in purple) and lysozyme (in yellow) from WT and *Xiap*$^{-/-}$ ileal sections.
B. Transmission electron microscopy (TEM) of ileal crypts. Type I and Type II Paneth cells are outlined in white lines, and representative images of mitochondria types A-D are shown.
C. Representative TEM images of WT and *Xiap*$^{-/-}$ organoids illustrating the distorted crypt-regions of organoid buds and disrupted ER-membranes and mitochondrial morphology in *Xiap*$^{-/-}$ Paneth cells.
D. Representative TEM images of PC morphology in WT and *Xiap*$^{-/-}$ ileal sections illustrating distorted vesicle, ER membrane, and mitochondrial morphology.
E. PC grading into type I and type II according to morphology and mitochondrial integrity of WT (n = 8), *Xiap*$^{-/-}$, (n = 8) chWT (n = 4), chXiap$^{-/-}$ (n = 4), *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$ (n = 4), and Tnfr2$^{-/-}$Xiap$^{-/-}$ (n = 4) mice from indicated housing. Data were analyzed by one-way ANOVA ($P < 0.0001$) and in figure are reported P values from post hoc analysis with Holm-Šidák's correction.
F. Representative images (scale bars, 100 μm) (F) and
G. quantification of TLR5 signal within the crypt of ileal tissues (n = 5 to 10) from WT, *Xiap*$^{-/-}$, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$, and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice from conventional housing. A score of 0 is equivalent to no detectable expression, and 3 is a high expression. Twenty crypts per animal were evaluated by an experimenter blinded to the genotype.
H. Morphometric analysis of organoids from WT, *Xiap*$^{-/-}$, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$, and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice stimulated with TNF (20 ng/ml) or flagellin (500 ng/ml). Data were obtained by at least three independent experiments, each including two to three domes, with 100 to 200 organoids per dome. Statistical analysis was performed using a chi-square test, with exact P values reported in the figures.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Next, we took a further step in characterizing Paneth cell integration using transmission electron microscopy, to obtain an in-depth assessment of their ultramicroscopic appearance. At first glance Paneth cells appeared to be present in two populations, subsequently termed type I or type II Paneth cells (**Figure 20B**). While type I Paneth cell appeared to show a normal mitochondrial morphology with predominantly mitochondria of type A and tightly stacked endoplasmatic Reticulum membranes, type II showed strong signs of mitochondrial damage (here predominantly mitochondria of type D were detected), as well as signs of widespread endoplasmatic reticulum stress, depicted as dislocation membranes and structure. Moreover, a defective vesicular compartment was observed supporting the data obtained by the UAE1-stain (**Figure 20A**). These data clearly supported the notion of Paneth cell stress in *Xiap*-deficiency, as reported in previous models of IBD, and so we proceeded to systematically quantify these defects across all our experimental and genotypical groups.

Lastly, transmission electron microscopy analysis of Paneth cells from cohoused *Xiap*$^{-/-}$ as well as *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$ mice exposed a marked reduction in the frequency of type II Paneth cells in these mice, compared with single housed *Xiap*$^{-/-}$. Surprisingly, the loss of *Tnfr2* did not increase the number of type I Paneth cells. Since our hypothesis of TNF-mediated Paneth cell impairment assumed TLR5 engagement, we looked at TLR5 expression in ileal crypts, precisely in Paneth cells. Semiquantitative analysis showed reduced expression of TLR5 in PCs of *Xiap*$^{-/-}$ and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice, whereas cohousing and the deletion of *Tnfr1* were independently sufficient to increase TLR5 expression. Thus, reduced TLR5 expression in PC correlated with their disordered organelle morphology (**Figure 20**).

Based on these observations, it appeared that the deletion of *Tnfr1* had the most impact on PC integrity, whereas *Tnfr2* likely exerted its function elsewhere. Therefore, to functionally test this hypothesis, we generated organoids from *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$ and *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice to assess the impact of TNF and flagellin on their growth (**Figure 20H**). Morphometric analysis showed that although both TNF and flagellin inhibited differentiation in *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ cultures, *Tnfr1*$^{-/-}$*Xiap*$^{-/-}$ cultures remained impervious to both TNF and flagellin (**Figure 20H**). These data, therefore, suggested that TLR5-mediated epithelial and PC dysfunction in *Xiap*$^{-/-}$ epithelium could be attributed to TNFR1-mediated signaling (**Figure 20**). *This paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

## TNFR2 targets an elusive TLR5 expressing (immune) cell compartment

Careful examination and comparison of the intestinal crypt compartment versus the overall phenotype of *Tnfr2*$^{-/-}$*Xiap*$^{-/-}$ mice revealed a stunning and seeming contradiction. While the overall phenotype was clearly indistinguishable from wildtype animals, ultramicroscopic analysis of the crypt compartment revealed a similar picture found in conventionally housed and therefore inflamed *Xiap*$^{-/-}$ animals (**Figure 20**). Clearly, the mechanism by which the TNF-TNFR2 axis led to inflammation, therefore, remained elusive. The hypothesis that a second population, beyond Paneth cells, expressed TLR5 and was exclusively targeted by TNFR2 was formulated. Thus, a conclusion was that similar to the Paneth cell compartment aberrant TNF signaling could target a second, yet-to-be-determined TLR5+ population, whose identity will be needed to be ascertained. Thorough literature research revealed that a relatively uncharacterized DC population in the gut also expressed TLR5 and whose definite identity will be elucidated in the upcoming inaugural thesis of Madeleine Müller, co-first author of **Wahida *et al.* Sci Immunol. (2021)**.

**RIPK3 but not MLKL drives inflammation**

Previous work published by Drs. Jost and Yabal revealed that in the absence of XIAP, TNFR2 activation alone could trigger RIPK3-dependent cell death modality in myeloid cell populations, prior to TLR (and for that matter MYD88) activation (**Yabal et al. 2014; Lawlor et al. 2017**). Based on these previous results, the next hypothesis was that RIPK3 could be the cellular effector attributable to cellular loss of the TLR5+ myeloid population in the absence of XIAP in our mouse model of intestinal inflammation. Thus, mice were generated by crossing *Xiap*$^{-/-}$ with *Ripk3*$^{-/-}$ mice to generate double knockout *Ripk3*$^{-/-}$*Xiap*$^{-/-}$ mice. Additionally, we generated *Mlkl*$^{-/-}$ *Xiap*$^{-/-}$mice, which lacked MLKL the downstream effector of RIPK3, and the canonical executor of the necroptosis (**Galluzzi et al. 2017**). First, we tested this hypothesis by examining the intestinal phenotype of these mice, by employing our set of readouts indicative of mild mucosal inflammation in the context of *Xiap* deficiency (**Figure 21A-C**).

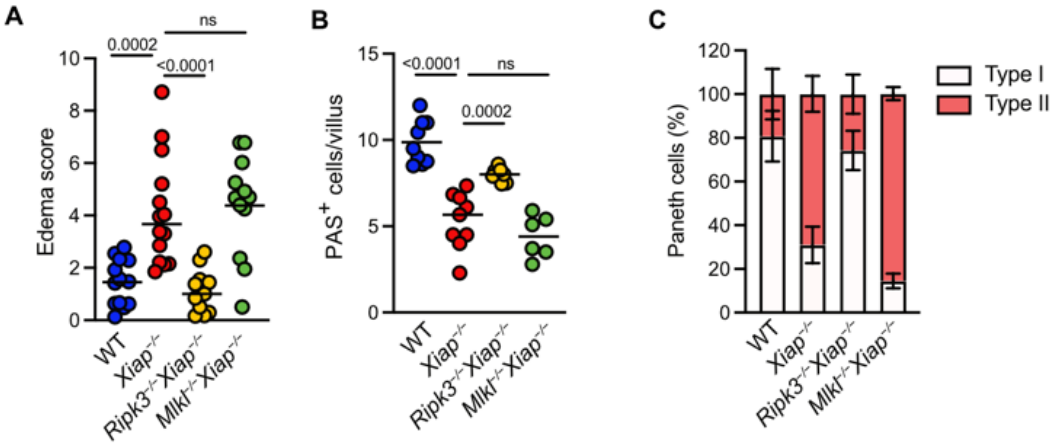

**Figure 21. Ripk3 deletion rescues ileitis in *Xiap*$^{-/-}$ mice**

A. Edema score or;
B. Goblet cells of wildtype, *Xiap*$^{-/-}$, *Ripk3*$^{-/-}$*Xiap*$^{-/-}$, and *Mlkl*$^{-/-}$*Xiap*$^{-/-}$ mice.
C. Quantification of type I and II PCs based on TEM of ileal crypts from indicated genotypes, based on 5 to 10 crypts per mouse per genotype, four to five mice per genotype.

All data were analyzed by one-way ANOVA (for all comparisons, $P < 0.0001$) and in the figure are reported P values from post hoc analysis with Šidák's correction.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Deletion of RIPK3 but not MLKL rescued the phenotype in terms of villous edema, a hallmark of inflammatory signaling in Xiap-deficient mice, but not in the case of co-deletion of MLKL. Furthermore, this correlated with a rescue of Paneth cells and goblet cells which were comparable to wildtype and again not phenocopied by deletion of MLKL. Thus, in *Mlkl*$^{-/-}$*Xiap*$^{-/-}$ mice, no real improvement of the inflammatory phenotype was observed compared to the deletion of RIPK3 in *Xiap*$^{-/-}$ mice. We concluded that these data showed that activation of RIPK3 in *Xiap*$^{-/-}$ mice was the main driver of inflammatory cues in the intestinal compartment, while MLKL did only play a minor role.

**Complicated disease course in human subjects is marked by high *TNFR2* expression**

In order to examine the contribution of TNF signaling in pediatric inflammatory bowel disease, we examined two distinct cohorts, the XLP-2 patient cohort (**Figure 22A-B**) and the RISK study (**Marigorta et al. 2017**) (GSE93624). The XLP-2 cohort consisted of mucosal biopsies from the colon of seven confirmed XLP-2 patients with diagnosed IBD (XLP-2), eight pediatric IBD patients (termed IBD), and 15 non-IBD (healthy cohort) pediatric controls. The non-IBD „healthy" control samples consisted of non-inflamed mucosa sampled from pediatric patients who underwent surgery for possible appendicitis, which was then excluded retrospectively.

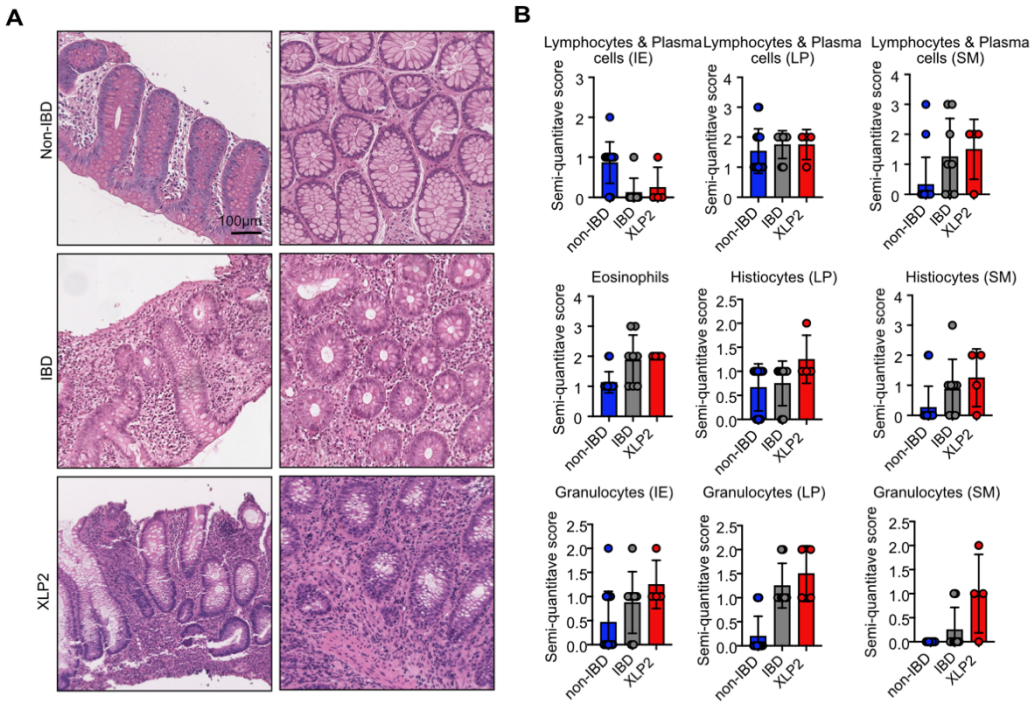


**Figure 22. Histopathological examination of intestinal inflammation in human XLP2 disease**.

A. Hematoxylin and eosin (H&E) staining of colonic biopsies from non-IBD, IBD, and XLP2 patients used for RNA-seq analysis.
B. Blinded pathological assessment of the tissues in (A) for cellular infiltrates illustrating no significant pathological differences between the IBD and XLP2 patient cohorts.

Samples were obtained from Prof. Hirokazu Kanegane affiliated at the Department of Child Health and Development at the Tokyo Medical and Dental University

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

First, we collaborated with PD Dr. med Julia Slotta-Huspenina (**Technical University of Munich**) to perform an unbiased blinded pathological examination which revealed that both the IBD and XLP-2 cohorts presented a similar histopathological appearance when comparing them to the healthy controls (**Figure 22**). As a validation cohort independent of XIAP-mutational status, we leveraged the RISK cohort (GSE93624) which included 27 patients with Crohn's Disease who progressed to a complicated clinical course (complicated disease; cCD) within the first three years, as well as a total of 210 treatment-naïve patients with CD, and 35 non-IBD healthy controls. Pathway analysis of gene expression data from these two cohorts revealed a similar pattern of deregulated inflammatory and metabolic pathways, characteristic of IBD (**Figure 23 and Figure 25**). When analyzing these pathways, we were reminded of the deregulation seen in single-housed *Xiap*$^{-/-}$ mice, which served as a layer of confirmation of the XIAP mouse model as a viable disease model of XLP2 disease. First, we noted several commonalities between cCD and XLP2, among which the TNF-dependent cytokine oncostatin M (*OSM*) and its receptor *OSMR*, as well as related genes *MMP9*, and *CX3CL1,* which ranked among the most up-regulated genes. OSM has been reported to play a major role in complicated IBD (**West et al. 2017**). Notably, the *OSM* pathway has been linked to resistance to anti-TNF therapy. Furthermore, we detected *TNFR2*, among the most upregulated genes, which prompted us further to understand the involvement of TNF in disease progression.

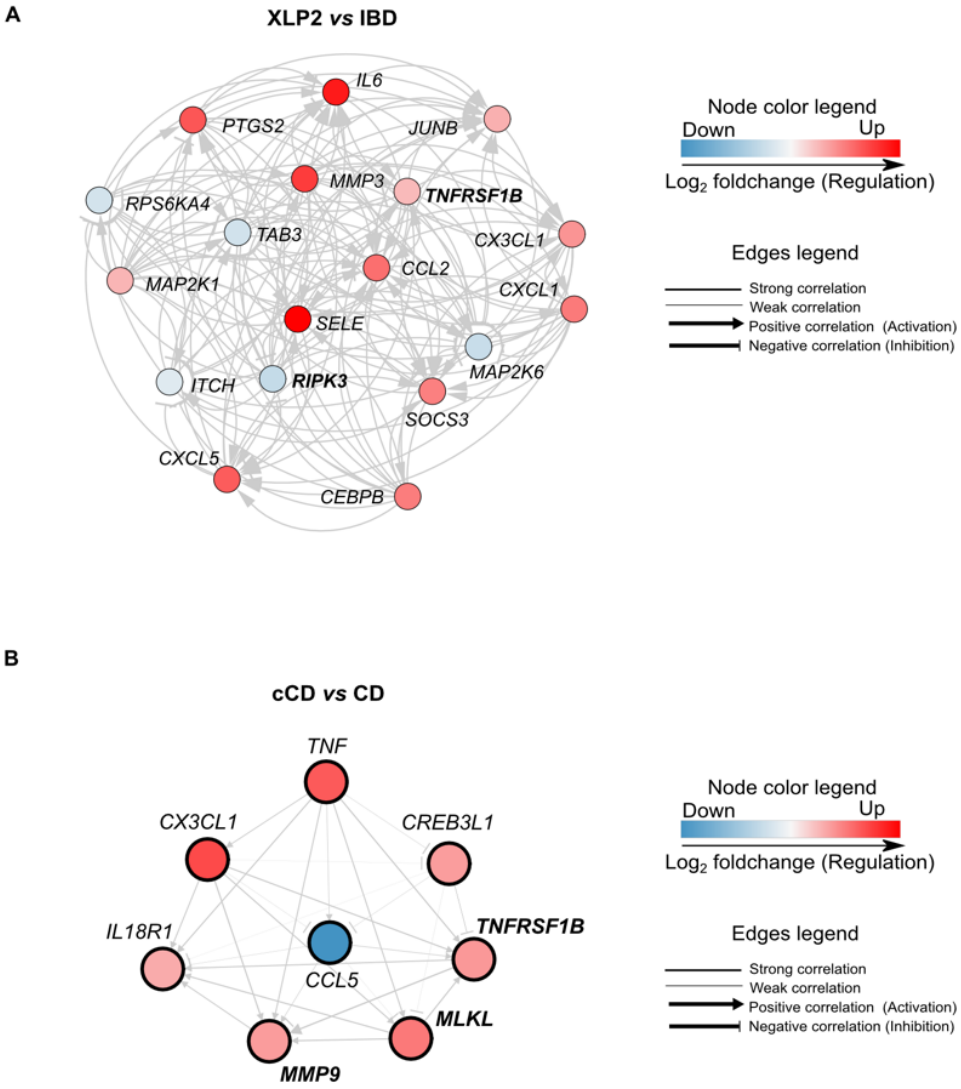


**Figure 23. Elevated TNFR2 correlates with complicated disease in pediatric IBD** Network analysis of upregulated TNF pathway genes in XLP2 (left) and cCD (right) compared to their controls (IBD and CD, respectively).

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Thus, we further analyzed the transcriptomic differences in the TNF pathway with special attention to the differences between the cCD and CD cohorts. We thereby identified eight significantly up-regulated genes; *TNF*, *CX3CL1*, *CREB3L1*, *IL18R1*, *MMP9*, *MLKL*, *TNFRSF1B*, and *CCL5* (**Figure 23 and 24***)*. The overexpression of MLKL was indicative of a necroptotic aspect within this particular clinically complicated disease cohort. When returning back to our in-house generated XLP-2 and IBD cohort, also several members of the TNF pathway were significantly upregulated in XIAP-deficiency, hinting towards some overlap with complicated course subjects; most excitingly though, was the overexpression of *TNFR2* and a concomitant correlation with *SELE* (E-Selectin), *IL6*, and *MMP3*, all of which are strong contributors of inflammatory tissue damage (**Figure 23**).

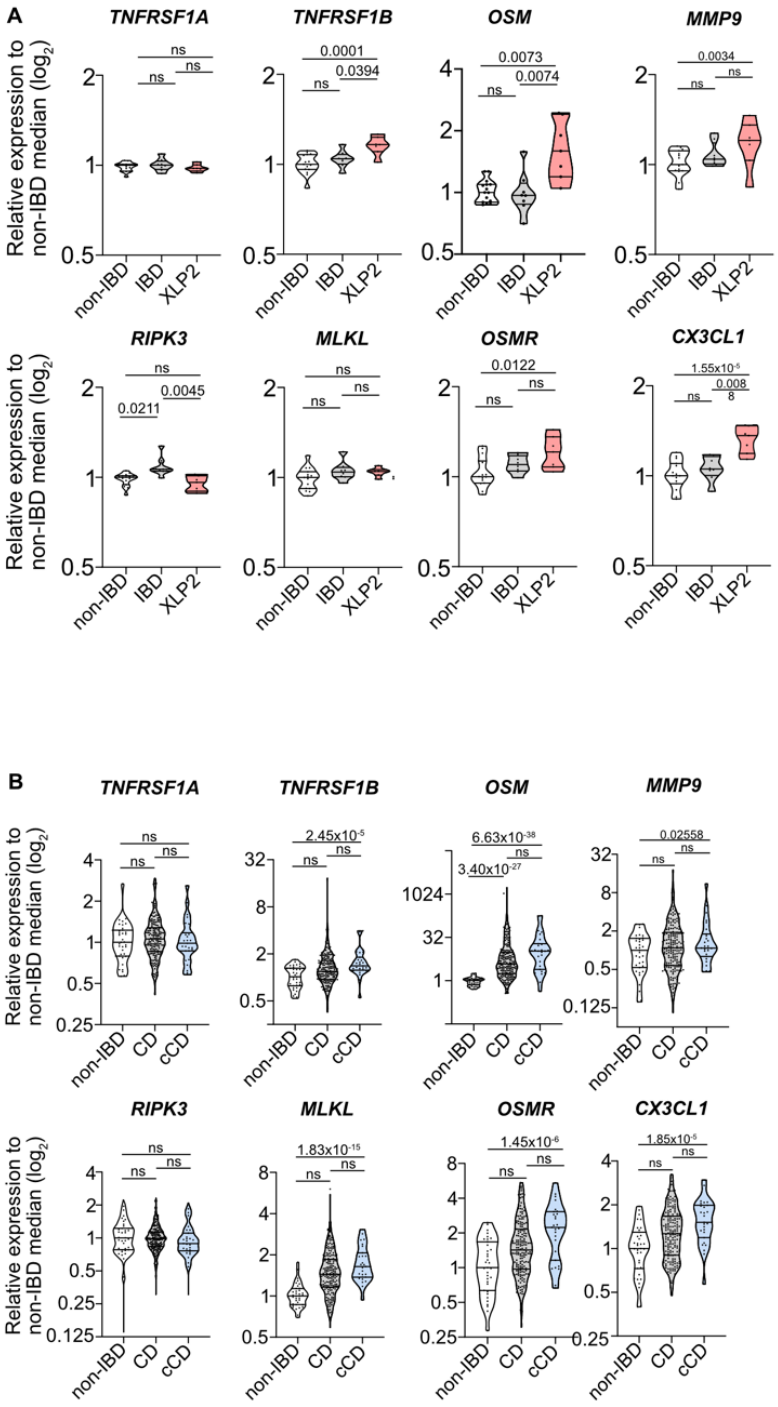


**Figure 24. Expression patterns of TNF pathway members in XLP2 disease and RISK cohort.**

A. Expression of TNFRSF1A, TNFRSF1B, RIPK3, MLKL, OSM, and OSMR in ileal biopsies from non-IBD, IBD, and XLP2 (A) and the RISK patient cohort (GSE93624)
B. The Wald test was used to generate the indicated P values from the pairwise comparisons.

*Figure and Legend were modified and taken from "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

All of these findings were indicative of the fact that intestinal inflammation in XLP2 was associated with markers of severe and complicated disease and characterized by elevated *TNFRSF1B* expression. Interestingly, *TNFRSF1B* was positively correlated to MLKL in the cCD cohort and negatively correlated to RIPK3 in the XLP2 cohort. These data are in line with observations in our murine studies of XIAP deficiency, in which aberrant activation of the necroptotic machinery, proceeded independently of MLKL. In summary, *TNFRSF1B* positively correlated with biomarkers disease severity in both cohorts hinting at common mechanisms, despite the different genetic backgrounds of these two patient cohorts.

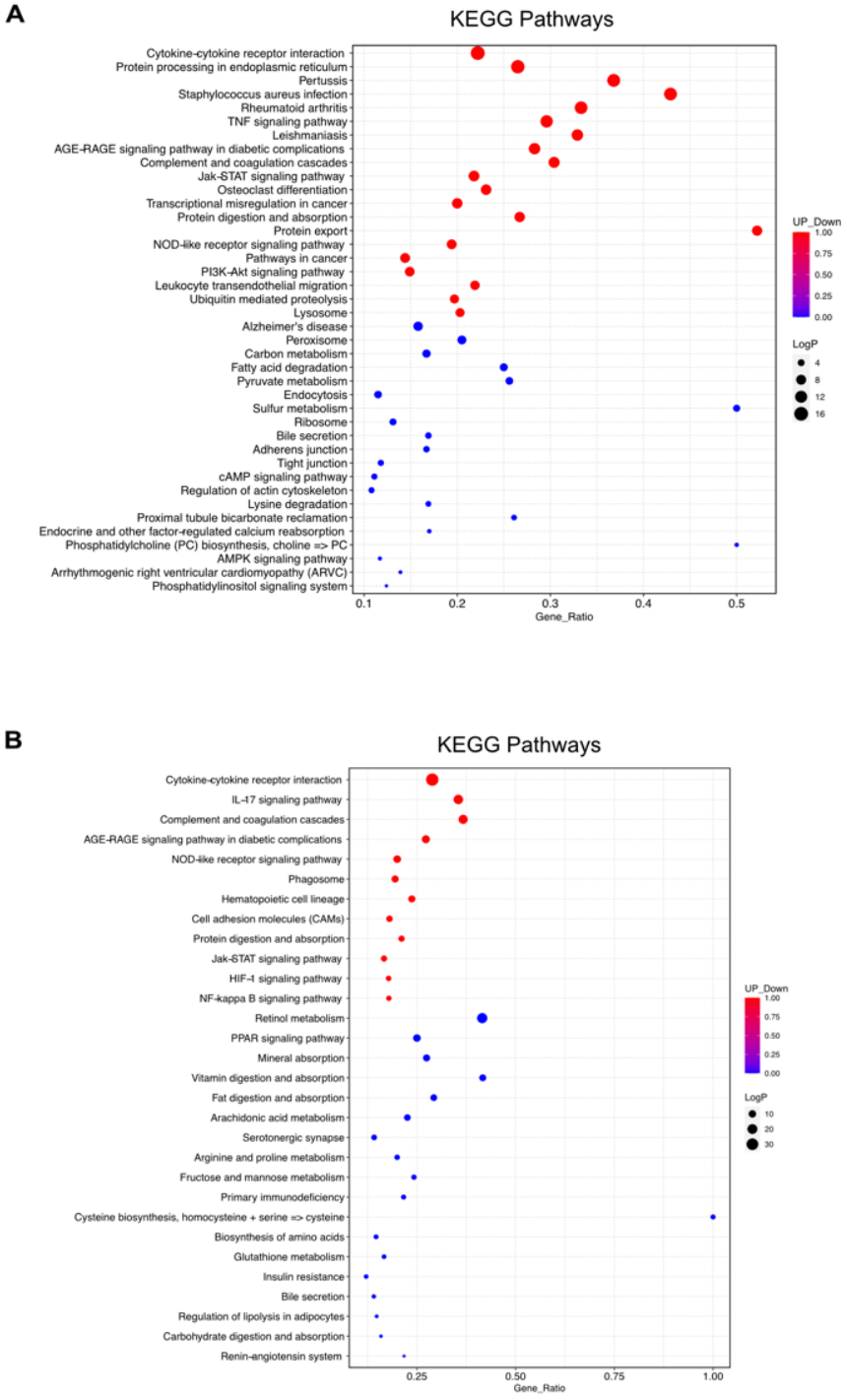


**Figure 25. Top deregulated KEGG pathways in XLP2 patients compared to non- IBD controls.**

A. and B. Top deregulated KEGG pathways in complicated Crohn's disease patients (cCD) compared to healthy controls based on GSE93624 (n = 35 non-IBD, 210 treatment-naïve patients of which 27 patients progressed to complicated disease).

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

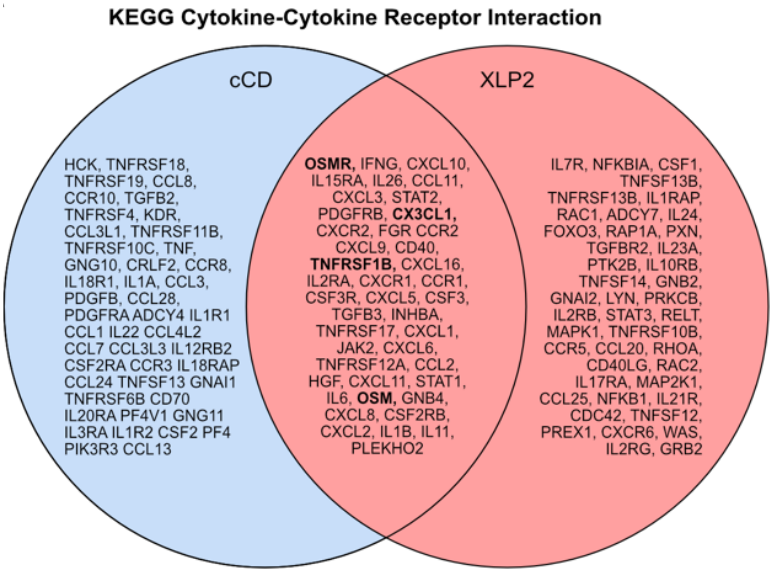


**Figure 26. Network analysis of upregulated genes.** Comparison of cytokine-cytokine receptor genes upregulated in the complicated CD group versus the XLP2 group. Data analysis was performed using the dataset GSE93624, and from transcriptomic analysis of of seven characterized XLP2 patients with a diagnosis of IBD (XLP2).

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

Concluding, we found a striking similarity in the complicated disease course in the RISK cohort with the XLP2 cohort which was marked by both markers of disease severity and strong expression patterns of TNFR2 (**Figure 26**). These findings suggest a common mechanism of disease amplification, which in the context of XIAP deficiency is masked by inflammatory death of these cells.

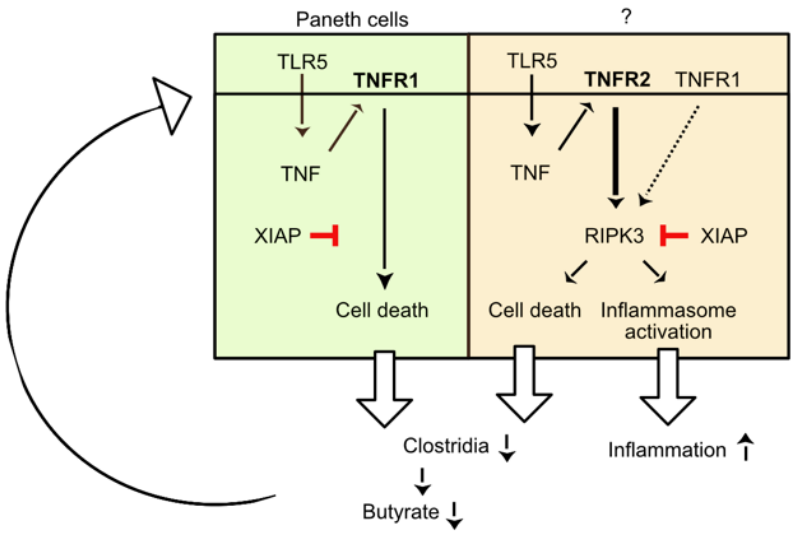


**Figure 27. Disease model**. Model illustrating the signaling events leading to abrogated TLR5 signaling in XIAP-deficiency.

*Figure and Legend were modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

**Contributions of Co-authors and Collaborators**

Without the extensive conceptual and experimental help of several co-authors, this work would not have been possible.

First and foremost, I would like to thank my mentor and supervisor **Philipp J. Jost**, who accepted me in his laboratory to conduct this exciting project. While I started off wanting to do research in hematology, I found myself dissecting aberrant mechanisms of host-microbiome interactions in the gut, only to find out later that this effect was driven by the hematopoietic system, fulfilling my initial desire, to understand a hitherto unexplored facet of hematology: shaping the microbiome. I thank him for his guidance and conceptual support, especially at times when this project hit dead ends.

Importantly, I would also like to thank **Monica Yabal** who essentially conceptualized the experimental approach to this project, i.e., using a hypothesis-driven murine set of experiments aimed at delineating the origins of intestinal inflammation in XIAP deficiency.

- **Madeleine Müller**: extensive experimental support throughout the project
- **Andreas Hiergeist, André Gessner**: 16S-sequencing and analysis
- **Bastian Popper, Katja Steiger**: analysis of murine tissue: TEM and Histology
- **Caterina Branca, Marie K. Pfautsch, Nicole Müller, Miguel Silva, Jan P. Böttcher, Martina Anton**: extensive experimental support
- **Markus Tschurtschenthaler, Timon E. Adolph, Peter Vandenabeele:** conceptual support and analysis of data

- **Thomas Engleitner, Sainitin Donakonda, Rupert Öllinger, Roland Rad**: bulk RNA sequencing
- **Jordy De Coninck, Geert Berx**: single cell RNA sequencing
- **Sinem Usluer, Tobias Madl**: Measurement and analysis of the metabolome
- **Nicole Pfarr, Julia B. Slotta-Huspenina, Hirokazu Kanegane, Andreas G. Nerlich**: Supply and analysis of Human Biopsies of the XLP2/(non)IBD cohort

**Table 1. Patient Characterization**

| *Patient cohorts* | | | | |
|---|---|---|---|---|
| **Non-IBD Control Cohort** | | | | |
| **Patient** | **Age** | **Sex** | **Anatomical Location** | **Histopathological diagnosis** |
| **A1** | 14 | male | proximal colon | healthy control tissue |
| **A2** | 11 | male | proximal colon | healthy control tissue |
| **A3** | 14 | male | proximal colon | healthy control tissue |
| **A4** | 11 | male | proximal colon | healthy control tissue |
| **A5** | 13 | male | proximal colon | healthy control tissue |
| **A6** | 12 | male | proximal colon | healthy control tissue |
| **A7** | 13 | male | proximal colon | healthy control tissue |
| **A8** | 11 | male | proximal colon | healthy control tissue |
| **A9** | 13 | male | proximal colon | healthy control tissue |
| **A10** | 13 | male | proximal colon | healthy control tissue |
| **A11** | 14 | male | proximal colon | healthy control tissue |
| **A12** | 8 | male | proximal colon | healthy control tissue |
| **A13** | 10 | male | proximal colon | healthy control tissue |
| **A14** | 10 | male | proximal colon | healthy control tissue |
| **A15** | 10 | male | proximal colon | healthy control tissue |
| **IBD Control Cohort** | | | | |
| **Patient** | **Age** | **Sex** | **Anatomical Location** | **Histopathological diagnosis** |
| **B1** | 12 | male | ascending colon | pediatric Crohn's disease |
| **B2** | 19 | male | transverse colon | pediatric Crohn's disease |
| **B3** | 13 | male | transverse colon | pediatric Crohn's disease |
| **B4** | 17 | male | sigmoid colon | pediatric Crohn's disease |
| **B5** | 17 | male | transverse colon | pediatric Crohn's disease |
| **B6** | 17 | male | ascending colon | pediatric Crohn's disease |
| **B7** | 15 | male | ascending colon | pediatric Crohn's disease |
| **B8** | 18 | male | transverse colon | pediatric Crohn's disease |
| **XLP-2 Disease Cohort** | | | | |
| **Patient** | **Age** | **Sex** | **Anatomical Location** | **Histopathological diagnosis** |
| **C1** | 14 | male | ascending colon | XLP-2 associated colitis (c.1021_1022delAA) |
| **C2** | 9 | male | intestine | XLP-2 associated colitis (c.847C>T) |
| **C3** | 5 | male | sigmoid colon | XLP-2 associated colitis (c.448_449insC) |
| **C4** | 9 | male | descending colon | XLP-2 associated colitis (deletion of exon 1) |
| **C5** | 11 | male | ascending colon | XLP-2 associated colitis (c.340C>T) |
| **C6** | 7 | male | sigmoid colon | XLP-2 associated colitis (c.1141C>T) |
| **C7** | 10 | male | transverse colon | XLP-2 associated colitis (c.1141C>T) |

*Table was modified and taken from "**Wahida, A**., Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235"*

*C1, 2, 3, 4, 5, 6, and 7 correspond to P6, P19, P15, P14, P16, P18, and P18 in

S. Ono, K. et al (2021), doi:10.1016/j.jaip.2021.05.045.

# III. MATERIALS AND METHODS

## Overall Study design

- The goal of this project was to delineate the sequence of events leading to intestinal inflammation in XIAP deficiency (XLP-2 Syndrome).
- Various methods including immunohistochemistry, confocal imaging techniques, and transcriptome analysis were utilized to investigate the origins of spontaneous ileitis in knockout mice.
- Throughout the study, both male and female were used in this study and different hygienic contexts and housing strategies were assigned to the experimental mice.
- All analyses were initially performed in a blinded manner. Sample sizes and biological or technical replicates are denoted throughout the figure legends.

**Mice**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

The mice employed: *Birc4*$^{-/-}$ (**Olayioye et al. 2005**), *Tnf*$^{-/-}$(**M. Pasparakis et al. 1996**), *Tnfr1*$^{-/-}$ (**Rothe et al. 1993**), *Tnfr2*$^{-/-}$(**Erickson et al. 1994**), *Ripk3*$^{-/-}$ (**Newton, Sun, and Dixit 2004**), and *Mlkl*$^{-/-}$ (**Murphy et al. 2013**). Mouse lines mice lacking *Birc4*, are described as *Xiap*$^{-/-}$ mice, and were subsequently crossed with *Tnf*$^{-/-}$, *Tnfr1*$^{-/-}$, *Tnfr2*$^{-/-}$, *Ripk3*$^{-/-}$, and *Mlkl*$^{-/-}$ mice in order to generate the respective double knockout lines.

For cohousing experiments mice were housed together for four weeks directly after weaning. For separation experiments, cohoused mice of both genotypes were separated for a period of four weeks before being subjected to analysis. The mice were housed in an environment with a 12-hour light:dark cycle, an ambient temperature of 24°C, and a humidity level of 55%. Following the experiment, the mice were euthanized, and their organs were harvested for analysis. All mice were bred and housed under either conventional or specific pathogen–free conditions according to the guidelines of the Federation of Laboratory Animal Science Association.

*Ethics protocols and approvals*: All mice experiments were authorized by permission of the District Government of Upper Bavaria (Vet_02-19-103). Ethics

approval for human samples was obtained from the ethics committee of the *Klinikum rechts der Isar*, TU München (339/20 S-KH).

**Human patient cohort**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

Patient material was sourced from three institutions: the Department of Pathology at Klinikum Schwabing in Munich, Germany provided non-IBD control tissue, the IBD cohort was obtained from the Institute of Pathology at Technical University of Munich, Germany, and the XLP2 cohort was procured from the Department for Child Health and Development at Tokyo Medical and Dental University, Japan. The XLP2 cohort comprised of colonic mucosal biopsies obtained from male pediatric patients with XIAP deficiency and confirmed colitis through endoscopy. The IBD control group included male pediatric patients of similar age range. Biopsies were collected during diagnostic colonoscopies from therapy-naïve disease stages. The study used colonic mucosa biopsies from 15 pediatric patients as non-IBD controls. The patients underwent endoscopy before appendectomy to rule out concurrent inflammation. Cases with macroscopically and histologically normal mucosa and localized acute appendicitis were included for analysis. The biopsies were evaluated through histopathological analysis using hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining. A pathologist used a semi-quantitative scoring system to assess inflammation, including quantification and distribution of lymphocytes, plasma cells, neutrophilic and eosinophilic granulocytes, and histiocytes. The pathologist also noted the presence or absence of PCs, crypt apoptosis, surface

epithelial necrosis, and regenerative changes. The study performed RNA-seq analysis on FFPE biopsy specimens by extracting RNA from 8- to 12 µ-thick sections.

**Histopathological analysis and confocal microscopy of murine ileal tissue sections**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

Ileal tissue was prepared according to the "swiss rolls" method as described previously in (**Moolenbeek and Ruitenberg 1981**) and stained with H&E, PAS reaction, or IHC antibodies (see table below).

| Antigen | Producer, Catalog no. |
|---|---|
| **TLR5** | Abcam, ab62460 |
| **CD3** | DCS Diagnostics, CI597C002 |
| **Ki-67** | ThermoFisher Scientific, RM-9106 |
| **5-bromo-2′-deoxyuridine (BrdU)** | Bu20a, Cell Signaling, no. 5292 |
| **beta-catenin** | E247, Abcam, no. ab32572 |

To quantify CD3+ cells and goblet cells (PAS+ cells along the villus lining) at least 20 villi per mouse were evaluated and the average per mouse used as a proxy for the overall goblet cell or t cell abundance in one particular organ.

To assess the degree of subepithelial edema, a semi-quantitative scoring system was devised. Subepithelial edema is characterized by the presence of a (focal) gap beneath the villous epithelial cells and the lamina propria at the edges or tips of the villi, and can be distinguished from autolysis. Autolysis of the small intestine typically involves detachment from the epithelium and concurrent contraction of the lamina propria

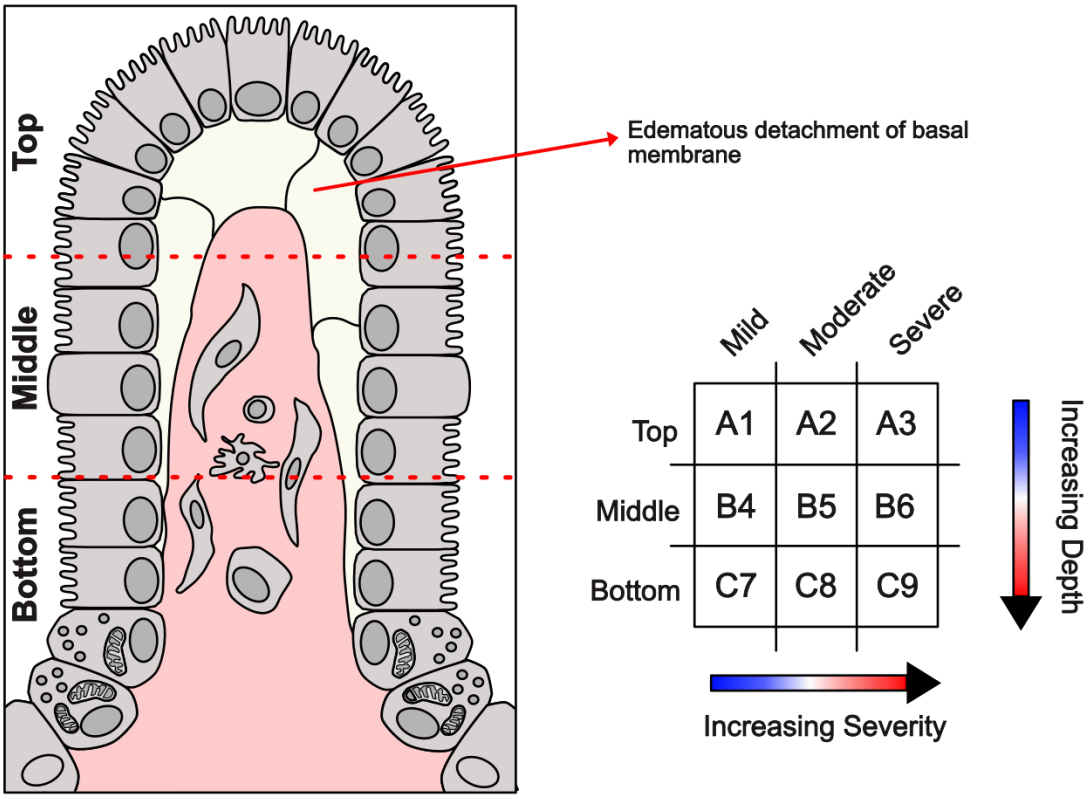


**Figure 28. Schematic of the edema score**.

For evaluating the extent of the edema, each countable villus is divided into three sections, and the edema in each level proportionally quantified. The deeper the edema stretches along the villus-axis, the higher the obtained score. The numerical score is calculated as follows: (0*Score 0+A1*1+A2*2+A3*3+B1*4+B2*5+B3*6+C1*7+C2*8+C3*9)/ (Total number of scored villi).

All confocal images were generated, by flushing approximately 1 cm of ileal tissue and fixing these in 4 % formaldehyde at 4°C. Cryopreservation was performed by incubating tissue for 24h in a 30% sucrose solution. Thereafter tissue was embedded in Tissue-Tek O.C.T. compound solution. Subsequently, sections were cut and stained an antibody against UAE I DyLight 649 (Vector Laboratories, DL-1068) or lysozyme EC 3.2.1.17/FITC (Dako, F0372) and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The stained sections were imaged on a TCS SP8 confocal microscope from Leica in conjuncture with a DMi Thunder Imager with a Pecon Incubator i8 setup also manufactured by Leica. IMARIS software was utilized (v9.5) for analysis.

**Transmission electron microscopy**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

The samples were fixed in Karnovsky's Fixative, which was freshly prepared by Electron Microscopy Sciences, and then embedded in Epon, supplied by Serva Electrophoresis. Ultrathin sections measuring 70 nm were cut and treated with UranyLess EM Stain from Electron Microscopy Sciences and 3% lead citrate from Leica using the Leica EM AC20 contrasting system. The JEOL 1200EX II TEM from JEOL in Akishima, Tokyo was used to image the samples at 80 kV. A digital camera (KeenViewII; Olympus, Germany) captured images that were processed using the iTEM software package (analySIS Five; Olympus, Germany). For TEM imaging, at least three random ultrathin sections were used for each biological replicate. The distribution of type I and II PCs was assessed by evaluating the extent of endoplasmic reticulum (ER) disruption and distortion of PC mitochondria, with grading of mitochondria following the method outlined by (**Cadwell et al. 2008**). At least 20 PCs were blindly quantified for each genotype.

**Intestinal microbiome analysis by 16S-rDNA semiconductor sequencing**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

***Isolation of DNA from stool specimens***: Mice cecum contents were collected, immediately cooled on dry ice, and stored at -80°C until processing. Upon thawing, each sample weighing 100 mg wet weight was mixed with a pool of three spike bacteria, namely *Salinibacter ruber, Rhizobium radiobacter, and Alicyclobacillus acidiphilus*, at a concentration of 1e+8, 8e+7, and 3e+8 16S rDNA copies per sample, respectively. These spike bacteria served as an internal control standard [Andreas H1]. To lyse cells, the samples were exposed to S.T.A.R. Buffer (Roche, Mannheim, Germany)/proteinase K, followed by five cycles of freezing in liquid nitrogen and boiling, and then repeatedly subjected to bead beating using a mixture of 0.1 mm silica spheres and two 1.4 mm titanium beads on the TissueLyser II (Qiagen) instrument. DNA was then purified using the MagNA Pure 96 instrument (Roche) and the MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche). The total nucleic acids were quantified using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

***Quantification of 16S rDNA copies by qPCR***: To determine the 16S rRNA gene copy numbers of total bacteria in the isolated DNA, qPCR was performed on a LightCycler 480 II Instrument (Roche) using universal eubacterial 16S rRNA gene primers S-D-Bact-0341-b-S-17 and S-D- Bact-0785-a-A-21, along with a universal

6FAM-labelled hydrolysis probe 506R and the LightCycler 480 Probes Master (Roche). Spike bacteria-specific qPCRs were also performed as described earlier.

The V4 to V6 hypervariable regions of bacterial 16S rRNA genes were amplified using the forward primer S-D-Bact-0517-a-A-17, which contains a 10-bp barcode sequence, and the reverse primer S-D-Bact-1046-b-A-19, from a total of 1e+7 bacterial 16S rDNA copies for each sample. The resulting ~600 bp amplicons were purified using MagSi-NGSPREP Plus beads (Steinbrenner Laborsysteme, Wiesenbach, Germany), and the copy numbers of amplicons containing A and P1-adaptors were determined using the KAPA Library Quantification IonTorrent Kit (Roche Diagnostics, Mannheim, Germany). The DNA library was prepared by pooling equimolar concentrations of adaptor-labeled amplicons and then re-amplified by isothermal amplification using the Ion PGM Template IA 500 Kit (Thermo Fisher Scientific, Darmstadt, Germany). Finally, the library was subjected to sequencing on an Ion TorrentTM PGM (Thermo Fisher Scientific, Darmstadt, Germany).

***Processing and analysis of 16S amplicon sequencing data*:** The Torrent Suite Software Version 5.10 was used for signal processing and base-calling without quality trimming of sequencing reads. Amplification primer and adapter sequences were removed from processed reads using cutadapt [Andreas H3] 1.16, and reads with an average quality below 15 were also removed. Low quality bases from the 3' ends were trimmed using sickle [Andreas H4] version 1.33 with a quality cutoff of 15 and a length cutoff of 250 bases in a sliding window approach. Cutadapt was used for demultiplexing of filtered reads with no allowance for errors. Subsequent analyses were performed using R v3.6.3. Demultiplexed reads were further processed and denoised using the dada2 v1.16 pipeline with a homopolymer gap penalty of -1 and

singletons retained before pooling of reads. Downstream analyses were conducted only on sequences with a maximum of 5 expected errors. The IDTAXA algorithm of the DECIPHER v2.18 package was applied for taxonomic classification of all detected amplicon sequence variants (ASV) using the SILVA 138 release 16S rRNA reference database. The alpha diversity measures and principal coordinates analyses based on weighted and unweighted UniFrac distances were calculated using the phyloseq v1.34 package (data not shown). Differential abundance analyses and generation of cladograms were carried out using the MicrobiotaProcess package with an alpha index cutoff of 0.01, and all plots were generated with ggplot2 v3.3.2. The species-level classification of ASVs that were mapped to Helicobacter spp. was manually verified using the IDNS3 Eubacteria 16S reference database within the web-based SmartGene IDNS3 pipeline (SmartGene GmbH, Zug, Switzerland).

**RNA-seq of human and murine tissue**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

***Crypt enrichment***: To enrich for murine crypts, single-housed and cohoused wildtype and *Xiap*$^{-/-}$ mice were euthanized in accordance with local ethical regulations, and their small intestines were removed. The small intestines were opened longitudinally and cleansed of mucus and fecal remnants by flushing with ice-cold PBS. Next, the intestines were cut into 0.5 $cm^2$ pieces and rigorously washed multiple times with ice-cold PBS until the supernatant was clear. The small intestinal pieces were then incubated with a 2mM EDTA solution in PBS for 30 minutes at room temperature. Several fractions were obtained by flushing the tissue pieces with ice-cold PBS using a transfer pipette, and the suspension was collected and further re-suspended in PBS. The fraction with the highest concentration of crypts and the least amount of debris was selected for analysis. RNA was extracted using the RNA isolation NucleoSpin® kit from Macherey-Nagel.

***RNA-isolation from patient FFPE tissue***: Sections of FFPE biopsy specimens, measuring approximately 8-12 µm thick, underwent deparaffinization and were digested with Proteinase K for one hour. An automated nucleic acid extraction system, the Promega Maxwell RSC16, was used to extract RNA along with the LEV RNA FFPE

Purification Kit from Promega. Tissue was scraped from the slide and then treated with the TURBO DNA-freeTM Kit from ThermoFisher Scientific to remove DNA and obtain DNA-free RNA. The QuBit 3.0 system and the QuBit RNA high-sensitivity kit from ThermoFisher Scientific were used to measure the concentration of RNA fluorometrically.

***Library Preparation***: The library preparation for bulk 3'-sequencing of poly(A)-RNA was carried out as previously described. Briefly, barcoded cDNA for each sample was generated using Maxima RT polymerase (ThermoFisher Scientific) with oligo-dT primers containing barcodes, unique molecular identifiers (UMIs), and an adapter. The 5' ends of the cDNAs were extended using a template switch oligo (TSO), and full-length cDNA was amplified by binding primers to the TSO-site and adapter after pooling all samples. The cDNA was then tagmented using the Nextera XT kit (Illumina), and 3'-end-fragments were amplified using primers with Illumina P5 and P7 overhangs. The P5 and P7 sites were exchanged compared to Parekh et al. to enable better cluster recognition by sequencing the cDNA in read1 and barcodes and UMIs in read2. The library was sequenced on a NextSeq500 (Illumina®) using 65 cycles for the cDNA in read1 and 16 cycles for the barcodes and UMIs in read2. The published Drop-seq pipeline (v1.0) was used to generate sample- and gene-wise UMI tables. Alignment was performed using the GRCh38 reference genome, and transcript and gene definitions were based on the ENSEMBL annotation release 75.

***RNAseq analysis and normalization***: The analysis and normalization of RNAseq data involved obtaining Gencode gene annotations v28 and the human reference genome GRCh38 from the Gencode homepage (EMBL-EBI). The Dropseq tool v1.12

was used for mapping the raw sequencing data to the reference genome, and the resulting UMI filtered count matrix was imported into R v3.4.4. Prior to differential expression analysis with DESeq2 v1.18.1, the dispersion of the data was estimated using the severity of the disease as a covariate. The Wald test was then used to determine the differentially regulated genes between all possible pairwise comparisons, with genes being considered differentially regulated if the adjusted p-value was less than 0.05. The differentially regulated genes were then subjected to gene set overrepresentation analysis using Enrichr within the KEGG gene set database. The data were then transformed using rlog transformation with the aforementioned model for further downstream processing.

For the mouse dataset, Gencode gene annotations m18 and the mouse reference genome GRCm38 were obtained from the Gencode homepage (EMBL-EBI). Mapping of the data was conducted as described above, and the dispersion of the data was estimated using a dummy variable that described all possible genotype-housing combinations. The LRT-test was used to compare the aforementioned model to a reduced Intercept-only model. Genes were considered differentially regulated if the adjusted p-value was below 0.05, and unsupervised clustering was used to determine sub-clusters. Gene set overrepresentation analysis with Enrichr within the KEGG pathway database was then performed on the genes in each cluster. Pathways were considered significantly associated with a cluster if the adjusted p-value was less than 0.05. The Wald-test was used to determine foldchanges between all pairwise comparisons, and the apeglm shrunken fold changes were calculated. GSEA of selected pairwise comparisons was then conducted with default settings in the pre-ranked mode using the apeglm shrunken folds as the ranking metric. A pathway was

considered significantly associated with an experimental group if the FDR was less than 0.05.

**TLR5 signature:** Raw data with accession number GSE117772 were obtained from NCBI GEO and aligned to the reference genome using STAR. The same downstream analysis was then performed as previously described, wherein the data dispersion was assessed using a parametric fit with the experimental group included as a covariate in the model. The TLR5 signature was identified using genes that were significantly differentially expressed between the untreated and flagellin-treated groups, with an adjusted alpha level of 0.05 as the threshold. This signature was used as a user-defined gene set in GSEA analysis for other experiments in this study.

**Correlation network analysis:** To distinguish the positive and negative relationships in the TNF signaling pathway, we used the R function rcorr to calculate the Pearson correlation coefficient between genes based on their expression values in the pathway. A correlation network was then generated based on these values, and Cytoscape v3.7.1 was utilized to visualize the network.

**Small intestinal organoid culture**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

Organoids derived from the small intestine (SI) were cultured following established protocols. Specifically, Matrigel (Corning Incorporated) was used to seed approximately 200 SI crypts, which were then cultured in IntestiCult® medium (StemCell®) containing 1% sterile penicillin-streptomycin (ThermoFisher Scientific). Organoid cultures were not stimulated until at least one passage had occurred. To induce stimulation, organoids were passaged and allowed to rest for 2 to 3 days before being treated with TNF (20 ng/ml), Na-butyrate (1 mM), or flagellin (500 ng/ml) in IntestiCult® medium. For morphometric analysis of organoid proliferation and differentiation, at least three domes per condition were used in three independent experiments per genotype and treatment group. Cultures were imaged at ×10 magnification using a Leica DMi8 Thunder Imager equipped with a Pecon Incubator i8 setup. The resulting organoids were categorized into four morphological profiles, including enteroids (fully differentiated organoids), enterospheres, or cystic (undifferentiated organoids).

CRISPR-Cas9–mediated deletion of Myd88 and Tlr5 in organoids was performed by transducing organoids with a lentiCRISPRV2 lentiviral system (Addgene

plasmid no. 52961; http://n2t.net/ addgene:52961; RRID:Addgene_52961) (**Sanjana, Shalem, and Zhang 2014**).

The sgRNA oligos were designed as follows using the in silico webtool [Benchling (2021), https://benchling.com].

| Gene | Forward primer |
|---|---|
| **Tlr5** | AGGGAGATATTACCAACACG (Exon 4) |
| **Myd88** | CCCACGTTAAGCGCGACCAA (Exon 1) |

For organoid transduction, lentiviral vectors were produced (**Wübbenhorst et al. 2010**), and organoids were treated as previously described (**Van Lidth de Jeude et al. 2015**). As a control, pHIV-7 SFeGFP IRES puromycin was used as control vector. Transduced organoids were then maintained for two to three passages under puromycin selection (5 g/ml; InvivoGen®).

In order to evaluate the existence of insertions and deletions (indels) in the genomic area aimed by the CRISPR-Cas9, Sanger sequencing was performed on the sgRNA target region of both *MyD88* and *Tlr5*. The efficiency of indels was evaluated by employing the webtool "TIDE: Tracking of Indels by Decomposition" (**Brinkman et al. 2014**).

| Gene | Forward primer | Reverse primer |
|---|---|---|
| **Tlr5** | TACTGGTGCCCGTGTGTAAA | ACAGCCGAAGTTCCAAGAGA |
| **Myd88** | GCCTAGTCCATCCACCTTGA | GCTAGCCTCGTTGATCCTTG |

For experiments, organoid cultures were expanded to reach a density of 100 to 200 organoids per Matrigel dome. For each independent experiment, four domes of each genotype were stimulated with either TLR5-ligand flagellin (FLA-BS Ultrapure, FPB-41-01, tlrl-pbsfla, InvivoGen®) at 500 ng/ml or recombinant mouse TNF- (BioLegend®, 575204) at 20 ng/ml, 2 days after passaging. After 4 days of stimulation, two to three wells per condition were imaged with Leica Thunder at ×10 magnification and with computational clearing. After imaging, organoids were harvested and subjected to real-time qPCR with the GoTaq qPCR (Promega®) according to the manufacturer's instructions, with the following primers from the following table. Relative expression was calculated using the $\Delta\Delta$Ct method. Each biological sample was run in LightCycler 480 II (Roche) with three technical replicates.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| ***Tnf*** | CCCTCACACTCAGATCATCTTCT | GCTACGACGTGGGCTACAG |
| ***Tnfrsf1a*** | CCGGGAGAAGAGGGATAGCTT | TCGGACAGTCACTCACCAAGT |
| ***Tnfrsf1b*** | ACACCCTACAAACCGGAACC | AGCCTTCCTGTCATAGTATTCCT |

**Metabolic studies**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

The study involved analyzing metabolites, which were measured using NMR following a previously described method (**Prokesch et al. 2017**). NMR experiments were conducted at 310 K using a Bruker Avance III 500 MHz spectrometer with a TXI probe head. The samples' spectra were processed automatically (0.3 Hz exponential line broadening), phased, and referenced to TSP at 0.0 ppm using Bruker Topspin 3.1 software (Bruker GmbH, Rheinstetten, Germany). The metabolites of interest corresponding to a specific number of protons and the external standard's integral regions were defined, normalized to the number of protons, and the concentration was determined using the external standard concentration. The Chenomx NMR Suite 8.2 was used to determine non-labeled compound concentrations by internal standard concentrations.

The concentration of Beta-hydroxybutyrate in the cecal content was measured using the Beta-Hydroxybutyrate Assay Kit (MAK041, Sigma-Aldrich), following the manufacturer's instructions.

**FITC-dextran permeability assay**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

To assess the permeability of the intestinal barrier, oral gavage of FITC-dextran (Sigma-Aldrich, no. FD4) in phosphate-buffered saline (PBS) was given to mice at a dose of 44 mg/100 g body weight. Four hours later, serum was collected from whole blood and mixed with PBS (pH 7.4) at a 1:1 ratio. The concentration of FITC in the serum was measured using a spectrophotofluorometer (BioTek®) by exciting at 485 nm and measuring the emission wavelength at 528 nm. The concentration was determined using serially diluted FITC-dextran in PBS as a standard.

**Statistical analysis**

*The following paragraph was adapted or partly taken from a previously published manuscript "**Wahida, A.**, Müller, M., Hiergeist, A., Popper, B., Steiger, K., Branca, C., Tschurtschenthaler, M., Engleitner, T., Donakonda, S., De Coninck, J., et al. (2021). XIAP restrains TNF-driven intestinal inflammation and dysbiosis by promoting innate immune responses of Paneth and dendritic cells. Sci Immunol 6, eabf7235."*

All statistical analyses except for omics experiments (R as described in the methods) were performed on GraphPad Prism (version 9.2.0). For datasets including one variable, a two-tailed nonparametric Mann-Whitney U test was used to test significance. For multivariable analyses, an analysis of variance (ANOVA) test was used with a Holm-Šidák post hoc analysis. All statistical details are described in the respective figure legends.

# IV. DISCUSSION

Throughout this dissertation, I have aimed to provide a molecular explanation for the sequence of events leading to intestinal inflammation in an ultra-rare hematological inborn condition termed X-linked lymphoproliferative syndrome Type 2 (XLP-2) or XIAP deficiency (**Mudde, Booth, and Marsh 2021**).

As detailed in the introductory part of this thesis, XIAP deficiency constitutes a mendelian cause of very-early onset inflammatory bowel disease (VEO-IBD) in affected boys (**Zeissig et al. 2015**). With a relatively high estimated reported penetrance of 30 % regarding the development of intestinal inflammation, the effect size of deleterious variants in *BIRC4,* the coding gene for XIAP, is considerably more pronounced than in sporadic IBD (**Graham and Xavier 2020**), which usually present in young adults. In the context of sporadic IBD, such as Crohn's disease and ulcerative colitis, GWAS studies have identified several genetic variations that may contribute to the development of the condition (**Graham and Xavier 2020**). Here, it is generally believed that inflammation is caused by a combination of genetic susceptibilities and non-genetic triggers, with the exact causes of the condition still not fully understood. These genetic variations, therefore, increase an individual's risk of developing IBD. Fine mapping these variants revealed that these affect the immune system or the way the host processes and responds to certain microorganismal components, such as those originating from bacteria in the gut.

However, it has been proven arduous to link the pathogenic effect of these variants with certainty to the probability of developing the disease. As such it has been posited

that only a minor fraction of people being carriers of these variants will develop IBD over the duration of their lives. Therefore, elucidating pathogenic mechanisms in the context of mendelian and early onset cases presents an opportunity to dissect genotype-phenotype causality and consequently bring novel therapeutic strategies to the fore.

## Microbiome signatures associated with inflammatory bowel disease

One point of convergence to mendelian and sporadic IBD is the development of dysbiotic alterations in the intestinal microbiome and concomitantly loss or blooming of bacterial families, more or less adapted to an inflammatory environment. While the intestinal flora strongly affects the host's physiology mounting evidence suggests that equivocally the host and potential inflammatory cues arising from him can shape the composition and function of the microbiota (**Sommer et al. 2017**).

Thus, next to converging features of TNF-mediated inflammation and cell death, one common aspect of IBD is a gradual change of intestinal microbial ecosystems. A wealth of knowledge acquired after the advent of high throughput sequencing methods such as 16S-ribosomal RNA sequencing has suggested that the blooming and vanishing of several bacterial families is a driving force of the clinical disease course, and thus may ultimately also serve as a therapeutic target (**Schirmer et al. 2019**). This has raised one critical question in the field of IBD concerning the debate of whether dysbiosis is a trigger or consequence of inflammation (**Stappenbeck and Virgin 2016**).

To date, two congruent theories have been galvanized which aim to link genetic susceptibility with the disease-associated changes in the intestinal microbiota. The first consideration is that genetic predisposition *per se* does not lead to changes in the microbiome alone. Rather, they collaborate with external triggers (so-called second hits) which in analogy to the Knudson hypothesis (Manolis Pasparakis and Vandenabeele 2015) then propagate an exaggerated inflammatory reaction in the gut,

which is the second congruent concept critical to this theory. Carriers of polymorphisms or even mutations that ultimately render these individuals susceptible to hyperinflammatory reactions also allow pathobionts, otherwise innocuous microorganisms, to create havoc. Identifying these microorganisms could pave the way for precision medicine approaches in such individuals.

Recent studies underscore this idea, which is unable to identify plausible genotype-microbiome associations when comparing the microbiome of diseased and healthy carriers of *NOD2* variants associated with IBD, but rather posit that dysbiosis is linked to disease status. The data presented in this thesis argue in favor of the model in which host genetics regulate the propensity for exaggerated inflammatory responses, which, in turn, ultimately drive microbial dysbiosis. In our model, phenotypic XIAP-deficient mice displayed a microbiome signature hallmarked by dysbiosis (loss of diversity), which was largely due to a decrease in various Clostridial species. This observation correlated with the presence of inflammation, which could not be intrinsically linked to the genetic loss of *Xiap* alone. Rather, the conclusion from the experimental lineup was that an inappropriate reaction to an environmental trigger, most likely a bacterial trigger, drove aberrant TNF-mediated destruction of homeostasis with encompassing loss of microbiome and mucosal integrity.

This concept has been supported by a study that was published colloquially with our study (**Strigli et al. 2021**). Here, colleagues focused on scrutinizing the necessity for a bacterial trigger against which XIAP-deficient host animal could not mount appropriate immune responses. Inoculation of these mice with *Helicobacter hepaticus*, otherwise innocuous in wildtype animals, led to the loss of mucosal integrity

in parts resembling the phenotype observed in our colonies. Next to the loss of Paneth cell viability and function, a clear pathogenic role for the TNF-TNFR1-RIPK3 axis mediating the pathology was established using gene-targeted mice, similar to our approach and thus supporting the overall pathomechanistic framework established by both studies.

Another important control experiment we performed to exclude the involvement of a pathogen also able to provoke intestinal inflammation in wildtype animals, according to the Koch postulates (**Singh, Proctor, and Willing 2016**), we performed cohousing experiments where *Xiap*$^{-/-}$ mice were housed together with same-sex wildtype animals. Due to the inherent murine behavior of coprophagy (**Klaasen et al. 1990**) which allows for the dissemination of dominant microbial strains across animals, we were able to show that the dysbiotic phenotype and ensuing inflammatory phenotype were not transferable to wildtype mice. We therefore concluded and also propose the concept of dysbiosis in this context being a recessive trait, leading to host phenotypes in genetically susceptible individuals. This notion serves as the basis for fecal matter transplantations (**Blaser 2019**), and the exact mechanisms thereof are the topic of my current scientific focus.

**Aberrant cell death in inflammatory bowel disease**

One of the most fascinating aspects of studying the molecular underpinnings of inflammatory bowel disease is the observation that irrespective of the host's genotype, be it a genetic polymorphism or highly penetrant mutation, or disease-promoting factors, the occurrence of aberrant cell death represents a common feature of pathology (**Patankar and Becker 2020**). Intriguing about this observation is furthermore that the inflammatory nature of the cell death modality involved often constitutes a disease driving force, therefore providing a molecular rationale why certain biologicals such as antibodies against tumor necrosis factor (TNF) do not only dampen cues amplifying the overall inflammatory component but also causally address the cell death component driven by aberrant TNF signaling (**Günther et al. 2011; Atreya et al. 2014**). Since apoptosis is generally understood to be immunologically silent, it is difficult to conceive how TNF-driven apoptosis might play a prominent role in this context. As elaborated in the introductory part, the outcomes of TNF signaling can be very diverse only leading to cell death in a minority of cases, with necroptosis most likely to play a role in gut inflammation (**Patankar and Becker 2020**).

In *Xiap*$^{-/-}$ mice, aberrant TNF signaling caused an impairment of Paneth cells which was shown by various complementary methods. Paneth cells are essential components of the intestinal crypt and therefore stem cell niche, but furthermore orchestrate – in an immune-like manner – how inflammatory responses are coordinated within this microanatomic compartment. Best known for their ability to produce antimicrobial peptides, the malfunction of these highly secretory and long-

lived cells; thus, particularly sensitive to ER stress, TNF signaling, and defective autophagy, and hence cell death, has been traced back to being the cellular cause of IBD (**Adolph et al. 2013; Azabdaftari and Uhlig 2021**).

In our model, TNF played a critical role and exerted its pathogenic function through both its receptors. This was exemplified through the use of gene-targeted mice, harboring deletions in *Tnf*, *Tnfr1*, or *Tnfr2*. Earlier, *in vitro* data suggested that in the absence of XIAP, beyond TNFR1 (**Yabal et al. 2014**) TNFR2 could also trigger inflammatory cell death (**Lawlor et al. 2017**), by yet-to-be-determined mechanisms, *in vivo* evidence linked to a relevant disease model was lacking. Our model now links these aspects as underlying rationale which levels with the search for clinically relevant therapies in XLP-2 disease.

Still, even with the certainty of a TNF-driven pathology, the exact source of TNF in the intestine was a continuing matter of concern since the gut represents an organ where the inflammatory response is tightly and cautiously regulated (**van Loo and Bertrand 2022**). The presence of a plethora of microorganisms could amount to up to several billion and thereby constitute a serious threat to trigger hyperinflammatory cues if it were not for tolerogenic immune cell subsets restraining pro-inflammatory bursts such as secretion of TNF. Next to regulatory T and B-cells, whose exact physiology – and for that matter role in disease – extends beyond the scope of this dissertation, various tolerogenic innate subsets collaborate with these adaptive populations to coordinate tolerance in the gut. Among these, dendritic cells act as master regulators informing subsequent immune responses by eliciting specific immune responses in reaction to sampling microorganismal components such as

bacterial cell walls or flagellar proteins, *i.e.*, flagellin. After these antigen-presenting functions, the secretion of specific cytokines then orchestrates an appropriate response or lowers the propensity for inflammation by inducing tolerance (**Cummings et al. 2016**). Next to these immune cell functions, specialized non-hematopoietic cells, most importantly Paneth cells, can also modulate and finetune immunogenic reactions in the gut, by secretion of soluble factors influencing adjacent and distant effector immune cells in the mucosa. One key family of molecules that are understood to shape these responses are Toll-like receptors (TLRs) which have been studied at large in the context of innate immunity (**Burgueño and Abreu 2020**). However, their exact role in mucosal immunity has been hampered by the lack of precise information regarding the expression pattern across the spectrum of immune (like) populations in the intestinal mucosa. Recently a resource paper, using reporter constructs for every different TLR in mice was published, with the goal of studying the expression patterns of TLRs in the gut (**Price et al. 2018**). This study revealed that as hypothesized the expression of TLRs is tightly regulated to prevent aberrant hyperinflammatory reactions in light of the myriad of microorganisms present in the gut. Along these lines, the expression of TLR4 was largely absent and only present dimly on certain infiltrating, probably patrolling, cells in the lamina propria of intestinal villi, thereby most likely constituting cycling immune cells such as monocytes of macrophages. Interestingly one major TLR family member – TLR5 – was seemingly strongly expressed, albeit only on a small fraction of cells, divided into two subgroups.

One subset of cells was located at the bottom of the intestinal crypt, another scattered around the lamina propria of the intestinal villi. The TLR5+ epithelial fraction residing at the bottom of the crypt was subsequently identified as Paneth cells, a cell

type that I discussed elaborately earlier. Validatory experiments showed that TLR5 engagement by its cognate ligand, flagellin, induced strong para- and autocrine TNF response. Since the destruction of Paneth cells was both an essential feature of the phenotype identified in XIAP deficiency (as well as overall in IBD) and genetic loss of TNF was able to reverse this, the hypothesis was generated as to how TLR5 as a critical source of TNF in the intestine was linked to this. Underscoring the overall importance of the question is that, dysfunctional Paneth cells associated with dysbiosis represent a recurrent hallmark of IBD (**Adolph et al. 2013**) and also independent of the risk allele, ultimately thus a cellular origin theory for IBD. Nevertheless, there are data which suggest the opposite: the presence of dysfunctional in non-IBD patients. These findings are suggestive that PC dysfunction alone is not sufficient for the development of IBD, but possibly a perquisite.

We were also able to observe this seemingly contradictory finding. Since deletion of *Tnf* abrogated intestinal inflammation and all ensuing phenotypes, we generated mouse models carrying deletions in either receptor mediating downstream signaling. Interestingly Tnfr1 deficiency protected both PC function and rescued TLR5+ immune cell numbers; loss of Tnfr2 only rescued LP TLR5+ cells. However, in line with human observation, we could not detect that in the absence of intestinal inflammation Paneth Cell integrity was restored. Essential to the question of which TNF receptor exerted which pathogenic role, the identity of the alleged immune cell compartment rescued by deletion of *Tnfr2* remained elusive. Except for their expression of TLR5, their exact identity remained unknown. While earlier in vitro work suggested these cells which were sensitive to TNFR2-mediated cell death after

priming with TLR engagement, were of innate ontogeny, the *in vivo* correspondent population was unknown.

Lastly, previous work revealed that in the context of *Xiap*$^{-/-}$ deficiency, inflammatory cell death occurred in a RIPK3-dependent manner (Yabal et al. 2014). Throughout the introduction, necroptosis as a highly immunogenic cell death modality was already introduced (**Linkermann and Green 2014**). However, it remained unclear whether these proposed mechanisms which were identified *in vitro* were also relevant *in vivo*. Again, murine genetic studies were the workhorse to test this hypothesis. Indeed, similar to in vitro findings, the deletion of RIPK3 was sufficient to abrogate the detrimental effects of TNF in XIAP-deficient mice. Restoration of Paneth cell integrity and restoration of intestinal integrity and TLR5+ populations showed that downstream of both TNF receptors RIPK3 acted as a central relay mediating tissue damage.

Additionally, we knew that TLR activation on myeloid cells is able to induce the expression of TNFR2 (**Lawlor et al. 2017**) on myeloid cells. One association found in our model could thus also be that the engagement of TLR5 specifically induced the expression of TNFR2 on myeloid cells, which are yet to be fully identified, and which would render these cells vulnerable to TNF-RIPK3 dependent cell death, which by virtue of engaging a pathway releasing intracellular contents, most importantly DAMPs (**Manolis Pasparakis and Vandenabeele 2015**), would be amplifying inflammation. Another interesting layer of complexity relates to the seemingly disparate expression pattern of MLKL in the XLP2 versus cCD cohort. While MLKL was strongly upregulated in XIAP-Wildtype cCD patients, the missing MLKL expression in the XLP2 patient

cohorts reflects its dispensable role in cell death induction in the absence of XIAP, as shown in our mouse experiments.

Beyond the scope of our findings, the role of TLR5 remains largely unknown, partly because of differing baseline hygienic microbial composition. For instance, loss of TLR5 was shown to govern the onset of spontaneous colitis (**Vijay-Kumar et al. 2007**), which has been further corroborated by cell type-specific deletions of TLR5, where TLR5 was shown to be important for maturation of the microbiome (**Fulde et al. 2018**). Interestingly only the deletion of TLR5 in intestinal epithelial cells provoked changes in relevant bacterial clades such as *Firmicutes* or *Bacteroides*, while the role of TLR5 in DCs was not equal (**Uematsu and Akira 2009**). Our data would support this notion since the deletion of *Tnfr1* but not *Tnfr2* could revert the microbiota changes more strongly. These data thus suggest that contrary to the loss of TLR5 epithelial cells, the loss of the elusive TLR5+ immune cell population is not pathogenic *per se*, rather their inflammatory death could be the reason for intestinal inflammation.

**Therapeutic implications**

This study has established a clear role for two axes driving pathology in a non-redundant manner; while TNFR2 drove immunopathology and its genetic deletion was sufficient to abrogate intestinal inflammation, TNFR1 was driving epithelial damage. This was clearly established using gene-targeted mice lacking the corresponding expression of either receptor or showed that based thereupon the changes in the microbiota otherwise observed in diseased mice were also absent. Published work based on XIAP-deficient patient studies that underwent hematopoietic stem cell transplantation (**Ono et al. 2021**), the only viable therapeutic option which leads to durable remissions in XLP-2 disease, monitored the microbiota's reaction before, during, and after HSCT. Supporting the findings of this thesis and the colloquially paper published upfront (**Wahida et al. 2021**), the pediatricians observed that with the decrease in clinical symptoms and intestinal inflammation, HSCTs could reverse the changes normally observed during inflammatory flares. Most importantly they were able to increase *Clostridial* abundance thereby underscoring the importance of the immune system in shaping the overall microbial landscape in the gut. The model presented here might serve as a blueprint for how epithelial and immune cells collaborate by distinct axes of the same pathway. Also, the XIAP model showcases the sequence of events leading to disease-driving dysbiosis which is a recurrent finding independent of genotype. The clearly established pathogenic role of TNFR2 is a novel and exciting context that on its own deserves attention as a potential target in future therapeutic endeavors. But more so re-establishing a population that is capable of generating appropriate TLR responses is a principle that might serve as a springboard for future interventions.

# V. ANNEX

## List of Figures and Tables

**ACKNOWLEDGEMENTS**

First, I would like to thank Philipp J. Jost for the opportunity to conduct this research project in his laboratory. Starting off from a research internship and clinical internship, to evolve into a Ph.D. thesis is something I will be eternally grateful for. His guidance was not just of high value regarding content-related issues but also my development into a clinician scientist would not have been conceivable without hour-long discussions of what it actually means to conduct research at the intersection of medicine and basic science. Most of all, our discussion started off from this line of research and has now evolved into more elaborate considerations regarding precision oncology. *To be continued.*

Florian Bassermann, Roland Schmid, and Sebastian Zeissig were key contributors to this project since their conceptual support as critical collaborators and members of my TAC committee was crucial for the execution of this project. Especially Florian Bassermann is credited with extraordinary dedication since he took over formal mentorship during a bureaucratically difficult transition period.

I share many exciting and happy memories with Monica Yabal, with whom I conducted this project “in tandem”. Starting off from a mild but spontaneous phenotype in mice, we went on to uncover an exciting pathomechanism, potentially relevant for patients affected by IBD, beyond XLP2. Most of all, I want to thank her for teaching me “scientific hygiene”, what it means to assemble meaningful figures, teaching me what proper controls are, and most of all being patient, at times were other obligations diverted my attention.

The list of persons having contributed to this project beyond the ones mentioned above is lengthy and is more or less completely reflected by the author list on (**Wahida et al. 2021**). Their input, technologically and conceptually, was critical to achieving this success and I will be thankful for the many hours of discussion, performing experiments, and answering anxious calls about what the data actually meant. Thanks to all of you.

A research project of this size requires also support beyond the scientific realm, I would like to therefore thank my parents who have supported me, even before I was thinking about XIAP-deficiency. It is to them that I dedicate this thesis because I know how important this journey was to them.

## REFERENCES


Adolph, Timon E., Moritz Meyer, Julian Schwärzler, Lisa Mayr, Felix Grabherr, and Herbert Tilg. 2022. “The Metabolic Nature of Inflammatory Bowel Diseases.” *Nature Reviews. Gastroenterology & Hepatology* 19 (12): 753–67.

Adolph, Timon E., Michal F. Tomczak, Lukas Niederreiter, Hyun-Jeong Ko, Janne Böck, Eduardo Martinez-Naves, Jonathan N. Glickman, et al. 2013. “Paneth Cells as a Site of Origin for Intestinal Inflammation.” *Nature* 503 (7475): 272–76.

Allaire, Joannie M., Shauna M. Crowley, Hong T. Law, Sun-Young Chang, Hyun-Jeong Ko, and Bruce A. Vallance. 2018. “The Intestinal Epithelium: Central Coordinator of Mucosal Immunity.” *Trends in Immunology* 39 (9): 677–96.

Atreya, Raja, Helmut Neumann, Clemens Neufert, Maximilian J. Waldner, Ulrike Billmeier, Yurdagül Zopf, Marcus Willma, et al. 2014. “In Vivo Imaging Using Fluorescent Antibodies to Tumor Necrosis Factor Predicts Therapeutic Response in Crohn’s Disease.” *Nature Medicine* 20 (3): 313–18.

Azabdaftari, Aline, and Holm H. Uhlig. 2021. “Paneth Cell Dysfunction and the Intestinal Microbiome in XIAP Deficiency.” *Science Immunology*.

Bagnoli, Johannes W., Christoph Ziegenhain, Aleksandar Janjic, Lucas E. Wange, Beate Vieth, Swati Parekh, Johanna Geuder, Ines Hellmann, and Wolfgang Enard. 2018. “Sensitive and Powerful Single-Cell RNA Sequencing Using McSCRB-Seq.” *Nature Communications* 9 (1): 2937.

Beumer, Joep, and Hans Clevers. 2021. “Cell Fate Specification and Differentiation in the Adult Mammalian Intestine.” *Nature Reviews. Molecular Cell Biology* 22 (1): 39–53.

Bigorgne, Amélie E., Henner F. Farin, Roxane Lemoine, Nizar Mahlaoui, Nathalie Lambert, Marine Gil, Ansgar Schulz, et al. 2014. “TTC7A Mutations Disrupt Intestinal Epithelial Apicobasal Polarity.” *The Journal of Clinical Investigation* 124 (1): 328–37.

Blaser, Martin J. 2019. “Fecal Microbiota Transplantation for Dysbiosis - Predictable Risks.” *The New England Journal of Medicine*. Massachusetts Medical Society.

Brinkman, Eva K., Tao Chen, Mario Amendola, and Bas van Steensel. 2014. “Easy Quantitative Assessment of Genome Editing by Sequence Trace Decomposition.” *Nucleic Acids Research* 42 (22): e168.

Burgueño, Juan F., and Maria T. Abreu. 2020. “Epithelial Toll-like Receptors and Their Role in Gut Homeostasis and Disease.” *Nature Reviews. Gastroenterology & Hepatology* 17 (5): 263–78.

Cadwell, Ken, John Y. Liu, Sarah L. Brown, Hiroyuki Miyoshi, Joy Loh, Jochen K. Lennerz, Chieko Kishi, et al. 2008. “A Key Role for Autophagy and the Autophagy Gene Atg16l1 in Mouse and Human Intestinal Paneth Cells.” *Nature* 456 (7219): 259–63.

Chichlowski, Maciej, Julie M. Sharp, Deborah A. Vanderford, Matthew H. Myles, and Laura P. Hale. 2008. “Effects of Helicobacter Typhlonius and H. Rodentium Infections in IL-10-deficient Mice.” *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 22 (S1). https://doi.org/10.1096/fasebj.22.1_supplement.899.11.

Cuchet-Lourenço, Delphine, Davide Eletto, Changxin Wu, Vincent Plagnol, Olivier Papapietro, James Curtis, Lourdes Ceron-Gutierrez, et al. 2018. “Biallelic RIPK1 Mutations in Humans Cause Severe Immunodeficiency, Arthritis, and Intestinal Inflammation.” *Science* 361 (6404): 810–13.

Cummings, Ryan J., Gaetan Barbet, Gerold Bongers, Boris M. Hartmann, Kyle Gettler, Luciana Muniz, Glaucia C. Furtado, Judy Cho, Sergio A. Lira, and J. Magarian Blander. 2016. “Different Tissue Phagocytes Sample Apoptotic Cells to Direct Distinct Homeostasis Programs.” *Nature* 539 (7630): 565–69.

Cutler, David J., Michael E. Zwick, David T. Okou, Sampath Prahalad, Thomas Walters, Stephen L. Guthery, Marla Dubinsky, et al. 2015. “Dissecting Allele Architecture of Early Onset IBD Using High-Density Genotyping.” *PloS One* 10 (6): e0128074.

Damgaard, Rune Busk, Ueli Nachbur, Monica Yabal, Wendy Wei-Lynn Wong, Berthe Katrine Fiil, Mischa Kastirr, Eva Rieser, et al. 2012. “The Ubiquitin Ligase XIAP Recruits LUBAC for NOD2 Signaling in Inflammation and Innate Immunity.” *Molecular Cell* 46 (6): 746–58.

Elinav, Eran, Jorge Henao-Mejia, Till Strowig, and Richard Flavell. 2018. “NLRP6 and Dysbiosis: Avoiding the Luring Attraction of Over-Simplification.” *Immunity* 48 (4): 603–4.

Erickson, S. L., F. J. de Sauvage, K. Kikly, K. Carver-Moore, S. Pitts-Meek, N. Gillett, K. C. Sheehan, R. D. Schreiber, D. V. Goeddel, and M. W. Moore. 1994. “Decreased Sensitivity to Tumour-Necrosis Factor but Normal T-Cell Development in TNF Receptor-2-Deficient Mice.” *Nature* 372 (6506): 560–63.

Flier, Laurens G. van der, and Hans Clevers. 2009. “Stem Cells, Self-Renewal, and Differentiation in the Intestinal Epithelium.” *Annual Review of Physiology* 71 (1): 241–60.

Fuchs, Yaron, and Hermann Steller. 2015. “Live to Die Another Way: Modes of Programmed Cell Death and the Signals Emanating from Dying Cells.” *Nature Reviews. Molecular Cell Biology* 16 (6): 329–44.

Fulde, Marcus, Felix Sommer, Benoit Chassaing, Kira van Vorst, Aline Dupont, Michael Hensel, Marijana Basic, et al. 2018. “Neonatal Selection by Toll-like Receptor 5 Influences Long-Term Gut Microbiota Composition.” *Nature* 560 (7719): 489–93.

Galluzzi, Lorenzo, Oliver Kepp, Francis Ka-Ming Chan, and Guido Kroemer. 2017. “Necroptosis: Mechanisms and Relevance to Disease.” *Annual Review of Pathology* 12 (1): 103–30.

GBD 2017 Inflammatory Bowel Disease Collaborators. 2020. “The Global, Regional, and National Burden of Inflammatory Bowel Disease in 195 Countries and Territories, 1990-2017: A Systematic Analysis for the Global Burden of Disease Study 2017.” *The Lancet. Gastroenterology & Hepatology* 5 (1): 17–30.

Glocker, Erik-Oliver, Daniel Kotlarz, Kaan Boztug, E. Michael Gertz, Alejandro A. Schäffer, Fatih Noyan, Mario Perro, et al. 2009. “Inflammatory Bowel Disease and Mutations Affecting the Interleukin-10 Receptor.” *The New England Journal of Medicine* 361 (21): 2033–45.

Graham, Daniel B., and Ramnik J. Xavier. 2020. “Pathway Paradigms Revealed from the Genetics of Inflammatory Bowel Disease.” *Nature* 578 (7796): 527–39.

Günther, Claudia, Eva Martini, Nadine Wittkopf, Kerstin Amann, Benno Weigmann, Helmut Neumann, Maximilian J. Waldner, et al. 2011. “Caspase-8 Regulates TNF-α-Induced Epithelial Necroptosis and Terminal Ileitis.” *Nature* 477 (7364): 335–39.

Hutchinson, Ezzie. 2001. “Alfred Knudson and His Two-Hit Hypothesis.” *The Lancet Oncology* 2 (10): 642–45.

Jost, Philipp J., and Domagoj Vucic. 2020. “Regulation of Cell Death and Immunity by XIAP.” *Cold Spring Harbor Perspectives in Biology* 12 (8). https://doi.org/10.1101/cshperspect.a036426.

Jostins, Luke, Stephan Ripke, Rinse K. Weersma, Richard H. Duerr, Dermot P. McGovern, Ken Y. Hui, James C. Lee, et al. 2012. “Host-Microbe Interactions Have Shaped the Genetic Architecture of Inflammatory Bowel Disease.” *Nature* 491 (7422): 119–24.

Kakiuchi, Nobuyuki, Kenichi Yoshida, Motoi Uchino, Takako Kihara, Kotaro Akaki, Yoshikage Inoue, Kenji Kawada, et al. 2020. “Frequent Mutations That Converge on the NFKBIZ Pathway in Ulcerative Colitis.” *Nature* 577 (7789): 260–65.

Klaasen, H. L. B. M., J. P. Koopman, P. M. Scholten, M. E. Van Den Brink, and A. G. M. Theeuwes. 1990. “Effect of Preventing Coprophagy on Colonisation by Segmented Filamentous Bacteria in the Small Bowel of Mice.” *Microbial Ecology in Health and Disease* 3 (2). https://doi.org/10.3402/mehd.v3i2.7518.

Kobayashi, Taku, Britta Siegmund, Catherine Le Berre, Shu Chen Wei, Marc Ferrante, Bo Shen, Charles N. Bernstein, Silvio Danese, Laurent Peyrin-Biroulet, and Toshifumi Hibi. 2020. “Ulcerative Colitis.” *Nature Reviews. Disease Primers* 6 (1): 74.

Kurilshikov, Alexander, Carolina Medina-Gomez, Rodrigo Bacigalupe, Djawad Radjabzadeh, Jun Wang, Ayse Demirkan, Caroline I. Le Roy, et al. 2021. “Large-Scale Association Analyses Identify Host Factors Influencing Human Gut Microbiome Composition.” *Nature Genetics* 53 (2): 156–65.

Lawlor, Kate E., Rebecca Feltham, Monica Yabal, Stephanie A. Conos, Kaiwen W. Chen, Stephanie Ziehe, Carina Graß, et al. 2017. “XIAP Loss Triggers RIPK3-

and Caspase-8-Driven IL-1β Activation and Cell Death as a Consequence of TLR-MyD88-Induced CIAP1-TRAF2 Degradation." *Cell Reports* 20 (3): 668–82.

Lehle, Anna S., Henner F. Farin, Benjamin Marquardt, Birgitta E. Michels, Thomas Magg, Yue Li, Yanshan Liu, et al. 2019. "Intestinal Inflammation and Dysregulated Immunity in Patients With Inherited Caspase-8 Deficiency." *Gastroenterology* 156 (1): 275–78.

Lei, Zili, Takako Maeda, Atsushi Tamura, Tetsuya Nakamura, Yuji Yamazaki, Hidetaka Shiratori, Kenta Yashiro, Sachiko Tsukita, and Hiroshi Hamada. 2012. "EpCAM Contributes to Formation of Functional Tight Junction in the Intestinal Epithelium by Recruiting Claudin Proteins." *Developmental Biology* 371 (2): 136–45.

Li, Yi, Luca Stocchi, Xiuli Liu, Yuanyi Rui, Ganglei Liu, Feza H. Remzi, and Bo Shen. 2015. "Presence of Granulomas in Mesenteric Lymph Nodes Is Associated with Postoperative Recurrence in Crohn's Disease." *Inflammatory Bowel Diseases* 21 (11): 2613–18.

Li, Yue, Marita Führer, Ehsan Bahrami, Piotr Socha, Maja Klaudel-Dreszler, Amira Bouzidi, Yanshan Liu, et al. 2019. "Human RIPK1 Deficiency Causes Combined Immunodeficiency and Inflammatory Bowel Diseases." *Proceedings of the National Academy of Sciences of the United States of America* 116 (3): 970–75.

Lin, Jian-Da, Joseph C. Devlin, Frank Yeung, Caroline McCauley, Jacqueline M. Leung, Ying-Han Chen, Alex Cronkite, et al. 2020. "Rewilding Nod2 and Atg16l1 Mutant Mice Uncovers Genetic and Environmental Contributions to

Microbial Responses and Immune Cell Composition." *Cell Host & Microbe*. Elsevier BV.

Linkermann, Andreas, and Douglas R. Green. 2014. "Necroptosis." *The New England Journal of Medicine* 370 (5): 455–65.

Loo, Geert van, and Mathieu J. M. Bertrand. 2022. "Death by TNF: A Road to Inflammation." *Nature Reviews. Immunology*, November, 1–15.

Maloy, Kevin J., and Fiona Powrie. 2011. "Intestinal Homeostasis and Its Breakdown in Inflammatory Bowel Disease." *Nature* 474 (7351): 298–306.

Mamantopoulos, Michail, Francesca Ronchi, Filip Van Hauwermeiren, Sara Vieira-Silva, Bahtiyar Yilmaz, Liesbet Martens, Yvan Saeys, et al. 2017. "Nlrp6- and ASC-Dependent Inflammasomes Do Not Shape the Commensal Gut Microbiota Composition." *Immunity* 47 (2): 339-348.e4.

Manichanh, Chaysavanh, Natalia Borruel, Francesc Casellas, and Francisco Guarner. 2012. "The Gut Microbiota in IBD." *Nature Reviews. Gastroenterology & Hepatology* 9 (10): 599–608.

Marigorta, Urko M., Lee A. Denson, Jeffrey S. Hyams, Kajari Mondal, Jarod Prince, Thomas D. Walters, Anne Griffiths, et al. 2017. "Transcriptional Risk Scores Link GWAS to EQTLs and Predict Complications in Crohn's Disease." *Nature Genetics* 49 (10): 1517–21.

Matsuzawa-Ishimoto, Yu, Yusuke Shono, Luis E. Gomez, Vanessa M. Hubbard-Lucey, Michael Cammer, Jessica Neil, M. Zahidunnabi Dewan, et al. 2017. "Autophagy Protein ATG16L1 Prevents Necroptosis in the Intestinal Epithelium." *The Journal of Experimental Medicine* 214 (12): 3687–3705.

Mishra, Vishwas, Avipsa Bose, Shashi Kiran, Sanghita Banerjee, Idrees A. Shah, Pooja Chaukimath, Mudasir M. Reshi, Swarna Srinivas, Anaxee Barman, and

Sandhya S. Visweswariah. 2021. “Gut-Associated CGMP Mediates Colitis and Dysbiosis in a Mouse Model of an Activating Mutation in GUCY2C.” *The Journal of Experimental Medicine* 218 (11). https://doi.org/10.1084/jem.20210479.

Moolenbeek, C., and E. J. Ruitenberg. 1981. “The ‘Swiss Roll’: A Simple Technique for Histological Studies of the Rodent Intestine.” *Laboratory Animals* 15 (1): 57–59.

Moriwaki, Kenta, Sakthi Balaji, Thomas McQuade, Nidhi Malhotra, Joonsoo Kang, and Francis Ka-Ming Chan. 2014. “The Necroptosis Adaptor RIPK3 Promotes Injury-Induced Cytokine Expression and Tissue Repair.” *Immunity* 41 (4): 567–78.

Mudde, Anne C. A., Claire Booth, and Rebecca A. Marsh. 2021. “Evolution of Our Understanding of XIAP Deficiency.” *Frontiers in Pediatrics* 9 (June): 660520.

Murphy, James M., Peter E. Czabotar, Joanne M. Hildebrand, Isabelle S. Lucet, Jian-Guo Zhang, Silvia Alvarez-Diaz, Rowena Lewis, et al. 2013. “The Pseudokinase MLKL Mediates Necroptosis via a Molecular Switch Mechanism.” *Immunity* 39 (3): 443–53.

Newton, Kim, Xiaoqing Sun, and Vishva M. Dixit. 2004. “Kinase RIP3 Is Dispensable for Normal NF-Kappa Bs, Signaling by the B-Cell and T-Cell Receptors, Tumor Necrosis Factor Receptor 1, and Toll-like Receptors 2 and 4.” *Molecular and Cellular Biology* 24 (4): 1464–69.

Olayioye, M. A., H. Kaufmann, M. Pakusch, D. L. Vaux, G. J. Lindeman, and J. E. Visvader. 2005. “XIAP-Deficiency Leads to Delayed Lobuloalveolar Development in the Mammary Gland.” *Cell Death and Differentiation* 12 (1): 87–90.

Ono, Shintaro, Kozue Takeshita, Yuko Kiridoshi, Motohiro Kato, Takahiro Kamiya, Akihiro Hoshino, Masakatsu Yanagimachi, et al. 2021. “Hematopoietic Cell Transplantation Rescues Inflammatory Bowel Disease and Dysbiosis of Gut Microbiota in XIAP Deficiency.” *The Journal of Allergy and Clinical Immunology in Practice* 9 (10): 3767–80.

Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. “Immune and Inflammatory Responses in TNF Alpha-Deficient Mice: A Critical Requirement for TNF Alpha in the Formation of Primary B Cell Follicles, Follicular Dendritic Cell Networks and Germinal Centers, and in the Maturation of the Humoral Immune Response.” *The Journal of Experimental Medicine* 184 (4): 1397–1411.

Pasparakis, Manolis, and Peter Vandenabeele. 2015. “Necroptosis and Its Role in Inflammation.” *Nature* 517 (7534): 311–20.

Patankar, Jay V., and Christoph Becker. 2020. “Cell Death in the Gut Epithelium and Implications for Chronic Inflammation.” *Nature Reviews. Gastroenterology & Hepatology* 17 (9): 543–56.

Pazmandi, Julia, Artem Kalinichenko, Rico Chandra Ardy, and Kaan Boztug. 2019. “Early-Onset Inflammatory Bowel Disease as a Model Disease to Identify Key Regulators of Immune Homeostasis Mechanisms.” *Immunological Reviews* 287 (1): 162–85.

Price, April E., Kiarash Shamardani, Kyler A. Lugo, Jacques Deguine, Allison W. Roberts, Bettina L. Lee, and Gregory M. Barton. 2018. “A Map of Toll-like Receptor Expression in the Intestinal Epithelium Reveals Distinct Spatial, Cell Type-Specific, and Temporal Patterns.” *Immunity* 49 (3): 560-575.e6.

Prokesch, Andreas, Franziska A. Graef, Tobias Madl, Jennifer Kahlhofer, Steffi Heidenreich, Anne Schumann, Elisabeth Moyschewitz, et al. 2017. “Liver P53 Is Stabilized upon Starvation and Required for Amino Acid Catabolism and Gluconeogenesis.” *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 31 (2): 732–42.

Purtilo, D. T., C. K. Cassel, J. P. Yang, and R. Harper. 1975. “X-Linked Recessive Progressive Combined Variable Immunodeficiency (Duncan’s Disease).” *The Lancet* 1 (7913): 935–40.

Rigaud, Stéphanie, Marie-Claude Fondanèche, Nathalie Lambert, Benoit Pasquier, Véronique Mateo, Pauline Soulas, Lionel Galicier, et al. 2006. “XIAP Deficiency in Humans Causes an X-Linked Lymphoproliferative Syndrome.” *Nature* 444 (7115): 110–14.

Roda, Giulia, Siew Chien Ng, Paulo Gustavo Kotze, Marjorie Argollo, Remo Panaccione, Antonino Spinelli, Arthur Kaser, Laurent Peyrin-Biroulet, and Silvio Danese. 2020. “Crohn’s Disease.” *Nature Reviews. Disease Primers* 6 (1): 22.

Rothe, J., W. Lesslauer, H. Lötscher, Y. Lang, P. Koebel, F. Köntgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. “Mice Lacking the Tumour Necrosis Factor Receptor 1 Are Resistant to TNF-Mediated Toxicity but Highly Susceptible to Infection by Listeria Monocytogenes.” *Nature* 364 (6440): 798–802.

Rothschild, Daphna, Omer Weissbrod, Elad Barkan, Alexander Kurilshikov, Tal Korem, David Zeevi, Paul I. Costea, et al. 2018. “Environment Dominates over Host Genetics in Shaping Human Gut Microbiota.” *Nature* 555 (7695): 210–15.

Sanjana, Neville E., Ophir Shalem, and Feng Zhang. 2014. “Improved Vectors and Genome-Wide Libraries for CRISPR Screening.” *Nature Methods* 11 (8): 783–84.

Sato, Toshiro, Robert G. Vries, Hugo J. Snippert, Marc van de Wetering, Nick Barker, Daniel E. Stange, Johan H. van Es, et al. 2009. “Single Lgr5 Stem Cells Build Crypt-Villus Structures in Vitro without a Mesenchymal Niche.” *Nature* 459 (7244): 262–65.

Saussure, Philippe de, and Claudio Soravia. 2005. “Crohn’s Disease.” *The New England Journal of Medicine* 352 (21): 2222–2222.

Schirmer, Melanie, Ashley Garner, Hera Vlamakis, and Ramnik J. Xavier. 2019. “Microbial Genes and Pathways in Inflammatory Bowel Disease.” *Nature Reviews. Microbiology* 17 (8): 497–511.

Singh, V. P., S. D. Proctor, and B. P. Willing. 2016. “Koch’s Postulates, Microbial Dysbiosis and Inflammatory Bowel Disease.” *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 22 (7): 594–99.

Sommer, Felix, Jacqueline Moltzau Anderson, Richa Bharti, Jeroen Raes, and Philip Rosenstiel. 2017. “The Resilience of the Intestinal Microbiota Influences Health and Disease.” *Nature Reviews. Microbiology* 15 (10): 630–38.

Stappenbeck, Thaddeus S., and Herbert W. Virgin. 2016. “Accounting for Reciprocal Host-Microbiome Interactions in Experimental Science.” *Nature* 534 (7606): 191–99.

Strigli, Anne, Shreya Gopalakrishnan, Yvonne Zeissig, Marijana Basic, Jun Wang, Tobias Schwerd, Shauni Doms, et al. 2021. “Deficiency in X-Linked Inhibitor of

Apoptosis Protein Promotes Susceptibility to Microbial Triggers of Intestinal Inflammation." *Science Immunology* 6 (65): eabf7473.

Takahashi, Nozomi, Lars Vereecke, Mathieu J. M. Bertrand, Linde Duprez, Scott B. Berger, Tatyana Divert, Amanda Gonçalves, et al. 2014. "RIPK1 Ensures Intestinal Homeostasis by Protecting the Epithelium against Apoptosis." *Nature* 513 (7516): 95–99.

Tegtmeyer, Daniel, Maximilian Seidl, Patrick Gerner, Ulrich Baumann, and Christian Klemann. 2017. "Inflammatory Bowel Disease Caused by Primary Immunodeficiencies-Clinical Presentations, Review of Literature, and Proposal of a Rational Diagnostic Algorithm." *Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology* 28 (5): 412–29.

Uematsu, Satoshi, and Shizuo Akira. 2009. "Immune Responses of TLR5(+) Lamina Propria Dendritic Cells in Enterobacterial Infection." *Journal of Gastroenterology* 44 (8): 803–11.

Van Lidth de Jeude, Jooske F., Jacqueline L. M. Vermeulen, Paula S. Montenegro-Miranda, Gijs R. Van den Brink, and Jarom Heijmans. 2015. "A Protocol for Lentiviral Transduction and Downstream Analysis of Intestinal Organoids." *Journal of Visualized Experiments: JoVE*, no. 98 (April). https://doi.org/10.3791/52531-v.

Venter, J. C., M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, et al. 2001. "The Sequence of the Human Genome." *Science* 291 (5507): 1304–51.

Vijay-Kumar, Matam, Catherine J. Sanders, Rebekah T. Taylor, Amrita Kumar, Jesse D. Aitken, Shanthi V. Sitaraman, Andrew S. Neish, et al. 2007. "Deletion of

TLR5 Results in Spontaneous Colitis in Mice." *The Journal of Clinical Investigation* 117 (12): 3909–21.

Wahida, Adam, Madeleine Müller, Andreas Hiergeist, Bastian Popper, Katja Steiger, Caterina Branca, Markus Tschurtschenthaler, et al. 2021. "XIAP Restrains TNF-Driven Intestinal Inflammation and Dysbiosis by Promoting Innate Immune Responses of Paneth and Dendritic Cells." *Science Immunology* 6 (65): eabf7235.

Wahida, Adam, Klaus Ritter, and Hans-Peter Horz. 2016. "The Janus-Face of Bacteriophages across Human Body Habitats." *PLoS Pathogens* 12 (6): e1005634.

Wahida, Adam, Fang Tang, and Jeremy J. Barr. 2021. "Rethinking Phage-Bacteria-Eukaryotic Relationships and Their Influence on Human Health." *Cell Host & Microbe* 29 (5): 681–88.

Weissbrod, Omer, Daphna Rothschild, Elad Barkan, and Eran Segal. 2018. "Host Genetics and Microbiome Associations through the Lens of Genome Wide Association Studies." *Current Opinion in Microbiology* 44 (August): 9–19.

West, Nathaniel R., Ahmed N. Hegazy, Benjamin M. J. Owens, Samuel J. Bullers, Bryan Linggi, Sofia Buonocore, Margherita Coccia, et al. 2017. "Oncostatin M Drives Intestinal Inflammation and Predicts Response to Tumor Necrosis Factor-Neutralizing Therapy in Patients with Inflammatory Bowel Disease." *Nature Medicine* 23 (5): 579–89.

Wübbenhorst, Daniela, Katja Dumler, Bettina Wagner, Gabriele Wexel, Andreas Imhoff, Bernd Gansbacher, Stephan Vogt, and Martina Anton. 2010. "Tetracycline-Regulated Bone Morphogenetic Protein 2 Gene Expression in

Lentivirally Transduced Primary Rabbit Chondrocytes for Treatment of Cartilage Defects." *Arthritis and Rheumatism* 62 (7): 2037–46.

Wullaert, Andy, Mohamed Lamkanfi, and Kathy D. McCoy. 2018. "Defining the Impact of Host Genotypes on Microbiota Composition Requires Meticulous Control of Experimental Variables." *Immunity* 48 (4): 605–7.

Yabal, Monica, Nicole Müller, Heiko Adler, Nathalie Knies, Christina J. Groß, Rune Busk Damgaard, Hirokazu Kanegane, et al. 2014. "XIAP Restricts TNF- and RIP3-Dependent Cell Death and Inflammasome Activation." *Cell Reports* 7 (6): 1796–1808.

Yeung, Frank, Ying-Han Chen, Jian-Da Lin, Jacqueline M. Leung, Caroline McCauley, Joseph C. Devlin, Christina Hansen, et al. 2020. "Altered Immunity of Laboratory Mice in the Natural Environment Is Associated with Fungal Colonization." *Cell Host & Microbe* 27 (5): 809-822.e6.

Zeissig, Yvonne, Britt-Sabina Petersen, Snezana Milutinovic, Esther Bosse, Gabriele Mayr, Kenneth Peuker, Jelka Hartwig, et al. 2015. "XIAP Variants in Male Crohn's Disease." *Gut* 64 (1): 66–76.

Zhu, Lei, Tingting Shi, Chengdi Zhong, Yingde Wang, Michael Chang, and Xiuli Liu. 2017. "IL-10 and IL-10 Receptor Mutations in Very Early Onset Inflammatory Bowel Disease." *Gastroenterology Research and Practice* 10 (2): 65–69.