Dysbalance of Intestinal Inflammation and Immunity in Patients with Inherited Caspase-8 Deficiency

Dissertation an der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

vorgelegt von

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Geboren am 02. Januar 1985 in Friedrichshafen

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften
Doctor rerum naturalium (Dr.rer.nat.)
an der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

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Diese Dissertation wurde angefertigt unter der Leitung von Prof. Dr. Dr. Christoph Klein und

Prof. Dr. Heinrich Leonhardt im Bereich der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

Erstgutachter: Prof. Dr. Heinrich Leonhardt

Zweitgutachter: Prof. Dr. Jürgen Lassak

Tag der Abgabe: 29. April 2020

Tag der mündlichen Prüfung: 27. November 2020

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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München, den 2. Dezember 2020 Anna Sophie Lehle, M.Sc.

As we know, there are known knowns; there are things we know we know. We also know there are known unknowns; that is to say we know there are some things we do not know. But there are also unknown unknowns – the ones we don't know we don't know.

-Donald Rumsfeld

Überall geht ein früheres Ahnen dem späteren Wissen voraus.
-Alexander von Humboldt

gewidmet in Liebe und Dankbarkeit

meinen Eltern, meiner Schwester und allen Patienten

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1. Summary

Inflammatory bowel disease (IBD) comprise a spectrum of complex and heterogeneous disorders characterized by chronic or relapsing inflammatory conditions of the gastrointestinal tract with a variable disease course. Children with rare forms of very early onset IBD (VEO-IBD; onset below six years) develop inflammatory conditions different from those observed in adults. Of note, VEO-IBD patients often show severe disease manifestations that fail to respond to conventional therapies. In paradigmatic studies, our laboratory had identified patients with IL-10R deficiencies as the first truly monogenic cause of IBD. However, the majority of VEO-IBD patients still lack a genetic diagnosis. It is of great relevance for their clinical management to decipher the pathophysiology.

To reveal novel genetic signatures of VEO-IBD, our laboratory has screened one of the largest international cohorts of VEO-IBD by whole exome sequencing. Our genetic screen has unveiled biallelic loss-of-function mutations in Caspase-8 as a novel cause for VEO-IBD. The aim of my thesis project work was to elucidate the molecular mechanisms of human Caspase-8 deficiency.

Detailed immune workup demonstrated that Caspase-8 deficiency is associated with impaired maturation, proliferation, and/or activation of T and B cells. Patient's monocytes and heterologous Caspase-8-deficient macrophages showed an altered inflammasome activity with increased IL-1β secretion upon LPS stimulation. In addition to innate and adaptive immune dysfunction, patient-derived 3D intestinal organoids ("3D mini-guts") and genetically engineered Caspase-8-deficient coloncarcinoma cell lines exhibited defective cell death responses, as indicated by abrogated TRAIL-induced apoptosis and increased necroptosis.

Taken together, my studies demonstrated that human Caspase-8 deficiency is associated with adaptive and innate immune dysfunctions as well as altered cell death responses in intestinal epithelial cells. My thesis provided first evidence for the critical role of Caspase-8 in controlling human intestinal inflammation and epithelial barrier integrity in children with VEO-IBD. The understanding of the underlying molecular pathomechanisms lays the foundation for the development of personalized therapies for children with life-threatening disease.

2. Zusammenfassung

Chronisch-entzündliche Darmerkrankungen (CED) umfassen ein Spektrum von komplexen und heterogenen Erkrankungen, die durch chronische oder wiederkehrende Entzündungen des Gastrointestinaltrakts charakterisiert sind. Patienten mit seltenen Verlaufsformern der frühkindlichen CED (Manifestationsalter unter sechs Jahren) entwickeln Entzündungen und Krankheitsverläufe, die sich von denen im Erwachsenenalter substantiell unterscheiden. Bedauerlicherweise weisen die Patienten mit einer frühkindlichen CED häufig einen sehr schweren Krankheitsverlauf auf und sprechen nicht auf konventionelle Therapien an. In paradigmatischen Studien ist es unserem Labor gelungen, IL-10R-Defizienzen als erste monogenetische Krankheitsursache mit vollständiger Penetranz zu identifizieren. Jedoch kann bei der Mehrzahl der Patienten mit einer frühkindlichen CED weiterhin keine definitive genetische Diagnose gestellt werden. Die Entschlüsselung der zugrundeliegenden Krankheitsmechanismen ist für die Behandlung von Patienten mit frühkindlichen CED von großer Bedeutung.

Um neue genetische Signaturen bei frühkindliche CED aufzudecken, hat unser Labor eine der international größten Patientenkohorten mittels Exomsequenzierungen analysiert. Dieser Screen hat biallelische *loss-of-function* Mutationen im Caspase-8 (CASP8)-Gen als neue genetische Entität für frühkindliche CED aufgezeigt. Das Ziel meiner Promotion bestand darin, die molekularen Mechanismen der humanen Caspase-8-Defizienz aufzuklären.

Im Rahmen einer detaillierten Immunphänotypisierung konnte ich zeigen, dass die Caspase-8-Defizienz zu Dysfunktionen im adaptiven und angeborenen Immunsystem führt, was mit einer eingeschränkten Differenzierung, Proliferation und Aktivierung von T- und B-Zellen assoziiert war. Darüber hinaus konnte als Zeichen einer gestörten Inflammasomenaktivierung eine erhöhten Sekretion des pro-inflammatorischen Zytokins IL-1β nach LPS-Stimulation in Patienten-abgeleiteten Monozyten und heterologen Caspase-8-defizienten Makrophagen-ähnlichen Zelllinien nachgewiesen werden. Neben den angeboren und adaptiven Immundysfunktionen konnten intrinsische Defekte in intestinalen Epithelzellen detektiert werden. Unter Verwendung von Patienten-abgeleiteten 3D intestinalen Organoidkulturen (3D Mini-Därme) und genetisch modifizierten Caspase-8-defizienten Kolonkarzinomzellen konnten wir defekte Zelltodreaktionen in Form einer erhöhten Nekroptose sowie der TRAIL-vermittelten Apoptose nachweisen.

Zusammenfassend zeigt meine Promotionsarbeit anhand von Patienten mit frühkindlicher CED die essentielle Funktion von Caspase-8 in der Regulation von intestinalen Entzündungsreaktionen und der Integrität der Epithelbarriere auf. Das Verständnis der zugrundeliegenden Pathomechanismen bietet die Grundlage, um zukünftig personalisierte Therapien für Kinder mit lebensbedrohlichen Erkrankungen zu entwickeln.

3. Objectives

Children with VEO-IBD often show a life- threatening disease that is refractory to conventional therapies. The elucidation of molecular pathomechanisms is crucial for optimizing clinical management of VEO-IBD patients. In a large genome-wide screen, we have identified germline mutations in the CASP8 gene as novel genetic cause for VEO-IBD.

The overall goal of the submitted PhD thesis was to characterize the underlying mechanisms of intestinal inflammation in patients with human Caspase-8 deficiency.

4. Specific aims

1) Functional validation of Caspase-8 deficiency (e.g., structural analysis, protein expression, signal transduction)
2) Analysis of lymphocyte dysfunctions in human Caspase-8 deficiency (e.g., immunophenotyping of peripheral blood leucocytes, lymphocyte proliferation and activation, signal transduction)
3) Evaluation of inflammasome phenotypes in patient macrophages and genetically engineered Caspase-8-deficient heterologous cellular models (e.g., proinflammatory cytokine secretion, signal transduction, cell death)
4) Assessment of intestinal epithelial dysfunctions in patient derived organoids ("3D mini- guts") and genetically engineered Caspase-8-deficient heterologous cellular models (e.g., signal transduction, cell death)

5. Introduction

5.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) comprises a spectrum of complex and heterogeneous disorders characterized by chronic or relapsing inflammatory conditions of the gastrointestinal tract with a variable disease course [1]. IBD affects 3 million people in the United States and 2.2 million in Europe, and thus is one of the most prevalent gastrointestinal diseases and a global disease burden [2].

IBD can be classified in Crohn's disease (CD), ulcerative colitis (UC), and IBD-unclassified (IBD-U) based on the clinical phenotype, endoscopic findings, and histological examination [3,4]. UC is the most common form of IBD and is confined to the colon characterized by relapsing and remitting inflammation affecting the colonic mucosa and submucosa [4]. The involvement of the colon is contiguous with proximal disease extension and can be classified according to the extent of colonic involvement. Patients manifest with (i) ulcerative proctitis in 30 – 60 %, (ii) left-sided colitis in 16 – 45 %, or (iii) extensive pancolitis in 14 - 35 % of the cases [5]. Clinically, UC patients primarily manifest with weight loss, abdominal pain, bloody diarrhea, tenesmus, and rectal bleeding. Serious complications in UC are the toxic megacolon and the development of colitis-associated colorectal cancer that is accounting for approximately 15% of all-cause mortality among IBD patients [6]. The medical management of UC includes treatment with 5-aminosalicylic acid, steroids, immunosuppressive drugs, and biologics targeting tumor necrosis factor (TNF-α), integrins (anti-α4β7), or the IL-12/IL-23 axis. Colectomy with ileal pouch-anal anastomosis is considered as curative treatment but associated with significant deterioration of life quality at various levels, including infections, pouch leakage, nerve damage, as well as psychological distress and impact on social life [7].

In contrast to UC, CD is typically characterized by transmural chronic inflammation of the intestine and can affect any part of the gastrointestinal tract from the mouth to the perianal region (discontinuous inflammation with “skip lesions”). In a population-based study, in 45 % of patients the disease was located in the terminal ileum, in 32 % in the colon, in 19 % in the ileocolonic region, and in 4 % in the upper gastrointestinal tract [8]. The disease behaviour can be categorized into non-stricturing and non-penetrating (81 %), stricturing (5 %), and penetrating (14 %) phenotypes [8]. Characteristic clinical symptoms of CD include weight loss, diarrhea, crampy abdominal pain, nausea, vomitting, and fever or chills. A majority of CD patients show disease progression and develop complications such as fistulas, abscesses, strictures, and penetration [9]. Despite advances in treatment using immunmodulators or biologics (e.g., anti-TNF, anti-α4β7, methotrexate (MTX), and thiopurines), most CD patients have to undergo surgical interventions [10].

5.1.1 Epidemiology of IBD

5.1.1.1. IBD – a global disease burden

IBD is a disorder of modern society with increasing incidence in developed countries since the mid of the 20th century. In the European population, IBD has an incidence of 12.7 per 100,000 persons and year, and a prevalence of 250 - 500 per 100,000 individuals [11]. Current data indicate an overall rising incidence of IBD in all age groups [12]. The mean age of onset of IBD is between 20 to 40 years of age [13], but IBD can manifest at any age [14-16].

A variation in the incidence and prevalence of IBD can be observed based on the geographic region, ethnic groups, and immigrant populations [11]. Industrialization has substantially affected people’s lives with improved domestic hygiene and sanitation adoption of sedentary

lifestyles, exposure to air pollution, and consumption of western diet containing excessive amounts of sugar and polyunsaturated fats [17]. The relevance of environmental factors in the pathogenesis of IBD has been suggested by increasing incidence rates of IBD in previously less affected ethnic groups (e.g., Asians, Hispanics) and in immigrants from low incidence regions moving to areas with a traditionally high incidence of IBD [12,17,18].

5.1.2. The multifactorial pathogenesis of IBD

IBD is a heterogeneous disorder with multifactorial etiology. The pathogenesis of IBD involves a complex interplay between environmental triggers, microbial dysfunctions, immune dysregulations, and epithelial barrier defects in genetic susceptible individuals (Figure 1)[19,20]. Despite tremendous advances in the understanding of the disease, the exact underlying molecular mechanisms and their interplays still remain largely elusive.

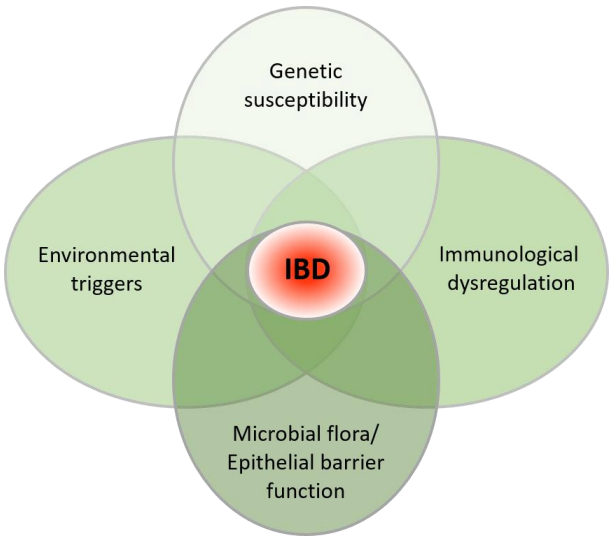

Figure 1: The pathogenesis of IBD.

IBD is a heterogeneous and multifactorial disorder that is triggered by environmental factors, imbalances of the microbial flora, defective epithelial barrier function, and immunological dysregulation in genetically susceptible individuals.

5.1.3. Microbial factors in IBD

The human intestine comprises of approximately 10^{14} bacteria and the majority are harmless commensals, when the mucosal barrier is intact [21]. However, emerging evidence suggests that commensal bacteria can trigger IBD, as enteric bacteria provide constant antigenic stimulation that may activate pathogenic immune cells and cause chronic intestinal inflammation [20,22]. A tightly controlled balance between complex immune responses, involving a broad range of cell types and effector cytokines, and tolerance to intestinal microbiota is required for the maintenance of intestinal homeostasis (Figure 2) [23,24]. The relevance of commensal enteric bacterial antigens in the development of immune-mediated chronic intestinal inflammation has been documented in several animal models [19]. Notably, most germ-free mouse models, except for IL-2-deficient or Samp-1/Yit mice, did not develop spontaneous intestinal inflammation or show hyperactivation of the immune system [25-28]. Furthermore, several studies have demonstrated beneficial effects of treatment with broad-spectrum antibiotics on onset, disease activity and reversion of intestinal inflammation [29].

In IBD patients, inflammations are particularly prominent in the colon and small intestine that contain the highest intestinal bacterial load. CD preferentially occurs in the small and large intestine, as well as other parts of the body (e.g., stomach, anus), whereas UC is limited to the colon and rectum [3]. Furthermore, there is growing evidence that differences in the composition and diversity of the gut microbiome can be observed between healthy individuals and IBD patients [30,31]. Moreover, genome-wide association studies (GWAS) have linked CD with genetic polymorphisms affecting the intracellular bacterial recognition (e.g., NOD2, TLR2, TLR4, TLR5), intracellular microbial processing (autophagy; e.g., ATG16L1), or NADPH-dependent bacterial killing (e.g., NCF4) [32-37].

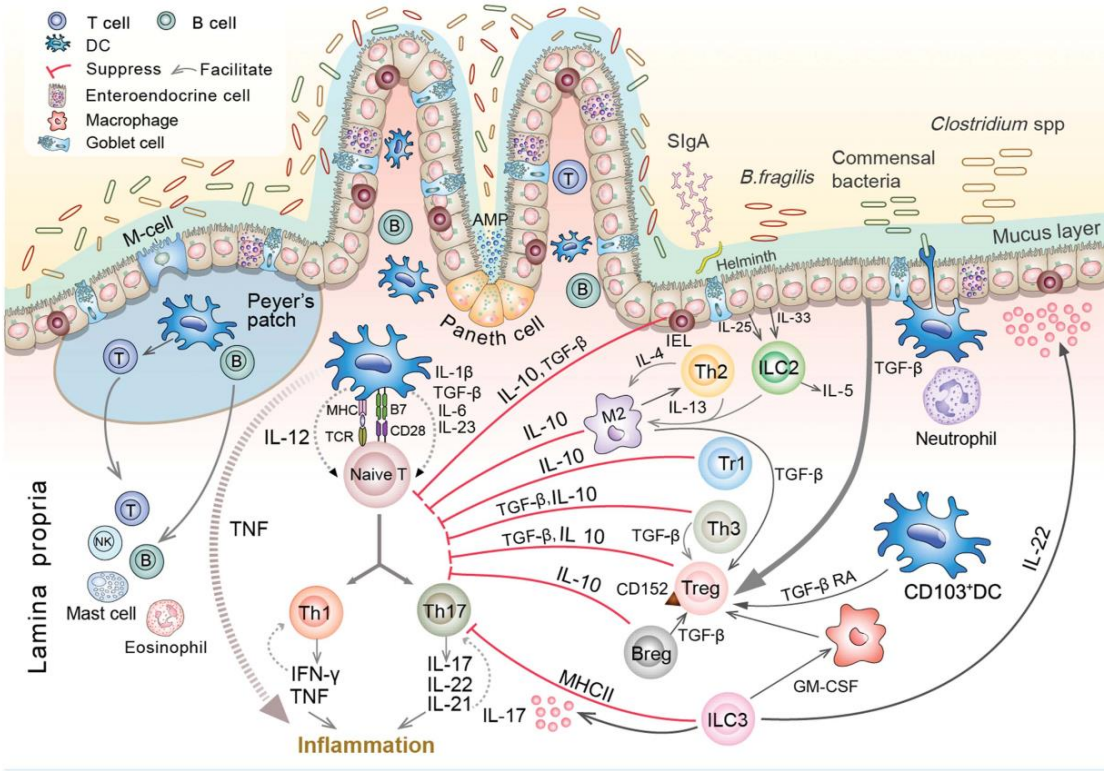

Figure 2: Complex immune responses are critical in maintaining intestinal homeostasis. The intestinal epithelial barrier is the first line of defense to prevent pathogen invasion, including the epithelial tight junction complexes, mucus layer secretion by goblet cells, secretory immunoglobulin A (sIgA), antimicrobial protein (AMP) secretion by plasma cells, and Paneth cells. Commensal and anti-inflammatory bacteria interact in the healthy gut with the mucosal layer, intestinal epithelial cells and immune cells to maintain gut homeostasis through recognition of pathogen-associated molecular patterns (PAMPs). In susceptible individuals, chronic and excessive inflammation is induced through an imbalanced interaction between host immune cells and microbes. Adapted from Sun et al. [24].

5.1.4. Mucosal immunology in IBD

The pathogenesis of IBD is poorly understood but dysregulated immune responses paired with environmental factors are key drivers of disease in genetically susceptible individuals [38]. Interestingly, UC and CD have been suggested to represent distinct forms of gut inflammation. While CD patients show characteristics of Th1-driven inflammation, UC has been proposed to be rather associated with dysregulated Th2-mediated immune responses [39,40]. Th17-associated responses have been also implicated in disease pathogenesis, because

Th17-related cytokines (IL-17, IL-10, IL-22) are known to play a critical role in modulating mucosal immunity.

5.1.4.1. Innate immunity in IBD

The innate immune system represents the first line of defense against external pathogens. Innate immune responses in the intestine involve a large variety of different cell types including epithelial cells, neutrophils, basophils, eosinophils, dendritic cells (DCs), monocytes, macrophages, and natural killer cells [41]. Unlike adaptive immunity, it provides a rapid, unspecific and short- term immune response of the host in response to pathogens by stimulation of diverse innate immune receptors including toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), C-type lectin receptors (CLRs), and retinoic acid-inducible gene 1-like receptors (RLRs) [42]. Upon sensing of microbial-derived PAMPs, intestinal macrophages and dendritic cells activate proinflammatory signaling pathways (e.g., NF-κB) to produce cytokines, chemokines, and anti-microbial peptides that modulate intestinal immune cell responses [43]. Previous studies have shown that the frequency and function of macrophages is impaired in IBD patients [44]. Whereas macrophages play a major role in phagocytosis and in the regulation of proinflammatory responses, activated DCs present intraluminal pathogens to naïve $CD4^+$ T cells in lymphoid organs and modulate polarization into regulatory T cells (Treg) and T helper cells [43]. Dysregulated activation of DC in IBD patients leads to an overexpression of proinflammatory cytokines (e.g., IL-6, IL-12), as well as disturbance of the balance between tolerance to commensal microorganisms and immune responses [45]. Treg cells suppress in turn pathogenic effector T cell differentiation and function through multiple mechanisms, including both contact-dependent and contact-independent (e.g., IL-10 and TGF-β) mechanisms. Moreover, anti-inflammatory macrophages

induced by IL-4 and IL-13 can inhibit effector T cells through IL-10 secretion and promote Treg cells suppressive function by producing TGF-β [24].

These complex interplays facilitate the maintenance of gut homeostasis by bridging innate immunity to adaptive immune protection. In a non-inflammatory environment, TLR signaling mediates down-regulation of pattern recognition receptors (PRRs) and tolerance towards luminal pathogens. However, defective innate immune responses might lead to persistent immune activation in IBD patients. For example, mucosal DC and macrophages of IBD patients show increased expression of immune and chemokine receptors (e.g., TLR2, TLR4, CD40, and C-C chemokine receptor 7 (CCR7)) and production of pro-inflammatory cytokines (e.g., TNF- α, IL- 1β, IL-6, and IL-18) [45-47]. Furthermore, GWAS have provided critical insights into the role of innate immunity in the pathogenesis of IBD. A variety of single nucleotide polymorphisms (SNPs) in genes controlling microbial sensing (e.g., *NOD2*, *IRF1*, *NFKB1*, *REL1*, *CARD9*) and elimination (e.g., *ATG16L1*, *IRGM*) have been identified as IBD risk factors [48-50].

These insights on genetic susceptibility, innate immunity and microbial imbalances have critical implications for the treatment of IBD patients. For example, the detection of altered TNF-α production by innate immune cells has provided rational arguments for the treatment of IBD with anti-TNF-α monoclonal antibodies (e.g., infliximab, adalimumab) [47]. This treatment option has been shown to be effective in inducing remission and mucosal healing in IBD patients [51-54] and has been established as standard care. In addition, other proinflammatory cytokines (e.g., IL-6 and IL-1β) that are critically involved in mediating innate immune responses may represent attractive therapeutic targets.

5.1.4.2 Adaptive immunity in IBD

In contrast to innate immunity, the adaptive immune system mediates highly specific responses that are critical in the induction, progression, and termination of chronic inflammation.

Upon thymic development, naïve T cells are activated by antigen-presenting cells and differentiate into T helper cells 1 (Th1), Th2, and Th17 effector cells or Treg cells cells in response to polarizing cytokine signals [55,56]. Immune dysbalances of Th1 and Th2 cells have been suggested to play an important role in IBD progression [57]. Increased mucosal level of Th1-mediated IFN-γ in CD patients have been shown to activate other immune cells (e.g., intestinal macrophages) resulting in enhanced TNF-α production and chronicity of IBD [58]. In contrast, key effector Th2-related cytokines (e.g., IL-5 and IL-13) are produced in large amounts in UC patients and have been demonstrated to impair epithelial barrier function by affecting epithelial apoptosis and tight junctions [59]. Based on the characteristic T-cell cytokine profile, CD has been characterized as a Th1-mediated disease, whereas UC has been suggested as Th2-mediated condition [60]. However, the distinction of Th1/Th2 responses in the intestinal mucosa of IBD patients is less clear and more dynamic, as the pathogenesis involves also other cell types such as Th17 cells.

The Th17 cells produce large amounts of IL-17A, IL-17F, IL-21, and IL-22 that are known to promote epithelial proliferation, mucosal healing, and expression of anti-microbial peptides in the mucus [61]. High levels of the Th17-associated cytokines have been detected in the inflamed mucosa of IBD patients [62,63]. In addition, GWAS have implicated genetic polymorphisms affecting Th17-related pathways (e.g., IL-23R and STAT3) in the pathogenesis of IBD [64]. Based on human genetic and mouse studies, IL-23 is considered as promising target for IBD therapy [65]. Accordingly, antibodies targeting the p40 subunit of IL-12 and IL-23 (Ustekinumab) have been shown to alleviate symptoms in CD patient refractory to

anti-TNF therapies [64]. In contrast, antibodies that inhibit IL-17A and IL-17RA failed to induce a robust improvement of disease and caused severe side effects (e.g., fungal infections) [66], underscoring the protective functions of Th17 cells in controlling mucosal immunity.

In addition to T helper cells, Treg cells play an important role in maintaining gut homeostasis by mediating tolerance to self-antigens and preventing autoimmunity. Treg cells are characterized by the expression of the transcription factor forkhead box protein (Foxp3), CD25, cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4), and (glucocorticoid-induced TNFR family related gene) GITR [67]. Treg cells dysfunctions caused by loss-of-function mutations in *FOXP3* have been shown to result in the development of severe multi-organ autoimmunity and intestinal inflammation in mice and humans [68-70]. In addition, several studies have proposed that IBD patients exhibit decreased frequencies and/or dysfunctions of Treg cells in comparison to healthy individuals [71]. Correspondingly, administration of Treg cells has been shown to ameliorate chronic intestinal inflammation in mice and has been promising in preliminary studies on CD patients [71-73].

Several mouse and human studies have demonstrated that the anti-inflammatory cytokines IL-10 and TGF-β are important for the regulatory property of Treg cells [74]. IL-10 has been shown to induce proliferation of Treg cells and mediate immunosuppressive effects on DCs and macrophages leading to inhibition of effector T cells [75]. Similarly, TGF-β leads to suppression of macrophages and effector T cells [76] but is also responsible for Th17 development [77-79].

5.1.5. Genetics in IBD

Population-based studies have provided compelling insights into the contribution of genetic factors to the pathogenesis of IBD.

First, several epidemiological studies have demonstrated that siblings of UC and CD patients have an 8 - 10-fold greater risk of IBD. Strikingly, twin studies have revealed 50 % concordance for CD in monozygotic twins as compared to below 10% in dizygotic twins [80]. Moreover, a higher incidence of IBD can be observed in defined ethnic groups (e.g., Ashkenazi Jews) [81,82]. The importance of genetics in the pathogenesis of IBD has been further underlined in large-scale GWAS on over 75.000 IBD patients by unravelling more than 200 genetic loci associated with increased susceptibility to IBD [83,84].

The identified GWAS loci encompass genes with several biological functions including PRRs (e.g., NOD2/CARD15 and TLRs), autophagy (e.g., ATG16L1, LRRK2 and IRGM), Th17-associated pathways (e.g., IL-23, CCR6, JAK2 and STAT3), intestinal epithelial barrier integrity (e.g., IBD5, DLG5, PTGER4, ITLN1 and DMBT1), and endoplasmic reticulum stress responses (e.g., XBP1) (Figure 3) [49,85-88]. These identified susceptibility gene loci highlight the importance of the genetic background as crucial factor in the pathogenesis of IBD. However, most GWAS do not provide insights into disease mechanisms and the functional significance of the genetic loci is still controversial [89].

Finally, the crucial role of genetic factors in the pathogenesis of IBD has been evidenced by paradigmatic studies reporting that germline biallelic mutations in IL-10R cause VEO-IBD [90,91].

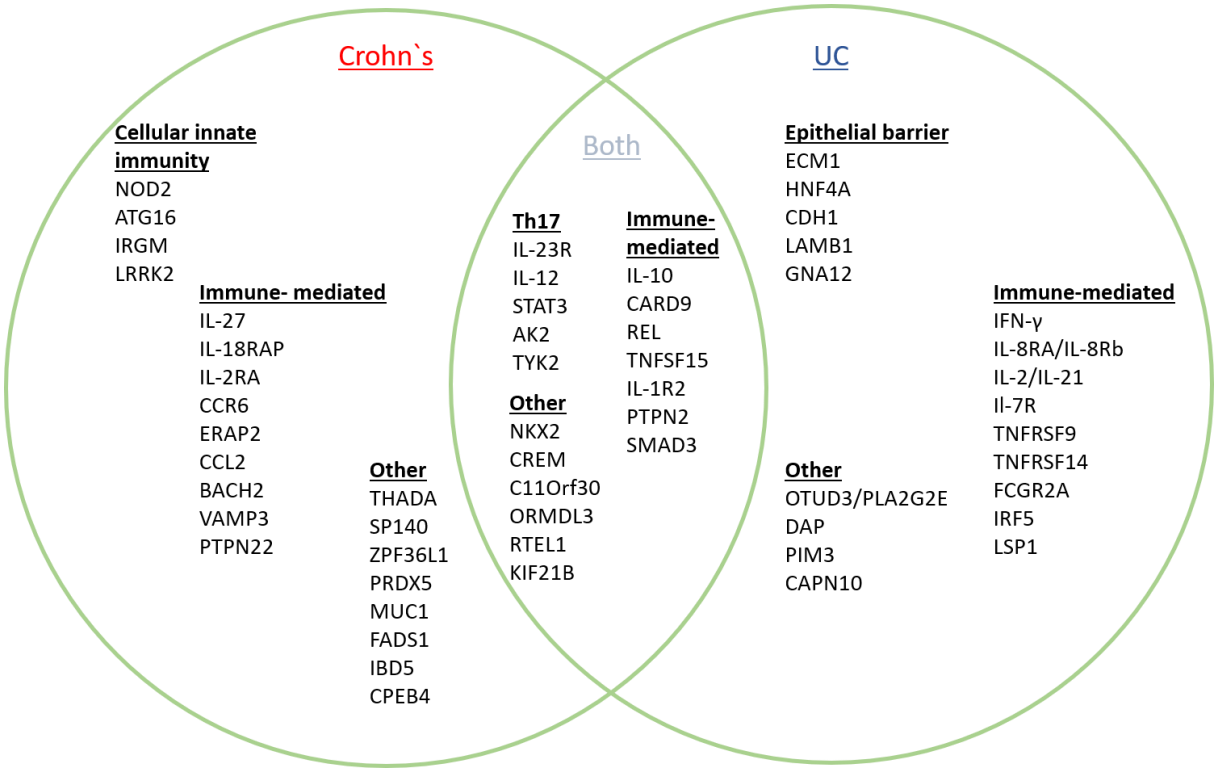

Figure 3: Genetic architecture of IBD.
GWAS have identified over 200 genetic loci with increased susceptibility to IBD.
Figure adapted from Lees et al. and Van Limbergen et al. [92,93].

5.1.6. Very early onset inflammatory bowel disease - A model to study key factors of intestinal homeostasis

The mean age of diagnosis of IBD is between 20 - 40 years, however IBD can manifest at any age [14,94,95]. Approximately 20 - 30 % of IBD cases are diagnosed below the age of 18 years [80,96]. The pathogenesis and disease behavior of IBD are heterogeneous and strikingly age-dependent. Children with VEO-IBD (disease onset below 6 years of age) develop inflammatory conditions different from forms observed in adults [97]. In particular, VEO-IBD patients present with predominance of colonic inflammation, predilection of perianal disease (fistula, abscesses) and extension of disease severity. VEO-IBD is a rare condition but the incidence has increased dramatically over the last decades worldwide. Epidemiological studies indicated that 15 % of pediatric IBD patients present at the age below 6 years [98]. Notably, a

higher frequency of VEO-IBD patients cannot easily be classified as CD or UC. These patients are categorized as IBD-U and often need a reclassification after some years. Of clinical relevance, VEO-IBD patients often show severe and even life- threatening courses that fail to respond to conventional therapies.

Based on the distinct phenotype and the early onset of disease, a predominant role of host genetics has been suggested in children with VEO-IBD in contrast to adult patients with higher contribution of environmental triggers [99,100]. In paradigmatic studies, our laboratory has identified patients with IL-10R deficiency as the first truly monogenic cause of IBD with complete penetrance [91]. Based on these insights, IL-10R-deficient patients were successfully treated by allogeneic hematopoietic stem cell transplantation (HSCT), an innovative therapeutic approach for patients with IBD [90]. This demonstrates the importance of cutting- edge genetic diagnostics for VEO-IBD patients and highlights that rare forms of IBD represent “exquisite models” to study key factors controlling intestinal homeostasis. Over the last years, IBD-like phenotypes have been associated with over 50 monogenic diseases, in particular characterized as inborn errors of immunity (Figure 4) [80,101]. However, the majority of VEO-IBD patients still lacks genetic diagnosis. It is of great relevance for the clinical management of these patients to decipher the underlying molecular pathophysiology.

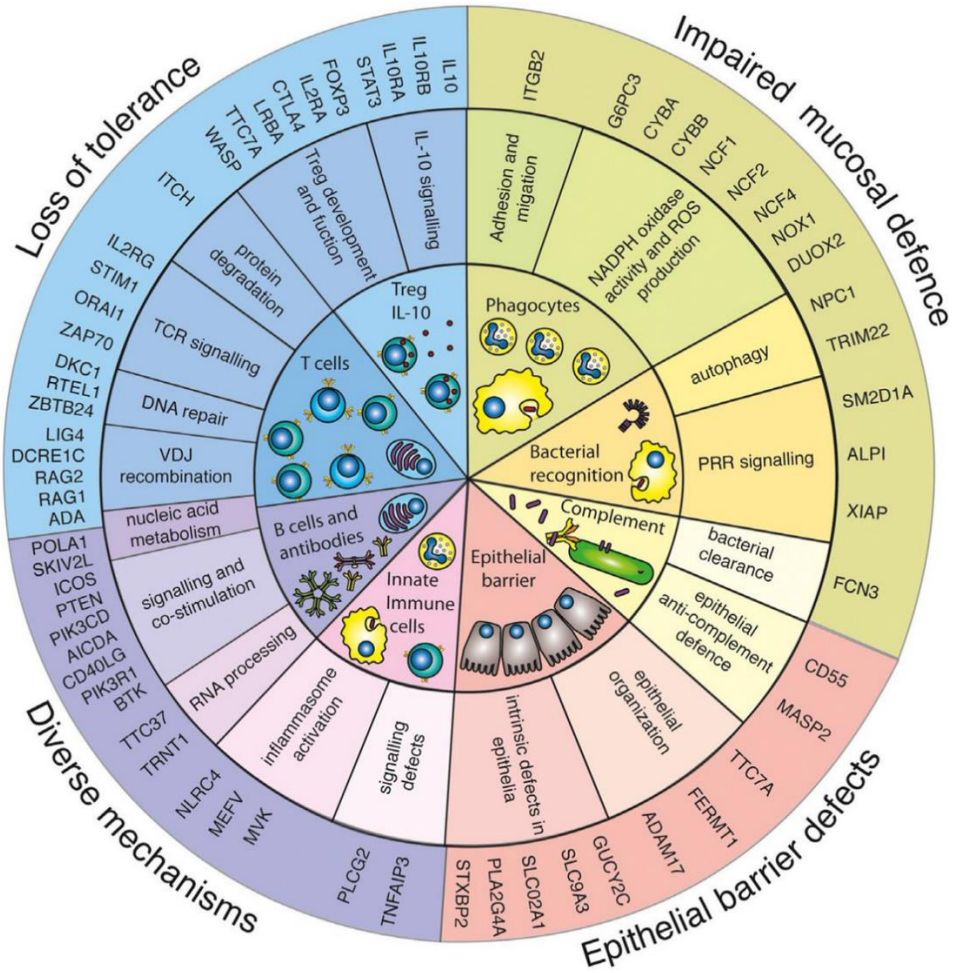

Figure 4: Cell types and molecular mechanisms involved in the pathogenesis of IBD.
The inner circle indicates cell types and cell components involved in the pathogenesis of IBD. The middle circle represents molecular mechanisms affected by genetic mutations in the context of IBD. The outer circle shows the molecular pathomechanisms leading to IBD. Figure has been adapted from Pazmandi et al. [101].

5.2. Caspases - gatekeepers of cell death and inflammation

Caspases are ubiquitously expressed and represent a group of evolutionary highly conserved cysteine-dependent aspartate-directed proteases that play an essential role in activation and execution of cell death [102]. Tightly regulated caspase-dependent intrinsic and extrinsic cell death pathways can be distinguished.

The extrinsic pathway relies on the stimulation of cell receptors through binding of extracellular death ligands such as TNF-α, Fas (CD95/APO-1) and TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1). Subsequently, death receptor signals are passed to the interior of the cell where activated apical caspases (Caspase-8 and Caspase-10) initiate apoptosis by cleavage and activation of the executioner Caspases-3, -6, and -7 [103,104]. In contrast, the intrinsic pathway is induced by various endogenous stimuli (e.g., DNA damage, growth factor withdrawal, cellular stress) and directly activates Caspase-3 resulting in mitochondrial membrane perturbation and release of cytochrome c into the cytoplasm [105]. As a consequence, the apoptosome is formed by the apoptotic protease activating factor 1 (APAF1) and the inactive form of Caspase-9 [106]. The initiator Caspase-9 cleaves and activates the executioner Caspases-3, -6, and -7, which in turn inactivate or activate specific cellular protein targets [107]. Ultimately, the downstream targets mediate the deactivation of homeostatic repair processes and cell cycle processes, as well as the inactivation of apoptotic inhibitors leading to cell death [108].

Caspases are key molecules in the apoptotic pathway and have been considered as potential drug targets. Altered caspase activity has been observed in several conditions such as stroke, sepsis, Alzheimer's disease, Parkinson's disease, and Huntington's disease [109-111]. In addition, targeting of inflammatory caspases has been proposed to control autoimmune disorders such as rheumatoid arthritis [112]. Furthermore, selective activation of caspases has been considered for the treatment of cancer and chronic viral infections [113].

5.2.1. Structure of Caspases

Caspases can be categorized into two subfamilies: interleukin-1β converting enzyme (ICE)- or *Caenorhabditis elegans* protein-3 (CED-3)-like caspases. Further subdivision can be made

based on their short (Caspase-3, -6, -7) or long (Caspase-1, -2, -4, -5, -8, -9, -10, -12) prodomains. To date, 14 mammalian Caspases have been described, while eleven Caspases are coded in the human genome, ten caspases are coded in the mouse genome [114]. An important characteristic of Caspases is the high substrate specificity with cleavage after an aspartic acid residue and a recognition sequence of at least four amino acids N-terminal to the cleavage site. This specificity is critical to control highly ordered and selective processes of cell death [115].

Caspases exist as inactive zymogenes and activation occurs by either of three distinct pathways: (a) recruitment-activation, (b) trans-activation, or (c) autoactivation. (a) For the recruitment-activation pathway, it has been shown that multiple homologous proenzymes are recruited to a common site, e.g., through ligation to the CD95 (Fas) receptor or receptors containing a death domain (e.g. TNF-R) that form the death-inducing complex through zymogen clustering [116,117]. For example, Procaspase-8 is recruited to an oligomeric activation complex using the adaptor protein FADD [118,119]. Following recruitment, the endogenous catalytic activity is sufficient to initiate full activation by proteolysis between the large and the small subunit of Procaspase-8 [120,121]. (b) In the trans-activation model, Caspases are sequentially activated. This process is initiated by activation of one of the activator Caspases (Caspase-6, -8, -9, -10) that in turn cleaves and activates downstream effector Caspases (Caspases-2, -3, -7) by proteolysis of the large and small subunit [122]. (c) The autoactivation of caspases depends on direct activation of Procaspase-3 but the underlying molecular mechanisms remain largely elusive [123].

In general, Caspases are activated by removal of N-terminal activation peptides through proteolytic cleavage as well as specific cleavage within the proteolytic domain [124]. Upon cleavage of the proenzyme at Asp (P1)-X(P1') the two originating subunits form the active enzyme. The active site with cysteine and histidine residues are located in the large subunit,

whereas the S1 subsite, essential for the binding of the P1 aspartic residue, is located at the interface between the large and small subunit of the Caspase.

5.2.2. Classification of Caspases

Based on the structural characteristics and function, Caspases can be divided into three groups: (i) inflammatory Caspases, (ii) initiator or apical Caspases, and (iii) effector or executioner Caspases (Table 1, Figure 5).

The group of inflammatory Caspases comprises Caspase-1, -4, -5, -12, and -14, which induce inflammasome activation or an inflammatory form of programmed cell death (pyroptosis) [125-127]. Whereas Caspase-1, -4, -5, -12 can be detected in humans, mice encode for caspase-1, -11, -12 with caspase-11 being a homologue of human Caspase-4 and -5.

Apoptotic Caspases initiate and execute apoptosis [128]. This family encompasses the apical or initiator Caspases-2, -8, -9, and -10 as well as the effector or executioner Caspases-3, -6, and - 7. Of note, Caspase-10 is expressed in humans but no homologue exists in mice. The initiator Caspases consist of a large N-terminal dimerization domain (death effector domain, DED) (Caspase-8, -10) or a CARD domain (Caspase-2, -9), whereas effector Caspases have a short N-terminal domain (Figure 5) [129-131]. These domains are important for the recruitment of the zymogens to specific activation platforms that facilitate autocatalytic processing. The activation platforms are the apoptosome (Caspase-9), inflammasome (Caspase-1, -4, and -5), PIDDosome (Caspase-2), and death-inducing signaling complex (DISC; Caspase-8, -10) [116,132-135]. Effector Caspases-3, -6, and -7 exist as pre-formed constitutive Procaspase dimers with a short prodomain and absence of self-activation ability [136,137]. They require

activation by initiator Caspases and mediate the morphological changes in apoptotic cells [130,131,138].

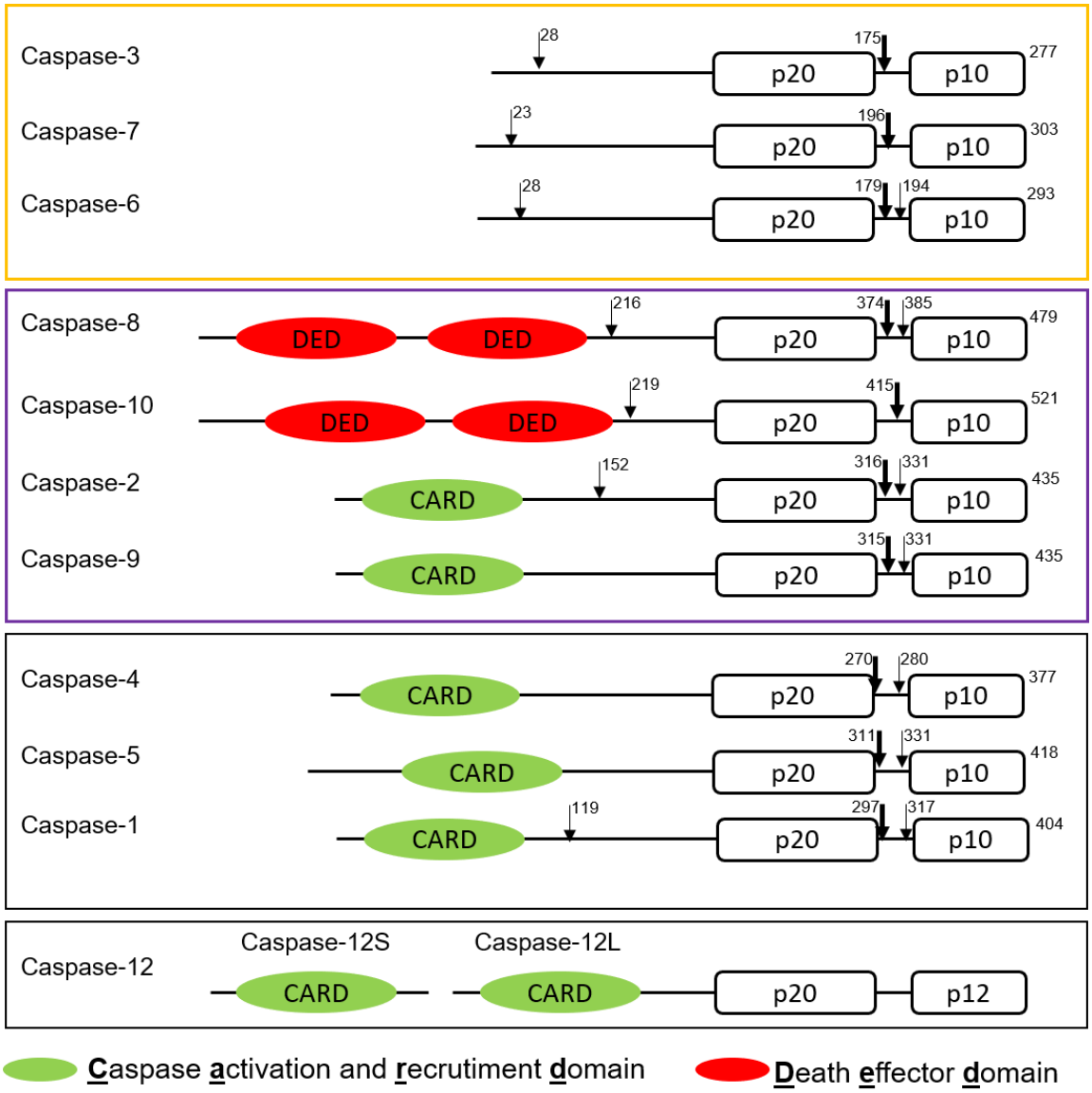

Figure 5*:* Classification of human Caspases.
The initiator Caspases are highlighted in orange, whereas the effector Caspases are labeled in purple. The inflammatory Caspases as well as the effector Caspase-2 and -9 possess a Caspase activation and recruitment domain (CARD) in their N-termini, while the initiator Caspases- 8 and -10 contain a death effector domain (DED), followed by a large (20 kDa) and small (10 kDa) catalytic subunit. The position of the first cleavage site is highlighted with a bold arrow, while additional sites are marked with a small arrow. Adapted and modified from Rodrigue-Gervais [139].

Table 1: Classification of Caspases

Caspase	Classification of function	Initiator or effector Caspase	Human locus	Murine locus	Specific Prodomain
Caspase-1	Inflammatory	Initiator	11q23	9 A1	CARD
Caspase-2	Apoptotic	Initiator	7q34-7q35	6 B2.1	CARD
Caspase-3	Apoptotic	Effector	4q34	8 B1.1	-
Caspase-4	Inflammatory	Initiator	11q22.2-11q22.3	Absent	CARD
Caspase-5	Inflammatory	Initiator	11q22.2-11q22.3	Absent	CARD
Caspase-6	Apoptotic	Effector	4q25	3 H1	-
Caspase-7	Apoptotic	Effector	10q25	19 D2	-
Caspase-8	Apoptotic	Initiator	2q33-2q34	1 B	DED-DED
Caspase-9	Apoptotic	Initiator	1p36.21	4 E1	CARD
Caspase-10	Apoptotic	Initiator	2q33-2q34	Absent	DED-DED
Caspase-11	Inflammatory	Initiator	Absent	9 A1	CARD
Caspase-12	Inflammatory	Initiator	11q22.3	9 A1	CARD
Caspase-14	Keratinocyte differentiation	Effector	19p13.1	10 C1	-

5.2.3. Caspase-8 – A master regulator of programmed cell death and immunity

Caspase-8 is an initiator cysteinyl aspartate-specific protease critically involved in mediating cell death receptor-induced apoptosis [118,119]. It was first described in 1996 as FLICE (FADD-like interleukin-1 beta converting enzyme), MACHα1 (Membrane Oligomerization and Cleavage Activates the Caspase-8), and MCH5 (Mono Carboxylate transporter Homolog) [118,119,140]. The gene encoding CASP8 is located on chromosome 2 band q33q34 and encompasses 10 exons. CASP8 is synthesized as inactive monomeric zymogen (Procaspase-8) consisting of two N-terminal death effector prodomains and the C- terminal protease domain composed of two catalytic subunits (p18, p10) [141]. Human Procaspase-8 is a 479 amino acid protein with a molecular weight of 53 or 55 kDa depending on the isoform. All isoforms share

a common 182 amino acid N-terminal region encoding the two DED domains but differ in their C-termini [119,142].

Caspase-8 plays an essential role in mediating apoptosis. Upon ligation of membrane-bound cell death receptors, FAS-associated death domain (FADD) adaptor proteins recruit inactive Procaspase-8 to the multimeric death-inducing signaling complex (DISC) at the cytoplasmic tail of the activated receptors [143]. Within the DISC, the interaction of Procaspase-8 molecules through the death effector domains (DED) facilitates dimerization and sequential proteolytic cleavage resulting in the formation of the catalytic active Caspase-8 heterotetramer $(p10\text{-}p18)_2$ (Figure 6) [144]. Subsequently, active Caspase-8 is released into the cytosol and induces apoptosis by triggering cleavage and activation of the downstream executioner Caspase-3 and Caspase-7 or the BCL2 family member BH3 interacting domain death agonist (BID) [144].

The essential role of Caspase-8 in controlling programmed cell death has been demonstrated in Casp8 knockout mice presenting with embryonic lethality accompanied by cardiac malformations, neural tube defects, growth retardation, and hematopoietic progenitor deficiency [145-147]. Of note, Caspase-8-deficient cells showed resistance to apoptosis upon death receptor stimulation [145,147].

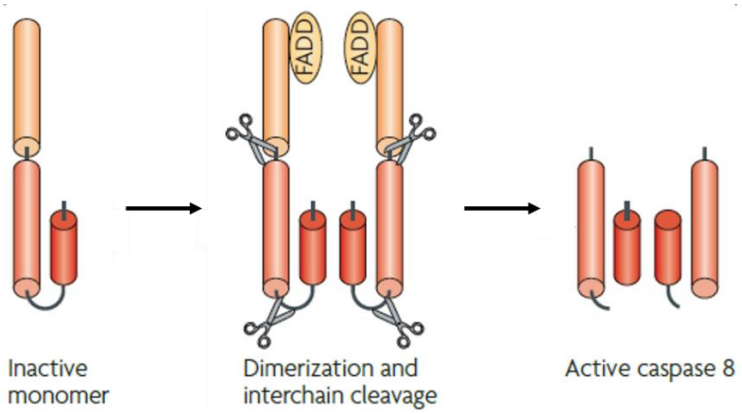

Figure 6: Activation of Caspase-8.
Caspase-8 is synthesized as an inactive monomer. Upon death receptor ligation, Procaspase-8 forms dimers by recruitment of monomers to the adaptor molecule Fas-associated death domain protein (FADD). The dimerization and auto-cleavage of the interdomain are required for the activation of mature Caspase-8 heterotetramers $(p10\text{-}p18)_2$.
Figure is adapted from Tait et al.[148].

Recent studies in mice have extended the knowledge on the function of Caspase-8 in controlling cell death responses to inhibitory regulation of programmed necroptosis upon death receptor or TLR signaling [149,150]. When Caspase-8 activity is compromised by chemical inhibition, pathogens, or genetic targeting, necroptosis is induced by oligomerization of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3 via the RIPK homotypic interaction motif (RHIM) [151]. Activation and autophosphorylation of RIPK3 leads to recruitment and phosphorylation of mixed lineage kinase domain-like (MLKL), a key executioner in programmed necrosis [152]. The catalytic activity of Caspase-8 has been shown to control necroptosis by terminating RIPK1 and RIPK3 complex formation via proteolytic cleavage, but the exact mechanisms remain unknown [149,153].

Emerging evidence highlights that Caspase-8 controls also several non-apoptotic cell functions such as development, growth, proliferation, differentiation, migration, and inflammation [154]. Constitutive Casp8 knockout mice show embryonic lethality accompanied by cardiac malformations, neural tube defects, growth retardation, and hematopoietic progenitor deficiency [145-147]. Studies on conditional knockout mouse models have demonstrated that Casp8 is critical in controlling skin, liver, and gut inflammatory conditions in response to injuries or infections [155-157]. Whereas skin inflammation was associated with enhanced responses to endogenous activators of interferon regulatory factor 3 (IRF3) in the epidermis [156], conditional deletion of Casp8 in the intestinal epithelium resulted in severe intestinal ileitis associated with TNF-α-mediated necroptotic cell death [157]. In addition, Caspase-8 has been shown to be implicated in controlling macrophage differentiation and function [145], T cell homeostasis [158], B cell proliferation [159], and NF-κB activation [160]. The exact mechanisms of the diverse physiological roles of human Caspase-8 remain largely unexplored and likely a tight regulation of Caspase-8 expression, subcellular compartmentalization, and activation is required for the maintenance of tissue homeostasis.

5.2.4. Human Caspase-8 Deficiency

The essential role of Caspase-8 in controlling human immunity has been highlighted by the identification of two siblings with features of an autoimmune lymphoproliferative syndrome (ALPS)-like disorder and a germline homozygous missense mutation in CASP8 affecting the p18 subunit domain (Arg248Trp) [161]. In contrast to typical ALPS caused by mutations in FAS and FASL [162,163], Caspase-8-deficient patients exhibited an immunodeficiency with defective activation of T, B, and natural killer (NK) cells, increased susceptibility to sinopulmonary and herpes simplex virus infections, as well as poor immunization responses [161]. In comparison to constitutive Caspase-8 knockout mice, no developmental retardation has been observed in Caspase-8-deficient patients. Recently, Niemala *et al.* reported two related patients presenting with the same CASP8 mutation and adult-onset multi-organ lymphocytic infiltrations with granulomas in the central nervous system, liver, spleen, bone marrow, and lungs [164].

In the context of intestinal diseases, dysfunctions of Caspase-8 have been controversially discussed in the pathogenesis of IBD and colorectal cancer [165]. For example, pediatric IBD or allergic colitis have been associated with necroptosis and reduced expression of Caspase-8 in a cohort study, but the clinical relevance of this finding has not been substantiated by functional studies [101].

5.3. Inflammasomes in health and disease

The human immune system has to fight against a huge variety of infections or tissues injuries, thus requiring a diverse set of sensors to recognize endogenous danger signals and pathogens. To orchestrate immune responses, human myeloid cells express large multimeric protein complexes that are central for a variety of immune responses controlling cytokine production,

cytoskeletal remodeling, and cell death [166]. In 2002, the group of Dr. Jürg Tschopp has introduced the term “inflammasome” for these protein complexes [167], based on structural and functional similarities with the complex found in the apoptotic signaling pathway, the apoptosome [168]. The inflammasome consists of three major components: a cytosolic PRR, the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), and Caspase-1. The activation of inflammasomes is a two-step process. The first step includes a priming via TLRs (e.g., LPS on TLR4) leading to the upregulation of pro-IL-1β [169,170]. Under steady-state conditions, NOD-like receptor family pyrin domain-containing 3 (NLRP3) is silenced through the interaction between the NACHT-domain and the leucine-rich repeat domain (LRR). Upon recognition of PAMPs or DAMPs (e.g., microbial components and sterile endogenous danger signals) [171,172], the silencing of NLRP3 is abolished resulting in the oligomerization of the adaptor protein ASC at the site of the NLR and the recruitment of pro-Caspase-1 to its CARD domain (Figure 7) [173,174]. In the second step, the activation of inflammasomes initiates a conformational change and oligomerization of the inflammasome receptor. Finally, dimerization of Procaspase-1 leads to autoproteolysis and cleaved Caspase-1 mediates processing of pro-IL-1β and pro-IL-18 into their mature forms. Several studies have demonstrated that IL-1β is predominantly produced by macrophages and DCs and represents a pleiotropic cytokine that exerts multiple roles in disease and health (Figure 7) [175,176]. For example, IL-1β has been shown to mediate recruitment of neutrophils to the sites of infection, promote endothelial cell adhesion, and stimulate T helper cell immune responses [177,178]. Upon activation of the IL-1β receptor, several pro-inflammatory cytokines (e.g., TNF-α, RANTES, IL-8) are expressed and altered inflammasome activity may lead to tissue damage [179-182]. The importance of functional inflammasome activity in human disease has been evidenced by the identification of patients with a rare IL-1β-mediated autoinflammatory condition (cryopyrin-associated periodic syndrome) caused by germline gain-of-function

mutations in *NLRP3* [183-185]. Furthermore, dysregulated IL-1β secretion has been implicated in a variety of acquired and hereditary autoinflammatory diseases such as gout, hereditary periodic fever syndromes, IBD, metabolic diseases, and type 2 diabetes [185-189]. A growing body of evidence suggested a critical role of inflammasomes in controlling intestinal homeostasis and innate immune responses during intestinal inflammation [190], since (i) genes involved in inflammasome activation were associated with increased susceptibility to IBD, (ii) patients with IBD exhibited increased IL-1β levels in the colon, and (iii) blockade of IL-1β ameliorated colitis in murine models [191]. Based on these findings, the inflammasome represents an attractive target for the treatment of IBD, however the complex molecular mechanism of inflammasome regulation need to be further defined to optimize therapies.

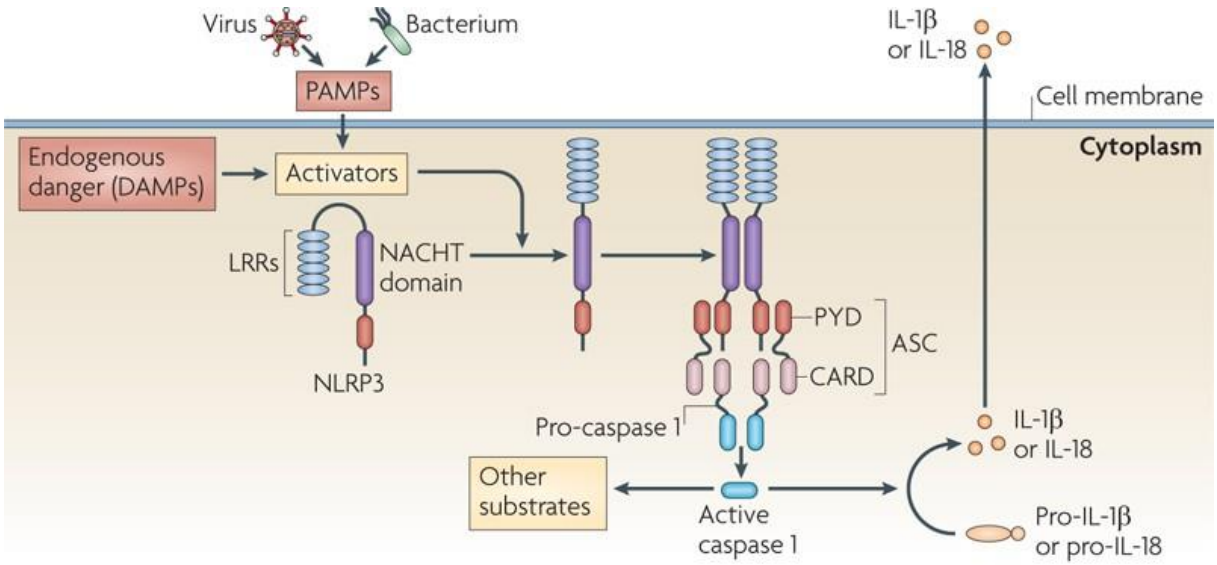

Figure 7: NLRP3-dependent inflammasome activation.
Upon stimulation with PAMPs from microorganisms or DAMPs from endogenous danger signals, the auto-repression of NLRP3 activity by the interaction of the NACHT domain and the LRRs is omitted. Subsequent maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18 upon cleavage of Caspase-1 occurs via NLRP3 oligomerization and recruitment of the adaptor protein apoptosis- associated speck- like protein containing a CARD (ASC) and the zymogen Procaspase 1. Figure has been adapted from Tschopp and Schroder [192].

The different types of inflammasomes are basically organized similarly and named according to sensors that mediate scaffolding functions for inflammasome assembly. The specificity of inflammasomes will be determined by a great variety of sensors, such as members of the NLR family (e.g., NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, and NLRC4), the HIN-200 (PYHIN) family (e.g., absent in melanoma 2 protein (AIM2) and interferon-inducible protein IF16), as well as the retinoic acid inducible gene-I-like receptors (RLR RIG-I) [193].

Some members of the NLR family mediate also functions other than controlling the inflammasome pathways. For example, (a) NLRC5 and NLRA are nuclear transactivator proteins responsible for the transcription of the antigen-presenting class I and class II human leucocyte antigen (HLA) molecules, (b) NLRC1/NOD1 and NLRC2/NOD2 act as cytoplasmic PRRs that mediate NF-κB activation, and (c) NLRP6 and NLRP12 have been reported to regulate gut microbiome and homeostasis by suppression of TLR-mediated NF- κB activation [194-200].

5.4. Cell death

The maintenance of tissue integrity and organ functions are major aspects of multicellular organisms and tissue homeostasis depends on a dynamic interplay of cell division, cell growth, proliferation and death [201]. In particular, programmed cell death has been demonstrated to be an integral process in several biological processes such as embryonic development, cell division and tissue homeostasis [202].

Based on morphological and biochemical changes, different modes of cell death can be distinguished [203]. Whereas apoptosis, autophagy and necroptosis belong to the category of programmed cell death which includes the release of DAMPs through membrane

permeabilization [204-206], necrosis is triggered by external and incidental factors (e.g., injuries, toxins or radioactivity) [207].

5.4.1. Apoptosis

The term apoptosis originates from Greek “leaves falling from a tree” and was first described by Kerr *et al*. in 1972 [204]. Apoptosis is the best characterized form of programmed cell death. It is an evolutionary conserved, tightly coordinated suicide mechanism of redundant and/or damaged cells. Apoptosis plays a pivotal role in controlling embryogenesis, immunity, tissue regeneration, and cellular homeostasis [208-210].

Apoptotic pathways are initiated upon cell death signals such as cellular stress or damage to cellular organelles by heat shock, radiation, cytotoxic drugs, and infections. In response to these triggers apoptotic cells undergo morphological and biochemical changes characterized by fragmentation of cell-adhesion and cytoskeletal proteins, nuclear envelope disassembly, chromatin condensation, DNA fragmentation and plasma membrane blebbing [211]. The fragments of the dying cells occur as compact membrane-enclosed structures, the so called “apoptotic bodies” that are engulfed and removed by phagocytes without causing inflammatory responses to the neighboring tissues [212]. The biochemical characteristics are energy- dependent mechanisms including protein cleavage, perturbance of mitochondrial function, DNA breakage, and phagocytic recognition signals [104]. As a consequence, mitochondrial membrane potential and integrity are altered leading to a stop of ATP production, rupture of the outer mitochondrial membrane with release of proapoptotic factors, and activation of inducer and effector Caspases [213]. Dysregulated apoptotic processes have been linked to a variety of diseases. Whereas excessive apoptosis could be shown to lead to neurodegenerative diseases, insufficient apoptosis has been reported in autoimmunity and

cancer [213,214]. The relevance of functional apoptosis in immunity has been especially demonstrated in patients with ALPS who present with lymphoproliferation and autoimmune disease associated with defective apoptosis in lymphocytes [215].

5.4.1.1. The extrinsic pathway of apoptosis

Apoptosis can be distinguished by two distinct signal transduction pathways: intrinsic (mitochondrial-mediated) and extrinsic (death receptor-mediated) apoptosis [103,106].

The extrinsic pathway will be activated by binding of extracellular protein ligands to specific transmembrane cell death receptors. This pathway has several critical functions in human immunity including the regulation of (a) immune responses, (b) selection and maintenance of the immune repertoire, as well as (c) removal of infected, transformed or damaged cells [216,217]. Death ligands of the extrinsic pathway belong to the family of TNF receptors comprising of TNF-α, FasL, and TRAIL [218]. The binding of ligands induces trimerization and conformational changes of corresponding cell death receptors leading to the recruitment of adaptor proteins such as FADD and TRADD. FADD consists of a DD and a DED enabling the interaction with the DED of the N-terminus of inactive and monomeric Caspase-8 or Caspase-10 [118,119,219,220]. Upon recruitment of Caspase-8 to the FADD complex, an oligomeric death- inducing signaling complex (DISC) is formed [221-223] which in turn allows oligomerization, proteolytic cleavage and activation of Caspase-8 [103,224-226], and results in execution of apoptosis by cleavage of effector Caspases-3, -6, and -7 [227,228].

5.4.1.2. The intrinsic pathway of apoptosis (mitochondria-mediated pathway)

The intrinsic apoptosis pathway is activated through a wide variety of intracellular stress stimuli such as oxidative stress, DNA damage, ischemia, and altered intracellular calcium concentrations. These signals lead to formation of pores in the mitochondrial outer membrane and subsequent mitochondrial outer membrane permeabilization (MOMP) [219,220,229]. This process is tightly regulated by Bcl-2 (B-cell lymphoma 2) family proteins. Active Caspase-8 cleaves Bcl-2 into truncated Bid (tBid) leading to the oligomerization of the Bcl-2 family member proteins Bak (Bcl-2 homologous killer) and Bax (Bcl-2 homologous X protein) [214]. Subsequently, cytochrome c, second mitochondria-derived activator of Caspases (Smac/Diablo), apoptosis inducing factor (AIF), and high temperature requirement A2 (HtrA2) are released from mitochondria [230,231]. Cytosolic cytochrome c associates with apoptosis protease activating factor-1 (APAF-1) enabling binding to ATP/dATP and formation of the apoptosome through oligomerization [232]. APAF-1 binds Caspase-9 via the CARD domain leading to its cleavage and dissociation from the apoptosome [232]. Finally, cleaved Caspase- 9 activates downstream effector Caspases-3, -6, and -7 [135,233-235], which collectively orchestrate the execution of apoptosis.

5.4.2. Necroptosis

Initially, necroptosis has been suggested as a form of cell death causing damage and inflammatory responses in the surrounding tissue [236,237]. Accumulating evidence suggests that necroptosis is a tightly regulated and programmed form of cell death [238,239]. Necroptotic cells show characteristic morphological changes, including swelling of organelles, plasma membrane rupture, and release of intracellular contents, while the nuclei of necroptotic cells remain intact in contrast to apoptosis [240,241].

TNF-α is the most commonly used stimulus for the induction of necroptotic pathways. When Caspase-8 activity is chemically blocked or genetically depleted, TNF-α is able to induce necroptosis in a wide range of cell lines and primary cells. The combination of TNF-α with antagonists of cellular inhibitor of apoptosis proteins (cIAPs) has been shown to aggravate the necroptotic signaling [242-244]. In addition, necroptosis can be induced by several other triggers, including ligation of CD95 or TRAIL, intracellular ATP depletion, mitochondrial depolarization, poly-(ADP-ribose) polymerase (PARP) activation, reactive oxygen species (ROS), as well as vaccination or viral infections [245].

RIPK1 and RIPK3 are critical regulators of cell death receptor-induced necroptosis [246-249]. Upon binding of TNF, the TNFR1 changes its conformation for trimerization and induces the formation of the TNF complex I by sequential recruitment of TRADD, TNF receptor-associated factor 2 (TRAF2), TRAF5, RIPK1, cIAP1/2, and the LUBAC complex consisting of haem- oxidized IRP2 ubiquitin ligase-1 (HOIL-1), HOIL-1-interacting protein (HOIP), and SHANK-associated RH domain-interacting protein (SHARPIN) [250-253]. In complex I, RIPK1 will be poly-ubiquitinated leading to the assembly of transforming growth factor-β-activated kinase-I (TAK1)-binding protein 2 and 3 (TAB2/3) complex, which in turns activates NF-κB, Inhibitor of nuclear factor kappa-B kinase subunit beta (IKK) and Mitogen-activated protein kinase 1 (MAPK) [254,255].

Compromised poly-ubiquitination of RIPK1 leads to deubiquitination of cylindrimatosis (CYLD) or IAP antagonists which in turn results in disassembly of complex I and formation of complex IIa consisting of TRAF2, RIP1, TRADD, Caspase-8, cFLIP and FADD [250,256]. When Caspase-8 is compromised, RIPK1 and RIPK3 are not cleaved and interact through their RHIM domains leading to autophosphorylation. Activated RIPK3 phosphorylates MLKL at the residues Thr357 and Ser358 [257] triggering oligomerization of MLKL and translocation of the necrosome from the cytosol to the plasma and intracellular membranes, where it disrupts

membrane integrity [258]. The importance of the RIPK1/RIPK3/MLKL-dependent pathway in necroptosis could be demonstrated by genetic targeting or pharmacological inhibition of these components leading to an arrest of necroptosis in cell lines challenged by TNF-α, Smac mimetic and z-VAD-fmk [152]. The role of RIPK3 and MLKL in necroptosis could be further confirmed by the generation of respective knockout mice [259-261].

Several mouse studies have demonstrated that dysregulation of RIPK1/RIPK3/MLKL-mediated necroptosis plays a pivotal role in pathophysiological conditions such as CD, microbial infections, pancreatitis, skin inflammation, myocardial infarction, atherosclerosis, as well as liver, retinal, and renal injury [151,262]. Based on these mouse studies, several inhibitors targeting RIPK activity have been developed to study the underlaying mechanisms and therapeutic potential of targeting necroptosis [263]. Based on these mouse studies, small- molecule inhibitors targeting RIPK1 (GSK2982772, DNL747) have been even tested in phase I studies for the treatment of chronic psoriasis, rheumatoid arthritis, and ulcerative colitis [264,265]. In addition, RIPK3 inhibitors (GSK`840, GSK`843, and GSK`872) have been proposed as attractive targets but they have been shown to trigger apoptosis and thus this targeted approach needs further refinement [266]. Moreover, MLKL inhibitors (e.g., necrosulfonamide (NSA), compound 1, TC13172) have been tested as potential new therapeutic targets for disturbed necroptosis, but the therapeutic success needs to be evaluated in preclinical models and clinical trials [267-269].

6. Materials and Methods

6.1. Materials

The below-mentioned materials were used for the experimental procedures.

6.1.1. Enzymes

Enzyme	**Catalog number**	**Company**
BshTI (AgeI)	ER1461	ThermoFisher Scientific
BcuI	FD1254	ThermoFisher Scientific
BamHI	FD0054	ThermoFisher Scientific
dNTPs	80192	ThermoFisher Scientific
HindIII	FD0504	ThermoFisher Scientific
MluI	FD0564	ThermoFisher Scientific
PmlI	R0532S	New England Biolabs
Q5® High-Fidelity DNA Polymerase	M0491S	New England Biolabs
ScaI	FD0434	ThermoFisher Scientific
SpeI	R0133	New England Biolabs
SmaI	FD0663	ThermoFisher Scientific
T4 DNA Ligase	EL0011	ThermoFisher Scientific
XhoI	FD0694	ThermoFisher Scientific

6.1.2. Antibodies

Antibodies	**Catalog number**	**Company**
ASC, pAb (AL177)	ab209384	Abcam
Caspase-1 (human) polyclonal antibody	ab196436	Abcam
Caspase-8 (human), mAb (12F5)	ab184718	Abcam
Caspase-1 (p20) (human), mAb (Bally-1)	AG-20B-0048-C100	Adipogen Life Sciences
Caspase-3 monoclonal antibody (31A1067)	AG-25B-0006-C100	Adipogen Life Sciences
Caspase-8 (1C12) Mouse mAb	9746S	Cell Signaling Technology
CD3 mAb (OKT3), Functional Grade	ALX-804-242	Enzo Life Sciences, Inc.
Cleaved Caspase-8 (D391) 18C8 Rabbit mAb	9496 S	Cell Signaling Technology

Cleaved Caspase-3 (D175) (5A1E)	BML-SA101-0100	Enzo Life Sciences, Inc.
Human IL-1 beta /IL-1F2 Antibody	16-0037-85	ThermoFisher Scientific
IL-1β (3A6) Mouse mAb	9664L	Cell Signaling Technology
MLKL antibody [EPR17514] (ab184718)	AF-201-NA	R&D Systems
MLKL (phospho S345) antibody [EPR9515(2)]	12242	Cell Signaling Technology
NLRP3 (D2P5E) Rabbit mAb	13158 S	Cell Signaling Technology
NF-κB Pathway Sampler Kit	9936	Cell Signaling Technology
Phospho-RIPK1 (Ser166) (D1L3S)	65746S	Cell Signaling Technology
Phospho-MLKL (Ser358) (D6H3V) Rabbit mAb	91689 S	Cell Signaling Technology
RIPK1 (D94C12) XP® Rabbit mAb	3493S	Cell Signaling Technology
RIPK3 (E1Z1D) Rabbit mAb	13526	Cell Signaling Technology
RIP3 (phospho S227) antibody [EPR9627]	ALX-804-305-C100	Enzo Life Sciences, Inc.

6.1.3. List of antibodies used for flow cytometry

Antibody (anti-)	**Clone**	**Catalog number**	**Company**
Cell surface antigens			
CCR4-PE-Cy7	L291H4	359410	BioLegend
CCR6-BV786	11A9	563704	BD Biosciences
CCR6-PE-Cy7	11A9	560620	BD Biosciences
CCR7-BV421	150503	562555	BD Biosciences
CD3-APC-H7	SK7	560176	BD Biosciences
CD3-BUV395	SK7	564001	BD Biosciences
CD3-BUV496	UCHT-1	564809	BD Biosciences
CD4-APC	SK3	565994	BD Biosciences
CD4-BB515	RPA-T4	564419	BD Biosciences
CD4 BUV395	RPA-T4	564724	BD Biosciences
CD8-BUV496	RPA-T8	564804	BD Biosciences
CD8-BUV737	SK1	564628	BD Biosciences
CD8-Pacific Blue	RPA-T8	558207	BD Biosciences
CD10-BV421	HI10a	562902	BD Biosciences
CD10-PE	HI10a	340921	BD Biosciences
CD11c-BV421	B-Ly6	562561	BD Biosciences

CD11c-BV650	B-ly6	563604	BD Biosciences
CD14-APC-Fire780	M5E2	301853	BioLegend
CD14-BV786	M5E2	563698	BD Biosciences
CD16-APC	3G8	557758	BD Biosciences
CD19-BUV395	SJ25C1	563549	BD Biosciences
CD19-BV737	SJ25C1	564303	BD Biosciences
CD19-PE	HD37	RO80801-2	Dako
CD19-PerCP Cy5,5	HIB19	302230	BioLegend
CD20-PE-Cy7	2H7	560735	BD Biosciences
CD21-BUV737	B-Ly4	564437	BD Biosciences
CD21-PE	B-Ly4	555422	BD Biosciences
CD25-PE	M-A251	555432	BD Biosciences
CD27-APC-R700	M-T271	565116	BD Biosciences
CD27-BV786	L128	563327	BD Biosciences
CD28-BB700	L293	745905	BD Biosciences
CD28PerCP-Cy5,5	CD28.2	302922	BioLegend
CD38-APC	HB7	340439	BD Biosciences
CD33-PE-Cy7	P67.6	333946	BD Biosciences
CD38-BV650	HB-7	356620	BioLegend
CD45-BV480	HI30	566115	BD Biosciences
CD45-V500	HI30	560777	BD Biosciences
CD45RA-APC	HB7	550855	BD Biosciences
CD45RA-BUV737	HI100	564442	BD Biosciences
CD45RA-PE	HI100	555489	BD Biosciences
CD45RO-BB515	UCHL1	564529	BD Biosciences
CD45RO-BV786	UCHL1	564290	BD Biosciences
CD56-PE-CF594	NCAM16.2	562289	BD Biosciences
CD57-BB515	NK1	560845	BD Biosciences
CD57-FITC	NK-1	555619	BD Biosciences
CD62L-BV650	DREG-56	563808	BD Biosciences
CD69-APC-Cy7	FN50	310914	BioLegend
CD123-BV421	9F5	562517	BD Biosciences
CD123-BV786	7G3	564196	BD Biosciences
CD127-APC	A019D5	351316	BioLegend
CD127-BUV737	HIL-7R-M21	564300	BD Biosciences
CXCR3-BV421	1C6	562558	BD Biosciences
CXCR3-PE-CF594	1C6/CXCR3	562451	BD Biosciences
HLA-DR BV711	G46-6	563696	BD Biosciences
HLA-DR PE	L243	307606	BioLegend
HLA-DR-PE-Cy7	G46-6	560651	BD Biosciences
IgD-BB515	IA6-2	565243	BD Biosciences
IgM-BV421	G20-127	562618	BD Biosciences
TCRαβ-APC-Fire780	IP26	306736	BioLegend

TCRγδ-APC-R700	11F2	657706	BD Biosciences
Phospho Flow antibodies /Intracellular antigens			
IκBα-AlexaFluor647	25/IkBa/MAD-3	560817	BD Biosciences
Phospho-Akt (Ser473)		9271	CST
Phospho-Erk1/2	(D13.14.4E)	4370	CST
Phospho-LAT (Tyr191)		3584	CST
Phospho-NFκB p65 (Ser529)	K10-895.12.50	558422	BD Biosciences
Phospho-NF-κB2 p100 (Ser866/870)		4810	CST
Phospho-PKCθ (Thr538)		9377	CST
Phospho-Zap-70 (Tyr319)/Syk (Tyr352)	(65E4)	2717	CST
Secondary antibodies			
Rabbit IgG-AlexaFluor488		4412	CST

6.1.4. Kits

Name	**Catalog number**	**Company**
Annexin V Apoptosis Detection Kit APC	88-8007-74	ThermoFisher Scientific
Applied Biosystems™ FAST SYBR™ Green Master Mix	4385617	ThermoFisher Scientific
BD Cytofix/Cytoperm™	554722	BD Biosciences
BD Perm/Wash™	554723	BD Biosciences
ExoSAP-IT PCR Product Cleanup Reagent	78201.1.ML	ThermoFisher Scientific
Ficoll paque plus	17-1440-03	GE Healthcare Bio-Sciences
FastAP Thermosensitive Alkaline Phosphatase	EF0654	ThermoFisher Scientific
HEK-Blue™ selection for mycoplasma	609911	InvivoGen
High-Capacity cDNA Reverse Transcription Kits	4374966	ThermoFisher Scientific
Human IL-1 beta/IL-1F2 DuoSet ELISA	DY201	R&D Systems, Inc.
Pan T Cell Isolation Kit, human	130-096-535	Miltenyi Biotec GmbH
pGEM®-T-Vector kit	A1360	Promega GmbH
QIAquick gel extraction kit	28706	Qiagen
QIAmp DNA blood mini kit	51106	Qiagen
Qiagen MaxiPrep Kit	12965	Qiagen
RNeasy Plus Mini Kit	74136	Qiagen
Zyppy Plasmid Miniprep Kit	D4020	Zymo Research Europe GmbH

6.1.5. Supplements

Name	Catalog number	Company
Bovine Serum Albumin	A-3059	Sigma- Aldrich
Brefeldin A	B7651	Sigma- Aldrich
Dulbecco's Modified Eagle's Medium (DMEM)	11960085	ThermoFisher Scientific
Roswell Park Memorial Institute (RPMI) 1640	61870044	ThermoFisher Scientific
Dimethylsulfoxid (DMSO)	5879	Sigma- Aldrich
Fetal Bovine Serum (FBS)	16000-044	ThermoFisher Scientific
Penicillin-Streptomycin (5,000 U/mL)	15070-063	ThermoFisher Scientific
L-Glutamine (200 mM)	25030-081	ThermoFisher Scientific
Phosphate-Buffered Saline (PBS)	14190-094	ThermoFisher Scientific
Ionomycin	A2177	AppliChem GmbH
Lipopolysaccharide (LPS)	L2654-1MG	Sigma- Aldrich
Milk powder blotting grade	T145.2	Carl Roth GmbH
Protease inhibitor cocktail	P8340	Sigma- Aldrich
Phorbol 12-myristate 13- acetate (PMA)	524400	Merck KGaA
Polyehtylenimine, linear (PEI)	23966-2	Polysciences, Inc.
Human TNF-α	300-01A-50	Peprotech
Human IL-4	200-04	PeproTech
Human TNF-α	300-01AB	PeproTech
SuperKiller TRAIL (soluble)(human)(recombinant)	ALX-201-115-C010	Enzo Life Sciences GmbH
Tween-20	1.423.121.611	AppliChem GmbH

6.1.6. Consumables

Name	Catalog number	Company
Amersham Hybond P0.45 PVDF	10600023	GE Healthcare Bio-Scie
Agarose Basic	A8963,1000	AppliChem GmbH
Ethidiumbromid-Lösung 1%	A1152,0010	AppliChem GmbH
NEB 5-alpha competent *E.coli*	C2988J	New England Biolabs
Page Ruler Prestained Protein ladder	26616	ThermoFisher Scientific
Mid Range DNA ladder (1000bp-3000bp)	M-203L	JenaBioscience
Rotilab syringe filters 0.45µm	KH55.1	Carl Roth GmbH
Restore Western Blot Stripping buffer	21059	ThermoFisher Scientific
Super Signal West Dura Extended Duration	34076	ThermoFisher Scientific
Tryphan blue (0.4%)	15250-061	Gibco
Trypsin	T3924	Sigma- Aldrich
Whatman® gel blotting paper, Grade GB003	WHA10426890	Sigma- Aldrich
10x cell lysis buffer	9803	Cell Signaling Tech
100bp DNA ladder	07-11-00050	Solis BioDyne

6.2. Methods

6.2.1. Cell culture

Cells were cultured in sterile cell culture flasks, multi-well plates or dishes, and were kept in a standard humidified incubator at 37 °C in a 5% CO_2 atmosphere. All cell culture media and solutions were pre-warmed to 37 °C before usage.

Cell cultures were regularly tested negative for mycoplasma contamination using the HEK-Blue™ Detection kit (InvivoGen).

Cell lines and culture conditions

Name	Cell type	Origin	Medium
HCT 116 (ATCC® CCL-247™)	colorectal carcinoma cells, male	ATCC	DMEM (ThermoFisher), 1 % L-glutamine(Life technologies), 10 % v/v fetal calf serum (FCS, Gibco), and 1% penicillin (ThermoFisher)
HT-29 (ATCC® HTB-38™)	colorectal adenocarcinoma cells, female	ATCC	DMEM (ThermoFisher), 1 % L-glutamine (Life technologies), 10 % v/v fetal calf serum (FCS, Gibco), and 1 % penicillin (ThermoFisher)
Human BLaER1 with CASP4/CASP8 knockout	immortalized human B cells	kind gift of Dr. Veit Hornung [270]	RPMI-1640 medium (ThermoFisher) supplemented with 1 % L-glutamine (Life technologies), 10 % v/v FCS (Gibco), and 1 % penicillin (ThermoFisher). For transdifferentiation into monocytes, the culture medium was supplemented with recombinant human (rh) IL-3 (10 ng/ml), rh M-CSF (10 ng/ml, both PeproTech, USA), and 100 nM β-Estradiol (Sigma-Aldrich, Germany)
Human EBV-transformed B cell lines (EBV-LCL)	generated from peripheral blood mononuclear cells (PBMC)	patients, parents and healthy individual	RPMI-1640 medium (ThermoFisher) supplemented with 1 % L-glutamine (Life technologies), 10 % v/v FCS (Gibco), and 1 % penicillin (ThermoFisher)

For passaging of adherent cell lines, cells were split at 80 % confluency by removing medium, washing with PBS, detaching with Trypsin-EDTA, and pelleting at 1200 rpm for 5 minutes. In case of suspension cultures, cells were passaged by removing the medium using centrifugation. The quantity of living cells was determined using a Neubauer counting chamber after staining with Trypan blue. Cells were resuspended in fresh medium and plated in respective cell culture flasks.

For long-term storage of cell lines, cells were trypsinized, the pellet was dissolved in freezing medium (FCS + 10 % DMSO) and transferred to cryo vials. Dimethyl sulphoxide (DMSO) has been used as cryoprotectant to avoid crystallization of water that could lyse cells during the freezing and thawing cycle. Cryoboxes filled with isopropanol were used to freeze the cells to -80°C with a rate of cooling of 1°C per minute, and subsequently cells were transferred to liquid nitrogen (-210°C) for long-term storage.

To thaw cryopreserved samples, cells were quickly thawed at 37°C, transferred to a falcon containing pre-warmed cell culture medium, and centrifuged at 1200 rpm for 5 minutes to remove DMSO-containing medium. Cell pellets were resuspended in fresh cell culture medium and transferred to cell culture flasks.

6.2.2. CRISPR/Cas9-knockout of Caspase-8 in HT-29 cells

For genetic engineering of a knockout of CASP8 *in* HT-29 cells, sgRNAs with low predicted off-target sites were designed by employing the online CRISPR guide design tool developed by Feng Zhang's Lab (http://crispr.mit.edu/) in order to generate a large deletion. The pSpCas9(BB)-2A-GFP (PX458) plasmid was purchased from Addgene (48138) and sgRNAs were inserted into the plasmid according to published protocols [271]. HT-29 cells were

transfected with the two targets using the Lonzo Nucleofector™ Technology. Targeted knockout of CASP8 in HT-29 cells was confirmed by Sanger sequencing and analysis of Caspase-8 protein expression using immunoblotting.

6.2.3 Intestinal Organoid Cultures

Human organoid cultures from intestinal biopsies were generated using previously published protocols [272]. Control healthy donor intestinal biopsies were collected from non-pathological mucosa. Biopsy specimens were manually dissected and the mucosal layer was cut into small pieces. Isolated intestinal crypts were embedded in Matrigel (Matrigel, Corning), seeded in 24-well plates, polymerized for 10 min at 37 °C and overlaid by organoid culture medium containing the following components: Advanced Dulbecco's modified Eagle medium/F12, 1 % penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, 1× N2, 1× B27 (all from Invitrogen, USA), 12,5 mM N-acetylcysteine (Sigma-Aldrich, Germany), 10 mM nicotinamide (Sigma-Aldrich), 500 nM A83-01 (Tocris), 10 μM SB202190 (Sigma-Aldrich), 50 % Wnt3A conditioned medium, 50 ng/ml m-EGF, 20 % RSPO1 conditioned medium, and 10 % Noggin conditioned medium. The medium was changed every two days and organoids were passaged every week by employing mechanical disruption and pelleting by centrifugation. Next, cell pellets were resuspended in Matrigel and fresh culture medium was added after polymerization. For stimulation experiments, cells were treated with TRAIL-SK (100 ng/ml. AdipoGen, USA) or TNF-α (20 ng/ml, PeproTech, USA) 5 days after seeding. Cell death was monitored by time-lapse videos recorded on a SP5 confocal microscope (Leica Microsystems, Germany) using a 10X objective and 15 min intervals. To analyze cleavage of Caspase-3 by immunohistochemistry, stimulated organoid cultures were collected in warm medium, without disrupting the Matrigel, and fixed overnight in 4 % formalin at 4 °C. After two washes with 70

% ethanol, organoids were embedded in HistoGel (Thermo Fisher Scientific) and paraffin. Five µm sections were dewaxed and rehydrated and antigens were retrieved in 10 mM sodium citrate with pH of 6 (15 min autoclaving). Following blocking in 1 % BSA/PBS the sections were incubated with rabbit anti-cleaved Caspase-3 (Cell Signaling, #9661; 1:400, overnight at 4 °C), followed by incubation with BrightVision Poly-HRP-Anti Rabbit reagent (ImmunoLogic, 1 h at room temperature) and detection using 3,3'-diaminobenzidine (DAB) as substrate. Sections were counterstained with hematoxylin before mounting. To evaluate cell death in intestinal organoids upon treatment with TNF-α or TRAIL we quantified cleaved Caspase-3-positive nuclei in ratio to the total number of nuclei using ImageJ software [273].

6.2.4. Site-directed mutagenesis and molecular cloning

Standard molecular biology techniques were used for cloning. To introduce genes of interest into a plasmid backbone, a recombinant PCR was performed using specific primers pairs (Table **2**). All vectors used were confirmed by Sanger sequencing using the MWG Eurofins service (Ebersberg). The pRB-Caspase-8 WT plasmid was a kind gift of Prof. Dr. Veit Hornung (Gene Center LMU Munich, Germany).

To generate a lentiviral vector system, the Caspase-8 WT gene was subcloned into the pGEM- T plasmid backbone (Promega) using specific restriction enzyme cutting sites. Next, Caspase-8 WT was released from the pGEM-T vector backbone and inserted into the 3rd generation lentiviral vector pRRL-MCS90-IRES-GFP using a PCR amplification with primers specific for Caspase-8 WT as well as *Age*- and *Spe*- cutting sites (Table 2).

For site-directed mutagenesis of the patient-specific mutant variant 1 pRRL-MCS90-Caspase-8^{A659G}-IRES-GFP was created by using primer sets for the amplification of the first 800 base pairs encompassing one part of Caspase-8 including the mutation site with *SmaI*- and

SpeI- restriction enzyme sites and for the amplification of the other part of Caspase-8, the 724 base pairs with a *SmaI*- and *Bam/AgeI*- restriction enzyme sites were amplified (Table 2).

For subcloning, both PCR products were purified by gel extraction and then ligated into the pGEM-T vector backbone. The pGEM-T vectors containing either the 800 or 724 bp fragments were digested with respective restriction enzymes and both fragments were ligated to obtain the pGEM-T-Caspase-8 Mutation 1 plasmid. For the generation of the CASP8-encoding lentiviral plasmid, full length mutant CASP8 was released from pGEM-T-Caspase-8 Mutation 1 plasmid and inserted into the pRRL-MCS90-IRES-GFP backbone using the restriction site *AgeI* and *SpeI*.

For site-directed mutagenesis of the patient-specific mutant variant 2 pRRL- MCS90- Caspase- 8^{C742T}- IRES-GFP was created by using primer sets for the amplification of 730 base pairs of the first part of CASP8 encompassing the mutation and *HindIII*- and *SpeI*- restriction enzyme sites and for the amplification of the second part of CASP8, the 785 base pairs were amplified with *HindIII*- and *AgeI*- restriction enzyme sites (Table 2). For subcloning of patient-specific mutant variant 2, both purified PCR products were ligated into the pGEM-T vector backbone. The pGEM-T vectors containing either the 730 or 785 bp fragments were digested with respective restriction enzymes and both fragments were ligated to obtain the pGEM-T-Caspase-8 Mutation 2 plasmid. For the generation of the CASP8- encoding lentiviral plasmid, full length mutant CASP8 was released from pGEM- T- Caspase-8 Mutation 2 plasmid and inserted into pRRL-MCS90-IRES-GFP backbone using the restriction site *AgeI* and *SpeI*.

Table 2: Primer Sequences

Primer	**Sequence (5'-3')**
C8 - Bam/Age fw	GGATCCACCGGTGCCACCATGGACTTCAGCAGAAATCTTTATGATATTG
C8 - Spe/Pml rev	TTATTCACGTGTCCGGACTAGTTAATCAGAAGGGAAGACAAGTTT
C8 - c.A659G Sma1 fw	GAATCACGGACTTTGGACAAAGTTTACCAAATGAAAAGCAAACCCCGGGGATACT
C8 - c.A659G - Sma1 rev	AGTATCCCCGGGGTTTGCTTTTCATTTGGTAAACTTTGTCCAAAGTCCGTGATTC
C8 – c.C742T Hind3 fw	ACAATTTTGCAAAAGCTTGGGAGAAAGTGCC

All PCR amplification steps for cloning were done using Q5 DNA polymerase (NEB) or Hifi polymerase (ThermoFisher Scientific) following manufacturers' protocols (Table 3).

Table 3: PCR amplification steps

5x Q5 Reaction Buffer or	10 µl
Buffer no2	3µl
10 mM dNTPs	1 µl
10 µM Forward Primer	2.5 µl
10 µM Reverse Primer	2.5 µl
Template DNA	0.5- 1 µl (1 ng- 1 µg)
Q5 High- Fidelity DNA Polymerase or	0.5 µl
Hifi DNA Polymerase	0.15 µl
Nuclease-free water	to 50 µl

The polymerase chain reaction (PCR) was started with the initial denaturation at 98 °C for 30 seconds, followed by 30 cycles of denaturation at 98 °C, annealing at the primers specific melting temperature (50 - 72 °C) for 30 seconds and elongation at 72 °C for 20 - 30 seconds per kb. The final extension of the amplicon was done at 72 °C for two minutes. The specificity of amplicons was confirmed by gel electrophoresis.

6.2.5. Preparation of DNA for ligation

Prior to the ligation of subcloned fragments into the plasmids, DNA was digested with appropriate restriction enzymes (1 U/ µl) and buffers following manufacturers protocols. Digestion was performed for one hour at 37 °C in the thermocycler and confirmed by agarose gel electrophoresis. To remove enzymes after the restriction digestion or PCR, five volumes of PB buffer were added to one volume of the reaction mix. The DNA was eluated in water from the gel using a QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions.

For the ligation of DNA fragments, a ratio of plasmid and insert of 5:1 was mixed with 1 µl T4 ligase, 5 µl of 2x buffer and 2 µl water. The ligation reaction was incubated at 4°C for 12 h.

6.2.6. Transformation

For the reproduction of the vectors with the cloned DNA fragment, aliquots of competent *E. coli* (NEB) were thawed on ice and the DNA plasmid was added. The mix was incubated for 30 minutes on ice, followed by heat shock for 40 seconds at 42 °C and immediate cool down on ice. Next, 250 µl of LB medium was added to the reaction mix and bacteria were allowed to grow for one hour at 37°C and 550 rpm in a thermocycler and then plated on a LB agar plate containing ampicillin cultured at 37°C overnight. Bacterial colonies selected by the ampicillin resistance, were transferred into 5 ml of LB-medium supplemented with ampicillin and incubated overnight in an incubator shaker (250 rpm, 12 h, 37 °C).

6.2.7. Extraction and purification of plasmid DNA from bacteria

Plasmid DNA was extracted from transformed bacteria that were grown in LB media supplemented with ampicillin in a shaking incubator at 37 °C overnight.

Minipreps or Maxipreps were carried out using the manufacturers' protocol of the Zyppy plasmid Miniprep kit (Zymo) or Qiagen MaxiPrep kit (Qiagen).

The concentration and purity of extracted DNA was measured using NanoDrop (Thermo Fisher) by determining the nucleic acid concentration by the absorbance of UV light.

6.2.8. Colony PCR

For screening of recombinant bacteria, single colonies were picked and resuspended in 18 µl water together with 3 µl 2mM dNTPs, 1.5 µl DMSO, 3 µl reaction buffer, 1.5 µl forward primer (10 µM), 1.5 µl reverse primer (10 µM), and 0.15 µl Hifi polymerase. The reaction was started with denaturation for five minutes at 95 °C, followed by 30 cycles of denaturation for one minute at 95 °C, annealing for two minutes at 56 °C and elongation for three minutes at 72 °C. In the final step, elongation was conducted for seven minutes at 72 °C.

6.2.9. Generation of stable cell lines with transgenic Caspase-8 expression by lentiviral gene transfer

For lentivirus production, the polyethylenimine (PEI) transfection method was used in HEK 293T cells. Plasmids encoding either (i) the Vesicular Stomatitits Virus Glycoprotein (ENV), (ii) group antigen and reverse transcriptase (GAG/POL) as well as (iii) mRNA export

helper protein (REV) were used as packaging mix. One day prior to transfection, HEK 293T cells were plated onto 10 cm cell culture dishes with approximately 50 - 60 % confluency. Two hours before transfection, the medium was replaced with fresh DMEM and the transfection mix was prepared according to the following scheme (Table 4):

Table 4: Transfection mix

	µg	**volume**
pRRL-plasmid [µl]	40	x µl
REV [µl]	7.0	x µl
GAG/POL [µl]	10.0	x µl
ENV [µl]	7.0	x µl
PEI (2 mg/ml) [µl]	128	64.0
dH_2O [µl]		x µl
Total		**1000 µl**

First, dH_2O was added as dissolvent to a 1.5ml Eppendorf tube, followed by addition of plasmid DNA and PEI. The mix was incubated for 30 minutes at room temperature before it was given dropwise to the cells. The cell culture plates were incubated at 37 °C for at least 8 hours before the medium was gently removed and replaced with 13 ml fresh medium. The cells were incubated for additional 48 - 60 hours. Next, the medium containing lentiviral particles was harvested and cell debris was removed by filtration through a syringe filter (0.45 µm). To concentrate lentiviral particles, ultracentrifugation (2.5 hours, 4 °C, 24.000 rpm, SW32Ti rotor) of supernatants was conducted. the pellets of viral particles were resuspended Tin 500 µl of plain RPMI medium and aliquots were stored at -80 °C.

For transduction of adherent cells, 30.000 cells were added directly into the virus tube and incubated for one hour at 37 °C. After incubation the tube was rinsed with fresh medium and transferred to a 48-well plate. Transduction efficacy was assessed by GFP expression using

flow cytometry. The pRRL-MCS90-IRES-GFP plasmid without the gene of interest (CASP8) was used as control plasmid.

6.2.10. DNA Electrophoresis and DNA recovery

Separation of DNA based on fragment size was performed using agarose gels (Applichem). Briefly, 1 % agarose was dissolved in TRIS-Acetat-EDTA-buffer (TAE), heated up and poured into the DNA gel electrophoresis cassettes (Biorad) containing ethidiumbromide (5 µg/ ml). The DNA was mixed with 6x DNA loading dye (home- made) and separated by electrophoresis in TAE buffer according to the size of the DNA molecules. The mid- range or 100bp DNA ladders (Solis BioDyne) were used for size estimation.

When DNA was needed for subsequent usage, the DNA bands of interest were excised under the UV lamp and purified with the QIAquick gel extraction kit (Qiagen). Briefly, the desired gel piece was mixed with binding buffer, heated up to 56°C to dissolve it. Subsequently it was put on a DNA binding spinning column, washed and eluted in water.

6.2.11. Sanger sequencing

The Sanger sequencing method was used to determine the exact sequence of nucleotides in plasmids or patient's samples.

First, DNA was extracted from samples using the Qiamp DNA blood mini kit (Qiagen) according to instructions. For PCR amplification of the DNA region of interest, primers were designed for each target employing the AmplifX software/algorithm (Table 5). Upon amplification, excess primers and nucleotides were removed by gel electrophoresis and extraction using the QIAquick gel extraction kit or by enzymatic cleanup of the amplified PCR

fragment with the shrimp alkaline phosphatase ExoSAP (ThermoFisher). The sequencing reaction was performed using the Eurofins Genomics (Ebersberg) service. Sequencing data were analyzed using the ApE software (version 2.0.49).

Table 5: List of primer sequences for Sanger Sequencing

DNA Sanger sequencing	
Primer	**Sequence (5'-3')**
CASP8 P1/2_fw	CAGGATAGTGAATCACGGACTTTGGACAAA
CASP8-1 P1/2_rev	CTTTGTCCAAAGTCCGTGATTCACTATCCT
CASP8 P3_fw	TGCAAAAGCATGGGA
CASP8 P3_rev	TGGGCACTTTCTCCC
CRISPR/Cas9-mediated genetic engineering	
Primer	**Sequence (5'-3')**
sgRNA CASP8_fw	GTTAGGCAGGTTAGGGGACT CGG
sgRNA CASP8_rev	CTCTTCGTTGTTTGCAGGGG AGG
Site-directed mutagenesis	
Primer	**Sequence (5'-3')**
CASP8 - wt Bam/Age_fw	GGATCCACCGGTGCCACCATGGACTTCAGCAGAAATCTTTAT GATATTG
CASP8 - wt Spe/Pml_rev	TTATTCACGTGTCCGGACTAGTTAATCAGAAGGGAAGACAA GTTT
CASP8 - c.A659G Sma1_fw	GAATCACGGACTTTGGACAAAGTTTACCAAATGAAAAGCAA ACCCCGGGGATACT
CASP8 - c.A659G Sma1_rev	AGTATCCCCGGGGTTTGCTTTTCATTTGGTAAACTTTGTCCA AAGTCCGTGATTC
CASP8 - c.C742T Hind3_fw	ACAATTTTGCAAAAGCTTGGGAGAAAGTGCC
CASP8 - c.C742T Hind3_rev	GGCACTTTCTCCCAAGCTTTTGCAAAATTGT

6.2.12. Cell death assays

6.2.12.1 Cytotoxicity Assay

Cell death of stimulated cell lines was measured by detection of the lactate dehydrogenase (LDH) release using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions. While supernatants from unstimulated cells were used to determine background of LDH release, stimulated cells which had been lysed by lysis buffer were used for determination of Maximum LDH release. The percentage of cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = \frac{\text{experimental LDH release } (OD_{490}) - \text{background LDH release } (OD_{490})}{\text{Maximum LDH release } (OD_{490}) - \text{background LDH release } (OD_{490})} \times 100$$

6.2.12.2 Flow cytometry-based detection of Apoptosis

Under normal physiologic conditions phosphatidylserine can be found in the inner plasma membrane. A characteristic feature of apoptosis is the appearance of the phospholipid phosphatidylserine in the outer leaflet of the plasma membrane, where it marks the cells as target for phagocytosis [274]. In early-stage apoptosis, phosphatidylserine on the outer membrane can be measured by fluorescently labelled Annexin V in a calcium dependent manner, while cells cannot be stained by a viability dye such as Propidium iodide (PI). In late- stage apoptosis, the cells lose integrity allowing both Annexin V to bind to phosphatidylserines in the inner cell membrane and PI staining. Thus, the combined staining of Annexin V and PI can be used to distinguish early-stage apoptotic cells (Annexin V positive, PI negative) from late-apoptotic cells or necrotic cells (Annexin V and PI positive).

To detect cell death in HT29 cells upon treatment with SuperKiller TRAIL or TNF-α ± BV6, the Annexin V Apoptosis Detection Kit APC (eBioscience™, 88-8005-72) was used according to the manufacturer instructions. In brief, cells were stained with Annexin V APC in 100 µl binding buffer, incubated for 15 min at room temperature, and washed once with binding buffer before staining with PI. Cells were then analysed using the flow cytometer BD LSRFortessa™ (BD Biosciences). Data were analysed using the FlowJo version 9.9.5, LLC software (Tree Star, Inc).

6.2.13. Flow cytometry-based analysis of intracellular cytokines

Flow cytometry is a useful tool to determine intracellular proteins that are expressed by specific cell types. To determine the production of TNF-α in patient- derived cells, are washed two times with PBS containing 0.5 % FCS and stimulated in 200 µl complete medium supplemented with 50 ng/ml PMA and 1 µg/ml Ionomycin for 2.5 hours at 37 °C in cell culture incubators. To enhance intracellular cytokine staining by blocking cellular transport processes, 10 µg/ml Brefeldin A was added and cells were incubated for additional 2.5 hours. To stop the reaction/stimulation, cells were washed two times with PBS containing 0.5 % FCS, fixed and permeabilised in 100 µl Fixation/Permeabilization Solution (Cytofix/Cytoperm, BD), and incubated for 20 minutes on ice. Following two washing steps with BD Perm/Wash™, cells were incubated with antibodies diluted in BD Perm/Wash™for 20 minutes on ice (Table 6). After the incubation, cells were washed two times with PBS containing 0.5% FCS, resuspended in 100 µl PBS containing 0.5% FCS and subjected to flow cytometry. Data from 2x 10^5 to 1x 10^6 cell were acquired on a LSR II BD LSRFortessa™ (BD Bioscience) with BD FACSDiva Software v8.0.3 (BD Bioscience) and analysed using FlowJo version 9.9.5, LLC software (Tree Star, Inc).

Table 6: Antibody dilution for intracellular cytokine staining

IFN-γ (clone 4SB3) BV711, 100 µg/ml	BD Pharmigen
IL-4 (clone MP4-25D2) BV421, 100 µg/ml	BD Pharmigen
IL-17A (clone N49-653) PE, 100 µg/ml	BD Pharmigen

6.2.14. Isolation of Peripheral Blood Mononuclear Cells

For the isolation of PBMCs from whole blood of patients, parents, and healthy donors, the Ficoll-Paque (GE healthcare) method was used. This density gradient centrifugation method is based on the principle of differential migration of blood cells through the Ficoll.

In brief, anticoagulant blood samples were diluted with PBS and carefully layered on top of 12 ml Ficoll solution in a 50 ml conical tube, followed by centrifugation (room temperature, 25 minutes, 1600 rpm, without brake, acceleration 3, deceleration 0 in a swinging-bucket rotor) to form different layers containing the different type of cells. Without disturbing the upper layer containing plasma, the PBMCs at the interphase was aspirated, transferred to a new 50 ml conical tube and washed with PBS to remove platelets, Ficoll and plasma. Cell pellets were resuspended in the appropriate medium and counted using a Neubauer counting chamber prior to functional assays.

6.2.15. Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA was used to measure secretion of cytokine in the supernatant of stimulated patient cells and heterologous cell lines according to manufacturers' protocol (R&D). In brief, a known quantity of capture antibody was immobilized to a 96-well microplate at 4 °C overnight. After washing the plate with PBS/0,05 % Tween-20, free binding sites were blocked with 1 % BSA/PBS for one hour at room temperature. Subsequently, supernatants from stimulated cells were diluted with 50 µl 1 % BSA/PBS and transferred to the antigen coated

plate. After incubation for two hours at room temperature, plates were washed three times and 100 µl of diluted horseradish peroxidase- conjugated detection antibody was added. Plates were incubated for one more hour at room temperature and washed three times. Finally, 100 µl of substrate was added to each well for 30 minutes and reaction was stopped using 50 µl 5 M H_2SO_4. The absorption was read at 490 nm BioTek™ using a ELx800™ Absorbance Microplate Reader. The calculation of ELISA assay values was done by using a protein standard curve followed by the estimation of the assay value from the absorbance of the sample.

6.2.16. Analysis of Protein Expression

6.2.16.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For the generation of total protein cell lysates, adherent cells were trypsinized, washed with PBS, and lysed for 1 hour on ice with 1x cell lysis buffer (Cell Signaling Technology) containing PMSF (Phenylmethylsulfonyl fluoride,1 mM, Roche) that Inhibits serine proteases. The whole cell lysate was centrifuged (10 minutes, 15000 rpm, 4 °C) and the supernatants containing the protein lysate were collected in a new tube. The total concentration of isolated proteins were quantified using the Bradford protein assay reagent (BioRad) and BioTek™ ELx800™ Absorbance Microplate Reader. Required volumes of protein samples were supplemented with 5x Laemmli sample buffer containing β-mercaptoethanol and denatured by boiling for 10 minutes at 95 °C in a thermocylcer. PageRuler™ Prestained Protein Ladders (ThermoScientific) were loaded on each gel as a standard for the determination of the molecular weight of proteins (Table 7).

Table 7: Seperating and stacking gel for SDS-PAGE

Seperating-Gel (10%)	
1.5 M Tris HCl (pH 8.8)	2,9 ml
Acryl-Bisacrylamid	2,6 ml
10% SDS	77,50 µl
Temed	5,75 µl
H_20	3,10 ml
20% APS	37,60 µl
Stacking-Gel (6%)	
0.5 M Tris HCl (pH 6.8)	360,00 µl
Acryl-Bisacrylamid	487,50 µl
10% SDS	28,75 µl
Temed	2,87 µl
H_20	1950 µl
20% APS	18,80 µl

For the separation of proteins based on their molecular weight, the gels were run at 80 V till the samples reached the separating gel. Next, voltage was raised to 120 V and the samples were run until the loading front reached the bottom of the gel.

6.2.16.2. Western Blot analysis

After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the gels were soaked in 1x transfer buffer for 5 min. Using a wet blot system (BioRad) the following components were layered bubble- free in a transfer cassette: fibre pad, two pieces of Whatman filter paper™ 3mm, gel, PVDF membrane (Gellifesciences), two pieces of Whatman filter paper™ 3mm, fibre pad. The cassette was then placed into a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (BioRad) together with an ice unit and the tank was filled up with 1x transfer buffer. The transfer was stopped after one hour at 100 V. PVDF membranes

were blocked for one hour at room temperature in 5 % BSA or milk containing 0.1 % Tween (PBS-T), incubated with primary antibodies (12 hour, 4 °C), washed three times with PBS-T, and incubated with corresponding HRP-conjugated isotype-specific secondary antibodies (1 hour, room temperature).

After washing with PBS-T, the membranes were visualized by enhanced chemiluminescence using the Super Signal West Dura Extended Duration Substrate (ThermoFisher) and the ChemiDoc XRS+ System (BioRad). For multiple probing, stainings of primary and secondary antibodies were removed by incubating the membranes in Restore Western Blot Stripping buffer (ThermoFisher) for 20 minutes at room temperature, followed by wash steps with PBS- T. Subsequently, membranes were blocked and probed with respective primary and secondary antibodies as described above.

6.2.17. Analysis of mRNA expression

To analyse the expression of CASP8, RNA was isolated from cells using the RNeasy kit (Qiagen) according to manufacturers' protocol. In brief, cells were lysed in RLT buffer supplemented with 1 % β-mercaptoethanol to immediately inactivate RNases. The lysate was passed through a gDNA eliminator column to remove genomic DNA. After adding ethanol to the lysate, the sample was transferred onto a RNeasy spin column to bind RNA to the silica membrane. Subsequently, total RNA was eluted in water and the concentration was measured using a NanoDrop spectrophotometer.

To synthesize cDNA from isolated RNA, the high-Capacity cDNA Reverse Transcription Kit (ThermoFisher) was used according to manufacturers' instruction. To conduct the reverse transcription reaction, 1 µg of RNA was mixed with 2x reverse transcription master mix and put in the cycler.

6.2.18. Quantitative real-time PCR

Gene expression was quantified by performing SYBR green-based quantitative real time PCR (qRT-PCR). All samples were run in two technical replicates and the reactions were carried out in a 96-well format. qRT-PCR was performed in a final volume of 10 µl, consisting of 1 µl cDNA, 5 µl SBYR green reagent, 1 µl primer mix and 3 µl nuclease-free water. The specific amplification was confirmed by gel electrophoresis of the PCR amplicons. To determine RNA expression levels of the genes of interest, comparative ΔΔCt method was used and all results were normalized to respective GAPDH housekeeper values. The relative quantification was calculated by subtracting the Ct value of the endogenous reference gene from Ct values of the target gene to obtain the ΔCt value for each duplicate.

6.2.19. Statistical Analysis

Statistical significance of experimental data was determined by two-tailed paired Student's t test using GraphPad Prism version 6 (GraphPad Software, USA). No method of randomization or blinding was used and no statistical method was used to predetermine sample size for analyses. Graphical data are indicated as mean ± SEM. *P* values < 0.05 were considered as statistically significant. Unless indicated, data are statistically not significant.

6.2.20. Ethics

WES of 480 patients with different ethnic background was conducted and sequencing data were screened for rare monogenic disorders. Caspase-8-deficient patients have originally been referred for genetic testing to our institute by the Departments of Pediatrics at the Dr. von Hauner Children's Hospital of the Ludwig-Maximilians-Universität (LMU) Munich, Germany

(P1), the Infectious Diseases Research Core Facility, American University of Beirut-Medical Center, Lebanon (P2), and the Safra Children's Hospital, Sheba Medical Center, Tel Aviv University, Tel Hashomer, Israel (P3). Patient material from P1 and P2 were referred for further functional studies to the LMU Munich. The investigation was conducted in accordance with current ethical and legal frameworks and the study protocol was approved by Institutional Review Boards at the LMU. For genetic and functional experiments, biospecimens from patients, unaffected first- degree relatives, and healthy volunteers were obtained upon written consent.

7. Results

7.1. Identification of patients with Caspase-8 deficiency and infantile inflammatory bowel disease

Our index patient 1 (referred to as P1 or A.II-6) with VEO-IBD was born to a large consanguineous family with Lebanese origin and referred to us for genetic diagnosis at the age of 8 years (Figure 8A).

7.1.1. Clinical manifestations of the index patient

The patient P1 presented with VEO-IBD within the first months of life characterized by failure to thrive, bloody diarrhea, and perianal disease accompanied by fissures and tags (Figure 8B, i). At the age of two years, P1 developed rectal stenosis and rectovaginal fistula. Colonoscopy confirmed the diagnosis of a discontinuous severe colitis with deep ulcerations (Figure 8B, ii). Histological analysis revealed epithelial degeneration and regeneration accompanied by crypt hyperplasia, loss of goblet cells, and increased mitotic rate at the crypt base (Figure 8B, iii and iv). Further studies showed multiple food allergies associated with mild peripheral eosinophilia as well as chronic eosinophilic infiltration of the antrum and duodenum in the absence of *Helicobacter pylori*. P1 tolerated only amino acid-based formula feeding and very few oral foods. Her colitis was refractory despite exclusive feeding with amino acid-based formula and anti-inflammatory treatment (steroids, immunosuppressive drugs, anti-TNF-α antibody). An ileostomy at the age of 8 months improved the perianal lesions, but continuous colitis, stenosis and fistula formation required a total colectomy at the age of 6 years.

Extra-intestinal manifestations included chronic eczema with secondary infections, pityriasis amiantacea, and peripheral hypothyroidism (Figure 8, v-vii). Since the first months of life, P1

also presented with increased susceptibility to infections with recurrent fever and pneumonia, otitis media, conjunctivitis, herpes zoster, purulent dermatitis, and molluscum contagiosum. In addition, she had local infections (e.g, *Klebsiella pneumoniae*, *E. coli*, *Candida spec*.) with abscess and fistula formation at the site of the percutaneous endoscopic gastrostomy and stoma requiring temporary parenteral nutrition. Moreover, she developed progressive hepatosplenomegaly and showed a generalized lymphadenopathy (Figure 8B, viii) associated with granulomatous lymphadenitis and necrosis at the age of 8 years. However, positron emission tomography-computed tomography (PET-CT) at the age of 14 years did not indicate increased metabolic activity and lymphoma (Figure 8B, viii). Histology of bone marrow biopsies did not reveal signs of ALPS or lymphoproliferation. A 12 months' trial of treatment with rapamycin (Sirolimus) showed some improvement of symptoms but no worsening after weaning.

P1 is currently 15-years old and shows a stable intestinal situation and growth along the 10th percentile with tube feeding using amino acid-based formula and only few oral foods.

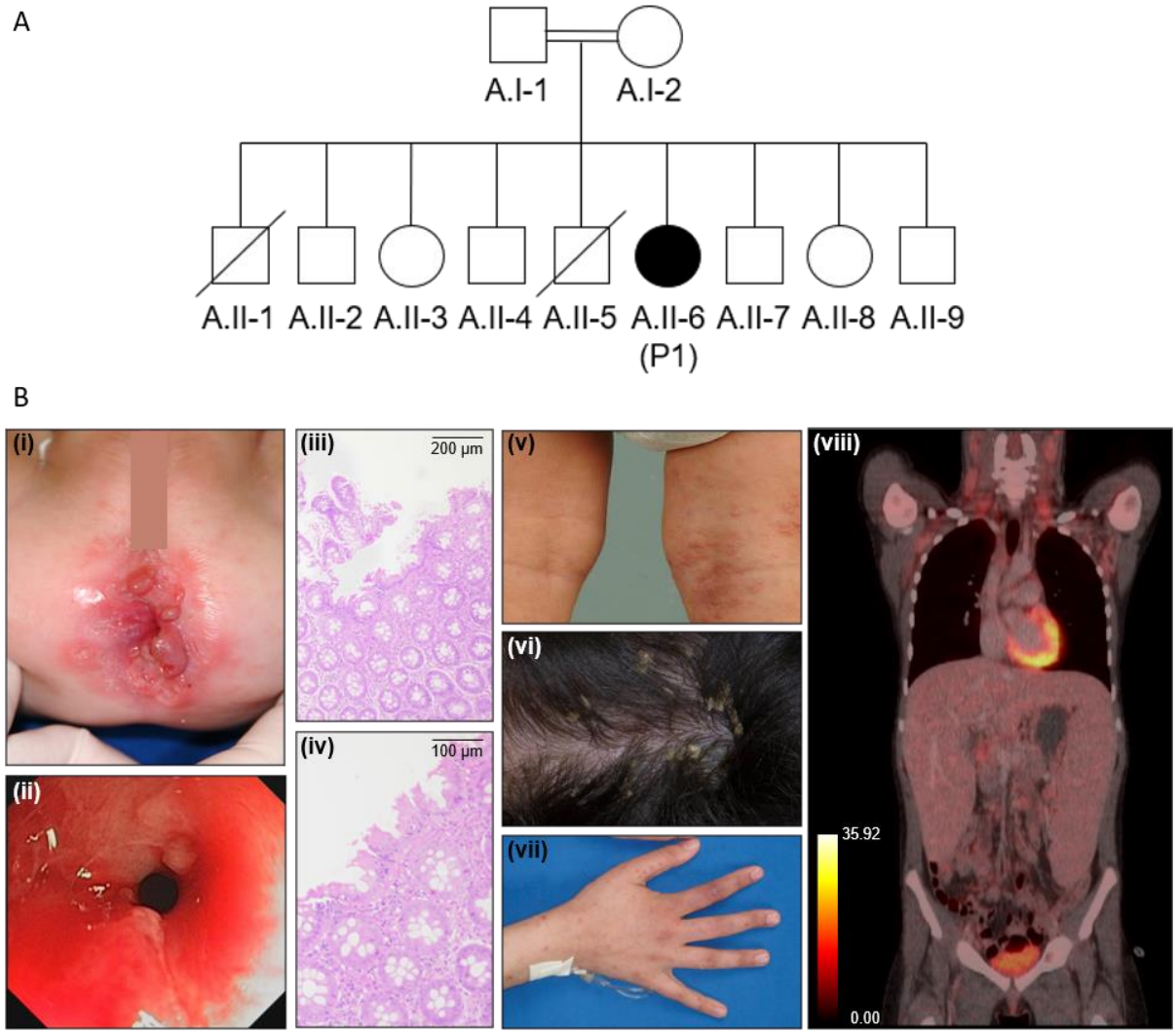

Figure 8: Clinical manifestations of the index patient P1.
(A) Pedigree of P1 who presented with VEO-IBD and was born to a large consanguineous family with Lebanese origin. (B) Clinical manifestations of P1: Pancolitis was associated with severe perianal disease (i), anorectal stenosis (rectoscopy, (ii)), retained crypt architecture and epithelial degeneration in the colon (histology, (iii and iv)), skin manifestations (chronic eczema, (v); pityriasis amiantacea, (vi); and Mollusca contagiosa, (vii)), as well as marked hepatosplenomegaly and lymphadenopathy (PET-CT, (viii)).

7.1.2. Identification of a germline biallelic Caspase-8 mutation in the index patient

To unravel the genetic cause for VEO-IBD and primary immunodeficiency in P1, we conducted whole exome sequencing revealing a homozygous missense mutation in the CASP8 gene (c.710A>G, p.Q237R; *NM_001228, ENST00000264275.9*). The identified ultra-rare sequence variant of CASP8 has not been reported in the gnomAD database [275]. Segregation of the

sequence variant with the disease phenotype of P1 was confirmed by Sanger sequencing (Figure 9B), because all available first-degree family members of the consanguineous family showed either a heterozygous or homozygous wild-type CASP8 sequence.

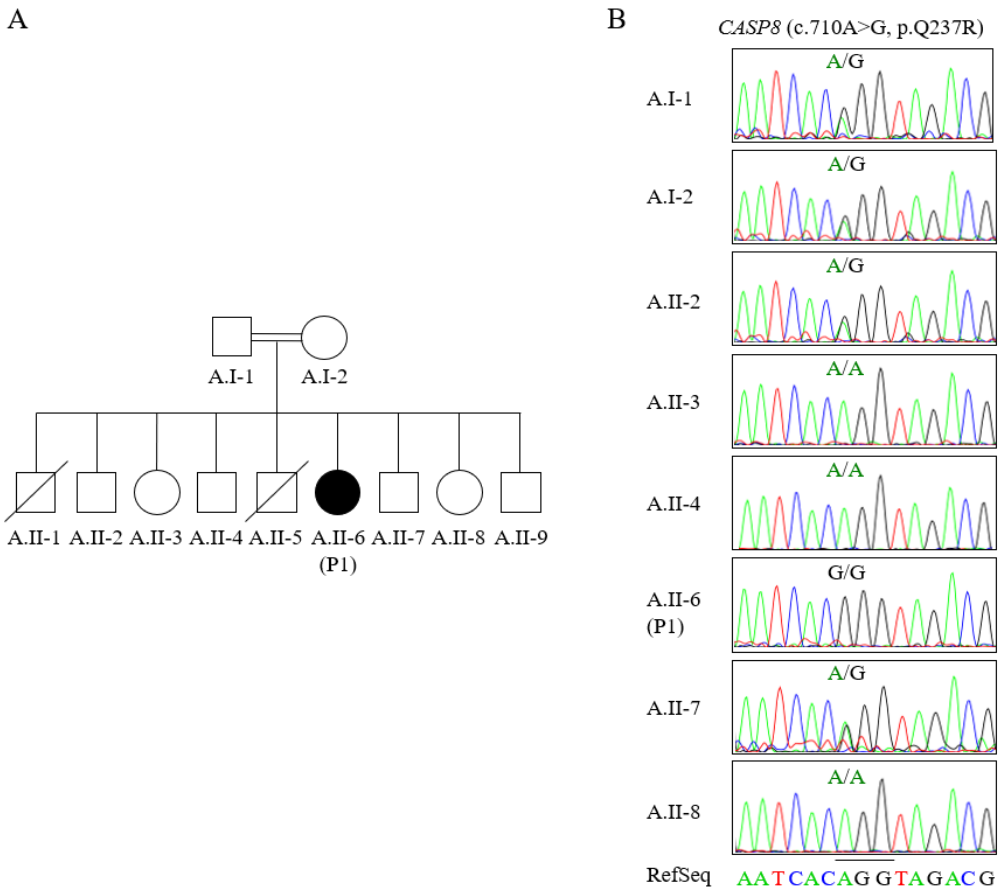

Figure 9: Sanger sequencing of the Caspase-8 variant in P1.
(A) Pedigree of P1 who presented with VEO-IBD and was born to a large consanguineous family with Lebanese origin. (B) Whole exome sequencing of genomic DNA from P1 identified a homozygous missense mutation in the CASP8 gene (c.710A>G, p.Q237R). Sanger sequencing confirmed the segregation of the variant with the VEO-IBD phenotype in available first-degree relatives of the consanguineous family with Lebanese origin.

7.2. Identification of additional Caspase-8-deficient patients

During the course of our study whole exome sequencing screening of more than 480 VEO-IBD patients revealed two additional children from unrelated families with biallelic CASP8 missense mutations.

Patient 2 (referred to as P2, B.II-4) was born to healthy Lebanese parents with a consanguineous background (first-degree cousins) and had the same mutation as our index patient P1 (c.710A>G, p.Q237R) (Figure 10A,B). Patient P3 (referred to as P3, C.II-2) has a Palestinian background and carried the mutation c.793C>T, p.R265W that has been previously associated with ALPS-like disease (Figure 10D,E) [161]. Similar to P1, immunoblotting revealed reduced protein expression level of Caspase-8 in EBV-LCL derived from P2 (Figure 10C). Both patients P2 and P3 manifested with refractory pancolitis accompanied by deep ulcerations and disrupted colonic architecture within the first weeks of life. Clinically, the patients showed recurrent fever and infections, failure to thrive, bloody (P2) or non-bloody (P3) diarrhea, and perianal disease. P3 suffered from intestinal obstruction, requiring right hemicolectomy in the first year of life. Noteworthily, the sister of P2 (B.II-3) also presented with recurrent episodes of severe diarrhea and infections and succumbed due to sepsis at the age of 4 months, but genetic testing could not be conducted because of lack of genomic patient's material. Unfortunately, P3 was not available for functional studies but showed a refractory course despite anti-inflammatory treatment (e.g., steroids, azathioprine, infliximab, adalimumab).

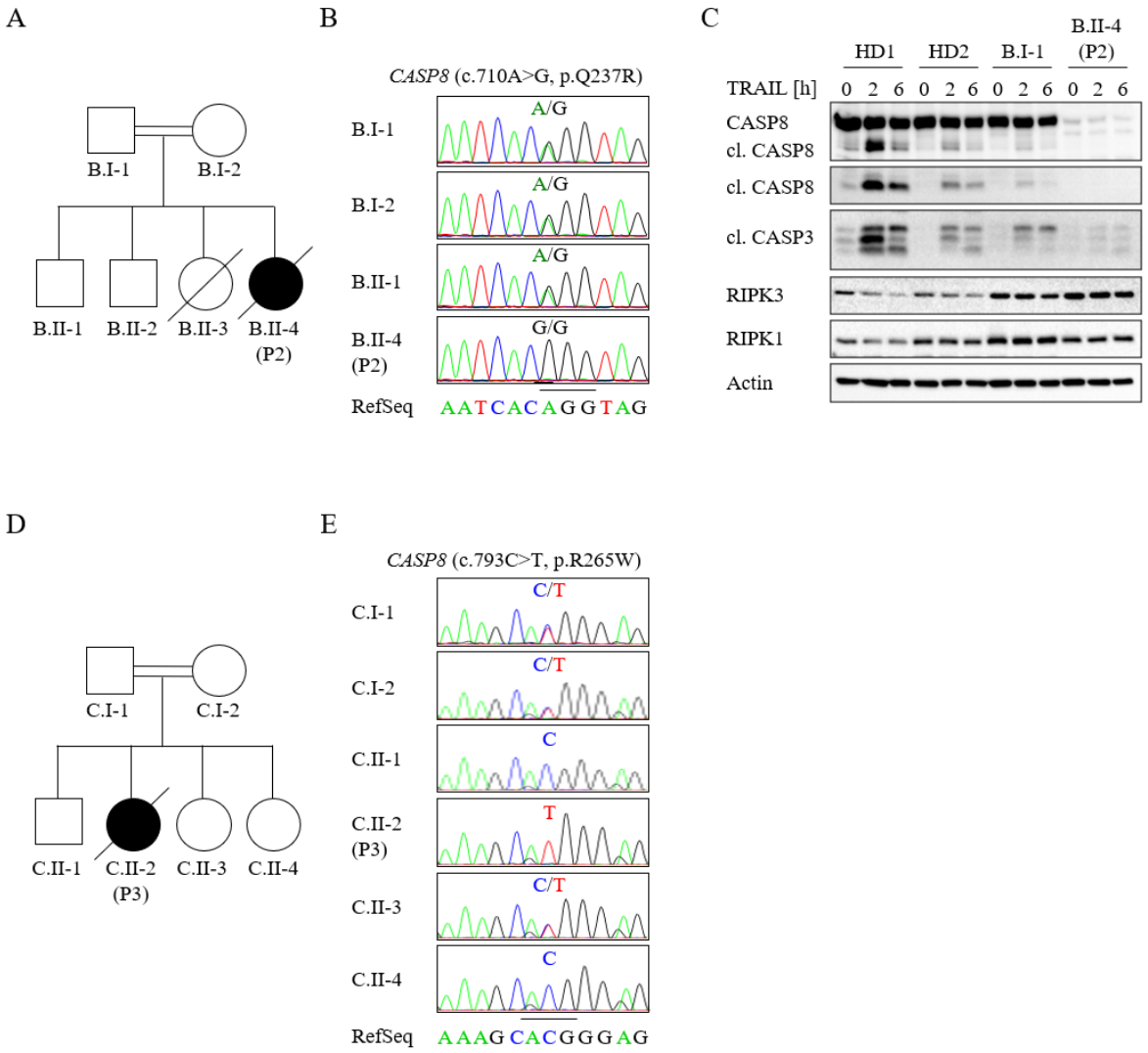

Figure 10: Identification of patients 2 and 3 with VEO-IBD and biallelic germline mutations in CASP8.
(A) Pedigree of the consanguineous Lebanese family B with patient 2 (P2, B.II-4) suffering from VEO-IBD. The child B.II-3 presented also with signs of infantile IBD, but succumbed due to sepsis prior to genetic testing. (B) Sanger sequencing confirmed the homozygous CASP8 missense mutation (c.710A>G, p.Q237R) in P2 and its segregation with the disease phenotype in available first-degree relatives of pedigree B. (C) Immunoblotting of EBV-LCL showed a reduced protein expression of Caspase-8 in P2, as compared with healthy donors (HD) and her father (B.I-1). (D) Pedigree of the Palestinian family C with one affected child (P3, C.II-2) presenting with VEO-IBD. (E) Sanger sequencing chromatograms indicate the homozygous missense mutation of CASP8 (c.793C>T, p.R265W) in P3 segregating with the phenotype of VEO-IBD.

7.3. Structural analysis of the identified Caspase-8 mutations

Caspase-8 is synthesized as inactive monomeric zymogen (Procaspase-8) consisting of two N-terminal death effector prodomains and the C-terminal protease domain composed of the two catalytic subunits (p18 and p10) (Figure 11A) [141]. Procaspase-8 molecules interact through the DED facilitating dimerization and sequential proteolytic cleavage which in turn result in the formation of the catalytic active Caspase-8. The identified sequence variant of P1/P2 (c.710A>G, p.Q237R) is located close to the cleavage site-2 of Caspase-8 which consists of three consecutive potential recognition sequences (D227, D233 and D240) [276], whereas the identified sequence variant of P3 (c.793C>T, p.R265W) is located within the p18 subunit (Figure 11A). The substrate specificity of Caspase-8 is mainly determined by hydrogen bond interactions in the specificity pockets S3 and S4 (Figure 11B) and effective cleavage of substrate requires an aspartic acid or glutamine at the residues P3 or P4 [277]. Our structural analysis suggested that the mutation Q237R might disrupt or alter the cleavage and activation of Caspase-8 at site-2, since the arginine is presumably not accepted in S4 and/or the P3 threonine is not able to establish the critical hydrogen bond interaction in S3.

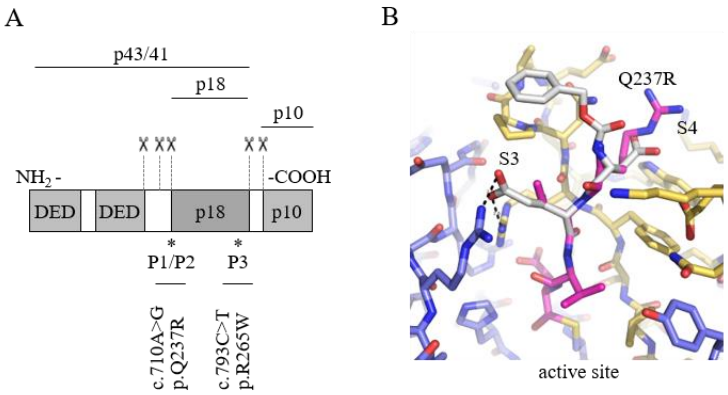

Figure 11: Structural analysis of the Caspase-8 mutation.
(A) Schematic illustration of DNA and protein sequences of the identified mutations in P1, P2, and P3 relative to the Caspase-8 structure depicting the death effector domains (DED) and the subunits p43/p41, p18 and p10. (B) Structural model of the Q237R mutation shown in pink bound in the substrate binding pocket S4 of the active site of Caspase-8. The model was prepared based on the crystal structure of Caspase-8 in complex with the Z-DEVD peptide inhibitor shown in grey (PDB accession 1F9E).

7.3.1. Analysis of the expression pattern of the Caspase-8 mutation identified in our index patient

To assess whether the identified Caspase-8 mutation (c.710A>G, p.Q237R) has any consequences on the mRNA expression level in PBMC, we conducted real-time PCR using specific primers. qPCR showed that P1 exhibited mRNA expression of CASP8 comparable to parents and healthy donors (not shown). In contrast, immunoblotting demonstrated reduced protein expression and cleavage of Caspase-8 in patient's EBV-LCL and PBMC as compared to healthy donors (Figure 12). Of note, the parents with heterozygous genotypes for the Caspase-8 mutation (c.710A>G, p.Q237R) showed an intermediate expression level of Caspase-8, consistent with an autosomal recessive inheritance pattern.

Previously, Guenther et al have shown that conditional knockout of Casp8 in mouse intestinal epithelial cells exhibited enhanced TNF-α induced cell death associated with increased expression of RIPK3 [157]. Notably, deletion of *Ripk3* could rescue the lethal phenotype in Casp8-deficient mice [157]. In contrast, our Western blot analysis could not reveal altered expression levels of RIPK1, RIPK3 or Caspase-3 in patient-derived EBV-LCL neither under conditions without stimulation (time point 0 hour) nor upon stimulation with TRAIL (Figure 12).

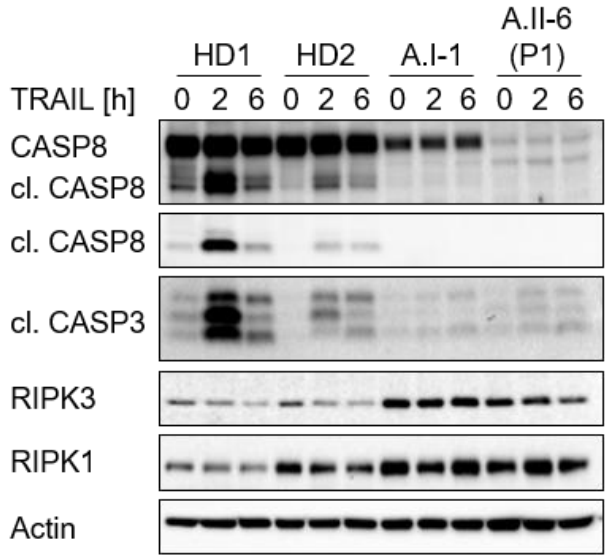

Figure 12: Reduced protein expression of Caspase-8 in P1.
Immunoblotting (n = 3) of EBV-LCL from healthy donors (HD1 and HD2), A.I-1 (father), and P1 demonstrated reduced expression of full length and cleaved Caspase-8 as well as cleaved Caspase-3. Expression levels of RIPK3 and RIPK1 in P1 were comparable to A.I-1. Cells were stimulated for two hours with 100 ng/ml TRAIL.

7.4. Caspase-8 deficiency is associated with T and B cell dysfunctions

Routine immune workup of P1 revealed leukocytosis, hypochromic microcytic anemia, and anisocytosis. Serum levels of IgA (796 mg/dl; normal: 46 -304) and IgE (1076 IU/ml; normal: <90) were increased since early childhood. Neutrophil oxidative burst (DHR assay), NK cell degranulation (assessment of CD107a surface expression), and perforin expression were normal. Absolute numbers of peripheral T, B, and NK cells were within normal range. To characterize the immunological compartment of P1 in greater detail, PBMC were analyzed by multicolor flow cytometry. Immunophenotyping revealed an altered T cell subset distribution with a relative increase of $CD45RO^{+}CCR7^{-}$ effector memory $CD4^{+}$ and $CD8^{+}$ T cells accompanied by a decrease of $CD45RO^{+}CCR7^{+}$ central memory T cells (Figure 13). In addition, P1 showed a decreased proportion of $CD45RO^{+}$HLA-DR^{-} memory regulatory T cells

and CCR6^{+}CXCR3^{-} Th17 cells, whereas a substantial increase of CCR6^{-}CXCR3^{+} Th1 cells could be detected (Figure 13).

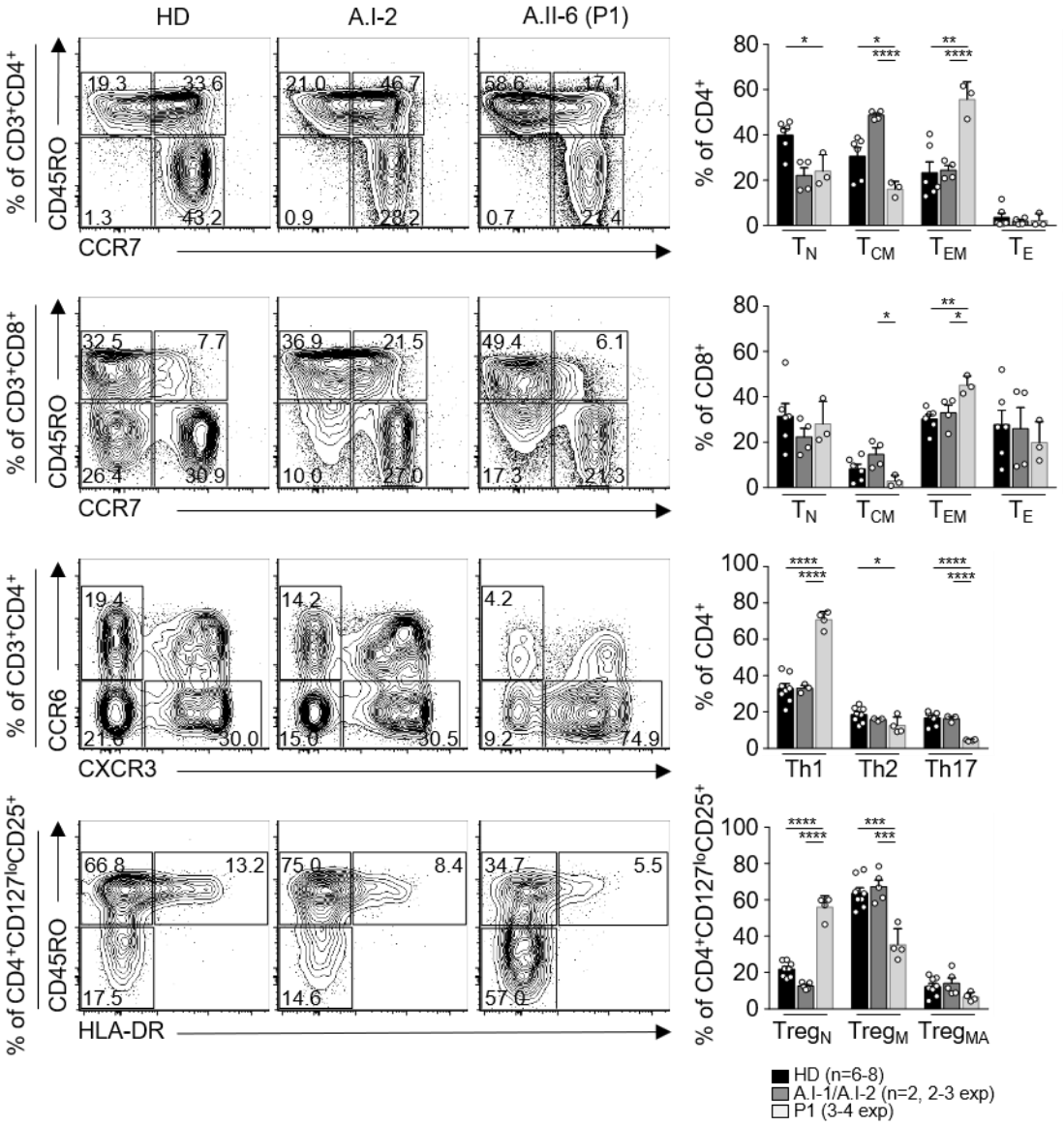

Figure 13: Caspase-8 deficiency in patient P1 is associated with T cell dysfunctions.
Representative FACS-based immunophenotypical characterization of naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and effector (T_E) CD4^{+} and CD8^{+} T cells (based on CD45RO and CCR7 expression), as well as CXCR3^{+}CCR6^{-} Th1, CXCR3^{-}CCR6^{+} Th17, and CD127^{lo}CD25^{+} naïve (Treg cells$_N$), memory (Treg cells$_M$) and activated memory (Treg cells$_{MA}$) Treg cells cells in PBMC from healthy donors (HD), parents (A.I-1, A.I-2), and P1 (A.II-6) (left panel).
Graphical representation of 3-4 independent experiments depicts the percentage of indicated cell populations (mean ± SEM) with individual data points as overlays (right panel). *P* values were calculated using two-tailed unpaired t tests: *p<0.05, ** p <0.01, *** p <0.001, and **** p<0.0001.

To study the function of peripheral T cells in P1, we assessed the T cell proliferation and activation in response to mitogens and specific antigens. CFSE dilution assays revealed a decreased T cell proliferation in P1 upon stimulation with anti-CD3 for 4 days, whereas the proliferative response upon co-stimulation with anti-CD28 was comparable to healthy controls (Figure 14A, B). Correspondingly, we could detect reduced surface expression of CD25 and CD69 in response to anti-CD3 suggesting reduced T cell activation (Figure 14B). Similar to defective T cell response in P1, thymidine incorporation assays showed impaired T cell proliferation in response to PHA (9342 cpm, control: 65,640) and anti-CD3 (702 cpm, control: 13,000) in P3.

Previous data on T cells from the first reported Caspase-8-deficient patients have suggested that T cell activation in response to anti-CD3 was decreased due to impaired NF-κB signaling. To assess whether the defective T cell activation and proliferation was associated with altered TCR-dependent signaling, we conducted phospho flow cytometry assays in $CD4^+$ and $CD8^+$ T cells from healthy donors (HD), father (A.I-1), and P1 upon stimulation with anti-CD3 and anti-CD3/CD28 for 0, 2, 5, 15 and 20 minutes (Figure 14C). FACS-based analysis showed normal TCR-dependent signal transduction with respect to phosphorylation of LAT, ZAP, PKC, ERK, AKT, as well as NF-κB signaling molecules (NF-κB p65, IκBα, p100) in P1 as compared with healthy donor controls and her father (Figure 14C).

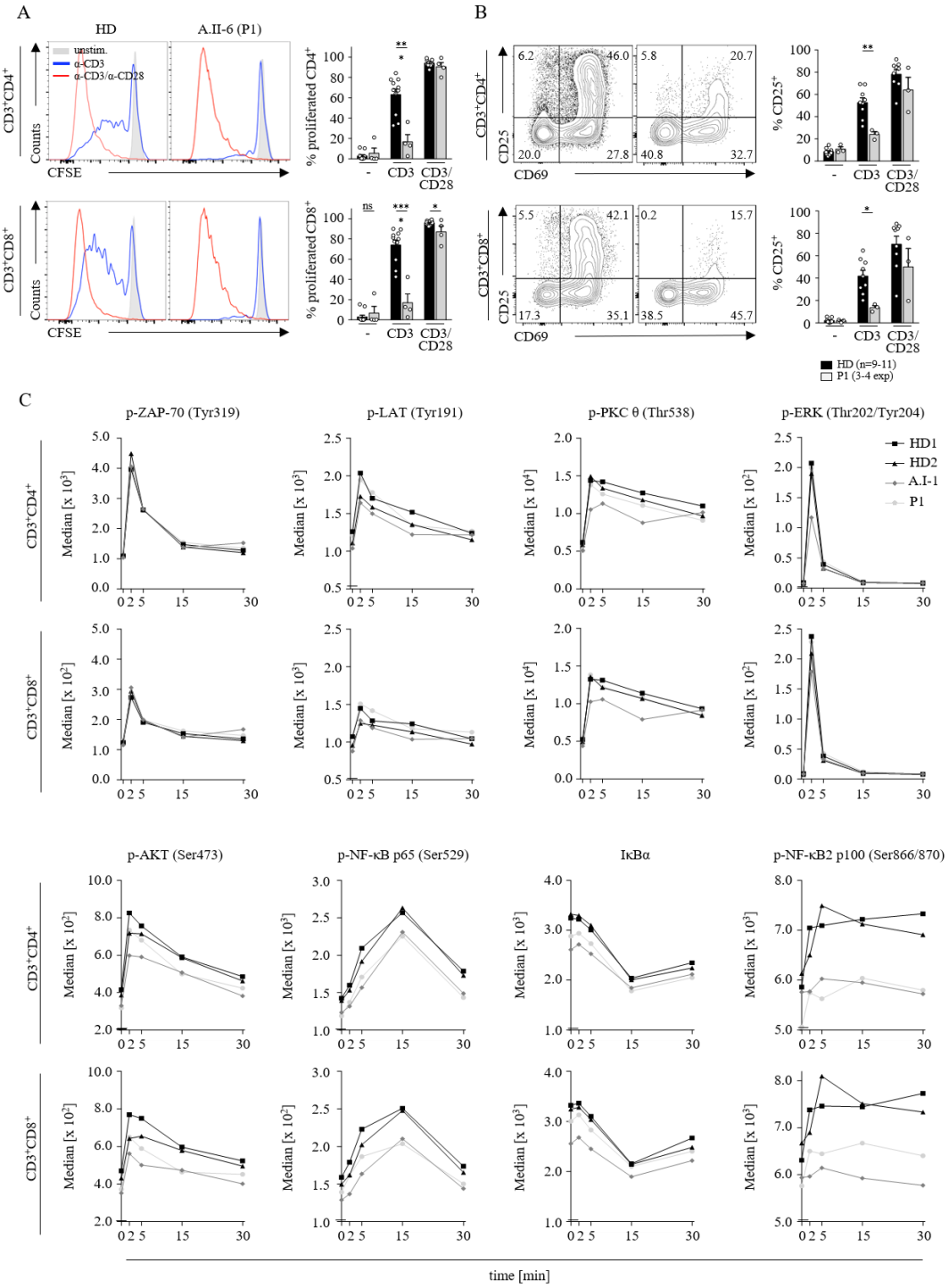

Figure 14: Caspase-8 deficiency in patient P1 is associated with altered T cell proliferation and activity.

(A,B) Flow cytometry-based analysis of $CD4^+$ and $CD8^+$ T cell proliferation (CFSE dilution assay, A) and activation (CD25 and CD69 expression, B) in response to anti-CD3 ± anti-CD28 (left panel). Graphs display the mean ± SEM with individual data points of the percentage of proliferated (n = 4) or activated ($CD25^+$, n = 3) $CD4^+$ and $CD8^+$ T cells (right panel). (C) Phospho flow cytometry-based assessment of TCR-mediated downstream signaling in $CD4^+$ and $CD8^+$ T cells from healthy donors (HD), father (A.I-1), and patient 1 (P1) stimulated with anti-CD3 and anti-CD3/CD28 for 0, 2, 5, 15 and 30 min. *P* values were calculated using two-tailed unpaired t tests: * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001.

In addition to the T cell compartment, we assessed the distribution of B cell subsets in the peripheral blood from P1. Multicolor FACS analysis showed reduced frequencies of marginal zone $CD19^{+}IgD^{+}CD27^{+}$, class-switched $CD19^{+}IgD^{-}CD27^{+}$ as well as transitional $CD19^{+}IgM^{hi}CD38^{++}$ B cells in P1 (Figure 15A). Notably, P1 developed a continuous reduction of the mean fluorescence intensity (MFI) of cell surface IgD expression on $CD19^{+}$ B cells at the age of 13 years (460 ± 41,3; 3 independent experiments) in comparison to her parents (4112 ± 485; 2 independent experiments) and healthy donors (3018 ± 396; 7 unrelated HD, 3 experiments). *In vitro* B cell proliferation assays revealed normal responses upon stimulation with CD40L, IL-4, and IL-21. However, patient sorted naïve B cells or PBMC showed a non-significant reduction in the differentiation of $CD19^{+}CD27^{+}CD38^{+}$ plasmablasts (Figure 15B), affecting in particular the differentiation of plasmablasts with high MFI expression of CD27 and CD38.

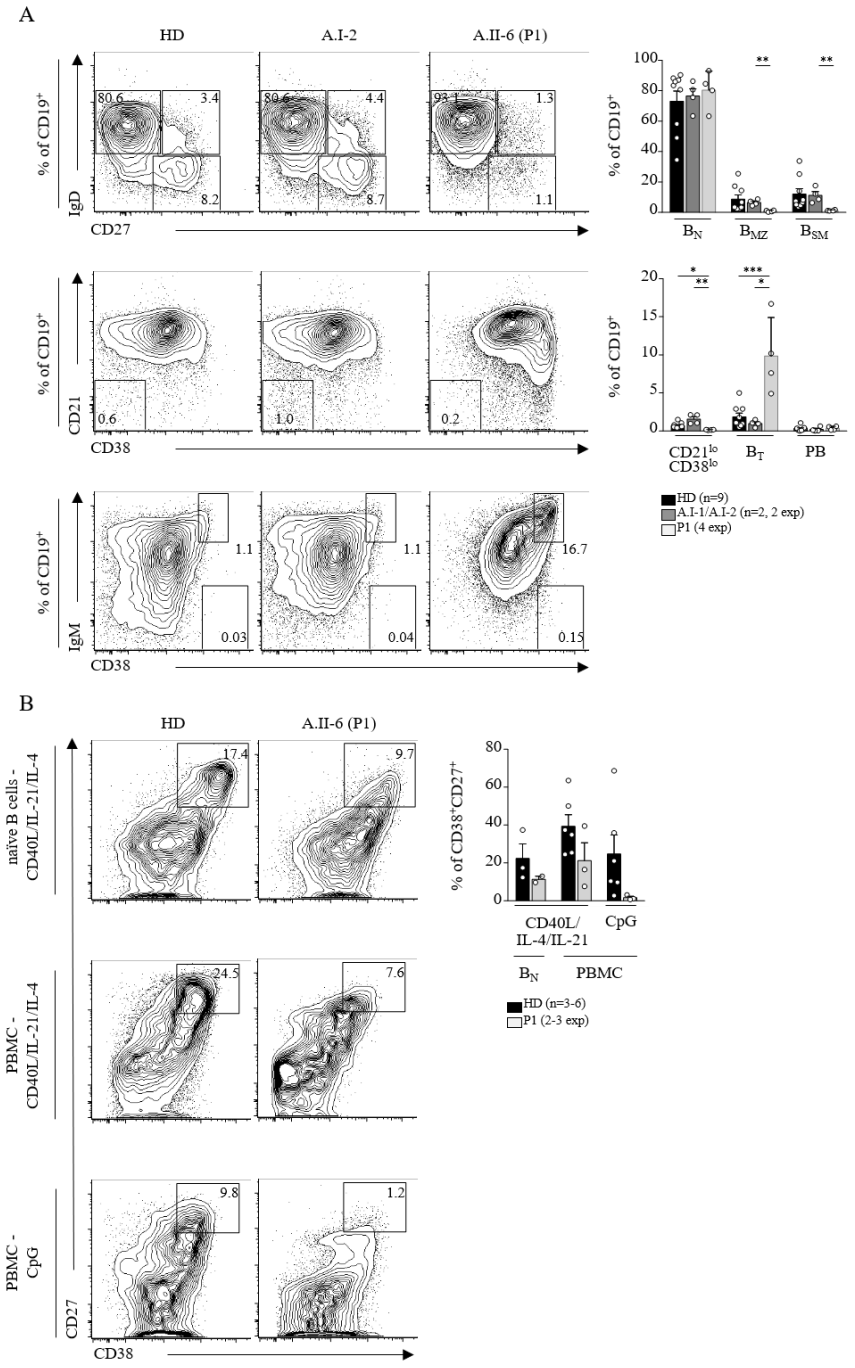

Figure 15: Caspase-8 deficiency in patient P1 is associated with impaired B cell differentiation. (A) Immunophenotypical analysis of peripheral blood IgD^+CD27^- naïve (B_N), IgD^+CD27^+ marginal zone (B_{MZ}), IgD^-CD27^+ switched memory (B_{SM}), $CD21^{lo}CD38^{lo}$, $IgM^{hi}CD38^{++}$ transitional (B_T), and IgM-$CD38^{+++}$ plasmablast (PB) B cells from healthy donors (HD), parents (A.I-1, A.I-2), and P1 (A.II-6) (left panels). Graphical representations indicate the mean ± SEM of the frequencies of B cell populations from 4 independent experiments (right panel). (B) Analysis of the *in vitro* B cell differentiation of sorted naïve B cells (B_N) and PBMC from healthy donors or P1 into $CD27^+CD38^+$ plasmablasts upon stimulation with CD40L, IL-4, and IL-21 or CpG (left panel). Graphs show the mean ± SEM of 2 (naïve B cells) or 3 (PBMC) independent experiments with individual data points as overlays (right panel). *P* values were calculated using two-tailed unpaired t tests. *p<0.05, **p<0.01, and ***p<0.001.

7.5. Human Caspase-8 controls inflammasome activity in macrophages

Mouse bone marrow-derived macrophages and DCs from conditional Casp8 knockout mice have been previously shown to exhibit increased NLRP3 inflammasome activity [278]. Based on these mouse data, we have studied the release of IL-1β from macrophages upon stimulation with LPS and ATP. Interestingly, monocytes from P1 secreted elevated IL-1β levels in response to LPS priming without the requirement for the secondary stimulus of inflammasome activity (e.g., ATP, Nigericin) (Figure 16A). Notably, additional stimulation with ATP did not show robust induction of inflammasome activity in Caspase-8-deficient monocytes from P1, as compared with healthy controls. The increased LPS-stimulated inflammasome activity was not associated with significant alterations of cytotoxicity, as assessed by LDH release assays (Figure 16B).

To evaluate whether the enhanced IL-1β release is mediated by enhanced inflammasome activity, we studied the NLRP3-dependent inflammasome pathway. Unexpectedly, immunoblotting showed reduced protein expression of key inflammasome mediators in cell lysates such as NLRP3, ASC, and cleaved Caspase-1 (Figure 16C). Correspondingly, immunoblotting revealed that the secretion of mature IL-1β was reduced in protein lysates and supernatants of P1 upon LPS priming and ATP stimulation as compared with healthy controls (Figure 16C). Taken together, these data suggested increased secretion of unprocessed IL-1β upon inflammasome activation in primary patient's cells.

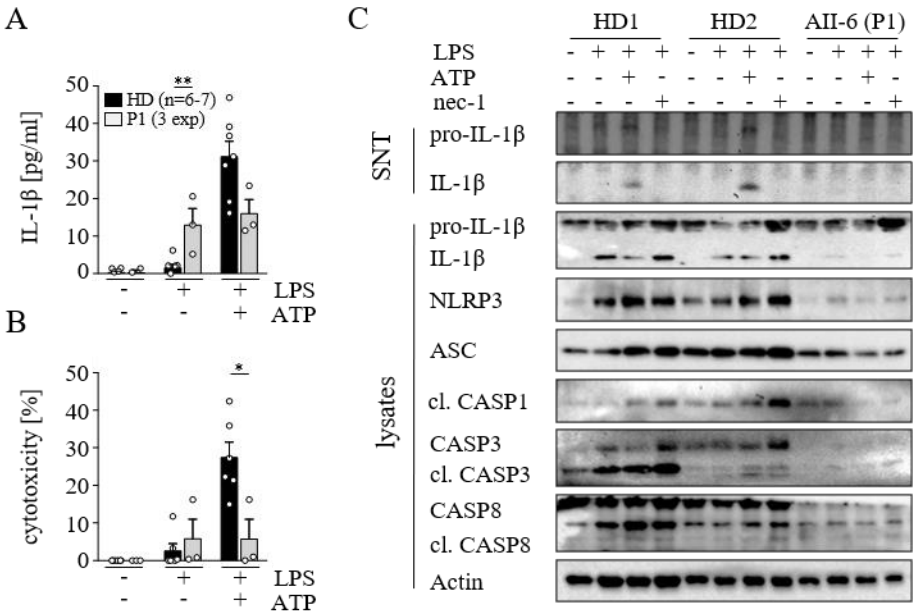

Figure 16: Caspase-8-deficient monocytes and macrophages showed an increased inflammasome activity upon LPS priming.
(A,B) ELISA of IL-1β secretion (A) and LDH release (B) in PBMC-derived monocytes from healthy donors (HD) and P1 in response to LPS ± ATP. Graphs display mean ± SEM of 6-7 HD and P1 from 3 independent experiments. (C) Representative immunoblotting (n=3) of Caspase-8 and proteins involved in inflammasome activation and downstream signaling (IL-1β, NLRP3, ASC, CASP1, CASP3).

To shed further light on inflammasome biology in the context of the identified CASP8 mutations, we used the recently published BLaER1 monocyte transdifferentiation model with knockout of CASP4 and CASP8 [270] and lentivirally overexpressed wild-type Caspase-8 or the mutant Caspase-8 variants Q237R (P1, P2) and R265W (P3). Similar to primary patient's cells, increased IL-1β secretion could be detected in BLaER1 cells with CASP8 knockout or overexpression of the Caspase-8 mutant Q237R upon LPS priming, as compared with wild-type Caspase-8 reconstituted BLaER1 cells (Figure 17A). However, no increase of IL-1β secretion or altered inflammasome activity could be detected in BLaER1 cells expressing the variant R265W that has been identified in P3. Whereas no elevated cytotoxicity could be detected in patient's primary cells, BLaER1 cells with CASP8 knockout or overexpression of

the Q237R variant showed increased cell death as determined by LDH release assay (Figure *17*B).

To evaluate whether the enhanced inflammasome activity is caused by NLRP3 inflammasome activity and/or necroptosis, we analyzed the LPS-triggered IL-1β production of BLaER1 cells upon treatment with the small molecule inhibitors MCC950 and necrosulfonamide (NSA), respectively. MCC950 is a selective inhibitor of canonical and noncanonical NLRP3 inflammasome activation and has been considered as potential therapeutic for NLRP3- associated syndromes causing autoinflammation and autoimmunity [279]. Necrosulfonamide is a small molecule that inhibits human MLKL adaptor function and thereby prevents MLKL-RIPK1-RIPK3 necrosome complex formation [152]. Treatment of Caspase-8-deficient LPS-primed BLaER1 cells with MCC950 or NSA resulted in decreased IL-1β secretion suggesting that both altered NLRP3 inflammasome activity and necroptosis at least partially mediate increased proinflammatory responses of macrophages in the absence of Caspase-8 (Figure 17A).

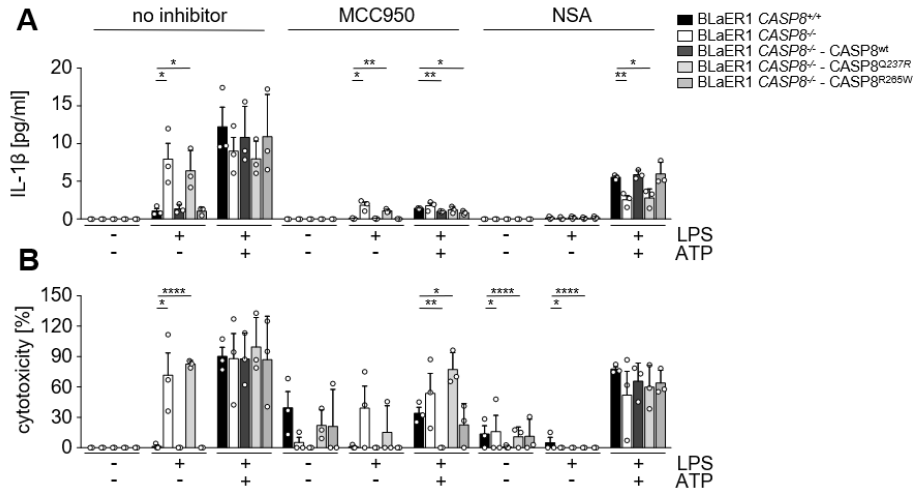

Figure 17: Analysis of inflammasome activation and necroptosis in Caspase-8-deficient BLaER1 cells.
BLaER1 cells with CASP8 knockout that stably overexpress green-fluorescent protein (GFP), wild-type (WT) Caspase-8, or mutant Caspase-8 variants Q237R (P1, P2) and R265W (P3) were treated with LPS ± ATP ± inhibitors for NLRP3 (MCC950) or MLKL (NSA) (n=3 independent experiments). (A) Assessment of IL-1β secretion (ELISA, n=3) and (B) cytotoxicity (LDH release assay, n=3). *P* values were calculated using two-tailed unpaired t tests. **p*<0.05, ***p*<0.01, ****p*<0.001, and *****p*<0.0001.

Execution of necroptosis is based on the activation of RIPK3 which in turn phosphorylates MLKL. Activated MLKL forms oligomers and translocates to plasma and intracellular membranes, where it directly disrupts membrane integrity and induces necroptotic cell death [280]. Immunoblotting of Caspase-8-deficient BlaER1 cells revealed a distinct pattern of MLKL oligomerization after LPS priming (Figure 18A), suggesting altered necroptosome signaling. Accordingly, inhibition of MLKL oligomerization with NSA led to a decreased secretion of mature IL-1β in Caspase-8-deficient macrophages (Figure 18B).

Taken together, studies on the BLaER1 cell model (i) confirmed that human Caspase-8 deficiency alters NLRP3-dependent inflammasome activity and (ii) facilitated the elucidation of the underlying mechanisms by showing that increased secretion of IL-1β is associated with altered MLKL-dependent necroptosome activity.

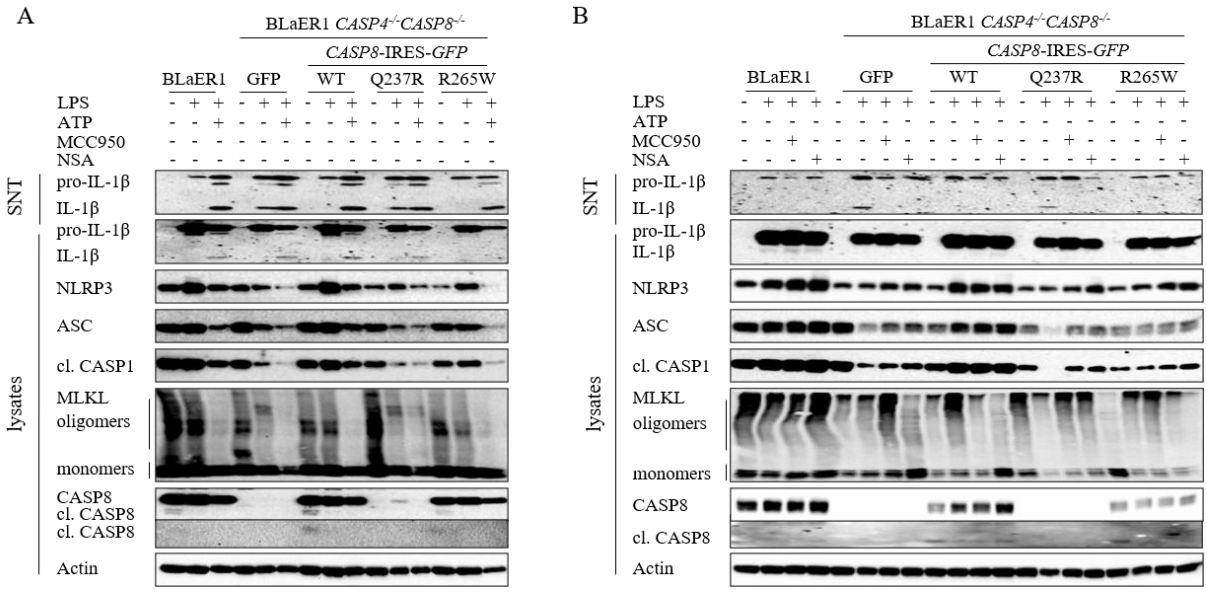

Figure 18: Analysis of inflammasome activation and necroptosis in Caspase-8-deficient BLaER1 cells.
CASP8 knockout BLaER1 cells with stable overexpression of green-fluorescent protein (GFP), wild-type (WT) Caspase-8 or mutant Caspase-8 Q237R and R265W variants (n=3 independent experiments) were treated with LPS ± ATP ± inhibitors for NLRP3 (MCC950) or MLKL (NSA). (A, B) Immunoblotting of key molecules of the NLRP3-dependent inflammasome in supernatants (SNT) and lysates of BLaER1 cells upon treatment with LPS ± ATP (A, n=3) ± inhibitors for NLRP3 (MCC950) or MLKL (NSA) (B, n=3). (A, B) MLKL oligomerization was assessed by SDS-PAGE under non-reducing conditions.

7.6. Human Caspase-8 deficiency alters cell death responses in intestinal epithelial cells

Previous studies have shown that dysregulated epithelial cell death is a critical pathogenic factor in intestinal inflammation [157]. Mice with conditional deletion of Caspase-88 in the intestinal epithelium spontaneously developed inflammatory lesions in the terminal ileum [157]. As underlying mechanism, Guenther *et al.* reported that increased TNF-α-induced cell death was associated with enhanced RIPK3 expression and that altered cell death responses could be inhibited by blockage of necroptosis.

To evaluate epithelial cell death responses in P1, we generated intestinal organoids from colorectal biopsies. These *in vitro* 3D dimensional models recapitulate the cellular composition and architecture of *in vivo* intestinal tissues and exhibit patient-specific characteristics. To test whether human Caspase-8-deficient organoids exhibit increased cell death responses, we conducted immunohistochemical analysis of cleaved Caspase-3 upon stimulation with TNF-α and TRAIL. In contrast to the published conditional Caspase-8-deficient mice, we were not able to detect significant differences in the cleavage of Caspase-3 upon stimulation with TNF-α between Caspase-8 wild-type and mutant intestinal organoids using immunoblotting or immunohistochemistry (Figure 19A). However, treatment with TRAIL induced rapid and robust cleavage of Caspase-3 and cell death in healthy donor organoids within two hours, whereas organoids derived from P1 were unresponsive to TRAIL stimulation (Figure 19B, C). The abrogated response to TRAIL confirmed defective Caspase-8-mediated signaling in P1.

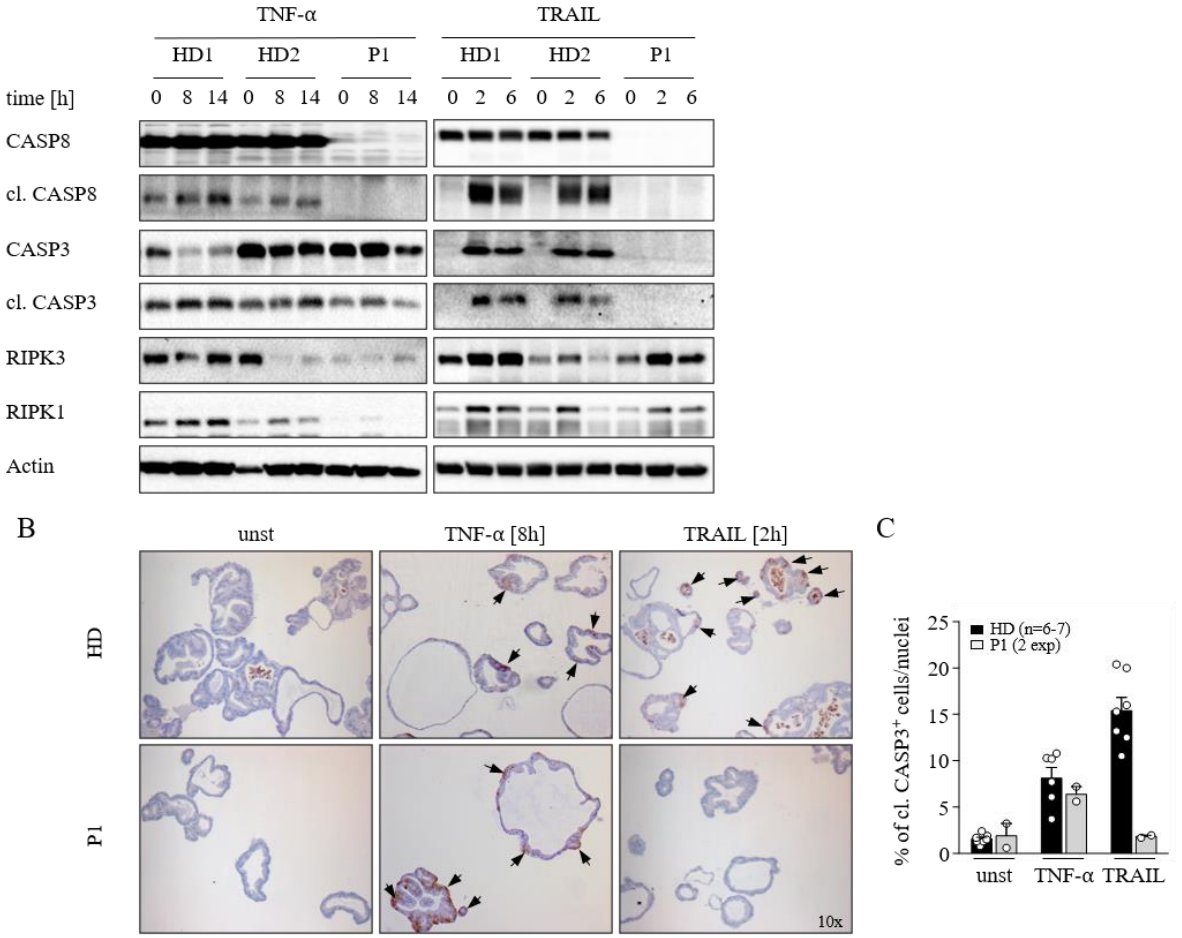

Figure 19: Impaired TRAIL-induced cell death responses in Caspase-8-deficient intestinal organoids.
(A) Representative immunoblotting (n=3) of Caspase-3 and Caspase-8 cleavage as well as RIPK1 and RIPK3 expression in intestinal organoids from healthy donors (HD) and P1 upon treatment with TNF-α or TRAIL. (B, C) Analysis of cell death in TNF-α- and TRAIL-treated intestinal organoids from HD (n = 6-7) and P1 by immunohistochemistry-based detection of cleaved Caspase-3^+ cells (indicated by arrows; B). Data are representative of 2 independent experiments with evaluation of 960 ± 467 nuclei for HD (n=6-7) and 773 ± 480 nuclei for P1 (C).

To study the effects of the newly identified Caspase-8 mutations on the necroptotic pathway and cell death signaling in intestinal epithelial cells in greater detail, we genetically engineered HT-29 coloncarcinoma cell lines with CRISPR/Cas9-mediated knockout of Caspase-8 and subsequent lentiviral overexpression of wild-type or mutant Caspase-8. To induce necroptotic cell death, cells were treated with a mimetic (BV6) of the second mitochondrial activator of apoptosis (SMAC), a pro-apoptotic mitochondrial protein that suppresses the inhibitors of

apoptosis proteins (IAPs). Treatment of HT-29 cells with TNF-α and BV6 revealed enhanced phosphorylation and a distinct oligomerization pattern of MLKL in cells with knockout of Caspase-8 and overexpression of the variant Q237R (P1 and P2) (Figure 20). In addition, we could detect enhanced phosphorylation of RIPK1 and expression of RIPK3 in Caspase-8-deficient cells suggesting altered activation of RIPK3/MLKL-dependent necroptosis. Similar to the inflammasome phenotype in BLaER1 cells, coloncarcinoma cells overexpressing the variant R265W (P3) showed activation of necroptosis comparable to wild-type reconstituted cells indicating genotype-phenotype differences.

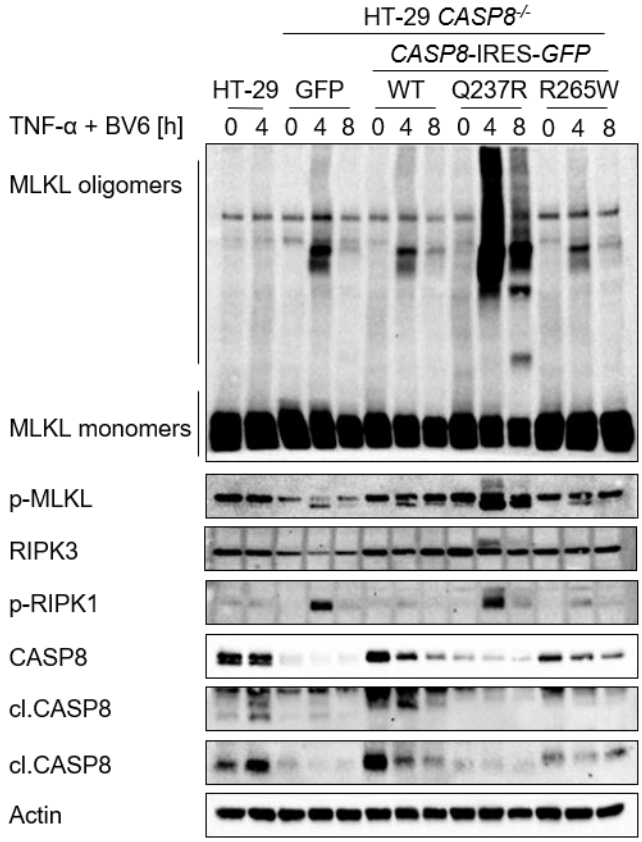

Figure 20: Distinct pattern of MLKL oligomerization in Caspase-8-deficient HT-29 coloncarcinoma cells.
Immunoblot detection (n=2) of key molecules involved in necroptosis upon treatment with TNF-α ± BV6 in heterologous coloncarcinoma HT-29 cells with knockout of CASP8 and subsequent lentiviral-mediated overexpression of GFP, wild-type (WT) Caspase-8 or mutant Caspase-8 variants Q237R and R265W. SDS-PAGE under non-reducing conditions was used to detect MLKL oligomerization. The disulfide bonds of the oligomerized MLKL proteins were formed by oxidation during cell lysis [259].

To evaluate whether the altered MLKL signaling is associated with dysregulated necroptosis, we assessed cell death responses in Caspase-8-deficient HT-29 cells upon TNFR1-mediated signaling by employing FACS- based Annexin V/PI staining. While we could not observe differences in the frequencies of Annexin V^+ cells upon stimulation with TNF-α alone, HT-29 cells with a Caspase-8 knockout or overexpression of the patient-specific mutation Q237R (P1, P2) showed increased cell death upon treatment with TNF-α and BV6 in comparison to cells with expressing wild-type Caspase-8 or the mutation R265W (P3) (Figure 21).

Collectively, our data on heterologous HT-29 cells demonstrated altered MLKL oligomerization patterns and necroptotic cell death in cells with Caspase-8 KO or transgenic expression of the mutation Q237R (P1. P2). Taken together, studies on patient-derived organoids and genetically engineered HT-29 cells suggested that human Caspase-8 deficiency perturbs cell death responses in intestinal epithelial cells with respect to TRAIL and TNF-α/BV6 signaling pathways.

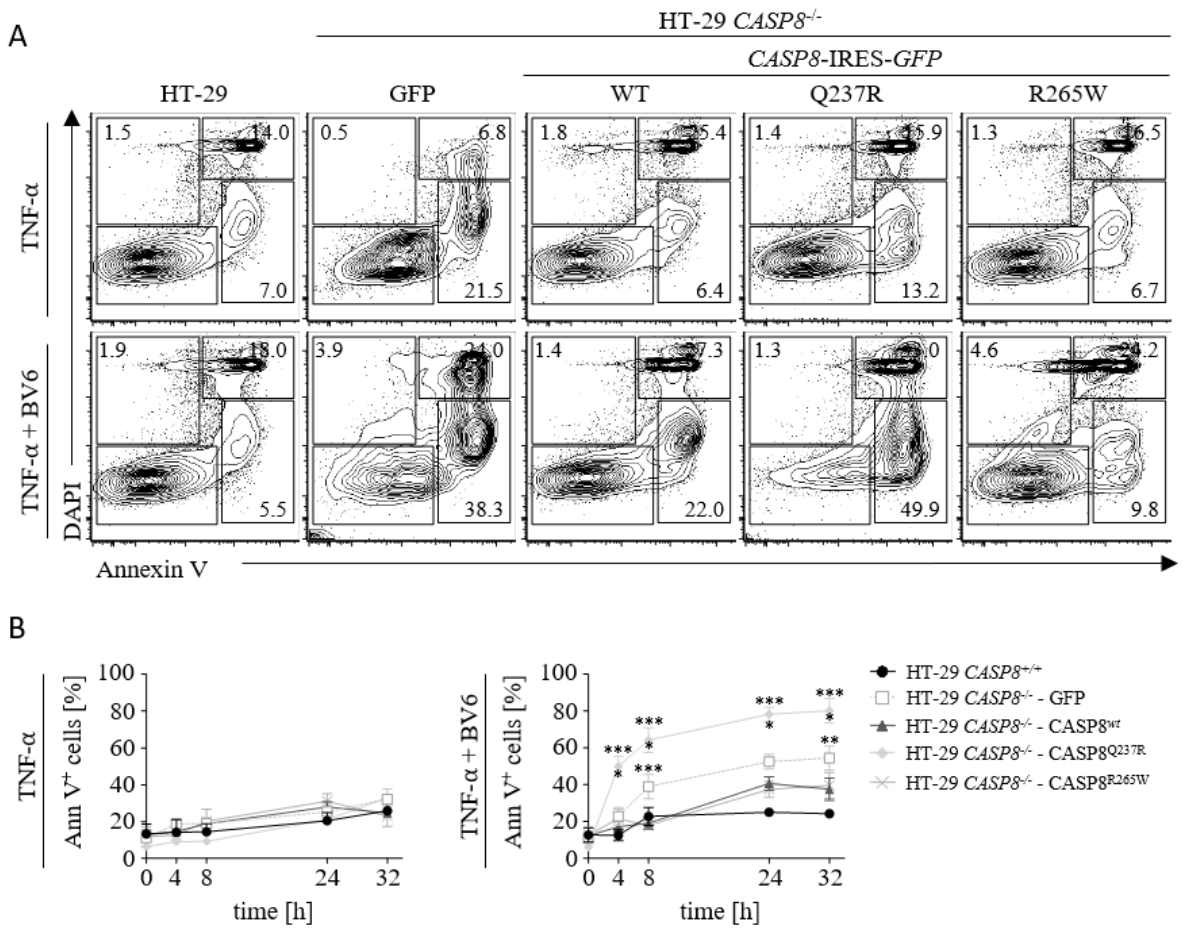

Figure 21: Increased necroptotic cell death in Caspase-8-deficient HT-29 cells.
(A) Flow cytometry-based analysis of Annexin V^+ heterologous coloncarcinoma HT-29 cells with knockout of CASP8 and lentiviral-mediated overexpression of GFP, wild-type (WT) or mutant Caspase-8 Q237R and R265W variants in response to TNF-α ± BV6. (B) Data indicate the mean percentage of Annexin V^+ cells ± SEM of 4 independent experiments. Statistical analysis was performed using two-way analysis of variance (ANOVA) for repeated measures with Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 compared with WT.

8. Discussion

8.1. Caspase-8 deficiency - A novel molecular cause for VEO-IBD

Children with VEO-IBD (age of onset below 6 years) develop inflammatory conditions different from forms observed in adults [97], e.g., predominance of colonic inflammation, predilection of perianal disease (fistula, abscesses) and extension of severity. VEO-IBD is a rare condition but the incidence has increased dramatically over the last decades worldwide and it has been reported in 15 % of pediatric IBD patients [98]. Monogenic diseases provide evidence that genetic factors and immune dysfunctions are critical pathomechanisms in children with VEO-IBD [99,100]. Advances in next-generation sequencing have discovered more than 50 monogenic diseases associated with IBD-like phenotypes [99]. To study novel genetic entities our laboratory has conducted a whole exome sequencing screen on one of the largest international cohorts of ~480 patients with VEO-IBD.

Our genome-wide screen of VEO- IBD patients unraveled biallellic loss-of-function mutations in Caspase-8 in three unrelated children as novel monogenic entity for IBD. The patients manifested with a severe refractory disease course associated with failure to thrive, bloody diarrhea, and perianal disease within the first few months of life. The phenotype of our patients could not be explicitly classified as UC or CD and thus was rather defined as IBD unclassified. Our findings highlighted the functional relevance of Caspase-8-mediated signaling on controlling innate and adaptive immunity and intestinal inflammation.

In 2002, the first evidence that Caspase-8 plays an important role in controlling human immunity was shown by the Leonardo group [161]. They reported two siblings with a homozygous missense mutation in Caspase-8 presenting with an ALPS-like disorder and immunodeficiency, characterized by relapsing infections [161]. In contrast to classical ALPS

phenotypes, patients with the Caspase-8 mutation exhibited defective activation of T, B, and NK cell compartments. Furthermore, they have shown that Caspase-8 regulates functions in lymphocyte activation independently of CD95 [161]. The clinical spectrum of Caspase-8 deficiency has been further expanded by the characterization of two additional patients presenting with the same Caspase-8 mutation and adult-onset-organ lymphocytic infiltration with granulomas in the central nervous system, liver, spleen, bone marrow, and lungs [164].

In contrast to these previous reports, our Caspase-8-deficient patients showed a disease that was strikingly different from patients with ALPS. Of note, all three children primarily presented with severe refractory VEO-IBD that manifested within the first months of life [162,163,281]. Thus, our clinical and molecular studies indicated for the first time a critical role of Caspase-8-mediated signaling for the establishment of a balanced immune-milieu in the human intestinal tract. In independent investigations, our findings have recently been confirmed by a case report describing a patient with a germline Caspase-8 mutation (NM_001228:c.1232C>T, p.P411L) who presented with immunodeficiency and early onset of gastrointestinal involvement accompanied by failure to thrive, recurrent enterorrhagia, rectal prolapse, rectal polyps and perianal fistula [282]. Caspase-8 deficiency in this patient was associated with reduced FAS-mediated apoptosis in effector memory T cells and impaired TLR3-mediated production of pro-inflammatory cytokines in innate immune cells. In contrast to our studies, TLR4 engagement did not alter cytokine production in patient-derived innate immune cells and intestinal epithelial cell defects were not investigated.

8.2. The influence of Caspase-8 deficiency on the immune system

First line of evidence showed that immunodeficiency is associated with enhanced susceptibility to infections due to dysfunctions of T, B, and NK cells [283]. Furthermore, mouse studies have

provided evidence that Caspase-8 has notably a major role in mediating apoptosis but also in controlling of T cell proliferation and activation [158,284]. Moreover, mice with T cell-specific deletion of Casp8 have been shown to develop an age-dependent lethal lymphoproliferative and lymphoinfiltrative immune disorder [285].

Basic immunophenotyping of peripheral blood cells from the previously reported Caspase-8-deficient patients showed increased activated T cells, low level of naïve T cells, and decreased mature B and NK cells [161]. However, these studies did not provide detailed immunophenotypical analysis. In our index patient P1, absolute numbers of peripheral T, B, and NK cells were within normal range. However, multicolor immunophenotyping of PBMC revealed an altered distribution of T cells subsets and a decreased proportion of Treg cells and Th17 cells, while Th1 cells were increased. Persistent inflammation in IBD is thought to be a result of abnormal Th1 responses, which are induced by the presentation of commensal bacteria by DC and aggravated by impaired Treg cell function [286]. Similar to our Caspase-8-deficient index patient, it was shown that IBD patients often exhibit elevated level of Th1 cells and Th1- associated cytokines such as IFN-γ [20,59,287,288]. Furthermore, we could show that the level of memory Treg cells was decreased in our index patient. Since Tregs have key functions in sustaining the immune response of the intestine against gut microbes and antigens by suppressing naïve T cell proliferation and effector T cell function [289], the impaired Treg cell differentiation might represent another contributing factor of intestinal inflammation.

In addition, our detailed immune worked revealed a reduced T cell proliferation and activation in our index patient upon stimulation with anti-CD3. This impaired proliferation of Caspase-8-deficient T cells in response to anti-CD3 could be due to impaired regulation of IL-2, CD25, and cell cycle-associated proteins [290,291]. Furthermore, Su *et al.* have previously reported impaired nuclear translocation of NF-κB signaling in human Caspase-8-deficient T cells upon TCR stimulation [160], but previous later studies in mice have suggested that the defects in

NF-κB signaling may be a consequence of aberrant necroptosis [149,292]. Our studies could not detect altered NF-κB signaling as correlate for the defective T cell activation in patient-derived T cells. Therefore, future studies on the underlaying molecular mechanisms of altered T cell homeostasis using advanced human Caspase-8-deficient disease models are required.

As antigen-presenters and cytokine producers, B cells play a critical role in controlling intestinal homeostasis. However, the suppressive and exacerbating capacity of B cells in regulating gut inflammation are discussed controversially [293,294]. Our index patient showed reduced marginal zone and class-switched B cells as well as increased transitional B cells, indicating impaired B cell maturation. The relevance of this finding on intestinal inflammation needs to be addressed in future studies.

Collectively, current reports on patients with human Caspase-8 deficiency suggest a striking genotype-phenotype correlation [161,164,282,295]. These differences in the phenotypes of Caspase-8 deficiency might be due to the type of mutation, genetic modifiers, microbiome composition, infections, or medications. Furthermore, it is known that monogenic diseases, in particular those affecting immunity, can manifest with a substantial phenotypic variability despite mutations affecting the same gene [296]. Further studies are required to assess the genotype-phenotype correlations and molecular triggers of disease.

8.3. Human Caspase-8 controls inflammasome activity in macrophages

The human immune system has to combat a huge variety of infections, endogenous danger signals or cell damages to maintain homeostatic tissue function. The innate immune system has evolved different signaling receptors that recognize foreign molecular structures as well as altered self-molecules [297,298]. Inflammasomes are a group of multimeric protein complexes of the innate immune system responsible for the activation of inflammatory responses

[299,300]. The activation of the inflammasome and secretion of pro-inflammatory cytokines (IL-1β and IL-18) are triggered by molecules released upon infections, tissue damage or metabolic imbalances [167]. The importance of inflammasomes in human disease has been shown for patients with autoinflammatory and inflammatory diseases such as rheumatic arthritis [301-303]. In particular, previous studies have shown that altered inflammasome activity in immune cells is crucial for the pathogenesis of IBD, since (i) genes responsible for the activation of the inflammasome were associated with IBD susceptibility, (ii) IBD patients showed increased IL-1β levels in the colon, and (iii) blockage of IL-1β revised colitis in murine models [304].

Activated macrophages are known to be a major source of IL-1β production and altered macrophage function may promote intestinal inflammation [305]. Previous studies suggested that chemical inhibition or conditional deletion of Caspase-8 impairs macrophage differentiation and survival by non-apoptotic mechanisms [145,306]. Whereas IL-1α and IL-1β did not contribute to the cutaneous inflammation in mouse Caspase-8-deficient epidermis [156], Kang et al. demonstrated that loss of Casp8 in mouse bone marrow-derived DC facilitated LPS-induced NLRP3 inflammasome activity in a RIPK1/RIPK3-dependent manner [278]. In our studies, Caspase-8-deficient peripheral blood-derived macrophages from P1 showed elevated secretion of IL-1β in response to LPS priming, while addition of secondary stimuli (e.g., ATP) failed to induce robust canonical NLRP3 inflammasome activation. Unexpectedly, we could show a reduced expression of key mediators of inflammasome activation such as NLRP3, ASC, and cleaved Caspase-1 in cell lysates and reduced level of active IL-1β in supernatants of patient's cells as compared with healthy donors. These studies suggested increased secretion of unprocessed IL-1β upon inflammasome activation in primary patient cells but could not provide unambiguous insights into the molecular mechanisms of

Caspase-8 dysfunction. Notably, increased secretion of unprocessed IL-1β was not associated with enhanced cell death in primary patient samples.

To study the effects of the newly identified Caspase-8 mutations on inflammasome biology in greater detail, we used a recently published BLaER1 monocyte transdifferentiation model [307]. BLaER1 monocytes express a transcriptome comparable to primary monocytes and closely resemble physiological immune responses [270]. Studies on heterologous BLaER1 cells with CASP8 knockout or transgenic expression of the patient-specific mutation Q237R confirmed the critical phenotype observed in our index patient that loss-of-function of Caspase-8 in macrophages resulted in increased IL-1β secretion upon LPS priming. Furthermore, the BLaER1 cell model allowed us to link the increased IL-1β activity of Caspase-8-deficient macrophages to increased necroptosis, as demonstrated by increased LDH release and MLKL oligomerization. Similarly, Gaidt *et al.* have recently demonstrated that human BLaER1 monocytes failed to respond to the NLRP3 agonist Nigericin upon CASP8 knockout, while blockade of necroptosis by additional deletion of *RIPK3* rescued this defect [270]. Collectively, these data indicate that enhanced inflammasome activity in Caspase-8-deficient macrophages might be caused by altered necroptotic cell death.

IL-1β is known to mediate pleiotropic effects in acute and chronic inflammation and elevated IL-1β activity has been detected in experimental colitis [308] as well as intestinal tissues of patients with CD [309]. Interestingly, IL- 1β has been shown to mediate intestinal inflammation in children with IL-10R deficiency and the treatment of patients with IL-1R antagonist (Anakinra) induced marked clinical, endoscopic, and histologic responses [308]. Based on these data, increased IL-1β secretion in P1 might contribute to the IBD pathogenesis. However, treatment with IL-1β antagonists is not standard care for children with VEO-IBD and has yet not been tested in P1. Furthermore, we could not document an altered inflammasome activity

in the heterologous BLaER1 cells expressing the Caspase-8 mutant R265W that has been previously reported in the first Caspase-8-deficient patients with ALPS-like disease [161] and that has been identified in P3. Therefore, it is likely that pathomechanisms of human Caspase-8 deficiency are multifactorial and complex, involving innate and adaptive immune dysfunctions as well as epithelial barrier defects with impaired lymphocyte homeostasis as common phenotype in all reported Caspase-8-deficient patients. Furthermore, it is tempting to speculate that Caspase-8 might control inflammasome activity via scaffolding functions, because the Caspase-8 mutant variant Q237R was associated with substantial reduction in protein expression and showed a comparable phenotype to CASP8 KO cells, whereas R265W had a protein expression comparable to wild-type reconstituted cells. Further studies are required to determine the genotype-phenotype correlations that lead to the gut pathology and whether IL1-β is one of the driving factors of intestinal inflammation.

8.4. Caspase-8 deficiency causes intrinsic intestinal epithelial cell death defects

TNF-α plays a critical role in regulating host defense but can also be pathogenic in several inflammatory conditions [310]. The complexity of TNFR1 signaling is underlined by the transition between inflammation, survival, and programmed cell death [311]. While TNF-α-induced NF-κB nuclear translocation promotes cell survival and inflammation, modulation of signaling cascades can induce Caspase-8–mediated apoptosis or RIPK3-dependent necroptosis, as reviewed in [312]. Dysregulation of TNFR1 signaling can lead to chronic inflammation. Correspondingly, inhibition of TNF-α is an effective treatment for several autoinflammatory and autoimmune disorders. To study the molecular mechanisms of dysregulated intestinal epithelial cell integrity in the context of human Caspase-8 deficiency, we made use of the human intestinal organoid technology enabling us to grow patient-derived

3D mini-gut systems with physiological cellular architecture and diversity of intestinal cell types. In contrast to conditional Casp8 KO mice, we could not observe increased TNF-α-induced cell death or enhanced expression level of RIPK1 or RIPK3 in Caspase-8-deficient patient-derived intestinal organoids or heterologous coloncarcinoma cells, as indicated by normal cleaved Caspase-3 expression. Importantly, TNF-α blockade (Infliximab) failed to induce sustained clinical remission in our patients. However, we could detect unresponsiveness of patient-derived-Caspase-8-deficient organoids to TRAIL stimulation, whereas healthy donor organoids showed a rapid TRAIL-induced cell death. Since TRAIL is a direct interaction partner of Caspase-8, abrogated TRAIL signaling could validate functional Caspase-8 deficiency. Further studies need to address to which extend the defective TRAIL signaling and which other mechanisms contribute to intestinal inflammation.

To further assess whether the identified CASP8 mutants affect the TNF-α mediated cell death pathways, we genetically engineered HT-29 coloncarcinoma cell lines by CRISPR/Cas9-mediated knockout of CASP8 and subsequent lentiviral overexpression of wild-type or mutant CASP8 alleles. Previous mouse studies have shown that loss of Casp8 leads to aberrant necroptosis and that disease phenotypes can be rescued by knockout of *Ripk3* or *Mlkl* [149,292,313]. Whereas we could not observe differences in cell death upon challenge with TNF-α alone, Caspase-8-deficient coloncarcinoma cells showed (i) an enhanced pattern of MLKL oligomerization, (ii) phosphorylation of RIPK1 and RIPK3, as well as (iii) increased cell death upon treatment with TNF-α and BV6, a mimetic of SMAC.

Taken together, our studies on patient-derived intestinal organoids and heterologous coloncarcinoma cell lines suggested that human Caspase-8 deficiency leads to cell death dysfunctions in the intestinal epithelium.

8.5. Phenotypic similarities and differences of mouse and human Caspase-8 deficiency

The essential role of Caspase-8 in controlling embryogenesis and tissue homeostasis has been demonstrated in several knockout mouse models. Whereas heterozygous mice appeared phenotypically normal, mice with a complete knockout of Casp8 exhibited embryonic lethality at day 11.5 of gestation [147]. Embryonic lethality was accompanied by cardiac malformations, neural tube defects, growth retardation, and hematopoietic progenitor deficiency [145-147]. Mouse models have provided critical insights in basic mechanisms of innate and adaptive immunity, but substantial species-specific differences make it indispensable to study human immunology [314]. In contrast to Casp8 knockout mouse models, our Caspase-8-deficient patients with missense mutations showed no obvious developmental defects and the phenotype was primarily affecting the gut and immune system. Whereas conditional deletion of mouse Casp8 in hematopoietic cells resulted in embryonic lethality [157], humans showed overall normal hematopoiesis but exhibited a defective T, B and NK cell activation leading to common immunodeficiency [282,295].

Of relevance for our project, refractory colitis strongly resembled the phenotype in mice with targeted knockout of Casp8 in the intestinal epithelium [157]. However, Caspase-8-deficient mice developed inflammatory lesions in the terminal ileum with lack of Paneth cells and elevated expression of RIPK3 [157], while our patients exhibited pancolitis with unspecific signs of inflammation without granuloma formation or ileitis. Notably, mouse Caspase-8-deficient organoids showed increased enhanced RIPK3 expression [157] and TNF-mediated cell death responses that could be blocked by Necrostatin-1 (nec-1), an allosteric small-molecule inhibitor of RIPK1 [157]. In contrast to Caspase-8-deficient mice, we could not detect a significant difference in the frequency of cleaved Caspase-3-positive epithelial cells upon stimulation with TNF-α between Caspase-8 wild-type and mutant intestinal organoids.

Impaired cell death responses might contribute to the pathogenesis of VEO-IBD in our patients, but the physiological *in vivo* triggers and mechanisms perturbing cell death and intestinal homeostasis need to be defined in order to developed targeted therapies. Even though there are striking similarities between *Casp8* KO mice and Caspase-8-deficient patients, Caspase-8 represents another example that mutations in human and mouse orthologs result in phenotypic discrepancies [315]. The differences in the phenotypes and disease triggers might be due to the treatment of patients with anti-inflammatory and/or immunosuppressive drugs, the type of mutation, genetic background, the environmental factors, and microbiome. In addition, Caspase-10, a homolog of Caspase-8, is only present in human [281] and might have at least in part redundant functions in mediating human cell death receptor-dependent responses [316]. Loss- of-function mutations of CASP10 have been reported to cause ALPS with distinct features affecting multiple death receptor pathways and signs of immunodeficiency [317]. Overlapping functions of Caspase-10 and residual protein Caspase-8 expression due to the type of mutation in our patients (missense mutations versus targeted knockout in mice) might also explain that human Caspase-8 or Caspase-10 deficiency are compatible with life [318]. Comparative studies are required to further decipher the difference of Caspase-8-mediated death cell receptor signaling in human and mice.

8.6. Therapy of Caspase-8 deficiency

Studies in mice have emphasized that Caspase-8 is a key molecule in controlling immunity, inflammation, and cell death responses by scaffolding-dependent functions and cleavage of cell death substrates. Our own and previous studies by others showed that patients with Caspase-8 deficiency exhibit lymphocyte dysfunctions, impaired inflammasome activation in innate immune cells, and defective cell death responses in intestinal epithelial cells. The broad functions of Caspase-8 in different cell types and the potential multifactorial disease mechanisms are a major challenge for the development of therapeutic strategies for human Caspase-8 deficiency. Correspondingly, our identified Caspase-8-deficient patients showed a life-threatening, refractory colitis despite standard care with exclusive enteral nutrition, anti-inflammatory treatment (e.g., steroids, azathioprine, infliximab), and surgical interventions.

A potential approach for treatment might be the altered inflammasome activity in Caspase-8-deficient patients. IL-1β is known to mediate pleiotropic effects in acute and chronic inflammation and elevated IL-1β activity has been detected in experimental colitis [308] and mucosal tissues of IBD patients [309]. Accordingly, increased IL-1β secretion by innate immune cells in P1 might contribute to the IBD pathogenesis. As proof-of-principle, the

IL-1 receptor antagonist Anakinra has been beneficial in the treatment of IL-1β-mediated intestinal inflammation in IL-10R-deficient patients serving as successful bridge to allogeneic hematopoietic stem cell transplantation [308]. However, IL-1 receptor blockade may be less advantageous in Caspase-8 deficiency with elevated IL-1β level due to putative damage-associated molecular patterns that appear to be secondary to necroptosis.

Studies on patient-derived intestinal organoids and heterologous coloncarcinoma cell lines suggested that dysfunctions of human Caspase-8 perturb cell death responses of intestinal epithelial cells. In conditional Casp8 knockout mice, pre-treatment with an allosteric small-molecule inhibitor of RIPK1 (nec-1) ameliorated small intestinal tissue destruction [157]. Thus, therapeutics designed to target necroptosis might be an attractive approach, however more precise understanding on the specific mechanisms involved in necroptosis and approval for clinical studies are required. Since mice expressing catalytically inactive RIPK1 developed normal but did not show inflammatory phenotypes, alternative strategies might focus on small-molecule inhibitors targeting RIPK1 kinase activity [244,319,320]. Clinical phase I trials for the treatment of chronic psoriasis, rheumatoid arthritis, and ulcerative colitis (GSK2982772, DNL747) have been successfully conducted with promising results [264,265]. In addition, targeting of RIPK3 is a new idea to treat inflammatory diseases. However, concerns have been raised about toxic effects as knock-in mice expressing catalytically inactive RIPK3 D161N exhibited Caspase-8-dependent embryonic lethality [320]. Another possible treatment would be the blockade of MLKL (Necrosulfonamide, NSA), which is a compound that modifies Cys86 of human MLKL and inhibits its oligomerization. It has been suggested as a potential therapeutic for neurodegenerative diseases [267]. Furthermore, a new inhibitor (TC13172) targeting Cys86 of MLKL was recently shown to block the translocation of MLKL to cell membranes in cell lines [268].

In view of the refractory and life-threatening course, persistent immunodeficiency, and a risk of malignant transformation, an allogeneic hematopoietic stem cell transplant has been considered in P1. HSCT might cure immune and cytokine production defects, but not intrinsic epithelial defects, similar to NEMO-deficient patients [321]. The exact *in vivo* triggers perturbing epithelial integrity in mice or humans lacking Caspase-8 have not been defined. Our index patient P1 has been successfully transplanted with sustained remission regarding immune and intestinal phenotypes post-transplant. However, P1 has developed polyarthritis after HSCT and therefore further studies are required to determine whether phenotypes in non-hematopoietic studies can be cured long-term by HSCT.

There is an urgent need for children with Caspase-8 deficiency to decipher the underlaying disease mechanisms and identify druggable phenotypes targeting both immune and epithelial defects.

8.7. Caspase-8 deficiency as model - what do we learn from monogenic diseases?

A plethora of diseases are associated with dysregulation of cell death and/or inflammation. Caspases have emerged as potential drug targets and Emricasan, the first caspase inhibitor for clinical use, has been tested for potential application in liver diseases [322], acute myeloid leukemia [323], and viral infections [324]. The beneficial effects of this therapeutic approach in patients still remains unclear. Suppression of Caspase-8 expression or activity might be considered in conditions of severe or refractory diseases. However, our data emphasize that human Caspase-8 has pleiotropic apoptotic and non-apoptotic functions in specific cellular contexts and thus warrants awareness about potential toxicities of therapeutic strategies targeting Caspase-8. In particular, our studies have shown that Caspase-8 deficiency is

associated with enhanced necroptosis that might cause pro-inflammatory phenotypes. It has been shown that necroptosis is involved in malignancies and pathological inflammatory conditions [325]. RIPK1 and RIPK3 could also serve as an attractive therapeutic target for diseases with increased necroptotic activity. However, our laboratory has recently also discovered VEO-IBD patients with germline *RIPK1* mutations. Human RIPK1 deficiency was associated with defective lymphocyte differentiation, increased inflammasome activity, and impaired TNFR1-mediated cell death responses in intestinal epithelial cells [326]. These studies have highlighted pleiotropic cell and tissue-specific functions of RIPK1 and therefore warrant awareness of potential toxicities of therapies targeting RIPK1.

Further mechanistic studies in advanced human preclinical models are required to provide critical understanding of imbalanced inflammation and cell death in VEO-IBD in the context of Caspase-8 deficiency. This knowledge on rare monogenic disease will hopefully help to optimize personalized treatment for children with devastating diseases but will also prioritize new targets for drug development of common autoimmune and autoinflammatory diseases.

9. References

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10. Annex

10.1. Table of figures