Aus der Kinderklinik und Kinderpoliklinik
im Dr von Haunerschen Kinderspital
der Ludwig-Maximilians-Universität München

Dissertation
zum Erwerb des Doctor of Philosophy (Ph.D.)
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Mechanistic insights into the role of RIPK1 and NOD2 in Inflammatory Bowel Disease

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Jahr 2023

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Datum der Verteidigung:

22.09.2023

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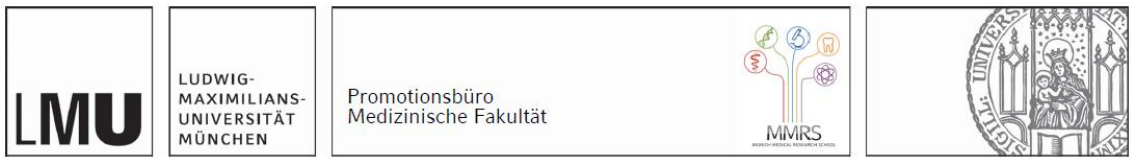

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Table of content

List of abbreviations

ALR	AIM2-like receptors
ATF6	activating transcription factor 6
ATG16L1	ATG16 Autophagy Related 16-Like 1
Bip	immunoglobulin-heavy-chain-binding protein
CARD	Caspase Recruitment Domain Family Member
Cas9	CRISPR associated protein 9
CD	Crohn's disease
CGD	chronic granulomatous disease
CHOP	C/EBP homologous protein
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
CTLA4	cytotoxic T-lymphocyte antigen 4
CYLD	cylindromatosis
DAMP	danger-associated molecular patterns
DD	death domain
DSS	dextran sulfate sodium
DTT	dithiothreitol
EGF	epidermal growth factor
ER	Endoplasmic reticulum
FADD	Fas Associated Via Death Domain
FOXP3	Forkhead Box P3
FUT2	Fucosyltransferase 2
GSDMD	gasdermin D
GVHD	graft-vs-host disease
GWAS	genome wide association studies
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
iE-DAP	gamma-D-glutamyl-meso-diaminopimelic acid
IKBa	NF-kB inhibitor-a
IKK	IkB kinase
IL	Interleukin

iPSC	induced pluripotent stem cell
IRE1α	inositol requiring 1α
IRF3	Interferon response factor 3
JAK	Janus kinase
LPS	lipopolysaccharide
LRR	leucine-rich repeats
LUBAC	linear ubiquitin chain assembly complex
MDP	muramyl dipeptide
MyD88	myeloid differentiation primary response 88
Nec-1s	Necrostatin-1s
NEMO	NF-kB essential modulator
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain protein
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PERK	PRKR-Like Endoplasmic Reticulum Kinase
PRR	pattern recognition recep
RA	rheumatoid arthritis
RHIM	RIP homotypic interaction motif
RIPK	receptor interacting protein kinase
RLR	RIG-I-like receptors
S1P	site-1 protease
S2P	site-2 protease
SCID	severe combined immunodeficiency
STAT	signal transducer and activator of transcription proteins
TAK1	transforming growth factor-b-activated kinase 1
TBK1	TANK binding kinase 1
TIR	Toll/IL-1 receptor
TLR	Toll-like receptors
TRADD	TNFRSF1A Associated Via Death Domain
TRAF	TNF receptor-associated factor
TTTC7A	Tetratricopeptide Repeat Domain 7A
TYK2	Tyrosine Kinase 2
UC	ulcerative colitis

UPR	unfolded protein response
VEO-IBD	very-early-onset inflammatory bowel disease
WAS	Wiskott-Aldrich Syndrome
WES	whole exome sequencing
XBP1	X-box-binding protein 1
XIAP	X-Linked Inhibitor Of Apoptosis
ZBP1	Z-DNA Binding Protein 1

List of publications

Manuscript I:

Li, Y.[*], Führer, M.[*], Bahrami E.[*], Socha, P., Klaudel-Dreszler, M., Bouzidi, A., Liu, Y., Lehle, A. S., Magg, T., Hollizeck, S., Rohlfs, M., Conca, R., Field, M., Warner, N., Mordechai, S., Shteyer, E., Turner, Boukari., R, Belbouab., R, Walz., C, Gaidt, M. M., Hornung, V., Baumann, B., Pannicke, U., Al Idrissi, E., Ali Alghamdi, H., Sepulveda, F. E., Gil, M., de Saint Basile, G., Honig, M., Koletzko, S., Muise, A. M., Snapper, S. B., Schwarz, K., Klein, C., Kotlarz, D. (2019). **Human RIPK1 Deficiency Causes Combined Immunodeficiency and Inflammatory Bowel Diseases**. *Proc Natl Acad Sci U S A* 116, 970-975. *** equal contribution.**

Manuscript II:

Ghalandary, M.[*], **Li, Y.**[*]. Frohlich, T., Magg, T., Liu, Y., Rohlfs, M., Hollizeck, S., Conca, R., Schwerd, T., Uhlig, H. H., Bufler, P., Koletzko, S., Muise, A. M., Snapper, S. B., Hauck, F., Klein, C., Kotlarz, D. (2022). **Valosin-containing protein-regulated endoplasmic reticulum stress causes NOD2-dependent inflammatory responses**. *Sci Rep* 12: 3906. *** equal contribution.**

Manuscript III:

Li, Y., Klein, C., Kotlarz, D. (2019). **Dysregulation of Cell Death in Chronic Human Inflammation**. *Cold Spring Harbor perspectives in biology* 12(7):a037036

1. Contribution to the publications

1.1 Contribution to paper I

Li, Y.*, Führer, M.*, Bahrami E.*, Socha, P., Klaudel-Dreszler, M., Bouzidi, A., Liu, Y., Lehle, A. S., Magg, T., Hollizeck, S., Rohlfs, M., Conca, R., Field, M., Warner, N., Mordechai, S., Shteyer, E., Turner, Boukari., R, Belbouab., R, Walz., C, Gaidt, M. M., Hornung, V., Baumann, B., Pannicke, U., Al Idrissi, E., Ali Alghamdi, H., Sepulveda, F. E., Gil, M., de Saint Basile, G., Honig, M., Koletzko, S., Muise, A. M., Snapper, S. B., Schwarz, K., Klein, C., Kotlarz, D. (2019). **Human RIPK1 Deficiency Causes Combined Immunodeficiency and Inflammatory Bowel Diseases**. *Proc Natl Acad Sci U S A* 116, 970-975. *** equal contribution.**

In this paper, we have identified RIPK1 deficiency as a novel monogenic cause of primary immunodeficiency and inflammatory bowel disease (IBD). For this study, I have (i) conducted segregation analysis of *RIPK1* mutants, (ii) assessed RIPK1 protein expression in patient cell lines, (iii) cloned the patients' mutations into lentiviral vectors, (iv) generated multiple stable cell lines (HCT116, HT-29, BLaER1, and Jurkat cell lines) overexpressing wildtype and mutant RIPK1 by lentiviral transduction and fluorescence activated cell sorting, (v) performed luciferase assays to assess the activity of NF-κB in HCT116 cells expressing patient mutations, (vi) performed immunoblotting to assess NF-κB and MAPK signaling in cells expressing RIPK1 variants upon TNF stimulation, (vii) analyzed inflammasome activation in BLaER1 monocytoid cells by immunoblotting and ELISA, as well as (viii) assessed cell death responses in heterologous HT-29 and Jurkat cell lines by immunoblotting and flow cytometry. Furthermore, I analyzed the experimental data, prepared the figures, and wrote the draft of the manuscript together with my supervisors. In addition, I took part in the submission and revision of the manuscript.

1.2 Contribution to paper II

Ghalandary, M.*, **Li, Y.***, Frohlich, T., Magg, T., Liu, Y., Rohlfs, M., Hollizeck, S., Conca, R., Schwerd, T., Uhlig, H. H., Bufler, P., Koletzko, S., Muise, A. M., Snapper, S. B., Hauck, F., Klein, C., Kotlarz, D. (2022). **Valosin-containing protein-regulated endoplasmic reticulum stress causes NOD2-dependent inflammatory responses**. *Sci Rep* 12: 3906. *** equal contribution.**

In this study we have identified a rare homozygous missense mutation in the first CARD domain of *NOD2* in a patient with immunodeficiency and enteropathy and identified ATPase valosin-containing protein (VCP) as novel interaction partner of NOD2. In particular, I performed experiments for the revision to (i) assess the ubiquitination and phosphorylation of RIPK2 in response to MDP stimulation by immunoprecipitation, (ii) determine IL-8 and TNF production upon MDP and tunicamycin stimulation by qPCR and ELISA, (iii) analyze the chemokine production profile (TNF, IL-6, CXCL1, CXCL2) upon tunicamycin stimulation, (iv) confirm the endogenous interaction of VCP and NOD2 by immunoprecipitation with an antibody to VCP and co-precipitation of NOD2 in HCT116 cell line with or without KO of NOD2, (v) study the effect of VCP knockdown on NOD2-RIPK2 interaction and RIPK2 activation by immunoprecipitation of Flag-NOD2 upon stimulation with L18-MDP in HCT116 cells with or without knockdown of VCP, and (vi) dissect different arms of the UPR responses (PERK, eIF2α, XBP1) upon stimulation of tunicamycin and thapsigargin in cells with knockdown of VCP. In addition, I analyzed the experimental data, revised the figures, and edited the manuscript as well as the rebuttal letter.

1.3 Contribution to paper III

Li, Y., Klein, C., Kotlarz, D. (2019). **Dysregulation of Cell Death in Chronic Human Inflammation**. *Cold Spring Harbor perspectives in biology* 12(7):a037036

In this paper, I wrote the manuscript with my supervisors, reviewing dysregulated cell death programs in the context of chronic inflammation and monogenic diseases, and drew the figure featuring TNF-mediated signaling.

2. Introductory summary

Inflammatory bowel disease (IBD) is a chronic relapsing disorder in the gastrointestinal tract [1]. Children with very-early-onset inflammatory bowel disease (VEO-IBD) often show life-threatening conditions refractory to conventional treatment [2, 3]. IBD are complex multifactorial disorders and the exact disease mechanisms and triggers are not fully understood.

In my PhD thesis, I evaluated genetic causes and risk factors of VEO-IBD identified through a whole exome sequencing screen on one of the largest international patient cohorts.

First, I have reported receptor interacting serine/threonine-protein kinase 1 (RIPK1) deficiency as novel monogenic disorder presenting with IBD and/or primary immunodeficiency. Rare biallelic loss-of-function mutations in *RIPK1* were detected in eight patients from six unrelated families [4]. These mutations led to reduced protein expression of RIPK1, impaired NF-κB activity, defective lymphocyte differentiation, altered activation of inflammasome, and impaired TNFR1-mediated cell death responses in intestinal epithelial cells [4]. Our study highlighted the crucial role of RIPK1 in controlling inflammation and immunity in humans, and provided critical insights into therapeutic strategies targeting RIPK1.

Second, I have characterized a rare homozygous missense mutation in the first CARD domain of nucleotide binding oligomerization domain containing 2 (*NOD2)* in a patient with immunodeficiency and enteropathy [5]. The mutation was associated with impaired NOD2-dependent signaling and subsequent production of proinflammatory cytokines in patient's primary cells and cellular models [5]. Of note, our study also revealed that ATPase valosin-containing protein (VCP) as a novel interaction partner of wildtype NOD2, while the interaction was abrogated with the mutated NOD2 [5]. Functional assays showed that knockdown of VCP lead to impaired inflammatory responses upon challenge with muramyl dipeptide (MDP) but enhanced tunicamycin-induced ER stress accompanied by increased cytokine and chemokine expression, which was abolished in the absence of *NOD2* [5]. This study has broadened the IBD disease spectrum and complemented our understanding of NOD2-mediated inflammatory signaling pathway.

My studies elucidating the pathomechanisms of RIPK1 and NOD2 deficiency on molecular and cellular level might help to develop novel treatment and diagnostic strategies for children with devastating VEO-IBD as well as other immune-related disorders.

Chapter 1. Inflammatory bowel disease - general introduction

Inflammatory bowel disease (IBD) is a chronic and/or relapsing inflammatory condition of the gastrointestinal tract, which can be classified as Crohn's disease (CD), ulcerative colitis (UC) or IBD unclassified [6]. The disorder typically manifests in early adulthood, but the incidence of pediatric onset IBD has been increasing worldwide [6].

Common symptoms of IBD usually involve abdominal pain, diarrhea, bloody stool, reduced appetite, unintended weight loss and fatigue [1]. Ulcerative colitis causes long-lasting, uniform and confined inflammation with ulcerations in the colon and rectum [7], whereas Crohn's disease manifests as a non-continuous transmural inflammation that may affect the whole digestive tract from mouth to the anus [8].

Environmental factors have been implicated in triggering IBD as the disease was previously more frequently found in industrialized countries [9]. With an emerging incidence recorded in developing countries since the twenty-first century [10], the pathogenesis of IBD has been recognized as complex interplay of multiple factors, such as genetic composition, immune dysregulation, epithelial barrier dysfunction, disturbed intestinal microbiota, as well as environmental risks [11].

IBD is a chronic and progressive disease that needs early intervention to prevent complications. Conventional therapies for IBD rely on controlling the disease symptoms through enteral nutrition or pharmacological therapies such as immune modulators (e.g., thiopurines, methotrexate, calcineurin inhibitors), aminosalicylates, or surgical interventions. Since conventional treatment may cause severe side effects and a large fraction of patients show a refractory course [12], there has been increasing interest in developing new therapies focusing on biologics (e.g., anti-TNF, anti-IL-12/IL-23, and anti-integrin therapies) and small molecules (e.g. JAK inhibitors) [13]. There are also alternative approaches that modulate the microbial balance/symbiosis by antibiotics, probiotics, prebiotics, postbiotics, symbiotics, and fecal microbiota transplantation to improve the composite of the intestinal microbiota [13].

Chapter 1.1. Very early onset inflammatory bowel disease

About 14 in 100,000 children have disease onset under the age of six years, which is defined as very early onset IBD (VEO-IBD) [14]. Notably, children with VEO-IBD more frequently present with predilection of colitis and higher incidence of severe fistulizing or perianal abscesses, resistance to immunosuppressive therapies, as well as increased lethality [2, 3]. It is difficult to precisely predict disease course and responses to therapies for VEO-IBD patients according to current classifications and researchers are seeking new avenues to treat the disease. Twin studies and the early onset of the disease suggested genetic predisposition as a critical factor for the development of VEO-IBD [15-17]. A paradigmatic study has identified IL-10R deficiency as the first truly monogenic cause of IBD [16] and allogeneic hematopoietic stem cell transplantation (HSCT) has been proven to be a novel therapeutic approach for VEO-IBD [17]. Since then, more than 100 monogenic entities have been attributed to IBD-causing genetic signatures [18]. These studies have highlighted the importance of genetic diagnosis to VEO-IBD patients and suggested that monogenic IBD represent promising models to study the key factors controlling immunological homeostasis in the intestine.

Chapter 1.2. Genetic contribution to inflammatory bowel disease

The genetic contribution to IBD has been first studied through twin studies and family-based linkage analysis [19]. The offspring of two affected parents showed more than 30 % chance of developing IBD [20-22] and positive family history has been more commonly observed in CD than UC patients [20]. Familial studies support the contribution of genetic factors to prognosis since similar patterns of disease behaviors are often observed within families [23, 24]. The approximated risk of developing IBD is 1.6% for the first-degree relatives of an UC patient,

and 5% for that of a CD patient in North American and European white non-Jewish populations [21, 25, 26], whereas in Jewish patients these risk probabilities are 5.2% and 8%, respectively [26]. Of note, recent genome wide association studies (GWAS) have identified more than 230 susceptibility loci for IBD, which have facilitated our understanding of genetic contributions to the diseases [27]. Furthermore, more rare genetic disorders could be detected benefiting from the advances of genomic sequencing technologies [28].

Chapter 1.2.1. GWAS studies in inflammatory bowel disease

For two decades, GWASs that screen the whole genome region for associations between disease status and common variants in large case-control cohorts have provided unprecedented insights into the pathogenesis of IBD [29]. Notably, *NOD2* has been first successfully identified as a susceptibility gene for CD in 2001 [30], followed by the description of other susceptible genes with more than 230 enriched risk loci, such as *Autophagy Related 16-Like 1* (*ATG16L1*) [31], *Interleukin* (*IL*) *23R* [32], *Caspase Recruitment Domain Family Member 9* (*CARD9*) [33], *Fucosyltransferase* (*FUT2*) [34], and *Tyrosine Kinase 2* (*TY2)* [35]. GWAS has highlighted multiple biological processes as crucial gatekeepers for intestinal homeostasis and disease development, such as autophagy, epithelial barrier function, reactive oxygen species (ROS) generation, epithelial restitution, microbial sensing and defense, endosplasmic reticulum stress, as well as regulation of innate and adaptive immunity [36, 37] (Figure 1).

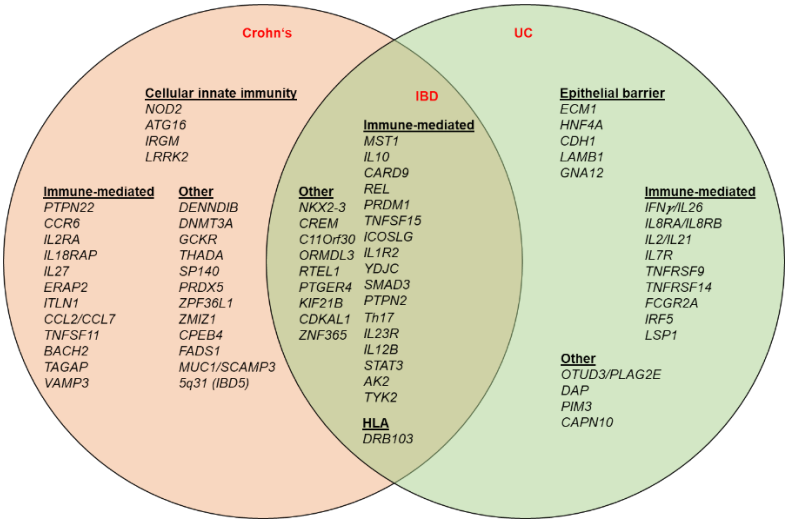

Figure 1. *GWAS identified risk loci associated with UC and CD [38].*

Chapter 1.2.2. Whole exome sequencing - a tool to identify monogenic causes for IBD

Whole exome sequencing (WES) has arisen as a strategy to identify rare monogenic Mendelian disorders [39]. Since whole exome sequencing has become the gold standard for identifying novel pathogenic mutations, an emerging number of novel candidate genes responsible for monogenic IBD have been identified in VEO-IBD patients such as *X-Linked Inhibitor of Apoptosis* (*XIAP*) [40], *Tetratricopeptide Repeat Domain 7A* (*TTC7A*), [41], *Spleen tyrosine kinase* (*SYK*) [42], *Syntaxin-Binding Protein 3* (*STXBP3*) [43], *Transforming growth factor beta 1* (*TGFB1*) [43], *caspase-8* [4], and *Receptor-interacting serine/threonine-protein kinase 1* (*RIPK1*) [4, 44]. To date, more than 80 monogenic entities have been characterized [45]. These monogenic disorders can be classified into different categories according to their biological mechanisms: immune dysregulation (e.g., IL-10 signaling defects), hyperinflammatory and autoinflammatory disorders, phagocytic defects, T- and B-cell defects, defects in the epithelial barrier, and others [46, 47] (Figure 2).

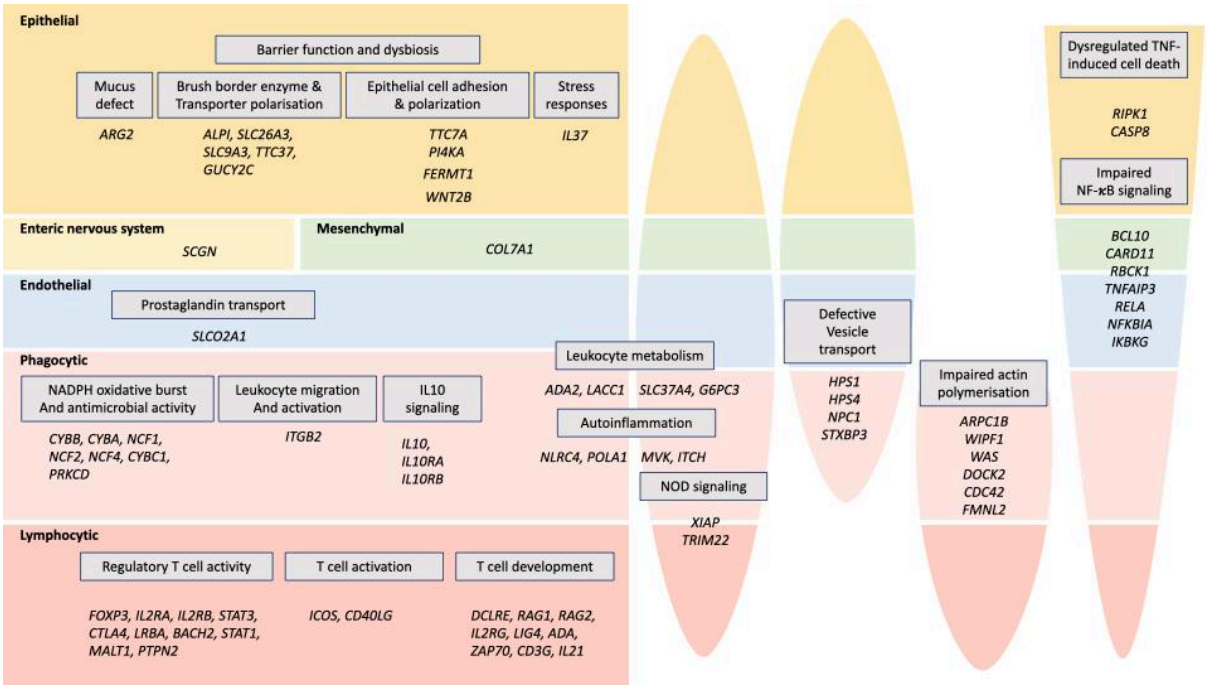

Figure 2. *An integrated taxonomy of monogenic IBD [45]*

Chapter 2. Key pathways in innate immunity underlying IBD pathology

Chapter 2.1. Innate microbe-sensing pathways

The human colon is constitutively colonized by microbiota. Some intestinal bacteria provide beneficial effects and are thus referred to as symbiotic microorganisms, whereas others can be detrimental and disease triggering, known as pathogens [48]. Recognition and distinction between pathogenic and symbiotic microorganisms by the innate immune system are crucial in maintaining intestinal homeostasis, and a disturbed interaction can lead to intestinal inflammation [49].

Innate immune recognition relies on distinct molecular structures of microorganisms [50]. The innate immune cells (e.g., monocytes, macrophages, NK cells, neutrophils, and dendritic cells) are able to recognize a large variety of microorganisms by diverse pattern recognition receptors (PRR) that can sense and interact with pathogen-associated molecular patterns (PAMP) or danger-associated molecular patterns (DAMP) [50]. Classical PRRs include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and NOD-like receptors (NLRs) [50] (Figure 3).

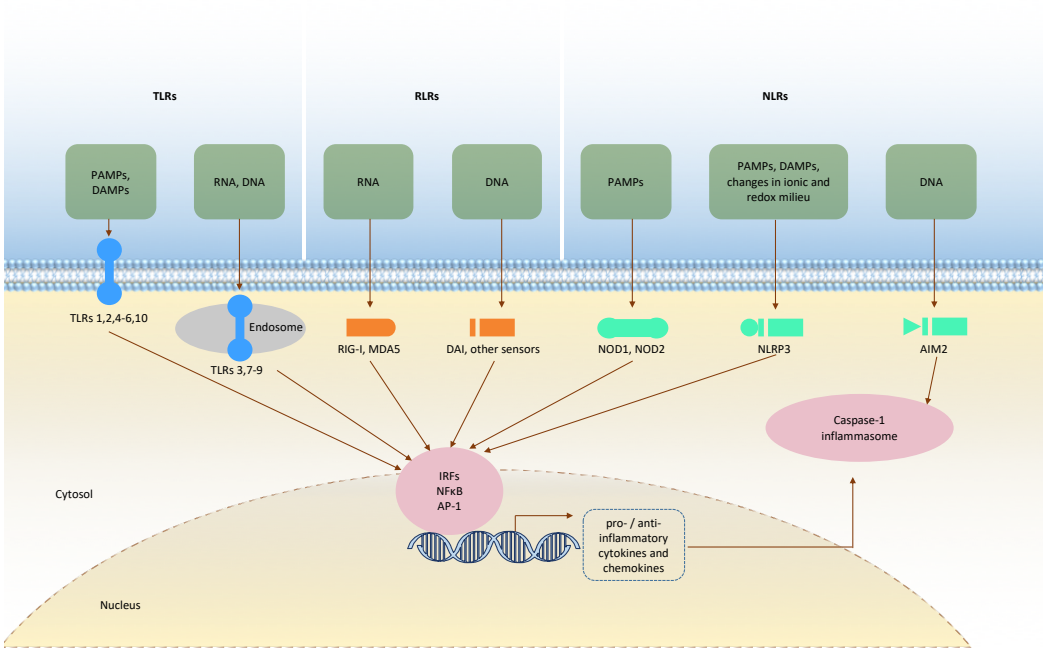

Figure 3. *A brief overview of sensors in the innate immune system including TLRs (Toll-like receptors, blue), RLRs (retinoid acid inducible gene-I-like receptors, orange), NLRs (nucleotide-binding and oligomerization domain-like receptors, green), as well as the corresponding ligands (dark green) and downstream signaling pathways (pink).*

Chapter 2.2. Toll-like receptors

TLRs are the first identified and best characterized group of PRRs [51]. These transmembrane receptors on innate immune cells are crucial in sensing PAMPs from various bacterial products [52]. Except for an ectodomain with leucine-rich repeats (LRRs) that recognize PAMPs, they usually also consist of a transmembrane domain and a signal-transducing Toll/IL-1 receptor (TIR) domain in the cytoplasm [52, 53]. Upon binding to PAMPs, TLRs recruit TIR domain-containing adaptor proteins such as TIR-domain-containing adaptor-inducing interferon-β (TRIF) and Myeloid differentiation primary response 88 (Myd88), and subsequently activate different downstream pathways [54]. While TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are localized on the cell surface, TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are localized to intracellular compartments and can recognize nucleic acids derived from viruses and bacteria [53, 55]. For example, (i) TLR2 works together with TLR1 or TLR6 to recognize a vast variety of bacterial components such as lipoproteins, lipotechoic acids, zymosan, and peptidoglycans [56], (ii) TLR4 is crucial for recognizing the gram-negative bacterial cell wall component lipopolysaccharide (LPS) [54], and (iii) TLR5 recognizes flagellin [57].

TLR signaling plays critical roles in multiple aspects. Notably, TLRs have been shown to be crucial sensors for mediating immune responses against microbes in the gut and their dysfunctions might trigger disease-associated inflammation. Emerging evidence has highlighted the TLR-mediated signaling as a critical player in the pathogenesis and a potential therapeutic target for several inflammatory diseases such as sepsis, rheumatoid arthritis, and systemic lupus erythematosus [58].

Chapter 2.3. NOD-like receptors

NOD-like receptors (NLRs) are a large protein family of intracellular sensors. They usually share a common central nucleotide-binding oligomerization domain (NOD) and a LRR-repeat region, but each member has a distinct N-terminal effector domain [59, 60]. In humans, 22 members of the NLR family have been described so far. According to their specific functions, NLRs are classified as adaptors (e.g., NLRP3, NLRC4, and NLRP6), regulators (e.g., NLRP1,

NLRC3, NLRC5 and NLRP4) and receptors (e.g., NOD1 and NOD2) [61]. Upon binding to microbial structures such as peptidoglycan or muramyl dipeptide (MDP), the auto-inhibitory LRR domain undergoes conformational changes resulting in the formation of large macromolecular scaffolds, which allow for the interaction with ASC. Upon subsequent recruitment of Caspase 1, multimeric complexes, called inflammasome, will be formed. While some NLRs can directly recruit caspases and form inflammasomes through scaffolding, other NLRs need to first activate transcription including NF-κB-, interferon regulatory factor (IRF)-, and mitogen-activated protein kinase (MAPK)-mediated signaling [62]. Mutations and polymorphisms in NLRs have been associated with various immune disorders such as IBD, multiple sclerosis, and asthma [63].

Chapter 2.4. NLRP3 inflammasome

The NLRP3 inflammasome is the best characterized inflammasome signaling complex. This multiprotein complex consists of the NLRP3 sensor protein, the adaptor, apoptosis-associated speck-like protein (ASC, also called PYCARD), and the effector protein, Caspase 1 [64]. Upon activation, NLRP3 forms oligomers through the central NACHT domains by homotypic interaction, followed by sequential recruitment of ASC and Caspase-1 to the macromolecular platform [64]. Upon self-cleavage and activation, the activated caspase-1 subsequently cleaves pro-IL-1β, pro-IL18, and Gasdermin D (GSDMD) into mature forms causing pore formation in the cell membrane and induction of pyroptosis [64] (Figure 4).

The NLRP3 inflammasome is a critical component to maintain homeostasis in the host immune system. Gain-of-function mutations in NLPR3 result in dysregulated inflammasome activity and have been shown to cause a rare inherited autoinflammatory condition, called cryopyrin-associated periodic syndrome (CAPS) [65]. In the context of IBD, a single nucleotide polymorphism (e.g., rs10754558) has been more frequently detected in UC patients than healthy controls [66]. Moreover, abnormal inflammasome activity has been observed in *in vivo* colitis models and IBD patients [67]. Of note, NLRP3-deficient mice showed defective epithelial integrity, leukocyte infiltration, as well as reduced anti-inflammatory cytokine production, causing severe colitis upon DSS challenge [68, 69].

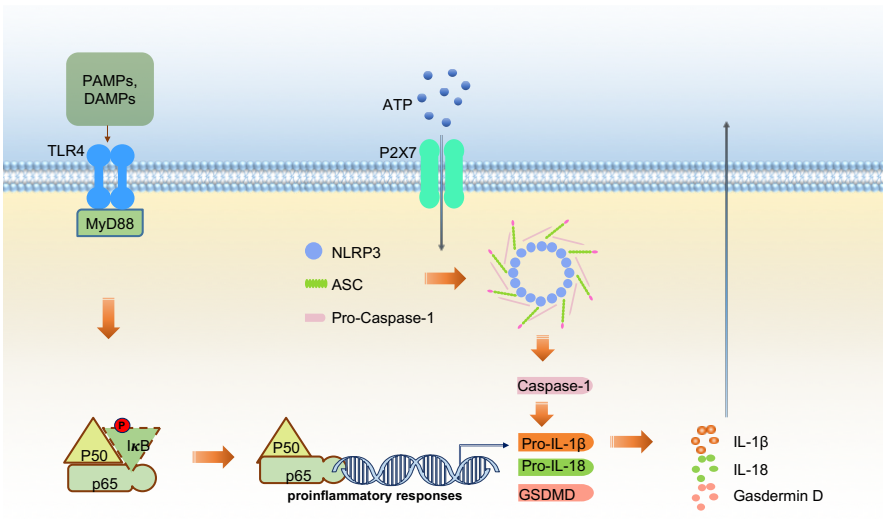

Figure 4. *Activation of NLRP3 inflammasome includes transcription of key components (e.g., pro-IL1B through NF-κB pathway,) and the assembly of the NLRP3 inflammasome comprising NLRP3, ASC, and Pro-caspase-1.*

Chapter 3. RIPK1 – A key molecule controlling immunity and intestinal homeostasis

Receptor-interacting serine threonine kinase1 (RIPK1) was first discovered for its ability to interact with the apoptosis-inducing death receptor FAS [70]. The RIPK1 protein consists of a carboxy-terminal death domain (DD), a RIP homotypic interaction motif (RHIM), and a kinase domain (KD) (Figure 5). In addition to Fas, the death domain can also bind to intracellular DD

of other proteins (e.g., TNFR1, TNFRSF1A associated via death domain (TRADD), as well as Fas associated via death domain (FADD)) [70-73]. RHIM mediates the interaction with other RHIM-containing proteins like RIPK3 [74], TRIF [75, 76] and Z-DNA Binding Protein1 (ZBP1) [77]. DD and RHIM have been both implicated in the cell death machinery [78]. On the other hand, the kinase domain is critical for its kinase activity and engages in signaling transduction [79-83].

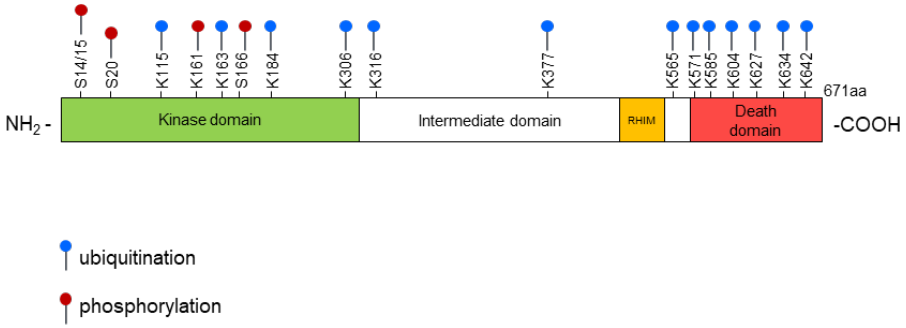

Figure 5. *Domain structure of human RIPK1 indicating the amino-terminal kinase domain (green), RIP homotypic interaction motif (RHIM; orange), and a carboxy-terminal death domain (DD; red). Autophosphorylation of S166 is crucial for the activation of the kinase domain, whereas ubiquitination on K377 and cleavage on D324 by caspase-8 could limit the activation of the kinase activity. In contrast, the function of ubiquitination on the death domain remains unclear.*

Chapter 3.1. The central role of RIPK1 in TNF signaling

RIPK1 mediates TNFR1 signaling in various cell types and context-dependent manners. Multimodal TNF signaling can induce NF-κB nuclear translocation and is critically implicated in promoting cell survival and inflammation, as reviewed in [84]. When TNF binds to trimeric TNFR1, they form a membrane-associated complex I containing the following proteins: TRADD, TRAF2, cIAP1/2, RIPK1, and Linear Ubiquitin Chain Assembly Complex (LUBAC) [78, 85, 86]. Posttranslational modification of RIPK1 (phosphorylation, Lys63-linked polyubiquitination and Met1-linked ubiquitination) and possibly other complex I components activates TGF-β-activated kinase 1 (TAK1) and IκB kinase (IKK) [78, 85, 87], which leads to translocation and activation of NF-κB signaling [85]. Alternatively, deubiquitylation of RIPK1 and TRAF2 can disrupt proinflammatory NF-κB signaling. This is mediated by A20- and cylindromatosis (CYLD)-induced disassembly of complex I [78, 87, 88], which compromises the pro-survival signaling and in turn induces the formation of complex IIa or IIb. The complex IIa contains TRADD, FADD, RIPK1, and pro-caspase-8 [78] and mediates apoptosis through subsequent cleavage of caspase-3 and caspase-7 [85, 87]. When complex IIa is inhibited, necroptosis will be initiated by the formation of complex IIb. RIPK1 recruits and activates RIPK3 to form oligomers [89, 90]. The oligomerized and autophosphorylated RIPK3 recruits and phosphorylates MLKL [91], which then forms large oligomers that translocate to the cell membrane and induce cell rupture [92] (Figure 5).

Chapter 3.2. RIPK1 in TLR signaling

TLRs are transmembrane proteins that detect distinct PAMPs derived from pathogenic microorganisms [52]. It has been shown that RIPK1 can be recruited to TLR3 and TLR4 [78]. While TLR3 detects double-strand RNA in the endosomal compartment, TLR4 detects bacterial LPS at the cell surface and in the endosome [75]. Most TLRs use MyD88 and TRIF as adaptor proteins to initiate downstream signaling and proinflammatory responses [54]. Upon binding to TRIF, RIPK1 can initiate IFN-β signaling [93], or bind to the E3 ubiquitin ligase TRAF6 resulting in TAK1- and LUBAC-dependent activation of IKK [75, 94-99] (Figure 6).

Furthermore, Ripk1 could protect inflammasome hyperactivation as fetal liver-derived macrophages from *Ripk1*-deficient mice exhibited enhanced inflammasome activity when engaged with LPS [78, 100].

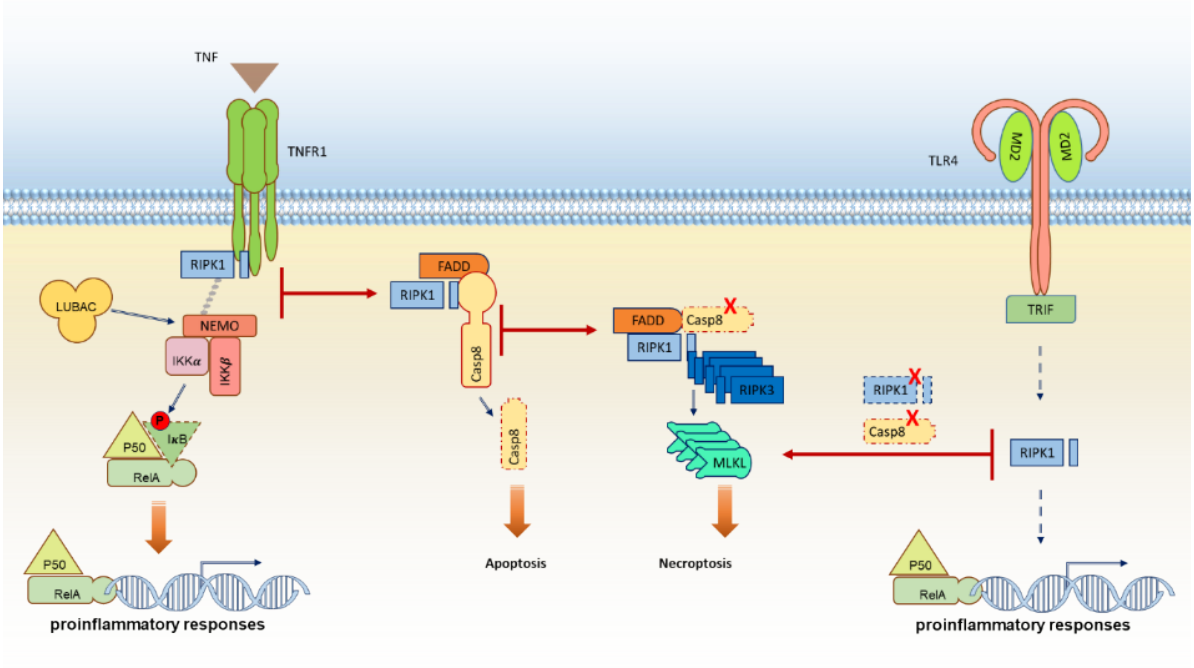

***Figure 6**. Overview of RIPK1-mediated signaling. RIPK1 plays an important role in mediating the activation of TNF-induced NF-κB inflammatory pathway, apoptosis and necroptosis. TLR4 detects lipopolysaccharide (LPS) on the cell surface, undergoes endocytosis and recruits the adaptor TRIF. Subsequently, TRIF recruits RIPK1 and induces the activation of NF-κB and MAPK pathways.*

Chapter 3.3. The role of RIPK1 in immunity

In RIPK1-deficient mice, perinatal lethality has been observed in association with multiorgan hyperinflammation due to aberrant apoptosis (Caspase-8-dependent) and necroptosis (MLKL-dependent) [78, 93, 101-103]. In addition, loss of *Ripk1* in mouse intestinal epithelial cells (IECs) resulted in severe inflammation in the gut due to increased apoptosis (FADD/Caspase-8-dependent) [78, 104, 105], whereas keratinocyte-specific knockout (KO) of *Ripk1* resulted in skin inflammation associated with *ZBP1/RIPK3/MLKL*-dependent necroptosis [78, 106]. Moreover, in immunodeficient recipient mice, *Ripk1*-deficient fetal liver cells showed less advantages to reconstitute the T-cell compartment, suggesting an essential role of RIPK1 in T cell development [78, 107, 108]. Accordingly, T cell-specific deletion of *Ripk1* in mice caused severe lymphopenia and defective T-cell proliferation, possibly due to increased apoptosis [78, 109]. Furthermore, RIPK1 also contributes to B cell development, as the number of peripheral B cells declined in immunodeficient recipient mice upon transfer of *Ripk1*$^{-/-}$ fetal liver cells [109]. In addition, proliferation responses of *Ripk1*$^{-/-}$ B cells were significantly reduced when stimulated with ligands for TLR-2, TLR-3, and TLR-4, possibly due to enhanced necroptosis [107].

Chapter 3.4. RIPK1 - a potential therapeutic target for inflammatory diseases

As TNFR1-mediated RIPK1 signaling is one of the most thoroughly studied inflammatory pathways, targeting of RIPK1 was considered to be a promising alternative treatment to anti-TNF blockade in some autoimmune pathologies. RIPK1 kinase activity has been associated with autoimmune and neurodegenerative conditions in previous studies, and experiments with small molecules aiming to inhibit RIPK1 kinase activity showed high efficacy in various animal

disease models [110-114]. As RIPK1 has been proposed to have a wider range of pro-inflammatory functions which are not only restricted to TNF signaling, RIPK1 blocking is expected to provide more benefits compared to anti-TNF treatment [114]. Moreover, RIPK1 inhibitors were shown to be safe in treating central nervous system inflammation [114, 115].

The small-molecule inhibitor Necrostatin-1s (Nec-1s) was the first to be developed to target RIPK1 kinase activity [114]. So far Nec-1s has been widely used to study RIPK1 mechanisms in cell death and inflammation as well as a wide range of diseases in animal models such as sepsis and the systemic inflammatory response syndrome [114, 116-120]. In addition, other RIPK1 inhibitors are also being evaluated for the treatment of various human diseases in clinical trials. DNL747, a brain-penetrant RIPK1 inhibitor, is currently in clinical trial phase Ib/IIa for amyotrophic lateral sclerosis (ALS) [114]. Furthermore, GSK´772 is in clinical trials for treating peripheral autoimmune diseases including UC, psoriasis, and rheumatoid arthritis (RA) [10, 114]. However, in view of pleiotropic cell- and context-specific functions of human RIPK1, awareness should be raised regarding potential toxicities of targeting RIPK1 [121].

Chapter 3.5. Characterization of human RIPK1 deficiency

By screening a large cohort of VEO-IBD patients, we have identified 8 patients with germline mutations in the *RIPK1* death domain or intermediate domain, associated with reduced protein expression in immune cells and fibroblasts [4]. These patients suffered from VEO-IBD and/or immunodeficiency [4]. Immunophenotyping suggested impaired T-cell maturation and B-cell class switch [4]. *In vitro* cellular models showed that cells expressing mutant RIPK1 presented with reduced responses to TNF-mediated NF-κB activity and cell death responses, but hyperactivation in response to LPS-mediated inflammasome signaling [4]. Inhibition of MLKL by small molecule inhibitors could attenuate the increased secretion of proinflammatory IL-1β [4, 44]. In parallel studies, Cuchet-Lourenco et al. reported patients with loss-of-function mutations in *RIPK1* who manifested life-threatening immunodeficiency defective lymphocyte differentiation, intestinal inflammation and/or arthritis [44]. Even though these authors suggested that the life-threatening disease could be curated by HSCT in a single patient [44], our study has rather argued for a non-redundant role of RIPK1 in controlling homeostasis in intestinal epithelial cells concluded by both HSCT and cellular assays [4]. Therefore, HSCT might rescue immune-related phenotypes, but not intrinsic intestinal defects of RIPK1 deficiency [4]. More recent studies have further expanded the spectrum of human *RIPK1* deficiency by demonstrating that heterozygous mutations affecting the cleavage of RIPK1 by caspase-8 cause early-onset periodic fever syndromes and severe intermittent lymphadenopathy associated with hypersensitivity to cell death [122, 123]. Taken together, these studies have complemented the research on transgenic mouse models by documenting the crucial role of RIPK1 in controlling human immune and intestinal homeostasis. Furthermore, these data suggested a critical genotype-phenotype correlation for monogenic *RIPK1* deficiencies and provided insights into the therapeutic efficacy and potential side effects of RIPK1 inhibitors. However, current studies could not yet provide a comprehensive view on the underlying disease mechanisms. Further studies are needed to elucidate whether IL-1 blockade or HSCT could be beneficial for patient management.

Chapter 4. NOD2 - A key molecule in sensing bacterial peptidoglycans

Nucleotide-binding and oligomerization domain containing protein 1 and 2 (NOD1 and NOD2) are NLRs that recognize gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) derived from bacteria, respectively [124].

NOD2 was the first gene to be firmly associated with IBD [33, 125-127]. NOD2 can sense MDP, a component from peptidoglycans derived from bacteria cell walls [128]. The NOD2 protein has two tandem N-terminal CARD domains that interact with downstream CARD-containing molecules, such as the receptor interacting protein kinase 2 (RIPK2) [129] (Figure

7). Activated RIPK2 subsequently facilitates the activation of NF-κB signaling, and initiates the transcription of inflammatory response genes [130, 131]. The IKK complex can also be activated by the transforming growth factor-β-activated kinase 1 (TAK1) followed by activation of MAP kinases and promotion of cell proliferation, differentiation and cell death [132, 133]. In addition, RIPK2 can also bind to TNF receptor-associated factor 3 (TRAF3) and activate TANK binding kinase 1 (TBK1) and IKKε, which further drives the phosphorylation and activation of Interferon response factor 3 (IRF3) for induction of IFN gene expression [134, 135]. Besides, NOD2 can also promote NLRP1-dependent IL-1β production upon bacterial infection [136, 137]. Independent of RIPK2 function, NOD2 can also recruit the protein ATG16L1 to the cell membrane for the initiation of autophagy upon bacterial encounter [138] (Figure 8).

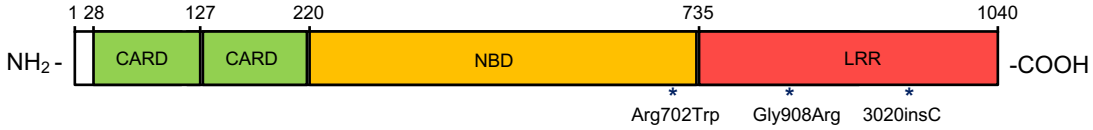

***Figure** 7. Structure of NOD2, including two caspase activation and recruitment domains (CARD; green), a nucleotide binding and oligomerization (NBD) domain (yellow), and leucine rich repeat (LRR) domain (red). Asteriks indicate three common IBD susceptibility loci identified by GWAS: R702W, G908R, and L1007fs.*

Chapter 4.1. NOD2 and Endoplasmic Reticulum stress

It has been suggested that endoplasmic reticulum (ER) stress-initiated unfolded protein responses (UPR) can induce inflammation [139]. The UPR can be initiated by receptors such as double-strand RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring 1α (IRE1α) [139]. The ER-luminal domains on these receptors sense the unfolded proteins and the cytosolic domains transmit signals causing transcriptional or translational attenuation [139]. At resting state, these receptors are sequestered by the ER chaperone protein immunoglobulin-heavy-chain-binding protein (BiP) [139]. Upon ER stress, BiP binds to unfolded proteins and releases these receptors resulting in activation of the UPR [139]. The activation process of PERK includes homodimerization and autophosphorylation, which then phosphorylates the eukaryotic translation-initiation factor 2α (eIF2α) and inhibits the assembly of the 80S ribosome; thereby halting the abundant protein synthesis [140]. Another arm activated under ER stress is autophosphorylation of IRE1α, which then executes its RNase function and removes a 26-base intron from the mRNA of X-box-binding protein 1 (XBP1), yielding a spliced active transcription factor [141]. Furthermore, ATF6 also dissociates from Bip and translocates to the Golgi apparatus, followed by cleavage by site-1 protease (S1P) and site-2 protease (S2P) and consequently enable it to migrate to the nucleus and start the transcription [141]. Downstream of the UPR, the C/EBP homologous protein (CHOP) will initiate apoptosis when homeostasis in the ER cannot be restored [141].

Apart from being a canonical cytosolic sensor for bacterial peptidoglycan ligands, NOD2 has also been associated to mediate ER-stress-induced inflammatory responses. For example, NOD2 activates the innate immune responses upon infection of influenza virus [135], which is known to trigger ER stress in host cells [142]. Furthermore, studies have shown that thapsigargin and dithiothreitol (DTT) can induce pro-inflammatory cytokine production (e.g., IL-6, TNF) in a NOD2-dependent way [143]. However, the specific mechanisms of how ER stress activates NOD1/2 remain elusive. It has also been proposed that NOD1 and NOD2 do not directly regulate ER stress-induced inflammatory responses, but possibly potentiate them

through other pathways that are activated by ER stress, which remain unidentified [144]. Therefore, more studies are needed to help understand the molecular mechanisms linking ER stress and NOD1/NOD2 activation, as well as their role in host defense and inflammatory diseases.

ER stress is associated with several inflammatory or autoinflammatory diseases such as diabetes [145], atherosclerosis [146], and CD [147]. Physiological UPR play an important role in intestinal homeostasis by regulating mucosal barrier function and modulating the microbiota [148, 149]. Several mouse studies have shown that abnormal UPR responses in intestinal epithelial cells are critically implicated in the pathogenesis of colitis. For example, knockout of IRE1 in mouse intestinal epithelial cells leads to increased ER stress and higher susceptibility to dextran sulfate sodium (DSS)-induced colitis [150]. Mice with conditional knockout of *Xbp1* in the intestinal epithelium have shown upregulated CHOP activity and developed spontaneous intestinal inflammation associated with loss of Paneth cells [147] [150]. Furthermore, *Atf6a* KO mice showed increased expression of CHOP in the colon epithelium associated with increased apoptosis [151]. *XBP1* polymorphisms in human have also been associated with both CD and UC [152-154].

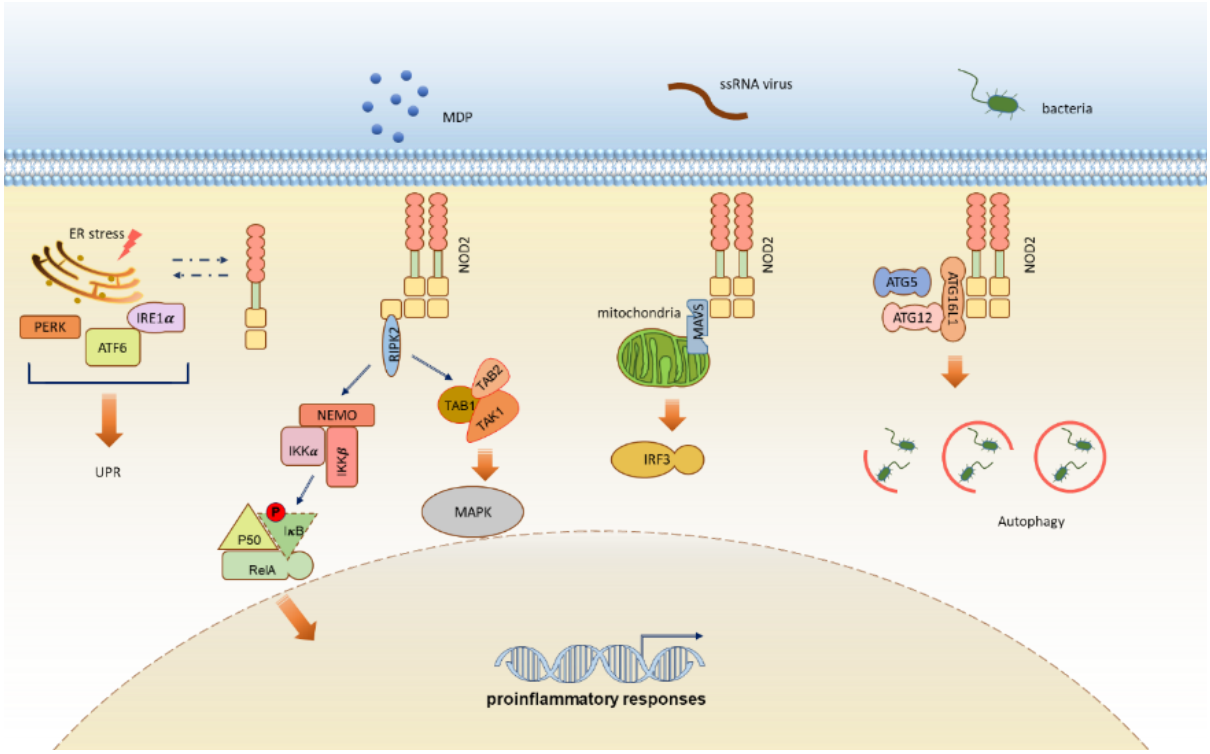

Figure 8. *Overview of NOD2-mediated signaling. MDP-mediated activation of NOD2 signaling first involves a conformational change and oligomerization of NOD2. Subsequently, NOD2 binds to the adaptor RIPK2 and results in the activation of NF-κB and MAPK signaling. In addition, viral ssRNA triggers NOD2 signaling independent of RIPK2. Instead, NOD2 interacts with MAVS, an adaptor protein associated with mitochondria, and activates the IRF3 which leads to type I interferon production. Furthermore, NOD2 can also interact with ATG16L1 and induce autophagy pathways. Moreover, NOD2 has also been shown to be associated with ER stress.*

Chapter 4.2. New evidence of NOD2-dependent ER stress-mediated inflammation in IBD

NOD2 is a crucial receptor in the innate immune system. Even though polymorphisms and mutations in *NOD2* have been associated with IBD for decades, the underlying disease mechanisms regarding the functional spectrum of NOD2 have been continuously expanded. Previously reported *NOD2* mutations in association with IBD are located near or in the LRR domain whose major function is ligand sensing and binding [5, 155]. In the second publication

of this thesis, we have identified a rare homozygous mutation in the *NOD2* CARD domain in a patient with enteropathy, uncovering a genotype-specific phenotype in human intestinal epithelial cells [5].

The patient with NOD2 p.E54K mutation showed reduced intracytoplasmic TNF production in PBMC-derived monocytes in response to MDP, while response to LPS was normal [5]. In addition, CD62L shedding was also reduced upon MDP stimulation in patient-derived neutrophils [5]. Correspondingly, patient PBMC showed impaired activation of NF-κB and MAPK signaling in response to MDP [5]. Further analysis in HEK293T and HCT116 cellular models suggested that the identified mutation p.E54K resulted in impaired expression of *IL8* upon stimulation of MDP and tunicamycin in contrast to the commonly reported mutation p.L1007fsX1008, [5]. Importantly, our studies on the p.E54K mutation have identified VCP as a novel interacting partner of NOD2 [5]. In heterologous cellular models, we could demonstrate that VCP represents a negative regulator of NOD2 activity during tunicamycin-induced ER stress, as silencing of VCP leads to increased NOD2-dependent expression of inflammatory cytokines and chemokines (e.g., *IL8*, *CXCL1*, *CXCL2*) [5].

Dysregulated UPR has been implicated in IBD [147, 150] and NOD2 was implicated in the regulation of ER stress [148]. However, the underlying mechanisms linking NOD2 function and ER stress remain largely unknown. Our findings provide a new mechanistic link between ER stress and altered NOD2-dependent proinflammatory cytokine responses in association with intestinal inflammation. Furthermore, we also uncovered a genotype-specific phenotype, as the p.E54K variant, but not the p.L1007fsX1008 variant in *NOD2*, showed altered *IL8* expression compared to WT cells [5].

Chapter 5. Outlook

Genetic studies on Mendelian disorders in IBD could provide rationale for available and new therapies such as the identification of targeted signaling and other biomarkers. Since the identification of IL-10R deficiency as the first truly monogenic cause of IBD, HSCT has become a standardized therapeutic approach for IBD patients with an underlying immune deficiency (e.g., *XIAP* deficiency, CGD, WAS) [156, 157]. However, the study of the disease mechanism is still challenging as the patient materials are not always accessible for multidisciplinary workups involving gastroenterology, immunology, and hematology, etc. In addition, the disease stages, medication, and confounding effects often interfere with the readouts. To overcome these difficulties, we have benefited from the development in the field of induced pluripotent stem cells (iPSC) and CRISPR/Cas 9 genomic editing. By using genetically engineered iPSC models, we have elucidated *LY96* (encoding MD2) as a novel genetic cause in patients with VEO-IBD [158]. The iPSC-derived macrophages with MD2 deficiency behaved physiologically identically with patient-derived macrophages as they both showed attenuated interaction with gram-negative bacteria [158]. This ground-breaking technology has unlimited potential for disease modeling as well as therapeutic screening.

Since a large fraction of patients do not show monogenic forms of IBD, a definitive diagnosis is still difficult, as the multifactorial pathophysiological complexity is yet to be fully understood on the molecular level. In this case, multi-omics analysis could provide a broader view on the genetic, environmental, microbiome, and immunological network using high-throughput data. Omics studies comprehensively study the gene and protein abundance as well as composition under cell-specific and context-specific conditions [159]. In addition, omics data facilitate the validation of identified genetic defects and targets. In the context of IBD, omics studies have already successfully identified new candidate biomarkers such as CXCL1, which showed low expression in normal or noninflamed mucosa, but high expression in inflamed CD mucosa [160].

Taken together, the described state-of-the-art techniques will enable depicting an integrative atlas of the disease mechanisms, predicting complications and progression, as well as guiding the therapeutic interventions to finally achieve personalized medicine.

3. Paper I

Title: Human RIPK1 deficiency causes combined immunodeficiency and inflammatory bowel diseases

Year: 2019

Journal: Proc Natl Acad Sci U S A

Volume: 116

Issue: 3

Pages: 970-975

DOI: 10.1073/pnas.1813582116

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Human RIPK1 deficiency causes combined immunodeficiency and inflammatory bowel diseases

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Edited by Daniel L. Kastner, National Institutes of Health, Bethesda, MD, and approved November 21, 2018 (received for review August 8, 2018)

Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is a critical regulator of cell death and inflammation, but its relevance for human disease pathogenesis remains elusive. Studies of monogenic disorders might provide critical insights into disease mechanisms and therapeutic targeting of RIPK1 for common diseases. Here, we report on eight patients from six unrelated pedigrees with biallelic loss-of-function mutations in *RIPK1* presenting with primary immunodeficiency and/or intestinal inflammation. Mutations in *RIPK1* were associated with reduced NF-κB activity, defective differentiation of T and B cells, increased inflammasome activity, and impaired response to TNFR1-mediated cell death in intestinal epithelial cells. The characterization of RIPK1-deficient patients highlights the essential role of RIPK1 in controlling human immune and intestinal homeostasis, and might have critical implications for therapies targeting RIPK1.

primary immunodeficiency | inflammatory bowel diseases | rare diseases

Significance

RIPK1 is a key signaling molecule controlling inflammation and cell death. Molecular targeting of RIPK1 is considered to be an attractive therapeutic strategy for inflammatory diseases or autoimmunity. However, the precise function of RIPK1 in human health and disease remains a matter of debate. Here, we report that human RIPK1 deficiency results in both immune and intestinal epithelial cell dysfunctions. Our studies provide insights into the pleiotropic functions of human RIPK1 and warrant awareness about potential toxicities of therapeutic strategies targeting RIPK1.

Single-gene inborn errors of immunity underlie diverse pathologies such as infection, allergy, autoimmunity, autoinflammation, and malignancy. Until now, the discovery of more than 350 monogenic immune disorders has opened unprecedented insights into genes and pathways orchestrating differentiation and function of the human immune system (1). Very early onset inflammatory bowel diseases (VEO-IBDs) may also result from inborn errors of immunity, as evidenced by IL-10R deficiency (2). Although the spectrum of monogenic disorders affecting the intestinal immune homeostasis has recently expanded, most patients with VEO-IBDs lack a genetic diagnosis. It is of great therapeutic relevance to define underlying genetic defects: Whereas disorders of the hematopoietic system can be cured by allogeneic hematopoietic stem cell transplantation (HSCT), intrinsic defects in epithelial or stromal cells require other therapeutic strategies. The discovery of patients with monogenic diseases highlights the functional relevance of genes and pathways, provides a basis for the development of targeted therapies for both rare and common diseases, and may add to a critical appraisal of anticipated effects or side effects of therapies (3).

The receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is a key signaling molecule controlling inflammation and cell death responses through both scaffolding- and kinase-specific

Author contributions: Y. Li, M. Führer, E.B., K.S., C.K., and D.K. designed research; Y. Liu, M.M.G., and V.H. contributed new reagents/analytic tools; Y. Li, M. Führer, and E.B. conducted experiments and analyzed the data; Y. Li, C.K., and D.K. wrote the paper; P.S., M.K.-D., A.B., S.M., E.S., D.T., R. Boukari, R. Belbouab, E.A.I., H.A.A., F.E.S., M.G., G.d.S.B., M.H., S.K., A.M.M., and S.B.S. recruited patients with RIPK1 deficiency; P.S., M.K.-D., A.B., S.M., E.S., D.T., R. Boukari, R. Belbouab, E.A.I., H.A.A., F.E.S., M.G., G.d.S.B., M.H., S.K., A.M.M., and S.B.S. were critical in the interpretation of the clinical data; Y. Liu assisted in CRISPR/Cas9-mediated genetic engineering; A.S.L. supported analysis of inflammasome activity; T.M. conducted T cell proliferation assays; M.R. performed whole exome sequencing; S.H., M. Field, N.W., U.P., and K.S. conducted the bioinformatics analysis of sequencing data; R.C. and M.H. supported immunophenotypical analysis; B.B. conducted the electrophoretic mobility-shift assays; C.W. performed pathological analysis; M.M.G. and V.H. provided the RIPK1-deficient BLaER1 cell model and experimental expertise; K.S., C.K., and D.K. conceived the study design and supervised Y. Li, M. Führer, and E.B.; and K.S., C.K., and D.K. recruited study participants.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The identified *RIPK1* mutations reported in this paper have been deposited in the ClinVar database, https://www.ncbi.nlm.nih.gov/clinvar/ (accession nos.: SCV000854770–SCV000854774). Information on the raw whole-exome sequencing data is not published to protect research participant privacy.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1813582116/-/DCSupplemental.

Published online December 27, 2018.

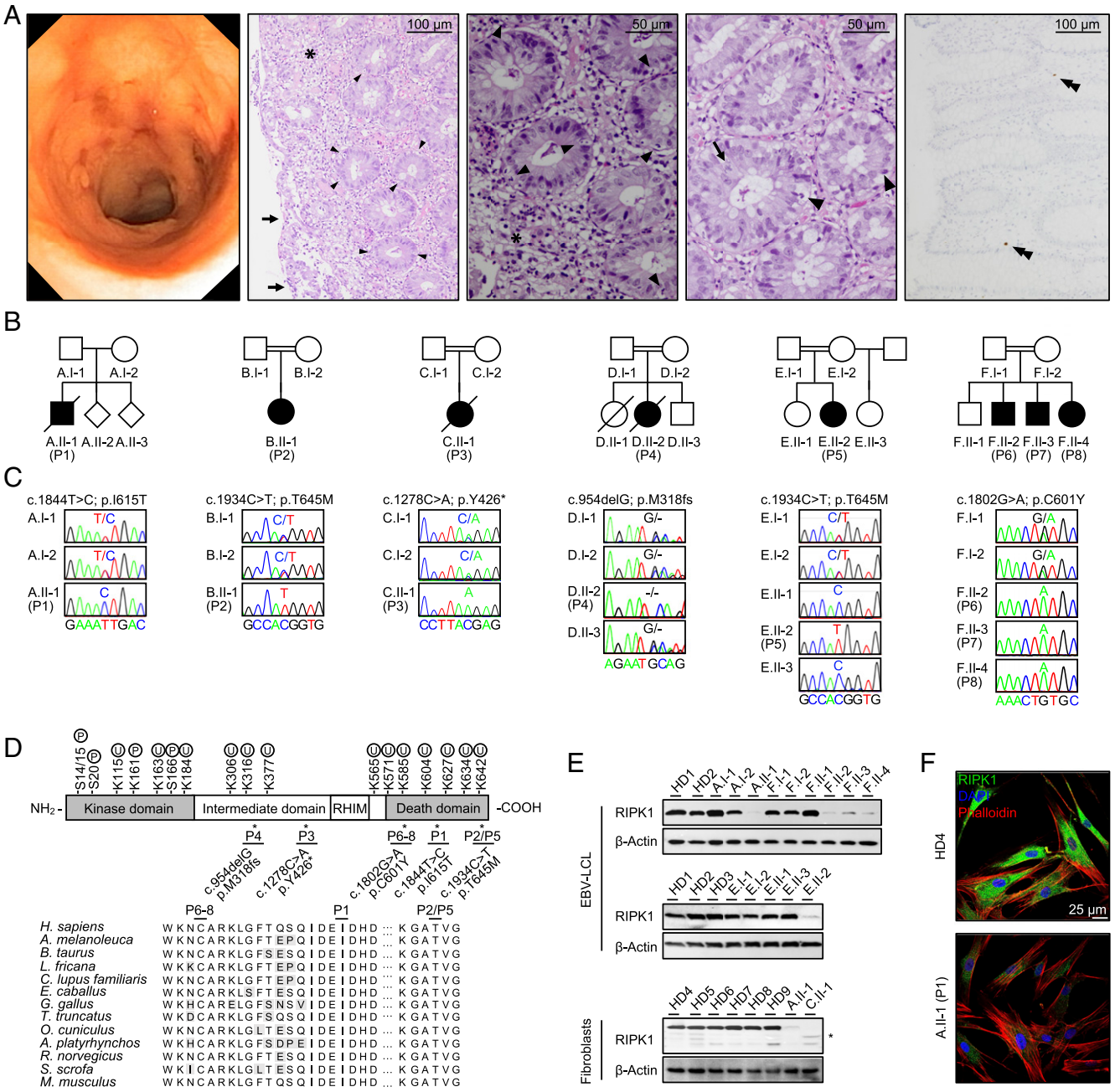

Fig. 1. Identification of biallelic *RIPK1* mutations in patients with combined immunodeficiency and pediatric inflammatory bowel disease. (*A*) Colonoscopy showing pancolitis with ulcers and granuloma in P1 (*Left*). Histology of colonic biopsies from P1 revealed chronic-active inflammation (asterisk) with erosions of the mucosal surface (arrow) (second image from *Left*) and epithelial degeneration. Higher magnification displays epithelial regeneration with increased mitotic activity (arrow) and apoptotic bodies (arrowhead) within the crypt epithelium (third and fourth images from the *Left*). Multiple myeloma oncogene 1 immunohistochemistry indicated subtotal depletion of plasma cells (*Right*, double arrowhead). (*B*) Pedigrees of six families (A to F) with patients (P1 to P8) presenting with primary immunodeficiencies and/or VEO-IBDs. (*C*) Sanger sequencing confirmed segregation of the biallelic *RIPK1* mutations with the disease phenotype in available first-degree relatives. (*D*) Schematic illustration of the RIPK1 protein domain architecture (NM_003804.3, NP_003795.2). Alignment of the human RIPK1 protein sequence showed that the mutated amino acids are conserved in orthologs. RHIM, RIP homotypic interaction motif. (*E*) Immunoblotting of three independent experiments revealing reduced protein expression of RIPK1 in patient-derived EBV-transformed B cells (P1, P5, P6, P7, P8) or fibroblast cell lines (P1, P3) in contrast to healthy donors (HDs) or patients' relatives. Truncated RIPK1 protein expression in P3 is indicated by an asterisk. (*F*) Representative confocal immunofluorescence microscopy images confirming reduced expression of RIPK1 in fibroblasts from P1, compared with HDs.

functions. In particular, RIPK1 is known to mediate multimodal signaling downstream of TNFR1 depending on cell type and biological context (4). While TNF-α–induced NF-κB nuclear translocation promotes cell survival and inflammatory signaling, modulation of intracellular signaling cascades can also induce caspase-8 (CASP8)–mediated apoptosis or RIPK3-dependent necroptosis in the absence of CASP8 (4). The exact mechanisms controlling the multimodal transition switches from RIPK1-mediated cell survival and inflammation to cell death remain largely unknown.

Mice with constitutive deletion of *Ripk1* die perinatally due to hyperinflammation and increased sensitivity to TNF-α–induced cell death and RIPK3-mediated necroptosis (5, 6). Depending on

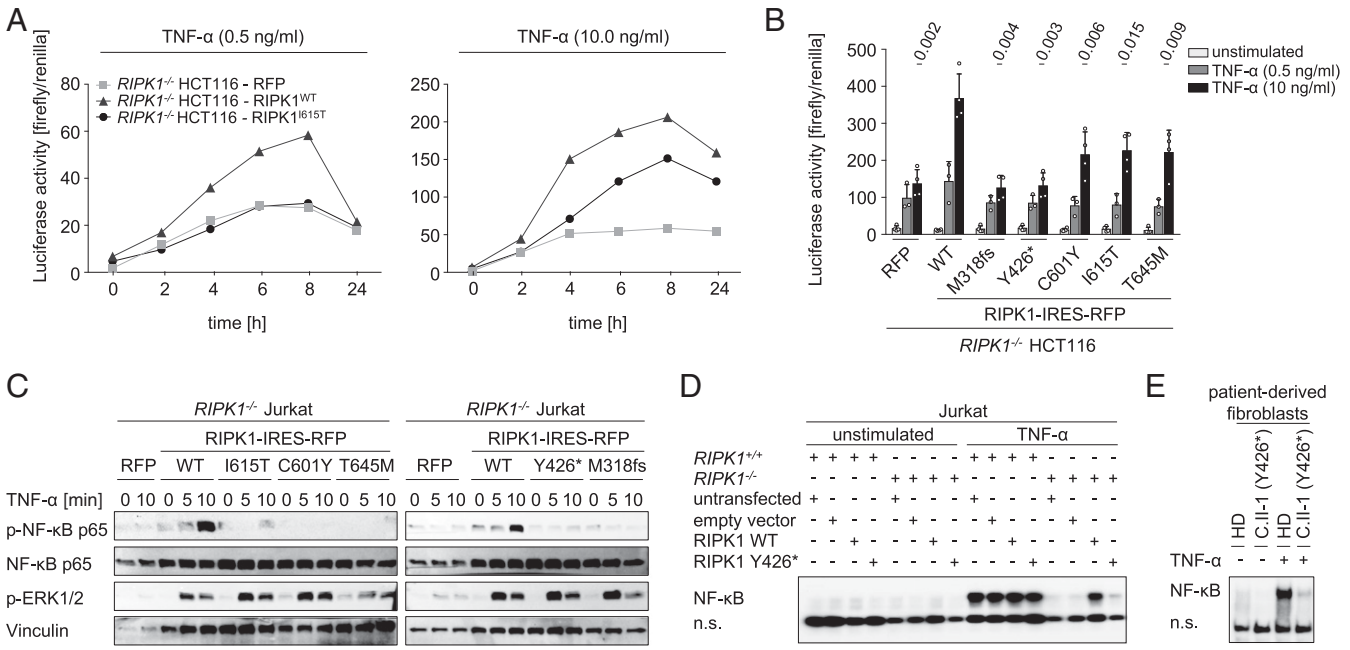

Fig. 2. Biallelic loss-of-function *RIPK1* mutations lead to impaired NF-κB–mediated signaling. (*A* and *B*) Representative luciferase reporter assays showed reduced NF-κB activation upon TNF-α stimulation in HCT-116 cells with *RIPK1* KO or lentiviral-mediated overexpression of RIPK1 mutants, compared with WT RIPK1. Data shown represent the mean ± SD. (*C*) Representative immunoblotting ($n = 3$) of serum-starved *RIPK1*$^{-/-}$ Jurkat cells with transgenic expression of mutant RIPK1 variants revealed decreased phosphorylation of the NF-κB p65 subunit (Ser536) in response to TNF-α (50 ng/mL), whereas phosphorylation of ERK1/2 was normal. (*D* and *E*) Representative EMSA ($n = 3$) showed reduced DNA-binding activity of the NF-κB p65 subunit in Jurkat cells overexpressing the RIPK1 mutation Y426* (*D*) or fibroblasts derived from P3 (*E*) after TNF-α stimulation (50 ng/mL) for 30 min. n.s., nonspecific bands.

the context, murine RIPK1 deficiency might be associated with increased sensitivity to both RIPK3-dependent necroptosis and TNF-α– and/or CASP8-dependent apoptosis (5–7). Studies on conditional *Ripk1* knockout (KO) mice have demonstrated that RIPK1 plays a critical role in controlling skin and intestinal inflammation, autoimmunity, and tissue fibrosis (8–11). RIPK3–MLKL–dependent necroptosis has been described as a common pathomechanism. However, the triggers and ligands relevant for activation of the necroptotic pathway in vivo remain poorly understood. Furthermore, RIPK1 has also been implicated in murine hematopoiesis (12), T and B cell homeostasis (13, 14), and inflammasome activity (5).

A pathogenic role of RIPK1 has been previously linked to multiple mouse models of disease, including colitis, skin inflammation, myocardial infarction, atherosclerosis, pancreatitis, and viral infections, as well as liver, retinal, and renal injury (15). Pharmacological inhibition of RIPK1 has been shown to block necroptosis and protect from ischemic organ damage (16). Small-molecule inhibitors of RIPK1 activity are currently being evaluated for patients with psoriasis, rheumatoid arthritis, and ulcerative colitis (17). Recently, RIPK1 has also been implicated in tumorigenesis and proposed as a therapeutic target in melanoma (18), colon cancer (19), and leukemia (20). However, the relevance of RIPK1 for human pathogenesis and the balance of anticipated effects and side effects of targeting RIPK1 are still discussed controversially.

Here, we report that biallelic loss-of-function mutations in human *RIPK1* cause impaired innate and adaptive immunity and predispose to VEO-IBD.

Results

Identification of Patients with Biallelic Mutations in RIPK1. Our index patient (P)1 (A.II-1) born to Caucasian parents from Poland presented with VEO-IBD characterized by growth failure, abdominal pain, chronic mucous and bloody diarrhea, oral aphthous lesions, and perianal lesions at the age of 6 mo. Endoscopy confirmed the diagnosis of pancolitis with ulcers and granuloma (Fig. 1*A*, *Left*), esophagitis, and gastric ulcers. Histology of gastric and colonic biopsies revealed chronic-active inflammation with erosions (Fig. 1*A*, second panel from *Left*), increased apoptotic bodies within the cryptic bases (Fig. 1*A*, third and fourth panels from *Left*), and subtotal depletion of lamina propria plasma cells (Fig. 1*A*, *Right*). Extraintestinal manifestations included hepatosplenomegaly, maculopapular skin and transient atopic skin lesions, recurrent fever, and infections (pneumonia, conjunctivitis), including episodes of deep-seated infections and sepsis in the neonatal phase. He showed a refractory course despite amino acid-based formula, parenteral nutrition, antibiotics, steroids, azathioprine, and ileostomy and succumbed to septicemia at the age of 4 y. To decipher the molecular cause of disease in this patient, we have conducted whole-exome sequencing and found a rare homozygous missense mutation in the *RIPK1* gene (NM_003804.3, c.1844T>C; NP_003795.2, p.I615T) (Fig. 1 *B* and *C*). Further screening for biallelic *RIPK1* mutations in more than 1,942 patients with VEO-IBDs and/or primary immunodeficiencies identified another seven patients from five unrelated pedigrees with homozygous germ-line mutations in *RIPK1* (Fig. 1 *B* and *C*). The sequence variants in *RIPK1* have been deposited in the ClinVar database (21) (accession nos.: SCV000854770–SCV000854774). While P3 (c.1278C>A, p.Y426*) and P4 (c.954delG, p.M318IfsTer194) primarily manifested with combined immunodeficiency associated with lymphopenia, P2 (c.1934C>T, p.T645M), P5 (c.1934C>T, p.T645M), P6 (c.1802G>A, p.C601Y), P7 (c.1802G>A, p.C601Y), and P8 (c.1802G>A, p.C601Y) were primarily referred for genetic testing due to signs of VEO-IBD. All patients suffered from recurrent bacterial and/or viral infections and had episodes of diarrhea. Perianal disease was reported in all patients except for P3. Further clinical details for the patients are summarized in *SI Appendix*, Table S1. Segregation of the *RIPK1* mutations with the disease phenotype could be confirmed by Sanger sequencing of available first-degree family members (Fig. 1*C*). In silico analysis using PolyPhen (22) and SIFT (23) predicted that the identified missense mutants in *RIPK1* are deleterious. These homozygous mutations have not been previously reported in the

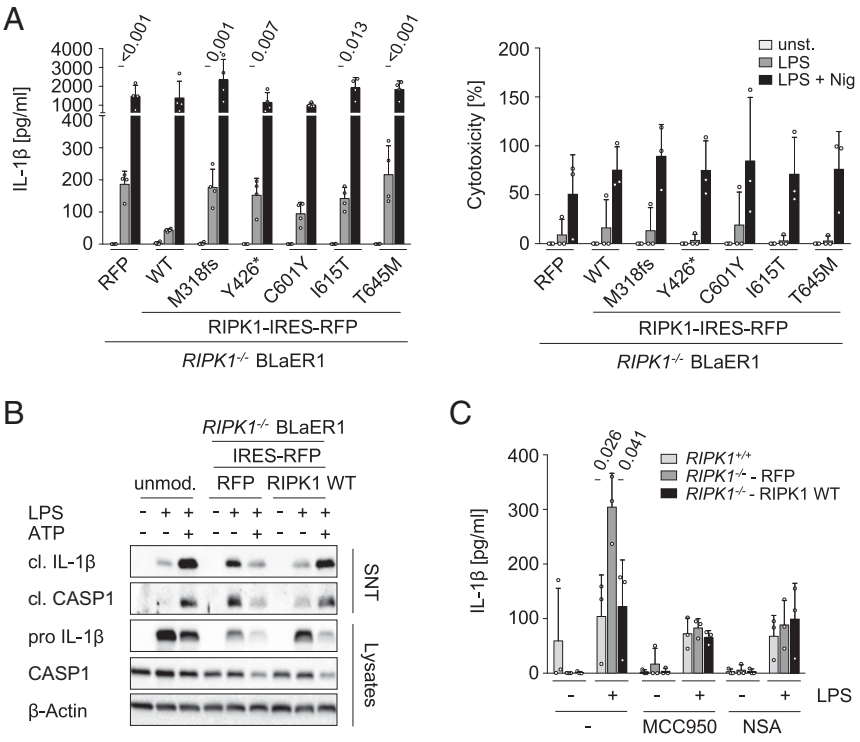

Fig. 3. RIPK1 deficiency is associated with increased inflammasome activity upon LPS priming. (*A*) ELISA showed increased release of IL-1β upon LPS priming (20 ng/mL, 3 h) in conditioned media from heterologous BLaER1 cells with *RIPK1* KO or overexpression of RIPK1 mutant variants. *P* values are analyzed in comparison with cells expressing WT RIPK1 (*Left*). No difference in inflammasome activation of RIPK1-deficient macrophages has been observed upon LPS + nigericin in contrast to WT RIPK1. Data are representative of four independent experiments. LDH release assays of three independent experiments showed no difference of cytotoxicity between RIPK1 WT and mutant BLaER1 cells upon stimulation with LPS ± nigericin (*Right*). (*B*) Representative immunoblotting ($n = 4$) of heterologous RIPK1-deficient BLaER1 cells confirmed increased secretion of the mature IL-1β and cleavage of CASP1 in comparison with unmodified or WT RIPK1-expressing cells (LPS, 200 ng/mL, 14 h). (*C*) Analysis of IL-1β release in *RIPK1*$^{-/-}$ BLaER1 cells overexpressing RFP or WT RIPK1 upon treatment with LPS ± small-molecule inhibitors for NLPR3 (MCC950) or MLKL (NSA) (three independent experiments). Data shown in *A* and *C* represent the means ± SD. cl., cleaved; SNT, supernatant.

Genome Aggregation Database (24). Sequence homology analysis revealed that the mutated amino acids in the death domain of RIPK1 are evolutionarily conserved (Fig. 1*D*). Immunoblotting of EBV-transformed lymphoblastoid cell lines (EBV-LCLs) from pedigrees A (P1), E (P5), and F (P6, P7, P8) and primary fibroblasts from pedigrees A (P1) and C (P3) (Fig. 1*E*), as well as confocal immunofluorescence microscopy of fibroblasts from pedigree A (P1) (Fig. 1*F*), demonstrated a reduced protein expression of mutated RIPK1. P3 carrying a frameshift mutation in *RIPK1* showed reduced expression of a truncated protein.

P3 and P4 presented with lymphopenia affecting T and B cells (*SI Appendix*, Table S2). Immunophenotyping of peripheral blood mononuclear cells from P1, P6, P7, and P8 showed a decreased frequency of $CD45RO^+CCR7^+$ central memory and $CD45RO^+CCR7^-$ effector memory $CD4^+$ and $CD8^+$ T cells (*SI Appendix*, Fig. S1 *A* and *B*), $CD45RO^+HLA\text{-}DR^+$ memory activated regulatory T cells (*SI Appendix*, Fig. S1*C*), and $CXCR3^+CCR6^-$ T-helper 1 (Th1) and $CXCR3^-CCR6^+$ T-helper 17 (Th17) populations (*SI Appendix*, Fig. S1*D*), as well as IgD^-CD27^+ class-switched B cells (*SI Appendix*, Fig. S1*E*), whereas P5 exhibited no measurable changes in these parameters (*SI Appendix*, Table S3). These data suggest that RIPK1 deficiency may lead to combined T and B cell dysfunction. However, T cell proliferation, activation, and cell death in response to anti-CD3, anti-CD3/CD28, or anti-PMA/ionomycin were normal. In addition, we could not observe a significant difference in cell death in RIPK1-deficient Jurkat cells upon treatment with FAS ligand, TNF-α ± BV6 (the second mitochondrial activator of apoptosis mimetic), or TNF-α ± cycloheximide in comparison with RIPK1 wild-type (WT) reconstituted cells (*SI Appendix*, Fig. S2).

Defective TNF-α–Mediated NF-κB Signaling in RIPK1-Deficient Cells. RIPK1 regulates multimodal signaling downstream of TNFR1 in a cell- and context-dependent manner (25). To assess the consequences of identified mutations for RIPK1 downstream signaling, we engineered colon carcinoma-derived HCT-116 cells with a CRISPR/Cas9-mediated *RIPK1* KO and subsequent lentiviral overexpression of WT or mutant RIPK1 variants. NF-κB luciferase reporter assays showed that cells expressing the RIPK1 variant I615T (identified in P1) exhibited impaired NF-κB activity in response to TNF-α, compared with cells with WT RIPK1 (Fig. 2*A*). Similarly, we could detect reduced luciferase activity for all five identified *RIPK1* mutants after TNF-α stimulation (Fig. 2*B*). Correspondingly, immunoblotting revealed reduced phosphorylation of the NF-κB p65 subunit (Ser536) in Jurkat cells with *RIPK1* KO or expression of mutant RIPK1 (Fig. 2*C*), whereas phosphorylation of ERK1/2 (Thr202/Tyr204) was normal. Electrophoretic mobility-shift assays confirmed reduced NF-κB DNA-binding activity in Jurkat cells expressing the RIPK1 mutant Y426* (Fig. 2*D*) and fibroblasts of P3 (Fig. 2*E*) in response to TNF-α, compared with WT RIPK1 reconstituted Jurkat cells and healthy donor fibroblasts, respectively. These data indicate that the identified mutations in *RIPK1* are associated with impaired TNF-α–induced NF-κB signaling.

Altered Inflammasome Activity in RIPK1-Deficient Macrophages. Previous studies have documented an altered inflammasome activity in conditional *Ripk1* KO mice (5, 26). To examine effects of the identified *RIPK1* mutations on inflammasome activity, we have adapted a BLaER1 monocyte cell model with KO of *CASP4* and *RIPK1* (27) and reconstituted the patients' mutations by lentiviral gene transfer. In contrast to cells with reconstitution of WT RIPK1, cells with KO of *RIPK1* or overexpression of the RIPK1 mutants (M318fs, Y426*, I615T, and T645M) showed increased IL-1β secretion without the requirement of a secondary stimulus for the processing of mature IL-1β (Fig. 3*A*). Increased inflammasome activity in RIPK1-deficient macrophages was not associated with increased cytotoxicity upon LPS priming for 3 h, as indicated by the LDH assay (Fig. 3*A*). Of note, no difference of IL-1β secretion could be observed upon addition of nigericin between cells with overexpression of WT and RIPK1 mutants (Fig. 3*A*). Immunoblotting confirmed increased release of mature IL-1β upon treatment with LPS in RIPK1-deficient macrophages (Fig. 3*B*). To test whether the altered IL-1β release is associated with increased NLPR3 activity and/or MLKL-dependent necroptosis in human RIPK1 deficiency, we assessed the inflammasome activation upon treatment with small-molecule inhibitors of NLRP3 (MCC950) and MLKL (NSA) (Fig. 3*C*). The inhibitors reduced IL-1β secretion in LPS-stimulated RIPK1-deficient macrophages, suggesting that both

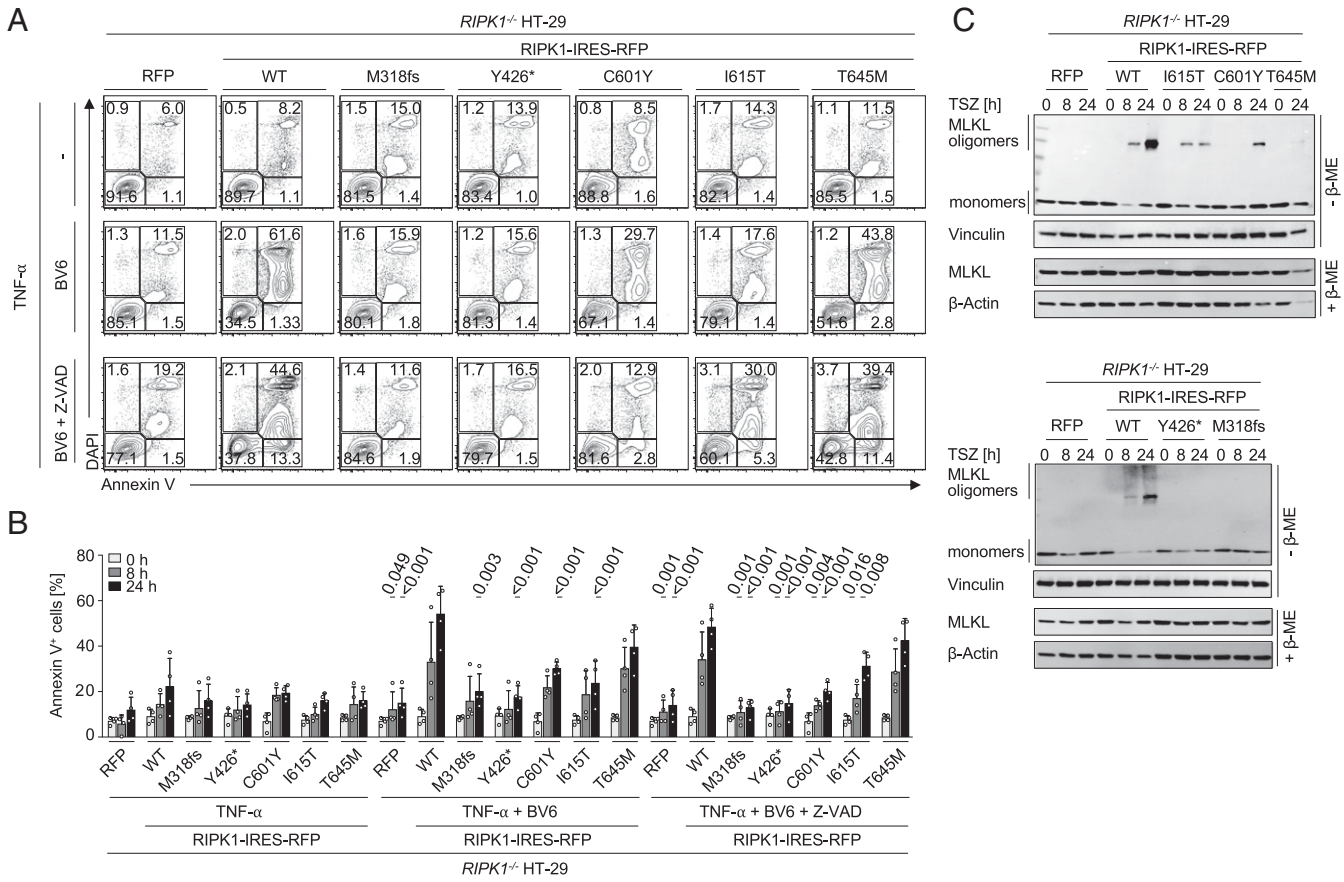

Fig. 4. RIPK1-deficient intestinal epithelial cells show altered cell death responses. (*A*) Representative FACS analysis of Annexin V/DAPI staining ($n = 4$) in HT-29 cells expressing mutant RIPK1 upon 24 h of treatment with TNF-α ± BV6 ± Z-VAD-FMK. (*B*) Graphical representation ($n = 4$) showing decreased frequencies of Annexin V$^+$ RIPK1-deficient cells compared with cells with WT RIPK1 after stimulation with TNF-α + BV6 ± Z-VAD-FMK. (*C*) Representative SDS/PAGE under nonreducing conditions ($n = 3$) revealed reduced MLKL oligomerization upon treatment with TNF-α + BV6 + Z-VAD-FMK in RIPK1-deficient cells. Data shown represent the means ± SD. ME, mercaptoethanol; TSZ, TNF-α + BV6 (SMAC mimetic) + Z-VAD-FMK.

pathways might be implicated in dysregulation of proinflammatory responses. Taken together, our results suggest that human RIPK1 plays a critical role in regulating LPS-mediated inflammasome activation.

Impaired TNF-α–Mediated Cell Death Responses in RIPK1-Deficient Epithelial Cells. RIPK1 and RIPK3 are critical regulators of cell death (28). To study the effect of patients' mutations on TNF-α–mediated cell death responses in epithelial cells, we engineered HT-29 colon carcinoma cells with KO of *RIPK1* and lentiviral reconstitution of WT or mutant RIPK1 variants. No alteration of cell death could be observed upon treatment with TNF-α in RIPK1-deficient HT-29 cells (Fig. 4 *A* and *B*). However, cell death responses were impaired upon treatment with TNF-α and BV6 ± the pan caspase inhibitor Z-VAD-FMK in cells expressing mutated RIPK1 variants (M318fs, Y426*, C601Y, and I615T) compared with cells overexpressing WT RIPK1 (Fig. 4 *A* and *B*). Correspondingly, immunoblotting showed reduced MLKL oligomerization in RIPK1-deficient HT-29 cells in response to TNF-α, BV6, and Z-VAD-FMK, suggesting impaired necroptosis under conditions of RIPK1 deficiency (Fig. 4*C*).

Discussion

The functional relevance of RIPK1 in human disease has been controversially discussed. We report RIPK1 deficiency as a Mendelian disorder predisposing to immunodeficiency and severe colitis. Whereas it may appear counterintuitive at first sight to associate immunodeficiency and hyperinflammatory responses, several monogenic diseases have a poorly understood Janus-faced appearance, for example autoimmune lymphoproliferative syndrome caused by TNFRSF6 (29) and CASP10 (30) deficiency or lymphoproliferation and autoimmunity caused by *IL2RA* null mutations (31).

Constitutive *Ripk1* KO mice appear to exhibit no developmental defects but show perinatal mortality associated with systemic multiorgan inflammation and apoptosis in lymphoid and adipose tissues (32). A potential role of RIPK1 in pathogenesis has been documented in several models of inflammation and tissue damage (16). In particular, conditional ablation of *Ripk1* has been reported to result in severe intestinal and skin inflammation associated with FADD-CASP8–dependent apoptosis of intestinal epithelial cells and ZBP1-RIPK3–MLKL–dependent necroptosis of keratinocytes, respectively (8, 11). Our patients with homozygous mutations in *RIPK1* showed no obvious developmental defects, and predominantly presented with immunodeficiency and diarrhea or colitis. Whereas children with complete loss of function of RIPK1 (P3, stop-gain mutation; P4, frameshift mutation) primarily manifested with combined immunodeficiency and diarrhea, patients with missense mutations in the death domain of RIPK1 were referred for genetic testing due to IBD-like conditions. Differences in clinical manifestation might be reflective of genotype–phenotype correlations and incomplete penetrance, or may be due to secondary factors such as genetic modifiers, infections, and treatment. Emerging evidence suggests that kinase-independent RIPK1 functions are critical in controlling intestinal epithelial homeostasis (5, 6, 8, 11). None of our identified patients had mutations directly affecting the

kinase domain of RIPK1, but the identified mutations perturbed total protein expression. Therefore, our study cannot unequivocally define whether the abrogated kinase activity is critical in mediating intestinal inflammation in our patients.

Mice with *Ripk1* KO in intestinal epithelial cells develop colitis accompanied by disrupted tissue architecture and increased apoptosis (8, 11). In parallel investigations, Cuchet-Lourenço et al. (33) identified four patients with loss-of-function mutations in *RIPK1* causing combined immunodeficiency and intestinal inflammation due to altered cytokine secretion and necroptosis of immune cells. Whereas these authors concluded that allogeneic HSCT may constitute a curative therapy, our studies suggest that RIPK1 plays a critical role in controlling cell death of the intestinal epithelium, and thus warrant awareness that HSCT might dampen intestinal inflammation but not rescue intrinsic intestinal phenotypes of human RIPK1 deficiency, similar to NF-kappa-B essential modulator deficiency (34). The exact triggers perturbing epithelial integrity in RIPK1 deficiency could not be fully determined in our studies or mouse models yet. Further studies are required to shed light on cell- and context-dependent functions of RIPK1 in controlling intestinal inflammation in vivo.

Necroptosis has been previously linked to the pathogenesis in various disease models such as atherosclerosis, myocardial infarction, ischemic brain injury, systemic inflammation, liver injury, and neurodegeneration (16). Targeting RIPK1 and RIPK3 represents an attractive therapeutic strategy for diseases with increased necroptotic activity. Necrostatin-1 allosterically inhibits RIPK1 activity and has been shown to block necroptosis in mouse models of ischemia (16). Recently, a small molecule (GSK2982772) has been developed as an inhibitor of RIPK1 to treat plaque-type psoriasis, rheumatoid arthritis, and ulcerative colitis in phase 2a clinical studies (17). The beneficial effects of this therapeutic strategy in patients still remain unclear. Inhibition of RIPK1 activity might be considered in patients with severe or refractory inflammatory or autoinflammatory diseases. Our study on RIPK1-deficient patients highlights that human RIPK1 has pleiotropic cell- and context-specific functions and thus warrants awareness about potential toxicities of targeting RIPK1.

Taken together, we report that patients with biallelic RIPK1 deficiency present with life-threatening combined immunodeficiency and/or intestinal inflammation associated with impaired lymphocyte functions, increased inflammasome activity, and altered TNF-α–mediated epithelial cell death responses. Thus, our study highlights the central role of RIPK1 in controlling human immunity and intestinal homeostasis.

Materials and Methods

Patients. Peripheral blood and skin biopsies from patients, first-degree family members, and healthy donors were acquired upon written consent. The study was approved by the respective institutional review boards of the University of Ulm, Necker Medical School, and University Hospital, LMU Munich and conducted in accordance with current ethical and legal frameworks.

Genetic, Immunologic, and Biochemical Analyses. Methods of genetic analyses, immunological studies, and biochemical and cell biological assays as well as statistics are described in *SI Appendix*.

ACKNOWLEDGMENTS. We are grateful to the interdisciplinary medical staff. We acknowledge the assistance of the Flow Cytometry and Care-for-Rare Genomics Core Facility at the Dr. von Hauner Children's Hospital, as well as the FACS facility of the Department of Pediatrics and Department of Molecular Diagnostics of the Institute for Clinical Transfusion Medicine and Immunogenetics Ulm and the Genomics and Bioinformatics Core Facility at the Imagine Institute and UFR Necker. We thank Dr. T. Graf (Center for Genomic Regulation, Barcelona) for providing BLaER1 cells, and Genentech for supplying BV6. Further, we acknowledge bioinformatics support by Dr. Jacek Puchalka. The work has been supported by The Leona M. and Harry B. Helmsley Charitable Trust, DFG (Gottfried-Wilhelm-Leibniz Program, Collaborative Research Consortium SFB1054 project A05), PID-NET (BMBF), BioSysNet, the Care-for-Rare Foundation, and INSERM. Y. Li has been supported by the China Scholarship Council, E.B. received a scholarship from the Care-for-Rare Foundation, and M. Führer received a scholarship from the "Landesgraduiertenförderungsgesetz." D.K. has been a scholar funded by the Daimler und Benz Stiftung, Reinhard-Frank Stiftung, and Else-Kröner-Fresenius Stiftung.

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4. Paper II

Title: Valosin-containing protein-regulated endoplasmic reticulum stress causes NOD2-dependent inflammatory responses

Year: 2022

Journal: Sci Rep

Volume: 12

Issue: 1

Pages: 3906

DOI: 10.1038/s41598-022-07804-1

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Valosin-containing protein-regulated endoplasmic reticulum stress causes NOD2-dependent inflammatory responses

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NOD2 polymorphisms may affect sensing of the bacterial muramyl dipeptide (MDP) and trigger perturbed inflammatory responses. Genetic screening of a patient with immunodeficiency and enteropathy revealed a rare homozygous missense mutation in the first CARD domain of NOD2 (ENST00000300589; c.160G > A, p.E54K). Biochemical assays confirmed impaired NOD2-dependent signaling and proinflammatory cytokine production in patient's cells and heterologous cellular models with overexpression of the NOD2 mutant. Immunoprecipitation-coupled mass spectrometry unveiled the ATPase valosin-containing protein (VCP) as novel interaction partner of wildtype NOD2, while the binding to the NOD2 variant p.E54K was abrogated. Knockdown of VCP in coloncarcinoma cells led to impaired NF-κB activity and *IL8* expression upon MDP stimulation. In contrast, tunicamycin-induced ER stress resulted in increased *IL8*, *CXCL1*, and *CXCL2* production in cells with knockdown of VCP, while enhanced expression of these proinflammatory molecules was abolished upon knockout of NOD2. Taken together, these data suggest that VCP-mediated inflammatory responses upon ER stress are NOD2-dependent.

The innate immune system has crucial functions in detection and eradication of pathogens. The recognition of microbial-associated molecular patterns (MAMPs) and induction of inflammatory responses depends on specific pattern recognition receptors (PRRs)[1]. The intracellular PRR nucleotide-binding oligomerization domain protein 2 (NOD2) senses muramyl dipeptide (MDP), an evolutionary conserved component of the bacterial

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cell walls[2]. Upon MDP challenge, intracellular NOD2 oligomerizes and recruits receptor-interacting serine/threonine-protein kinase 2 (RIPK2) through CARD-CARD homotypic interaction leading to activation of downstream signaling pathways such as nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling[3–7]. Dysregulated NOD2 signaling has been implicated in several inflammatory disorders such as Blau syndrome, sarcoidosis, allergy, asthma and autoimmunity[8–10]. In particular, *NOD2* has been recognized as the key susceptibility gene in Crohn's disease (CD)[8]. NOD2 polymorphisms associated with CD are mainly located in the leucine-rich repeat domain which is responsible for ligand sensing and binding.

Here, we report a rare missense mutation affecting the first CARD domain of NOD2 that has been identified in a child with enteropathy and was associated with defective MDP-dependent signaling, abrogated interaction with RIPK2, as well as impaired cytokine responses. The characterization of this rare mutation unveiled Valosin-containing protein (VCP) as novel interaction partner of NOD2. VCP is a ubiquitously expressed ATPase with pleiotropic functions in controlling Ubiquitin-proteasome system (UPS)-mediated protein degradation in endoplasmic reticulum (ER)-associated protein degradation (ERAD), apoptosis, and autophagy[11–13]. Our study highlighted that VCP-mediated proinflammatory responses during ER stress are NOD2-dependent.

Results

Defective NOD2 signaling caused by a biallelic germline mutation affecting the first CARD domain. Whole exome sequencing was conducted to elucidate the genetic etiology in a one-year-old female patient presenting with intractable diarrhea, recurrent perianal candida dermatitis, hemophagocytic lymphohystiocytosis (HLH), and prolonged Norovirus infection. Genetic screening revealed rare homozygous missense mutations in *NOD2* (ENST00000300589; c.160G > A, p.E54K) and *STXBP2* (ENST00000441779; c.949C > G, p.L317V). Even though the variant in *STXBP2* was predicted to be benign, this mutation likely has caused HLH associated with impaired NK cell degranulation (Supplementary Fig. 1). By contrast, the *NOD2* missense mutation has been proposed as deleterious based on PolyPhen and SIFT and thus, may contribute to the gastrointestinal phenotypes (intractable diarrhea, recurrent perianal dermatitis). Our study was not specifically designed to address whether the *NOD2* variant was the causal or risk factor of disease in our patient, but the distinct location of the variant triggered assessment of NOD2-mediated signaling and interacting networks, since previously reported CD-associated *NOD2* variants are mainly localized in or near the LRR domain (Fig. 1A)[14–16].

To elucidate the effects of the identified NOD2 mutation on the canonical NOD2-mediated signaling, we assessed intracytoplasmic TNF production in patient's peripheral blood mononuclear cells (PBMC)-derived monocytes upon stimulation with the ligand L18-MDP. While patient-derived monocytes showed a normal response to lipopolysaccharide (LPS), TNF expression was reduced in L18-MDP-treated cells as compared with healthy donors (Fig. 1B). In addition, patient-derived neutrophils showed reduced CD62L shedding upon MDP stimulation (Fig. 1C), confirming defective NOD2 signaling in patient innate immune cells. Correspondingly, patient's PBMCs revealed reduced phosphorylation of NF-κB p65, ERK1/2, and/or p38 upon stimulation with L18-MDP (Fig. 1D). Furthermore, luciferase reporter assays on HEK293T cells ectopically expressing the NOD2 variant p.E54K showed impaired NF-κB activity in response to L18-MDP comparable with the NOD2 variant p.L1007fsX1008 that has previously been associated with CD[15] (Fig. 2A).

To gain insights into the pathomechanisms of the mutation in the context of intestinal inflammation, we engineered coloncarcinoma-derived HCT116 cells with a CRISPR/Cas9-mediated NOD2 knockout (KO) and subsequent lentiviral overexpression of wild-type (WT) or mutant (p.E54K and p.L1007fsX1008) NOD2 variants. In contrast to WT reconstitution, HCT116 cells with NOD2 mutants showed reduced expression and secretion of IL-8 upon treatment with L18-MDP (Fig. 2B).

Emerging evidence highlights that NOD2 has critical functions apart from peptidoglycan (PGN) sensing. Notably, NOD2 has been implicated in mediating proinflammatory responses triggered by ER stress[17]. To assess the PGN-independent functions of NOD2, we evaluated the expression of the proinflammatory cytokine *IL8* in engineered HCT116 cells upon ER stress. Treatment with tunicamycin resulted in impaired *IL8* production in cells with expression of the NOD2 variant p.E54K as compared with WT NOD2 reconstituted cells. Interestingly, we could detect normal expression of *IL8* for the NOD2 variant p.L1007fsX1008 suggesting genotype-specific mechanisms of ER stress-induced proinflammation in the context of NOD2 deficiency (Fig. 2C).

Impaired RIPK2 binding and ubiquitination by the NOD2 mutant p.E54K. NOD2 is critical in mediating inflammatory signaling pathways in response to invading pathogens via the interaction of RIPK2 with its CARD domain[6]. Previously reported NOD2 polymorphisms associated with CD are mainly localized in the LRR domain[14,18–25], however two heterozygous variants (p.R38M and p.R138Q) affecting the first and second CARD domain have been suggested to alter RIPK2 recruitment and NF-κB signaling[26]. To assess whether the NOD2 variant p.E54K affects interaction with RIPK2, we conducted co-immunoprecipitation experiments using anti-FLAG beads in HEK293T cells ectopically expressing FLAG-tagged NOD2 WT or mutants along with WT RIPK2. While the NOD2 variant p.L1007fsX1008 showed normal binding to RIPK2, the interaction of the mutant p.E54K and RIPK2 was significantly reduced (Fig. 2D).

Previous studies have demonstrated that polyubiquitination and autophosphorylation of RIPK2 is triggered upon NOD2 activation[4,7,27,28]. Despite the different abilities of the NOD2 mutants p.E54K and p.L1007fsX1008 to interact with RIPK2, we could detect reduced phosphorylation of RIPK2 at position S176 by immunoblotting as well as impaired ubiquitination of RIPK2 by employing tandem ubiquitin-binding entities upon MDP stimulation in both NOD2 variants (Fig. 2E). These data suggest that defective polyubiquitination and/or autophosphorylation of RIPK2 may represent an underlying common mechanism for reduced MDP-dependent signaling associated with NOD2 polymorphisms.

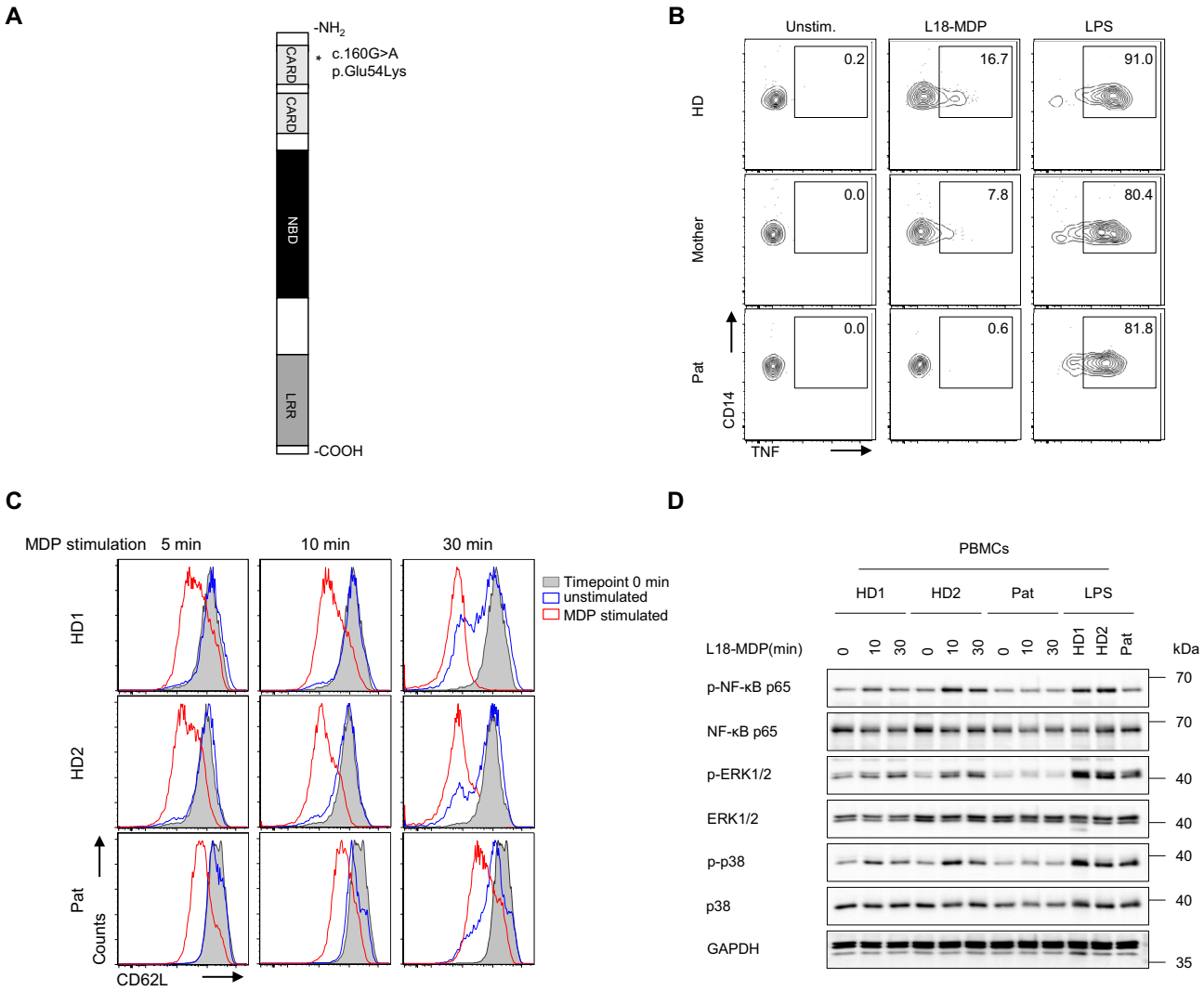

Figure 1. Defective NOD2-mediated signaling in patient primary cells. (**A**) Schematic illustration of NOD2 protein domains. The mutation identified in patient is depicted by an asterisk. (**B**) Representative FACS analysis of TNF staining (n = 3) on PBMC-derived monocytes ($CD14^+$) isolated from patient (Pat), mother and a healthy donor (HD) and stimulated with L18-MDP or LPS. (**C**) Representative FACS analysis of CD62L expression (n = 3) on neutrophils isolated from patient (Pat) and two healthy donors (HD) upon L18-MDP stimulation. (**D**) Representative immunoblotting of serum-starved PBMCs from patient (Pat) and two healthy donors (HD) stimulated with L18-MDP or LPS.

Identification of VCP as novel interaction partner of NOD2. To study the altered interactome of the NOD2 variant p.E54K, cell lysates from immunoprecipitation experiments on HEK293T cells with ectopic expression of WT or mutant NOD2 were subjected to SDS-PAGE. Silver staining revealed a band with a molecular weight of about 100 kDa only present in cells overexpressing WT NOD2 (Fig. 3A). Using co-immunoprecipitation and nano liquid chromatography tandem mass spectrometry (LC–MS/MS), we identified VCP as novel NOD2 interacting protein enriched in cells reconstituted with WT NOD2. In contrast, cells with expression of the variants p.E54K or p.L1007fsX1008 showed abrogated interaction of VCP with NOD2 (Fig. 3B and C). Notably, we also identified vimentin (VIM) and carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD) among the list of known NOD2 interacting proteins; thus increasing the confide in our screening approach. While vimentin was enriched in both NOD2 WT and p.E54K expressing cells as compared with RFP controls, CAD was found to be enriched only in cells overexpressing WT NOD2 (Fig. 3B). Finally, the interaction of endogenous VCP/NOD2 proteins in HCT116 cells was confirmed by immunoprecipitation with an antibody binding to VCP and co-precipitation of NOD2 (Supplementary Fig. 2).

VCP is an evolutionarily conserved AAA + ATPase governing diverse biological functions, in particular in the UPS and ER-associated degradation (ERAD)[29]. VCP mutations have been associated with several diseases such as myopathy, Paget's disease, dementia, amyotrophic lateral sclerosis and Huntington's disease[29,30]. However, the exact role of VCP in health and disease remains elusive. Previously, VCP has been listed as potential NOD2 interaction partner in one of three yeast two hybrid (Y2H) screens[31] and proteomic studies have revealed VCP in the group of proteins that are differentially expressed in HEK293T cells overexpressing the NOD2 variant

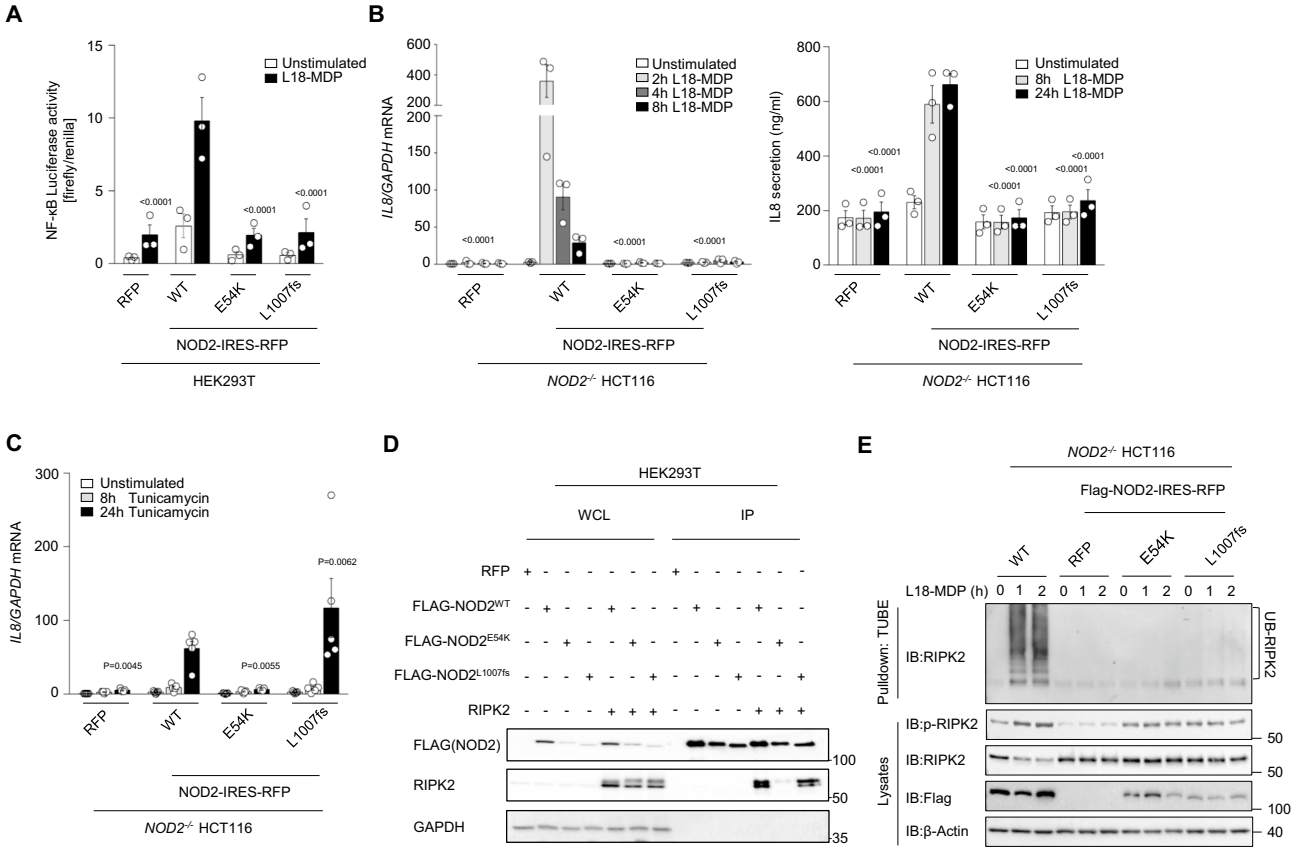

Figure 2. The NOD2 p.E54K variant leads to abrogation of both PGN-dependent and -independent signaling. (**A**) NF-kB luciferase reporter activity upon challenge with L18-MDP (7 h) in HEK293T cells overexpressing NOD2 wild-type (WT) or indicated mutants. (**B** and **C**) Quantitative RT–PCR analysis and ELISA of *IL8* expression upon stimulation with L18-MDP (**B**) or tunicamycin (**C**) in heterologous HCT116 cells. (**D**) Representative immunoprecipitation of FLAG-tagged NOD2 (n = 3) on HEK293T cells that were transiently transfected with Flag-NOD2 WT or indicated mutants alone or along with WT RIPK2. (**E**) Representative TUBE assay (n = 2) from L18-MDP-treated heterologous HCT116 cells. Data represent mean ± SEM of three (**A** and **B**) or five (**C**) independent experiments. P values for each treatment group are calculated in comparison to WT. WCL, whole cell lysate; IP, Immunoprecipitates.

p.L1007fsX1008[32]. These studies support our findings that VCP is an interaction partner of NOD2 but did not provide any functional links for the regulatory role of VCP in NOD2 signaling.

VCP controlled ER stress causes inflammatory responses in a NOD2-dependent manner. To study VCP function in the context of NOD2 signaling, we engineered heterologous HCT116 cells with siRNA-mediated knockdown of VCP and evaluated MDP-induced NF-κB activity and *IL8* expression. Luciferase reporter assays showed impaired NF-κB activation in cells with knockdown of VCP in response to L18-MDP treatment (Fig. 4A). Correspondingly, we could detect decreased expression of *IL8* in MDP-treated cells upon VCP knockdown, as compared with cells transfected with non-targeting siRNA (Fig. 4B). However, we could not observe a direct effect of VCP knockdown on phosphorylation of RIPK2 (S176) or binding of RIPK2 to NOD2 (Supplementary Fig. 3).

VCP plays a critical role in controlling UPR and inhibition of VCP resulted in increased ER stress[33]. Moreover, enhanced ER stress and activated UPR in intestinal epithelial cells have been reported in patients with CD and ulcerative colitis (UC)[34]. Recently, NOD1 and NOD2 have been proposed as molecular bridges linking ER stress to proinflammatory responses[17]. To examine the influence of NOD2 on VCP-mediated ER stress functions, we transfected HCT116 cells with NOD2 KO or lentiviral reconstitution of NOD2 WT with siRNAs targeting VCP. Knockdown efficiency was assessed by qPCR measurement of VCP mRNA as well as immunoblotting of VCP protein (Supplementary Figs. 4 and 5). Using this heterologous cellular model, we could confirm previous findings that VCP knockdown induces increased UPR, as demonstrated by enhanced expression of C/EBP homologous protein (CHOP) (Fig. 4C) as well as increased phosphorylation of PERK and eIF2α, while splicing of XBP1 appeared unaffected (Supplementary Fig. 5). Whereas additional knockout of NOD2 resulted in slight increase of the activated PERK-eIF2α axis (Supplementary Fig. 5), we could not observe a significant upregulation of the transcription of CHOP, which is a downstream target modulated by all three signaling branches of UPR (Fig. 4C). Moreover, treatment with tunicamcyin in NOD2 WT reconstituted cells induced enhanced *IL8*, *CXCL1,* and *CXCL2* expression upon knockdown of VCP, while increased proinflammatory cytokine and

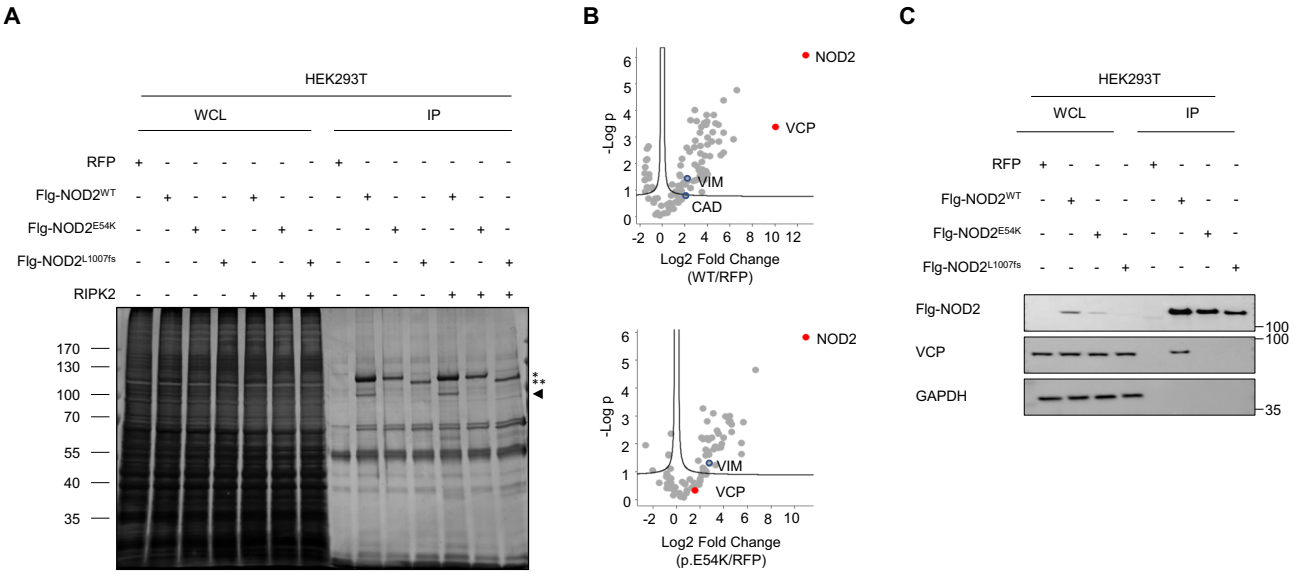

Figure 3. Identification of VCP as novel interaction partner of NOD2. (**A**) Representative SDS-PAGE and silver staining (n = 3) of cell lysates from immunoprecipitation experiments on HEK293T cells that ectopically expressed Flag-NOD2 WT (WT) or mutant alone or along with WT RIPK2. While the asterisks indicate expression of Flag-tagged NOD2 proteins (* full length, ** truncated), the arrow points to an interaction protein of Flag-NOD2 WT (molecular weight about 100 kDa) that was not detectable in the NOD2 mutants p.E54K and p.L1007fsX1008. (**B**) Volcano plots of proteins enriched in NOD2 WT or the mutant (p.E54K) versus the RFP control. (**C**) Flag IP on HEK293T cells transiently transfected with Flag-NOD2 WT or mutants (n = 3). WCL, whole cell lysate. IP, Immunoprecipitates.

chemokine expression could not be observed in NOD2 KO cells (Fig. 4D and E). Differences in *IL8*, *CXCL1,* and *CXCL2* expression between NOD2 knockout and WT reconstituted cells were not associated with increased cell death (Supplementary Fig. 6). Taken together, these findings suggest that inflammatory responses caused by VCP-regulated ER stress are NOD2-dependent.

Discussion

NOD2 is a key receptor of innate immunity and the first genetic locus that has been associated with inflammatory bowel disease (IBD)[14,15]. Since most CD-associated NOD2 variants are located in the LRR domain, the identification of a biallelic missense mutation affecting the CARD domain of NOD2 in a patient with enteropathy prompted us to investigate the signaling and interaction network of the mutant NOD2 protein in greater detail. Even though the HLH-associated phenotype in our patient is likely caused by the *STXBP2* mutation, the *NOD2* sequence variant may be a risk factor contributing to the gastrointestinal phenotypes (intractable diarrhea, recurrent perianal dermatitis). The function of NOD2 in pathogen recognition or peptidoglycan sensing has been previously acknowledged[35,36], however the role of NOD2 during ER stress remains still elusive. Our biochemical study showed impaired NOD2-governed PGN-dependent and independent signaling in primary patient cells as well as cellular models and unveiled VCP as novel interaction partner of NOD2 that regulates ER stress-mediated inflammatory responses.

Several studies have suggested the implication of dysregulated UPR in different inflammatory conditions such as neurodegenerative diseases and IBD[37–40]. A growing body of evidence indicate reciprocal relationships between inflammation and ER stress[41]. While inflammatory stimuli like pattern-recognition receptor (PRR) ligands or ROS can induce UPR, activation of the three main UPR pathways can trigger NF-κB- and MAPK-dependent inflammatory responses leading to the expression of proinflammatory cytokines such as IL-6 and TNF[17,42–44]. In the context of intestinal inflammation, mice with KO of *Ire1* and *Xbp* have been shown to have increased sensitivity to dextran sodium sulfate (DSS)-induced colitis[37,38].

NOD2 has been previously implicated in regulating ER stress[17]. For example, Laccase domain containing-1 (LACC1)-dependent induction of ER stress has been documented in macrophages upon MDP stimulation[45]. Furthermore, previous studies have shown that NOD1/NOD2/RIPK2-dependent inflammation can be triggered by ER stress in mouse bone-marrow-derived macrophages (BMDMs) via the IRE1α/TRAF2 pathway[17]. Recently, Pham et al. showed that mice lacking NOD1 and NOD2 or RIPK2 exhibit increased systemic bacterial burdens after infection with *Chlamydia* suggesting a relevant NOD2-dependent link between ER stress and bacteria-specific inflammatory responses[46]. However, the exact mechanisms of NOD2 activation and function during ER stress still remain largely unknown. Our study on NOD2-deficient epithelial cells suggested that the identified NOD2 germline mutation affecting the CARD domain showed compromised pro-inflammatory responses upon tunicamycin-induced ER stress. Interestingly, we could observe genotype-specific phenotypes, since overexpression of the NOD2 variant p.E54K in NOD2 knockout HCT116 cells resulted in altered *IL8* expression in comparison to cells expressing the NOD2 variant p.L1007fsX1008. Recently, Pei et at. have shown

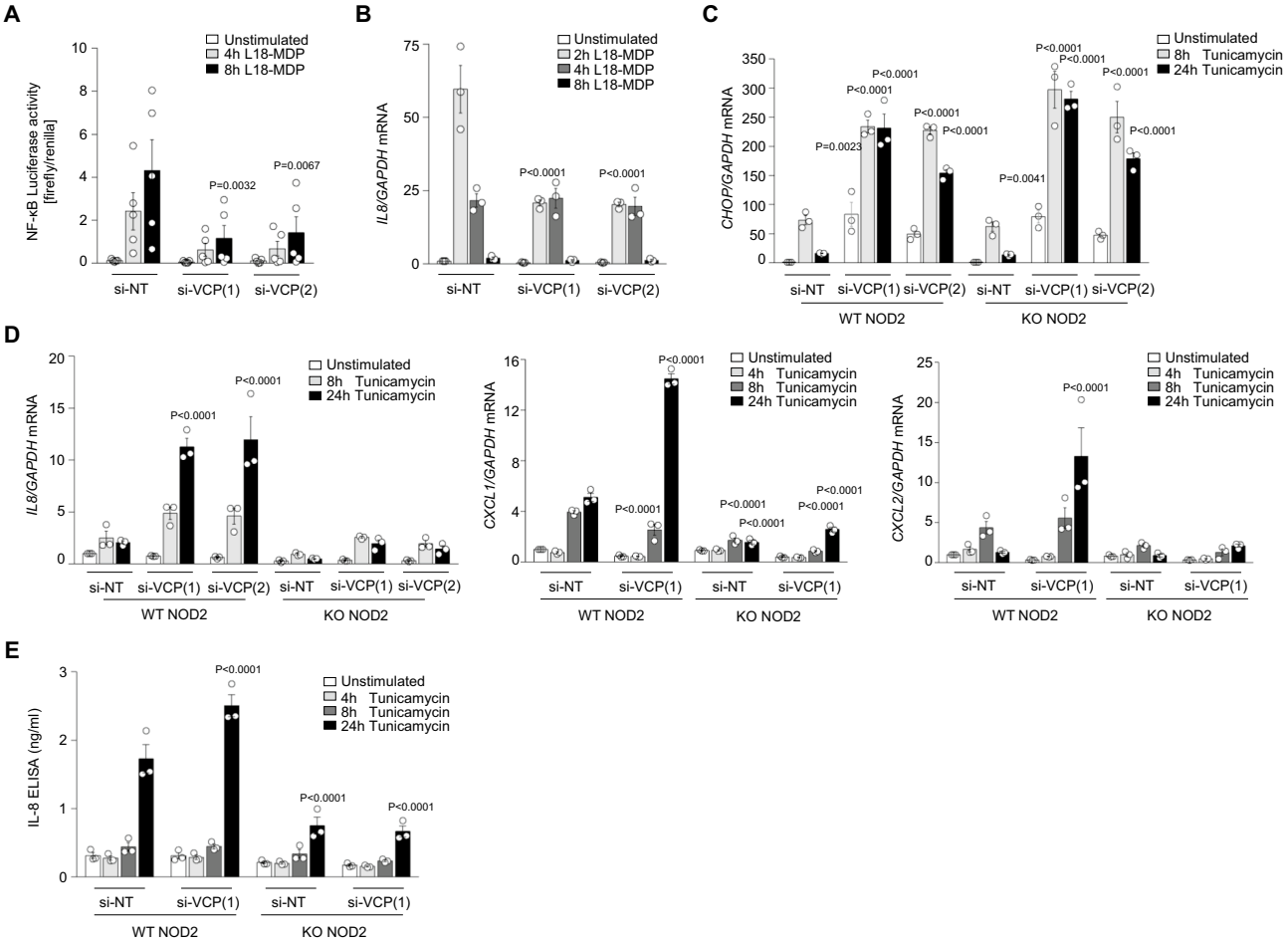

Figure 4. VCP-mediated ER-stress-induced proinflammatory responses are NOD2-dependent. (**A**) NF-kB-sensitive luciferase reporter activity (n = 5) and (**B**) quantitative RT–PCR analysis of *IL8* production in L18-MDP-treated HCT116 cells transfected with si-NT (non-targeting control) or si-VCP (see also Figure S4). (**C** and **D**) Quantitative RT–PCR analysis of *CHOP* (**C**) and *IL8*, *CXCL1*, *CXCL2* and *TNF* (**D**) transcriptional level on HCT116 cells transfected with si-NT or si-VCP upon tunicamycin stimulation (see also Figure S6). (**E**) ELISA of IL-8 secretion upon tunicamycin treatment in WT and NOD2 KO cells. (**B**, **C**, **D** and **E**) Data represent mean ± SEM of three independent experiments. P values in each treatment group were calculated in comparison to non-targeting control.

that NOD2 mediates proinflammatory responses upon different types of UPR via interaction of its nucleotide binding domains with sphingosine-1-phosphate[47]. Corresponding to their finding that ER stress activated NOD2 independent of the LRR domain, we could observe comparable transcriptional level of *IL8* in cells overexpressing the NOD2 p.L1007fsX1008 variant and WT NOD2. Thus, our investigation on a rare sequence indicated altered ER stress as possible mechanism how NOD2 polymorphisms may contribute to disease development and behavior.

The CARD domain of NOD2 is known to be important for the interaction with the adaptor protein RIPK2[48]. Our study revealed impaired RIPK2 interaction for the identified mutation p.E54K affecting the CARD domain but not the LRR domain variant p.L1007fsX1008. However, we could observe reduced phosphorylation and abrogated ubiquitination of RIPK2 as a potential common pathomechanism for the impaired MDP-triggered NF-κB activity in NOD2-deficient cells. Consistently, X-linked Inhibitor of Apoptosis (XIAP) E3 ubiquitin ligase activity mediating ubiquitination of RIPK2 has been previously reported to be indispensable for NF-κB activation initiated by NOD2 stimulation[49]. The relevance of this signaling axis for human disease has been demonstrated by loss-of-function XIAP mutations causing a severe immunodeficiency disorder[50,51]. In routine diagnostics, analysis of defective MDP-dependent NOD2 signaling is used to determine XIAP deficiency[52]. Since both mutations exhibited abrogated RIPK2 ubiquitination, future studies investigating the recruitment of XIAP to the NOD2 complex might provide further insights on the pathomechanisms of the NOD2 variants.

In view of the impaired RIPK2 interaction and posttranslational modification, we sought to decipher the interaction network of the NOD2 variant p.E54K. Using an immunoprecipitation-coupled mass spectrometry screen, we identified VCP as a novel NOD2 interaction partner that was associated with wild-type NOD2 protein but not with the NOD2 variants p.E54K and p.L1007fsX1008. VCP is an abundant ubiquitin-dependent ATPase that implicates in myriad of cellular processes such as ERAD, autophagy, DNA damage response, apoptosis and

ubiquitin–proteasome-dependent protein degradation[11–13,53,54]. Heterozygous germline mutations in *VCP* have been previously associated with Paget disease of bone and frontotemporal dementia, amyotrophic lateral sclerosis (ALS) and type 2 Charcot–Marie–Tooth disease[55–57]. Furthermore, increased level of proinflammatory cytokines have been observed in the plasma and myoblasts of patients with VCP mutations[58]. The function of VCP in the ERAD pathway has been reported to be regulated through interaction with the deubiquitinase ATAXIN3[59]. Interestingly phosphorylation of ATAXIN3 by NOD2 and TLR2 in myeloid cells has been recently shown to mediate mitochondrial reactive oxygen species production and bacterial clearance[60]. To evaluate plausible functions of VCP in NOD2 signaling, we used VCP-silenced cellular models that were stimulated with the NOD2 canonical stimuli MDP. Consistent with previous studies demonstrating impaired TNF- and IL-1β-triggered NF-κB signaling in VCP-deficient cells[61], we observed impaired NF-κB activity and proinflammatory cytokine responses in cells with knockdown of VCP upon MDP treatment comparable to NOD2-deficient cells. Strikingly, our data unveiled VCP as a negative regulator of NOD2 activity during tunicamycin-induced ER stress, as VCP silencing resulted in NOD2-dependent hyperinflammatory responses. While VCP-dependent CHOP transcription was not affected by knockout of NOD2, expression of the members of the CXC family of chemokines *IL8* (CXCL8)*, CXCL1, and CXCL2* was increased in a NOD2-dependent manner. These chemokines have been shown to be important in the regulation of neutrophil activation and migration as well as the induction of exaggerated angiogenesis at sites of inflammation[62]. Notably, the expression and activity of these molecules were positively correlated with the grade of inflammation in IBD patients[63–66]. Thus, altered NOD2-dependent proinflammatory cytokine responses upon ER stress may present a new link in the context of intestinal inflammation, however the exact mechanisms how VCP acts on NOD2 signaling remains elusive. Our data suggested that knockdown of VCP alters UPR but does not directly affect phosphorylation of RIPK2 or binding of RIPK2 to NOD2. Therefore, we propose that VCP-regulated UPR can be sensed by NOD2 and can trigger inflammatory responses in a NOD2-dependent manner. Previous studies have suggested that activation of NF-κB signaling is mediating ER stress-derived inflammation, however we could not observe substantial alteration of NF-κB p65 phosphorylation upon tunicamycin stimulation in VCP-silenced cells (data not shown). Further studies are required to profile ER stress-induced NOD2-dependent proinflammatory responses in greater detail and to decipher the underlying molecular mechanisms.

Taken together, molecular characterization of a rare germline mutation affecting the first CARD domain of NOD2 unveils VCP as novel interaction partner. Functional studies show that VCP controlled ER stress induces inflammatory responses in a NOD2-dependent fashion; thus, providing a new potential mechanistic link and therapeutic target in NOD2-related intestinal inflammation.

Methods

Patient. Written informed consent was obtained from the patient, first-degree relatives, and healthy donors for the collection of peripheral blood. The investigation was approved by the respective institutional review boards of the LMU Munich and conducted in accordance with current ethical and legal frameworks.

DNA sequencing. Next-generation sequencing was performed at the Dr. von Hauner Children's Hospital NGS facility. Genomic DNA was isolated from whole blood (Qiagen) for the generation of whole-exome libraries using the SureSelect XT Human All Exon V6 + UTR kit (Agilent Technologies). Barcoded libraries were sequenced with a NextSeq 500 platform (Illumina) to an average coverage depth of 90x. Bioinformatics analysis used Burrows-Wheeler Aligner (BWA 0.7.15), Genome Analysis ToolKit (GATK 3.6) and Variant Effect Predictor (VEP 89). The frequency filtering used allele frequencies from public (e.g. ExAC, GnomAD and GME) and in house databases. The potentially causative variants were confirmed by Sanger sequencing for the patient and informative family members.

Plasmids and retroviral-mediated gene expression. Full-length human WT *NOD2* was amplified from healthy donor (HD) cDNA. Patient-specific mutations (encoding p.E54K and P.L1007fsX1008) were introduced by site-directed PCR mutagenesis. WT and mutated *NOD2* cDNAs or fusion constructs with an N-terminal FLAG-tag were cloned into the IRES-EGFP or IRES-RFP bicistronic lentiviral pRRL vectors. Lentiviral particles were produced by transfection of HEK293T cells with viral packaging plasmids (psPAX2 and pMD2.G, kindly provided by Didier Trono, Geneva) together with lentiviral pRRL vectors enconding NOD2 WT or mutants. Supernatants were collected every 24 h for 3 days and filtrated prior to transduction of NOD2-deficient HCT116 cells in the presence of 8 µg/ml polybrene (Sigma-Aldrich). Sorting of transduced cells was conducted on a BD FACSAria cell sorter (BD Bioscience) based on RFP or EGFP mean fluorescence intensity. Human WT *RIPK2* was amplified from the verified cDNA sequence clone (GE Dharmacon, cat.no. MHS6278-202830678) and cloned into the pRRL-IRES-RFP plasmid.

Antibodies and reagents. Antibodies for phospho-NF-κB (p65) (Ser536) (3033, clone number 93H1), NF-κB (p65) (8242, clone number D14E12), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (4370, clone number D13.14.4E), p44/42 MAPK (Erk1/2) (9102), phospho-p38 MAPK (Thr180/Tyr182) (4511, clone number D3F9), p38 MAPK (9212), RIP2 (4142, clone number D10B11), phospho-RIP2 (Ser176) (14397S), VCP (2648), XBP-1S (12782 s), phospho-eIF2α (Ser51) (3597 s), and HRP-conjugated anti-rabbit IgG (7074) were purchased from Cell Signaling Technology. Phospho-PERK (T982) (ab192591) was purchased from Abcam. Beta-Actin-HRP (sc-47778, clone number c4) and GAPDH (sc-47724, clone number 0411) were purchased from Santa Cruz Biotechnology. Anti-Flag antibody (F1804, clone number M2), Anti-FLAG M2 Affinity Gel (A2220), lipopolysaccharide (LPS) (L2654) and tunicamycin Streptomyces sp. (T7765) were procured from Sigma-Aldrich. HRP-conjugated goat anti-mouse IgG (554002), PE mouse anti-human TNF (559321, clone

number MAb11) and anti-CD14-FITC (557153, clone M5E2), anti-CD14–BV786 (563698, clone M5E2), anti-CD3-BUV395 (564000, clone SK7), anti-CD3-PerCP (345766, clone SK7), anti-CD56-APC (341027, clone NCAM16.2), and anti-CD107a-PE (555801, clone H4A3) were procured from BD Biosciences. anti-CD3-pacific blue antibody (344823, clone SK7) was from Biolegend. L18-MDP (tlrl-lmdp) was from Invivogen. Agarose TUBE 2 (UM402) was purchased from Lifesensors. Lipofectamine™ 2000 (11668019) and Lipofectamine™ 3000 (L3000015) Transfection Reagents were procured from Thermo Fisher Scientific. IL-2 cytokine (2238131) was procured from Novartis.

Cell culture and stimulation. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll gradient centrifugation. PBMCs were maintained for 2–3 h in serum free RPMI-1640 medium (Gibco, Life Technologies) and then stimulated with L18-MDP (10 µg/ml) or LPS (1 µg/ml). To enrich for human primary monocytes, PBMCs were cultured in Iscove's Modified Eagle's Medium (IMDM) (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS) and kept overnight at 37 °C. The day after, non-adherent cells were washed off. To measure intracytoplasmic TNF, PBMC-derived monocytes were stimulated by adding either 200 ng/ml L18-MDP or 200 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich) in the presence of Golgistop (BD Biosciences) for 2.5 h. K562 cells (ATCC; CCL-243) were cultured in RPMI-1640 medium supplemented with 1% L-glutamine, 10% v/v FBS, and 1% penicillin/streptomycin. Human embryonic kidney HEK293T cells (ATCC, CRL3216) and coloncacinoma HCT116 cells (ATCC, CCL247) were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Thermo Fisher Scientific) supplemented with 1% L-glutamine, 10% v/v FBS, and 1% penicillin/streptomycin. For evaluating *IL8* transcriptional level, HCT116 cells stimulated with 1 µg/ml L18-MDP for 2, 4 and 8 h or with 5 µg/ml tunicamycin (Sigma-Aldrich) for 8 and 24 h. To analyze transcriptional level of *IL8*, *CXCL1*, *CXCL2* or *CHOP* in HCT116 cells 72 h post siRNA transfection, stimulation was performed with 1 µg/ml L18-MDP for 2, 4 and 8 h or with 5 µg/ml tunicamycin (Sigma-Aldrich) for 8 and 24 h.

NK cells Degranulation assay. PBMCs were either directly cultured in complete RPMI-1640 medium supplemented with anti-CD107a alone or together with K562 to induce NK cells degranulation. To investigate degranulation in activated NK cells, PBMCs were incubated for 2 days in complete RPMI medium containing 600 U/ml IL-2 before co-culturing with K562 and anti-CD107a. Centrifugation was performed at 30 g, RT for 3 min and cells were incubated for 3 h at 37 °C. Surface staining was performed with anti-CD107a, anti-CD3, and anti-CD56. Flow cytometry was conducted on the FACS Canto II (BD Biosciences) and CD107a surface expression was investigated in the $CD3^{-}CD56^{+}$ cell population. Analysis was performed with Flowjo V9 software (TreeStar).

Immunoblotting and silver staining. Cells were lysed in 1× cell lysis buffer (Cell Signaling Technologies) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1× protease inhibitors. Normalization of protein concentration was performed by Bradford assay and equal amount of proteins were subjected to 10–12% SDS–PAGE followed by immunoblotting using different antibodies. Chemiluminescence signals were detected using the SuperSignal West Dura detection kit (Thermo Fisher Scientific) on the ChemiDoc™ XRS + System (Bio-Rad) and analyzed with the ImageLab™ software (Bio-Rad). SDS–PAGE silver staining was performed using silverQuest™ (Invitrogen) according to the manufacturer's protocols.

Intracellular flow cytometry. Cells were washed with PBS and then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Bioscience) and stained with CD14, CD3 and TNF antibodies. Flow cytometry was performed on the LSRFortessa™ flow cytometer (BD Biosciences) and analyzed with the Flowjo V9 software (TreeStar)[52].

Engineering of NOD2 knockout cell lines using CRISPR/Cas9 genome editing. The Alt-R® CRISPR-Cas9 (IDT technology) genome editing system was used according to the manufacturer's instructions on HCT116 cells for the generation of knockouts. Electroporation was performed using the SE Cell Line 4D-Nucleofector® X Kit and the 4DNucleofector™ System (Lonza). Single cells were sorted into 96-well plates on a BD FACSAria (BD Bioscience) 48 h post transfection. In expanded clones, NOD2 knockout was functionally confirmed using NF-κB luciferase reporter gene assays.

Quantitative real-time PCR analysis and ELISA. Total RNA was isolated using the RNeasy plus Kit (Qiagen) and reverse-transcribed to cDNA according to the manufacturer's protocols (MultiScribe Reverse Transcriptase; Applied Biosystems). Relative transcriptional level were measured by SYBR Green dye-based quantitative real-time PCR (qRT–PCR) and analyzed using the ABI Prism 7500 Fast RT-PCR System (Applied Biosystems). GAPDH was used as a housekeeping marker. The list of primers is given in Supplementary Table 1. IL-8 secretion in the supernatant was quatified using the Human IL-8/CXCL8 DuoSet ELISA kit (R&D) and measured using a Synergy H1 microplate reader (BioTek Instruments) according to the manufacturer's protocol.

NF-κB luciferase reporter gene assays. HEK293T cells were transfected with the p55-A2-Luc luciferase reporter plasmid, internal control pTK-Green Renilla plasmid, NOD2 plasmids or control empty plasmids using Lipofectamine 2000™ (Thermo Fisher Scientific) according to manufacturer's recommendations. L18-MDP stimulation (200 ng/ml, Invivogen) was performed for 7 h followed by measurement of luciferase activity using the Dual Luciferase Assay Kit (Biotium). To study NF-kB activity in VCP knockdown HCT116 cells, cells were transfected with the p55-A2-Luc luciferase reporter plasmid and pTK-Green Renilla plasmid by

lipofectamine 3000™ (Thermo Fisher Scientific) 24 h after siRNA treatment. L18-MDP stimulation (200 ng/ml, Invivogen) was performed for 4 and 8 h after 72 h of siRNA transfection . To screen NOD2 KO HCT116, the NF-κB luciferase reporter assay was performed as described for HEK293T cells.

Co-immunoprecipitation assays. HEK293T cells were transfected with 10 µg FLAG-NOD2 WT and mutants alone or together with RIPK2 using polyethyleneimine (PEI; Polysciences). After 72 h, the cells were washed in PBS and lysed in the RIPA buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 10% glycerol) supplemented with 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride and 1 × protease inhibitors (Roche). Immunoprecipitation was performed by incubating the lysates with anti-FLAG M2 Affinity Gel (Sigma-Aldrich) for 7 h at 4 °C on the rocker platform. Beads were washed three times in 1 ml ice-cold RIPA buffer and bound proteins were eluted by boiling the beads in gel loading buffer. HCT116 cells stably expressing NOD2 constructs were directly treated with L18-MDP for indicated time points and lysed in RIPA buffer.

Purification of endogenous Ub conjugates. NOD2 KO HCT116 cells with WT or mutant NOD2 variants were stimulated with 200 ng/ml L18-MDP (Invivogen) for 1 and 2 h. Cells were washed in PBS and lysed in cell lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluorid, 1 × protease inhibitors (Roche) and 1 mM N-ethylmaleimide. Ubiquitinated proteins were isolated using Tandem Ubiquitin Binding Entities (TUBEs, LifeSensors) according to the manufacturer's instructions. Eluted samples were analyzed by western blotting on 10% SDS-PAGE.

siRNA transfection. Two siRNAs targeting human VCP and non-targeting (NT) siRNA oligonucleotides were designed and validated as described by Paola Magnaghi et al.[67]. HCT116 cells were transfected with 5 nM of siRNA oligonucleotides using Lipofectamine™ 3000 (Thermo Fisher Scientific) according to manufacturer's recommendations and incubated for 72 h. Knockdown efficiency was assessed by qPCR and western blotting. The sequences of oligonucleotides are provided in Supplementary Table 1.

Analysis of cell death in HCT116 coloncarcinoma cell lines. NOD2 knockout and lentiviral reconstituted NOD2 WT HCT116 cells treated with siRNAs targeting human VCP and non-targeting (NT) siRNA oligonucleotides were stimulated with 5 µg/ml tunicamycin (Sigma-Aldrich) for 24 h. To measure cell death, HCT116 cells were stained with Annexin V and DAPI (Thermo Fisher Scientific) and analyzed by flow cytometry.

Nano-LC MS/MS analysis. Samples were seperated by SDS-PAGE (SERVAGel TG PRiME 4–20%, Serva). Gels were Coomassie stained (Simply Blue, Expedeon) and the area containing proteins was excised. Gel slices were destained (50% acetonitril, 50 mM NH4HCO3) and subjected to in-gel digestion using the following steps: For protein reduction and alkylation, gel slices were first incubated in 45 mM DTE/50 mM NH4HCO3 for 30 min at 55 °C and then incubated for 30 min in 100 mM iodoacaetamide/50 mM NH4HCO3. In-gel digestion was done using 0.7 µg Trypsin at 37 °C overnight. Samples were analyzed by nano-LC MS/MS using an Ultimate 3000 nano liquid chromatography system (ThermoFisher Scientific) coupled to a TripleTOF 5600 + instrument (Sciex). As solvent A 0.1% formic acid and as solvent B acetonitrile with 0.1% formic acid was used. Peptides were separated at a flow rate of 200 nL/min on an Acclaim PepMap RSLC C18 column (75 µm × 50 cm, Thermo Fisher Scientific) with the following gradient: from 2% B to 25% B in 120 min followed by 25% B to 50% B in 10 min. For mass spectrometry, the ion source was operated at a needle voltage of 2.3 kV. Mass spectra were acquired in cycles of one MS scan from 400 m/z to 1250 m/z and up to 40 data dependent MS/MS scans of the most intensive peptide signals. For protein identification (FDR < 1%) and label free quantification, the MaxQuant software platform[68] was used in combination with the Human subset of the UniProt database. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD031539[69].

Statistical analysis. Prism version 6 (GraphPad Software, USA) was used for statistical analysis of experimental data. Probability (P) values were calculated using two-way repeated-measures ANOVA and P values < 0.05 were considered to be statistically significant. Statistical details of experiments can be found in the figures and figure legends. Biologically independent experiments are referred to as n.

Data availability

The data generated in this study is available upon request, please contact the corresponding author.

Received: 5 April 2021; Accepted: 24 February 2022
Published online: 10 March 2022

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Acknowledgements

We kindly acknowledge our index patient and family for the opportunity to study the molecular pathomechanisms of disease. We thank the interdisciplinary medical team at the Dr. von Hauner Children's Hospital. We acknowledge the assistance of the Flow Cytometry and Care-for-Rare Genomics Core Facility at the Dr. von Hauner Children's Hospital. The work has been supported by The Leona M. and Harry B. Helmsley Charitable Trust, DFG (Gottfried-Wilhelm-Leibniz Program, Heinz Maier-Leibnitz-Preis, Collaborative Research Consortium SFB1054 project A05), PID-NET (BMBF), BioSysNet, and the Care-for-Rare Foundation. Maryam Ghalandary has received scholarships from the Bayerische Forschungsstiftung and subsequently by the Care-for-Rare Foundation. Yue Li has been supported by the China Scholarship Council (CSC) and Care-for-Rare Foundation. Daniel Kotlarz has been a scholar funded by the Daimler und Benz Stiftung, Reinhard-Frank Stiftung, and Else-Kröner-Fresenius Stiftung.

Author contributions

M.G. and Y.L. designed and conducted the experiments and analyzed the data. T.F performed Nano-LC MS/MS assays and analysis. T.M. performed NK cell degranulation assay and MDP-dependent signaling on patient-derived cells. Y.L helped in CRISPR/Cas9-mediated genetic engineering. M. R performed whole-exome sequencing in the Next-Generation Sequencing facility at the Dr. von Hauner Children's Hospital. S. H. conducted the bioinformatics analysis of the sequencing. R.C supported the flow cytometry analysis. P.B., S.K., F.H., A.M.M., S.B.S. recruited and clinically characterized the patients and were critical in the interpretation of the human data. T.S. and H.H.U. provided expertise on NOD2 signaling studies. C.K. and D.K. conceived the study design, supervised M.G. and Y.L, and recruited study participants. M.G., Y.L, C.K., and D.K. wrote the draft of the manuscript. All authors interpreted the data and approved the final version of manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-07804-1.

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6. Appendix: Paper III

Title: Dysregulation of Cell Death in Human Chronic Inflammation

Year: 2020

Journal: Cold Spring Harb Perspect Biol

Volume: 12

Issue: 7

DOI: 10.1101/cshperspect.a037036

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Dysregulation of Cell Death in Human Chronic Inflammation

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Cold Spring Harbor Perspectives in Biology
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Inflammation is a fundamental biological process mediating host defense and wound healing during infections and tissue injury. Perpetuated and excessive inflammation may cause autoinflammation, autoimmunity, degenerative disorders, allergies, and malignancies. Multimodal signaling by tumor necrosis factor receptor 1 (TNFR1) plays a crucial role in determining the transition between inflammation, cell survival, and programmed cell death. Targeting TNF signaling has been proven as an effective therapeutic in several immune-related disorders. Mouse studies have provided critical mechanistic insights into TNFR1 signaling and its potential role in a broad spectrum of diseases. The characterization of patients with monogenic primary immunodeficiencies (PIDs) has highlighted the importance of TNFR1 signaling in human disease. In particular, patients with PIDs have revealed paradoxical connections between immunodeficiency, chronic inflammation, and dysregulated cell death. Importantly, studies on PIDs may help to predict beneficial effects and side-effects of therapeutic targeting of TNFR1 signaling.

Inflammation is a protective mechanism in host defense and wound healing during tissue damage or infection (Medzhitov 2008). The degree of inflammation depends on the infectious or toxic triggers and on host susceptibility. Inflammatory responses are complex processes involving vascular permeability, inflammatory mediators (e.g., chemokines, adhesion molecules, cytokines, enzymes), detecting sensors, and extracellular matrix components, as well as recruitment of circulating inflammatory cells, activation of resident immune cells, and adaptive immunity.

Inflammatory mediators, danger-associated molecular patterns (DAMPs), and hypoxia lead to recruitment and degranulation of platelets and resident mast cells as well as activation of tissue-resident immune cells. The release of chemoattractants orchestrates leucocyte migration to the site of inflammation (Medzhitov 2008). Neutrophils with phagocytotic and microbicidal functions are recruited from the circulation as well. Initially, neutrophils potentiate the proinflammatory environment to eliminate inflammatory agents, but apoptosis and clearance of neutrophils are central processes in the resolution of inflammation (Mantovani et al. 2011). Circulating monocytes enter the site of inflammation and differentiate into tissue macro-

Editors: Kim Newton, James M. Murphy, and Edward A. Miao
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Advanced Online Article. Cite this article as *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a037036

phages that phagocytose foreign particles, debris, and apoptotic cells. The clearance of apoptotic neutrophils triggers a switch from a pro- to an anti-inflammatory program in macrophages. In the late phase of inflammation, lymphocytes will be recruited and mediate adaptive immunity (Serhan and Savill 2005). Coordinated networks are required to resolve and control inflammatory processes. Excessive and uncontrolled inflammation caused by failure to remove noxious materials and apoptotic inflammatory cells may contribute to autoinflammation, autoimmunity, degenerative diseases, allergy, and malignancies (Silva et al. 2008).

Inflammation and cell death are intertwined biological processes sharing many receptors and effector molecules. The release of proinflammatory factors by dying cells may facilitate recovery or extension of inflammation, but accumulating evidence suggests that perturbed cell-death responses may actively contribute to inflammation (Rock and Kono 2008). Whereas necroptosis and pyroptosis release DAMPs (for example, ATP, DNA, and uric acid) through permeabilized membranes and are primarily considered to enhance inflammation, apoptosis contains cytoplasmic content and is thought to be critical in the termination process (Rock and Kono 2008). While different forms of cell death share morphological and biochemical similarities, the molecular characteristics and host responses can be drastically different depending on the biological context. The fate decision of cell death versus inflammation is tightly controlled by multiple pathways, including proinflammatory tumor necrosis factor receptor 1 (TNFR1) signaling. Mouse studies have unveiled mechanistic insights on the regulation of TNFR1 signaling and how it may contribute to disease (Fig. 1; Silke et al. 2015). The characterization of patients with monogenic primary immunodeficiencies (PIDs) has shown the critical role of TNFR1 signaling in human disease and highlighted paradoxical links between immunodeficiency and dysregulation of cell death in chronic inflammation (Table 1). Here, we review recent insights with a focus on novel inherited errors of human immunity.

MULTIMODAL TNFR1-DEPENDENT SIGNALING DETERMINES INFLAMMATORY AND CELL-DEATH FATES

TNF plays a critical role in regulating host defense, but can also be pathogenic in several inflammatory conditions (Monaco et al. 2015). TNFR1 signaling intertwines inflammation and cell death by engaging IKK/NF-κB and caspase-8/receptor interacting protein kinase 1 (RIPK1)/RIPK3 signaling (Fig. 1; Kalliolias and Ivashkiv 2016). TNF is produced by several immune, epithelial, endothelial, and stromal cell types (Grivennikov et al. 2005). Upon binding of TNF to trimeric TNFR1, a membrane-associated complex I is formed by recruitment of the adaptor protein TNFR1-associated death domain protein (TRADD), TNFR1-associated factor 2 (TRAF2), cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2), RIPK1, and linear ubiquitin chain assembly complex (LUBAC) (Micheau and Tschopp 2003; Kirisako et al. 2006). The latter is composed of heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), HOIL-1-interacting protein (HOIP), and SHANK-associated RH domain-interacting protein (SHARPIN) (Kirisako et al. 2006; Gerlach et al. 2011; Ikeda et al. 2011; Tokunaga et al. 2011). Modification of RIPK1 and possibly other complex I components with Lys63-linked polyubiquitin assembled by cIAP1/2, and Met1-linked ubiquitin assembled by LUBAC, mediates activation of TGF-β-activated kinase 1 (TAK1) and IκB kinase (IKK) (Micheau and Tschopp 2003; Wang et al. 2008). Activated TAK1 and IKK induce MAPK signaling and ubiquitin–protein system-mediated degradation of IκB leading to NF-κB activation.

Compromised prosurvival signaling emanating from complex I results in the formation of alternative cytosolic TNF-induced complexes mediating apoptosis and necroptosis (Van Antwerp et al. 1996). Proinflammatory NF-κB signaling can be terminated by disassembly of complex I through A20- and cylindromatosis (CYLD)-mediated deubiquitylation of RIPK1 and TRAF2 (Wertz et al. 2004; Wang et al. 2008). Formation of cytosolic complexes containing TRADD, Fas-associated protein with death domain (FADD), RIPK1, and procas-

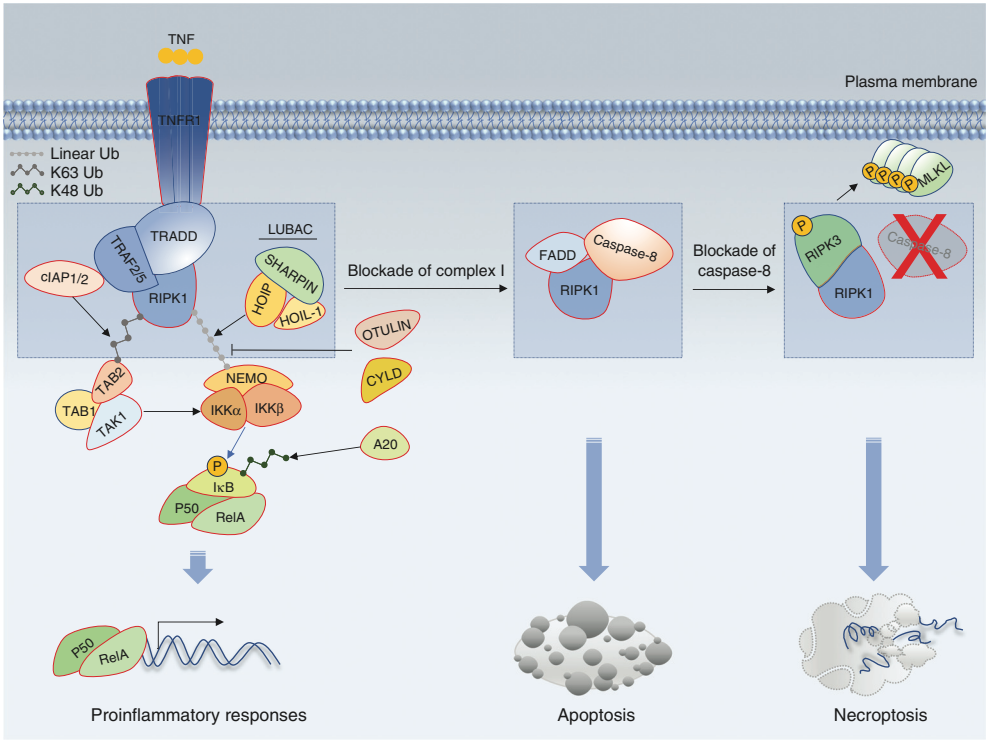

Figure 1. The tumor necrosis factor receptor 1 (TNFR1) signaling pathway as a master regulator of inflammation and cell death. TNFR1 encountering TNF nucleates complex I, which includes TNFR1-associated death domain protein (TRADD), receptor-interacting protein kinase 1 (RIPK1), TNFR1-associated factor 2 (TRAF2), cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2), and the linear ubiquitin chain assembly complex (LUBAC) composed of heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), HOIL1-interacting protein (HOIP), and SHANK-associated RH domain-interacting protein (SHARPIN). Polyubiquitinated RIPK1 recruits the IκB kinase (IKK) complex (composed of the NF-κB essential modulator [NEMO], IKKα, and IKKβ) and the TAK1 complex, which mediate NF-κB and MAPK signaling. Degradation of phosphorylated IκB mediates translocation of p50 and RelA to the nucleus and transcription of proinflammatory and prosurvival NF-κB target genes. The stability of complex I is regulated by deubiquitinating enzymes such as A20, cylindromatosis (CYLD), and OTU deubiquitinase with linear linkage specificity (OTULIN). Formation of complex II containing Fas-associated protein with death domain (FADD), caspase-8, TRADD, and RIPK1 can trigger apoptosis. If the activity of caspase-8 is compromised, RIPK3 interacts with RIPK1 via its RHIM domain. Autophosphorylated RIPK3 leads to recruitment, phosphorylation, and oligomerization of the pseudokinase mixed lineage kinase domain-like (MLKL). Translocation of activated MLKL to the plasma membrane results in necroptosis. Proteins highlighted by red frames indicate that mutations in the corresponding genes have been reported as monogenic causes for primary immunodeficiencies.

pase-8 (Micheau and Tschopp 2003; Wang et al. 2008) can lead to homodimerization and activation of caspase-8, with subsequent cleavage of caspase-3 and -7 mediating extrinsic apoptosis (Boatright et al. 2003; Micheau and Tschopp 2003). Apoptosis is the best-defined form of programmed cell death with characteristic morphological and biochemical changes such as nuclear envelope disassembly, cytoplasmic condensation and fragmentation, membrane blebbing, and formation of membrane-bound bodies (Green et al. 2009). Apoptosis plays a pivotal role in controlling immune cell development and homeostasis, by eliminating self-reactive, overactivated, and infected immune cells (Green et al. 2009). Apoptotic cells are ingested

Table 1. Phenotypes of constitutive knockout mouse models and patients with monogenic immune-related disorders affecting tumor necrosis factor receptor 1 (TNFR1) signaling

Genes (mouse/human)	Mouse	Human
Casp8/*CASP8*	Embryonic lethality; cardiac deformations; neural tube defects; hematopoietic progenitor dysfunctions (Varfolomeev et al. 1998)	ALPS-like disorder (lymphadenopathy, splenomegaly, immunodeficiency with defective activation of T, B, and NK cells (Chun et al. 2002) Late-onset multiorgan lymphocytic infiltrations with granulomas (Niemela et al. 2015) Immunodeficiency (increased susceptibility to viral and bacterial infections, defects in T and B cells) and VEO-IBD (Lehle et al. 2019)
Chuk/*CHUK* (IKKα)	Perinatal lethality; impaired limb outgrowth; skeletal morphogenesis; epidermal defects (Hu et al. 1999; Takeda et al. 1999)	Abortions; multiple fetal malformations (e.g., craniofacial abnormalities and absent limbs) (Lahtela et al. 2010)
Cyld/*CYLD*	Autoimmunity; abnormal thymocyte development; impaired lymphocyte activation; B-cell hyperplasia (Reiley et al. 2006; Zhang et al. 2006)	Phenotypic heterogeneity including cylindromatosis, multiple familial trichepithelioma type I, and Brooke–Spiegler syndrome (Bignell et al. 2000; Mathis et al. 2015)
Fadd/*FADD*	Embryonic lethality as a result of defective vascular development (Yeh et al. 1998)	Immunodeficiency (bacterial and viral susceptibility); functional hyposplenism; febrile episodes; encephalopathy; developmental abnormalities (Bolze et al. 2010)
Ikbkb/*IKBKB* (IKKβ)	Embryonic lethality; TNFR1-dependent hepatocyte apoptosis and degeneration (Li et al. 1999; Tanaka et al. 1999)	Severe combined immunodeficiency (hypogammaglobulinemia or agammaglobulinemia, peripheral T and B cells are exclusively of naive phenotype, absence of regulatory T cells and γδ T cells, impaired lymphocyte activation) (Pannicke et al. 2013)
Ikbkg/*IKBKG* (NEMO)	Males: embryonic lethality; liver degeneration; defective generation and/or persistence of lymphocytes Females: severe skin lesions with extensive granulocyte infiltration and hyperproliferation; hepatocyte and keratinocyte apoptosis (Schmidt-Supprian et al. 2000)	Loss-of-function mutations: incontinentia pigmenti (Smahi et al. 2000) Hypomorphic mutations: X-linked ectodermal dysplasia with immunodeficiency and diverse clinical manifestations (e.g., life-threatening infections, inflammatory diseases, osteopetrosis, lymphedema) (Zonana et al. 2000; Döffinger et al. 2001)
Mlkl/*MLKL*	No detectable abnormality in the development of immune cells (Wu et al. 2013)	No human disease identified
Nfkb1/*NFKB1*	Intestinal inflammation; B-cell dysfunction; defective adaptive immunity in response to infections (Sha et al. 1995)	Haploinsufficiency: common variable immunodeficiency with recurrent respiratory infections; hypogammaglobulinemia; autoimmunity; progressing pulmonary disease (Chen et al. 2013; Fliegauf et al. 2015) Loss of function: lymphadenopathy; splenomegaly; autoimmunity; defects in B-cell differentiation (Tuijnenburg et al. 2018)

Continued

Table 1. *Continued*

Genes (mouse/human)	Mouse	Human
Nfkbia/*NFKBIA* (IκBα)	Early neonatal lethality; severe inflammatory dermatitis; enhanced granulopoiesis (Beg and Baltimore 1996; Klement et al. 1996) Knockin mice (Ser32Ile): immunodeficiency; defective lymphoid organogenesis (Mooster et al. 2015)	Gain-of-function mutations: EDA-ID; T- and B-cell deficiencies with increased susceptibility to infections (Courtois et al. 2003; Boisson et al. 2017)
Otulin/*OTULIN*	Embryonic lethality; compromised craniofacial and neuronal development; impaired angiogenesis (Rivkin et al. 2013)	Fatal autoinflammation; recurrent nodular panniculitis; lipodystrophy; diarrhea; joint swelling; failure to thrive (Damgaard et al. 2016; Zhou et al. 2016b)
Rbck1/*RBCK1* (HOIL)	Embryonic lethality; disrupted vascular architecture and cell death in the yolk sac endothelium (Peltzer et al. 2018)	Immunodeficiency (susceptibility to pyogenic bacterial infections) and autoinflammation (hyperresponsiveness to IL-1β); amylopectinosis (Boisson et al. 2012)
Rela/*RELA*	Embryonic lethality; TNF-mediated cell death of hepatocytes, macrophages, and fibroblasts (Beg and Baltimore 1996)	Chronic mucocutaneous ulceration; increased apoptosis of fibroblasts in response to TNF; impaired NF-κB activation in fibroblasts and PBMCs; impaired stromal cell survival (Badran et al. 2017)
Ripk1/*RIPK1*	Perinatal lethality; massive apoptosis in lymphoid and adipose tissue; multiorgan hyperinflammation (Kelliher et al. 1998)	Life-threatening immunodeficiency (lymphopenia, recurrent infections, defective differentiation of T and B cells); VEO-IBD; arthritis (Cuchet-Lourenço et al. 2018; Li et al. 2019)
Ripk3/*RIPK3*	Viable and fertile (Newton et al. 2004)	No human disease identified
Rnf31/*RNF31* (HOIP)	Embryonic lethality; defective vascularization caused by aberrant endothelial cell death (Peltzer et al. 2014)	Multiorgan autoinflammation (hyperreactive monocytes in response to IL-1β); combined immunodeficiency (recurrent viral and bacterial infections, lymphopenia, antibody deficiency, impaired B-cell activation, and differentiation in response to CD40, impaired T-cell distribution and functions); subclinical amylopectinosis; systemic lymphagiectasia (Boisson et al. 2015)
Sharpin/*SHARPIN*	Liver inflammation; splenomegaly; severe eosinophilic skin inflammation; defective lymphoid organogenesis associated with excessive TNFR1-mediated death (Kumari et al. 2014; Rickard et al. 2014a)	No human disease identified
Tnfaip3/*TNFAIP3* (A20)	Perinatal lethality or lethality shortly after birth; severe multiorgan inflammation (e.g., liver, kidneys, intestines, joints, and bone marrow) (Lee et al. 2000)	Early-onset systemic autoinflammatory syndrome resembling Behcet's disease (Zhou et al. 2016a)

by phagocytes before they can release immunogenic intracellular contents, and this prevents activation of the innate immune system (Green et al. 2016). Impaired apoptosis has been implicated in the pathogenesis of immune-related disease conditions, as exemplified by autoimmune lymphoproliferative syndrome (ALPS) (Fisher et al. 1995; Rieux-Laucat et al. 1995; Drappa et al. 1996).

When the activity of caspase-8 is compromised, necroptosis is initiated by heterodimerization of RIPK1 and RIPK3 via their RIP homotypic interaction motif (RHIM) (Cho et al. 2009; He et al. 2009). Oligomerization and autophosphorylation of RIPK3 result in the recruitment and phosphorylation of the pseudokinase MLKL (Murphy et al. 2013). Subsequent oligomerization and translocation of MLKL to the plasma membrane lead to cell rupture (Petrie et al. 2019). The release of DAMPs from necroptotic cells may be highly immunogenic (Oberst et al. 2011). Altered necroptosis has been implicated in malignancies as well as several pathological inflammatory conditions, including infectious, cardiovascular, neurological, renal, and hepatic diseases (Weinlich et al. 2017). Moreover, several mouse studies have shown that deletion of *Mlkl* can partially ameliorate inflammation (Rickard et al. 2014a,b; Alvarez-Diaz et al. 2016), indicating that necroptosis may contribute to the pathogenesis of inflammatory disorders. In contrast, necroptosis may benefit host defense against viruses such as herpes simplex virus 1 by eliminating infected cells (Huang et al. 2015; Guo et al. 2018).

Multimodal TNFR1 signaling governs the transition between inflammation, survival, and programmed cell death. However, the exact physiological mechanisms triggering the transition from prosurvival to prodeath responses are still unclear. The complexity of TNFR1 signaling will be further modulated by cross talk with other signaling pathways that can engage with inflammatory and cell-death modules. Dysregulation of components involved in TNFR1 signaling can lead to chronic inflammation. Correspondingly, inhibition of TNF is an effective treatment for several autoinflammatory and autoimmune disorders.

INFLAMMATION IN MONOGENIC DISORDERS AFFECTING TNFR1-MEDIATED SIGNALING

Caspase-8 Deficiency

Caspase-8 is an initiator cysteinyl aspartate-specific protease critical for receptor-mediated apoptosis induced by TNF, TRAIL, and Fas ligand (FASL) (Boldin et al. 1996; Muzio et al. 1996; Ashkenazi and Dixit 1998). The zymogen procaspase-8 consists of two amino-terminal death effector domains (DEDs) and a carboxy-terminal protease domain with two catalytic subunits (p10 and p18) (Earnshaw et al. 1999). Procaspase-8 dimerization via the DED promotes proteolytic cleavage that generates active caspase-8 heterotetramers ($p10_2$-$p18_2$) (Earnshaw et al. 1999). Active caspase-8 then cleaves and activates the executioner caspases-3 and -7 to induce apoptosis (Earnshaw et al. 1999).

The essential role of caspase-8 in death receptor-induced apoptosis was shown using cells from *Casp8* knockout (KO) mice (Varfolomeev et al. 1998). These mice exhibited embryonic lethality associated with cardiac deformations, neural tube defects, and hematopoietic progenitor dysfunctions (Varfolomeev et al. 1998; Sakamaki et al. 2002). Conditional KOs of *Casp8* have revealed critical roles for caspase-8 in the response to tissue damage or infection. For example, caspase-8-deficient hepatocytes exhibited impaired proliferation after injury to the liver and this prompted chronic inflammation of the liver (Ben Moshe et al. 2007). Loss of *Casp8* in the epidermis also caused inflammation with hyperactive responses to activators of interferon regulatory factor (IRF)3 (Kovalenko et al. 2009). Furthermore, mice lacking caspase-8 in IECs developed spontaneous ileitis that was associated with TNF-induced necroptotic cell death (Günther et al. 2011).

When the activity of caspase-8 is hampered, RIPK1 initiates RIPK3/MLKL-dependent necroptosis (Cho et al. 2009; He et al. 2009; Zhang et al. 2009). Interestingly, most disease phenotypes associated with caspase-8 deficiency in mice were attributed to aberrant necroptosis because they were rescued by loss of *Ripk3* or *Mlkl*

(Kaiser et al. 2011; Oberst et al. 2011; Alvarez-Diaz et al. 2016). Mouse studies have also implicated caspase-8 in lymphocyte differentiation and function (Salmena et al. 2003; Kang et al. 2004; Beisner et al. 2005). T-cell-specific deletion of *Casp8* resulted in profound depletion of peripheral T cells associated with defective activation and/or survival upon engagement of the T-cell receptor (TCR) (Salmena et al. 2003). These defects impaired $CD8^+$ T-cell-mediated antiviral immunity. Proliferation of caspase-8-deficient T cells could be restored by inhibition of RIPK1 or genetic ablation of *Ripk3*, implying that caspase-8 suppresses necroptosis during T-cell activation (Bell et al. 2008; Ch'en et al. 2011; Kaiser et al. 2011; Oberst et al. 2011). B-cell-specific deletion of caspase-8 did not impact B-cell development but compromised B-cell activation by Toll-like receptor (TLR) agonists (Lemmers et al. 2007).

The relevance of caspase-8 for human immunity was originally recognized by studies involving two siblings with germline homozygous missense mutations in *CASP8* (Chun et al. 2002). Similar to ALPS patients with loss-of-function mutations in genes encoding Fas, FASL, and caspase-10, the patients with germline mutation in *CASP8* presented with lymphadenopathy and splenomegaly that was associated with defective Fas-mediated apoptosis in T cells (Chun et al. 2002). The homozygous mutation in *CASP8* (Arg248Trp) was located in the p18 protease subunit and it reduced protein stability and enzymatic activity. Unlike typical ALPS, the caspase-8-deficient patients also had defects in the activation of their T-, B-, and natural killer cells causing immunodeficiency (Chun et al. 2002). T-cell dysfunction was associated with impaired TCR-induced nuclear translocation of NF-κB (Su et al. 2005), but given the later studies in mice (Bell et al. 2008; Ch'en et al. 2011; Kaiser et al. 2011; Oberst et al. 2011), the question became whether the defects in NF-κB signaling were a consequence of aberrant necroptosis.

The clinical spectrum of caspase-8 deficiency was further broadened by the description of two patients with the mutation Arg248Trp. These patients presented with late-onset multiorgan lymphocytic infiltrations with granulomas (Niemela et al. 2015). By contrast, Lehle et al. recently described patients with homozygous missense mutations in *CASP8* (Gln237Arg) that affect the cleavage and activation of caspase-8 (Lehle et al. 2019). These patients had life-threatening very early-onset inflammatory bowel disease (VEO-IBD) and immunodeficiency that was accompanied by increased susceptibility to viral and bacterial infections, marked lymphadenopathy, reduced TCR-dependent T-cell proliferation and activation, and impaired B-cell maturation (Lehle et al. 2019). Mouse studies have previously shown that myeloid cells lacking *Casp8* exhibited increased NLRP3-dependent inflammasome activity with enhanced secretion of proinflammatory cytokines IL-1β and IL-18 (Kang et al. 2013). Correspondingly, caspase-8-deficient patient monocytes secreted more proinflammatory IL-1β than control monocytes in response to lipopolysaccharide (LPS) (Lehle et al. 2019). In caspase-8-deficient human BLaER1 monocyte/macrophage models, blockade of either NLPR3-dependent inflammasome activity or MLKL-dependent necroptosis attenuated IL-1β secretion (Gaidt et al. 2016; Lehle et al. 2019). Thus, both pathways are implicated in proinflammatory cytokine responses. These findings are consistent with the notion that necroptosis can activate the NLRP3 inflammasome (Vince and Silke 2016). Targeting necroptosis might present an attractive therapeutic approach in caspase-8-deficient patients with VEO-IBD, but more detailed mechanistic studies are required.

The identification of caspase-8-deficient patients with VEO-IBD underscores the critical function of caspase-8 in maintaining human intestinal epithelial homeostasis (Lehle et al. 2019). Whereas TRAIL triggered cell death in healthy donor-derived intestinal organoids, caspase-8-deficient cells were unresponsive to TRAIL. In contrast to mouse organoids with loss of *Casp8* (Günther et al. 2011), patient-derived caspase-8-deficient intestinal organoids did not exhibit a marked increase in TNF-induced cell death (Lehle et al. 2019). Further studies are needed to determine genotype–phenotype correlations of the mutations in human

CASP8. The physiological triggers of intestinal inflammation in human caspase-8 deficiency need to be further defined to identify targeted therapies.

FADD Deficiency

FADD is an adaptor protein that recruits caspase-8 to death receptors (Wilson et al. 2009). Mice lacking *Fadd* show RIPK3- and MLKL-dependent embryonic lethality similar to mice lacking caspase-8 (Yeh et al. 1998; Alvarez-Diaz et al. 2016). T-cell-specific loss of *Fadd*, similar to caspase-8 deficiency, caused defective T-cell proliferation that was rescued by inhibition of RIPK1 (Osborn et al. 2010). In addition, Osborn et al. observed enlarged lymph nodes and spleen with increased B cells and red blood cells, respectively. Mice lacking *Fadd* in epidermal keratinocytes or intestinal epithelial cells (IECs) developed severe inflammation (Bonnet et al. 2011; Welz et al. 2011), indicating that FADD is essential for homeostasis in the skin and intestine. Skin inflammation was triggered by RIPK3-dependent necroptosis, and was partially dependent on the catalytic activity of the deubiquitinating enzyme CYLD and/or TNFR1 signaling (Bonnet et al. 2011). Loss of *Fadd* in IECs caused spontaneous RIPK3-dependent colitis with epithelial erosions and crypt abscesses (Welz et al. 2011). Disease was prevented by deletion of *Cyld* or *Myd88,* or by elimination of the microbiota. Thus, TLR signaling activated by bacteria was a key driver of colitis (Welz et al. 2011).

In humans, a homozygous loss-of-function mutation in the death domain of *FADD* (Cys105Trp) was reported to impair Fas-induced apoptosis, as in patients with ALPS (Bolze et al. 2010). However, the related patients presented with immunodeficiency, bacterial susceptibility, and developmental abnormalities rather than autoimmunity (Bolze et al. 2010). In contrast to KO mouse models, FADD-deficient patients showed normal T-cell proliferation, but impaired interferon-dependent antiviral immunity, leading to increased susceptibility to viral diseases (e.g., varicella zoster, parainfluenza virus, and Epstein–Barr virus).

RIPK1 Deficiency

RIPK1 is a key molecule controlling both inflammation and cell-death responses via scaffolding-dependent and kinase-specific functions (Ofengeim and Yuan 2013). In particular, RIPK1 mediates multimodal TNFR1 signaling depending on the cell type and biological context. While TNF-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 Pasparakis and Vandenabeele (2015). RIPK1-deficient mice exhibited perinatal lethality because of multiorgan hyperinflammation that is driven by aberrant caspase-8-dependent apoptosis and MLKL-dependent necroptosis (Kelliher et al. 1998; Dillon et al. 2014; Kaiser et al. 2014; Rickard et al. 2014b). Conditional KO mice have demonstrated the essential role of RIPK1 in controlling immune and intestinal homeostasis. Mice with loss of *Ripk1* in IECs developed severe inflammation in the gut because of FADD/caspase-8-dependent apoptosis (Dannappel et al. 2014; Takahashi et al. 2014), whereas keratinocyte-specific RIPK1 KOs developed skin inflammation associated with ZBP1/RIPK3/MLKL-dependent necroptosis (Lin et al. 2016). T-cell-specific deletion of *Ripk1* in mice caused severe lymphopenia and defective T-cell proliferation (Dowling et al. 2016). RIPK1 also contributes to the maintenance of peripheral B cells (Zhang et al. 2011). RIPK1-deficient fetal liver-derived mouse macrophages exhibited enhanced inflammasome activity upon LPS priming (Lawlor et al. 2015).

In contrast to RIPK1-deficient mice, knock-in mice expressing catalytically inactive RIPK1 D138N or K45A showed no signs of tissue pathology and are protected from TNF-induced acute shock (Berger et al. 2014; Newton et al. 2014; Polykratis et al. 2014). Thus, the kinase activity of RIPK1 is dispensable for suppressing cell death. Necrostatin-1, a small molecule inhibitor of the kinase activity of RIPK1, has been shown to protect mice from retinal degeneration (Murakami et al. 2014), retinitis pigmentosa (Sato et al. 2013), ischemic brain injury (Deg-

terev et al. 2005; Northington et al. 2011), neurodegeneration (Zhu et al. 2011), myocardial infarction, cardiac hypoxia (Smith et al. 2007; Oerlemans et al. 2012), and renal ischemia-reperfusion injury (Lau et al. 2013).

Recently, studies on patients with monogenic defects of *RIPK1* have provided critical insights into the role of RIPK1 in human disease. The patients presented with early-onset, life-threatening immunodeficiency and intestinal inflammation (Cuchet-Lourenço et al. 2018; Li et al. 2019; Uchiyama et al. 2019). Some patients showed arthritis (Cuchet-Lourenço et al. 2018), but skin inflammation was not observed, which is in contrast to RIPK1-deficient mice. Human RIPK1 deficiency was associated with impaired T- and B-cell maturation, defective TNF-mediated activation of the NF-κB pathway, and dysregulated cytokine signaling in immune cells. Cuchet-Lourenço et al. (2018) suggested that inflammation in *RIPK1*-deficient patients was caused by altered cytokine secretion and necroptosis of immune cells. In parallel and independent experiments, Li et al. studied six pedigrees and demonstrated that *RIPK1*-deficient macrophages exhibited increased inflammasome activity in response to LPS. Inhibition of MLKL- and NLRP3-dependent pathways by small molecule inhibitors attenuated secretion of proinflammatory IL-1β (Li et al. 2019), but the underlying mechanisms are not completely understood. Blockade of IL-1 has not yet been tested in RIPK1-deficient patients.

Since histological examination of gastrointestinal biopsies revealed only occasional apoptotic morphology, Cuchet-Lourenço et al. proposed that dysfunction of the immune system was critical for disease development. The authors concluded that allogeneic hematopoietic stem cell transplantation (HSCT) may constitute a curative therapy and showed resolution of clinical symptoms in one patient (Cuchet-Lourenço et al. 2018). Li et al. (2019) studied RIPK1-deficient IECs as well as hematopoietic cells. RIPK1-deficient IECs were resistant to killing by TNF, suggesting that RIPK1 also plays a critical intrinsic role in controlling epithelial homeostasis. Differences in the observed phenotypes might be because of the treatment of patients with anti-inflammatory drugs and antibiotics, their genetic background, or environmental factors. HSCT might cure cytokine production defects in immune cells, but not intrinsic epithelial defects, similar to NEMO-deficient patients (Miot et al. 2017). The in vivo triggers perturbing epithelial integrity in mice or humans lacking RIPK1 have not been defined. Moreover, the currently reported RIPK1-deficient patients provided no insights into the role of the kinase domain of RIPK1, because the patient-specific mutations reduced expression of RIPK1 protein. Further studies are needed to define genotype–phenotype correlations, triggers, and molecular consequences of human RIPK1 deficiency.

MONOGENIC DEFECTS OF THE NF-κB SIGNALING PATHWAY

NF-κB is a master transcriptional regulator of cell survival and proliferation, innate and adaptive immunity, and inflammation. Consequently, NF-κB signaling must be tightly regulated for tissue and immune homeostasis (Hayden and Ghosh 2011). Abnormal NF-κB signaling might cause defective immune activation, immunodeficiency, autoimmunity, or lymphoid malignancies (Courtois and Gilmore 2006). Human monogenic defects in NF-κB signaling components have been shown to cause severe immune disorders (Hayden and Ghosh 2008) that may vary from phenotypes in mouse models.

The IKK complex is composed of catalytic subunits (IKK1/IKKα, IKK2/IKKβ) and a regulatory subunit (NF-κB essential modulator [NEMO]) (Chen et al. 1996; DiDonato et al. 1997; Yamaoka et al. 1998). Mice lacking IKKβ were embryonic lethal (Li et al. 1999; Tanaka et al. 1999), whereas impaired degradation of IκBα and delayed NF-κB signaling in IKKβ-deficient patients caused severe combined immunodeficiency (Pannicke et al. 2013). Mice lacking IKKα died at birth because of multiple severe malformations and skin defects (Hu et al. 1999; Takeda et al. 1999). Patients with IKKα deficiency manifested with similar phenotypes, but showed more severe craniofacial abnormalities (Lahtela et al. 2010).

Several mouse and human studies have documented that loss of NEMO, the regulatory subunit of the IKK complex, causes defective NF-κB activation. Loss of X-linked *Nemo*/*Ikbkg* caused embryonic lethality in male mice, whereas severe skin lesions were observed in heterozygous females (Schmidt-Supprian et al. 2000). Mice with IEC-specific KO of *Nemo* developed spontaneous colitis with enhanced apoptosis of Paneth cells and impaired expression of antimicrobial factors, which was dependent on the kinase activity of RIPK1 (Vlantis et al. 2016). Mutations in human X-linked *NEMO/IKBKG* cause varying phenotypes, in particular anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) (Zonana et al. 2000; Döffinger et al. 2001) or incontinentia pigmenti (Smahi et al. 2000). Notably, about 25% of patients develop colitis associated with poor HSCT outcome (Hanson et al. 2008; Kawai et al. 2012).

Similar to *NEMO* deficiency, autosomal-dominant gain-of-function mutations in *NFKBIA* (encoding IκBα) caused sustained inhibition of NF-κB signaling that leads to EDA-ID, T- and B-cell deficiency, and increased susceptibility to infections (Courtois et al. 2003; Boisson et al. 2017). Knockin mice that are heterozygous for the human *NFKBIA* mutation (Ser32Ile) developed EDA-ID and lacked lymph nodes, Peyer's patches, splenic marginal zones, and follicular dendritic cells. They also failed to develop contact hypersensitivity or form germinal centers, which are features characteristic of defective noncanonical NF-κB signaling through NF-κB2/RelB (Mooster et al. 2015).

Haploinsufficiency of *NFKB1* (p105/p50) or *NFKB2* (p100/p52) can cause common variable immunodeficiency with recurrent respiratory infections, hypogammaglobulinemia, and autoimmunity (Chen et al. 2013; Fliegauf et al. 2015). In addition, patients with loss-of-function mutations in *NFKB1* demonstrated noninfective complications, including lymphadenopathy, splenomegaly, and autoimmunity (Tuijnenburg et al. 2018). It remains to be shown whether the phenotype of pyoderma gangrenosum in patients with monoallelic *NFKB1* mutations is caused by dominant-active effects, loss-of-function, or haploinsufficiency. Of note, all patients showed defective B-cell differentiation. Similarly, *Nfkb1*-deficient mice developed intestinal inflammation that was associated with profound B-cell dysfunction, including defects in proliferation, class-switch recombination, maturation, humoral immunity, cytokine secretion, and susceptibility to infection (Sha et al. 1995; Bendall et al. 1999).

MONOGENIC DISORDERS OF UBIQUITINATION AND DEUBIQUITINATION IN THE TNFR1 SIGNALING CASCADE

The ubiquitin system plays a crucial role in balancing gene activation and cell death (Aksentijevich and Zhou 2017). Perturbed ubiquitination or deubiquitination can result in dysregulation of the immune system (Aksentijevich and Zhou 2017). LUBAC, the E3 ligase composed of HOIL-1, HOIP, and SHARPIN, inhibits TNFR1-mediated cell death by generating linear polyubiquitin chains on NEMO and other complex I components (Peltzer et al. 2014, 2018; Rickard et al. 2014a). Loss of *Rnf31* (encoding HOIP) caused embryonic lethality in mice (Peltzer et al. 2014) as a result of aberrant cell death (Peltzer et al. 2018), whereas excessive cell death in *Sharpin*-deficient mice caused severe eosinophilic skin inflammation and defective lymphoid organogenesis (Kumari et al. 2014; Rickard et al. 2014a). Mice with keratinocyte-specific depletion of LUBAC components developed severe dermatitis caused by FASL-, TRAIL-, and TNF-induced cell death (Taraborrelli et al. 2018).

No human loss-of-function mutations in *SHARPIN* have been reported yet, but HOIP or HOIL-1 deficiencies cause PID and autoinflammation with overlapping phenotypes such as susceptibility to infections and amylopectinosis (Boisson et al. 2012, 2015). Mutations in *RNF31* or *RBCK1* (encoding HOIL-1) that impaired the stability of LUBAC attenuated NF-κB signaling in fibroblasts or B cells treated with IL-1β or TNF. However, patient-derived monocytes were hyperresponsive to IL-1β, leading to up-regulation of inflammatory cytokines and chemokines. TNF-inhibitory treatment has

been shown to ameliorate pathology temporarily, but autoinflammation was controlled by HSCT in one HOIL-1-deficient patient (Boisson et al. 2012). It is unclear why HOIL-1 and HOIP are essential for embryogenesis in mice, but not humans. Heterogeneity in the genetic background of humans may be a factor, or there may be physiological differences between species.

The deubiquitinases A20, OTULIN, and CYLD are negative regulators of NF-κB signaling (Lork et al. 2017). However, emerging data have also suggested unexpected roles of these deubiquitinases in regulating cell death independent of NF-κB signaling (Draber et al. 2015; Heger et al. 2018; Polykratis et al. 2019). Defects in these genes lead to increased proinflammatory cytokine profiles (Lork et al. 2017). A20 can cleave Lys63-linked polyubiquitin chains on target proteins, such as RIPK1 and NEMO, to inhibit NF-κB signaling, but it is the binding of A20 to Met1-linked ubiquitin chains that appears to limit the formation of complexes that trigger proinflammatory cell death. For example, mice lacking A20 in myeloid cells developed arthritis that was driven by necroptosis and activation of the NLRP3 inflammasome. Analyses of A20 knockin mice indicated that the ubiquitin-binding ZnF7 domain in A20 is critical for preventing arthritis, whereas the deubiquitinating activity of A20 is dispensable (Draber et al. 2015; Polykratis et al. 2019).

A20-deficient mice die shortly after birth showing severe multiorgan inflammation (Lee et al. 2000). Tissue-specific deletion of *Tnfaip3* (encoding A20) in lymphocytes, enterocytes, dendritic cells, keratinocytes, mast cells, hepatocytes, and microglial cells has further demonstrated the crucial role of A20 in maintaining immune homeostasis and inhibiting inflammation (Cox et al. 1992; Tavares et al. 2010; Hammer et al. 2011; Wang et al. 2013; Vereecke et al. 2014; Drennan et al. 2016; Maelfait et al. 2016). Genetic variants of human *TNFAIP3* are associated with a broad range of inflammatory and autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, psoriasis, type I diabetes, celiac disease, Crohn's disease, coronary artery disease in type 2 diabetes, and systemic sclerosis (Ma and Malynn 2012; Zhou et al. 2016a). Mutations causing *TNFAIP3* haploinsufficiency led to early-onset systemic autoinflammatory syndrome, resembling Behcet's disease, because of increased NF-κB-mediated proinflammatory cytokine production (Zhou et al. 2016a). The authors did not specifically study cell-death responses, but patient cells treated with LPS showed enhanced cleavage of caspase-1 and secretion of mature IL-1. These findings are reminiscent of RIPK1 and CASP8 deficiencies, and thus it is tempting to speculate that enhanced inflammasome activation is mediated by aberrant necroptosis.

OTULIN cleaves Met1-linked polyubiquitin chains conjugated by LUBAC (Keusekotten et al. 2013). Recent studies have shown that OTULIN promotes rather than counteracts LUBAC activity. Specifically, OTULIN limits autoubiquitination of LUBAC, which would otherwise lead to RIPK1-dependent cell death (Heger et al. 2018). Consequently, *Otulin* KO mice were embryonic lethal (Rivkin et al. 2013) similar to mice lacking HOIP or HOIL-1 (Peltzer et al. 2014, 2018). Homozygous missense mutations in human *OTULIN* caused cell-type-specific alterations in NF-κB signaling, fatal autoinflammation with recurrent nodular panniculitis, lipodystrophy, diarrhea, joint swelling, and failure to thrive (Damgaard et al. 2016; Zhou et al. 2016b). Patient-derived monocytes and fibroblasts exhibited increased sensitivity to TNF-induced cell death (Damgaard et al. 2019). Moreover, treatment with anti-TNF neutralizing antibodies could ameliorate inflammation, whereas HSCT induced sustained remission in OTULIN-deficient patients (Damgaard et al. 2019).

CYLD has been extensively studied for its role in removing Lys63- or Met1-linked polyubiquitin chains from proteins mediating NF-κB signaling. For example, CYLD deubiquitinates proteins in TNFR1 complex I, which limits NF-κB signaling and promotes the assembly of cell-death signaling complexes (Draber et al. 2015). It has been suggested that CYLD regulates innate and adaptive immune responses via its negative regulation of NF-κB signaling components, but dysfunctional cell death might also contribute to immune-related phenotypes. Mice

lacking CYLD showed autoimmunity associated with abnormal thymocyte development, lymphocyte activation, and B-cell hyperplasia (Reiley et al. 2006, 2007; Zhang et al. 2006; Jin et al. 2007). CYLD deficiency in humans can lead to distinct phenotypes with cylindromatosis and skin manifestations such as multiple familial trichoepithelioma, type I (Mathis et al. 2015; Farkas et al. 2016). The phenotypic heterogeneity of human *CYLD* deficiency is likely the result of its diverse roles in controlling other NF-κB-independent pathways such as cell-death responses, cell-cycle progression, and microtubule dynamics (Sun 2010; Zhang et al. 2017a).

TNFR1 SIGNALING AS A THERAPEUTIC TARGET—WHAT DO WE LEARN FROM MONOGENIC DISEASES?

Several mouse and human studies indicate the critical role of TNFR1 signaling in health and disease, as reviewed in Brenner et al. (2015). TNF inhibition has proven effective as treatment for several autoinflammatory and autoimmune conditions (Kalliolias and Ivashkiv 2016). However, many patients with inflammatory disorders are refractory to anti-TNF therapy or develop side-effects (Kalliolias and Ivashkiv 2016). Thus, alternative strategies targeting the TNFR1 pathway are needed to expand the therapeutic armamentarium.

RIPK1/RIPK3/MLKL-dependent necroptosis has been implicated in malignancies and several pathological inflammatory conditions (Weinlich et al. 2017). Small-molecule inhibitors targeting RIPK1 kinase activity present attractive therapeutic potential, because mice expressing catalytically inactive RIPK1 develop normally without inflammatory phenotypes (Berger et al. 2014; Newton et al. 2014; Polykratis et al. 2014). The therapeutic potential of RIPK1 inhibitors has been demonstrated in various mouse disease models (Silke et al. 2015). Based on these studies RIPK1 inhibitor programs have successfully passed clinical phase I trials for the treatment of chronic psoriasis, rheumatoid arthritis, and ulcerative colitis (GSK2982772, DNL747) (Harris et al. 2017; Mullard 2018).

Targeting of RIPK3 is a new idea to treat inflammatory diseases, particularly since mice lacking *Ripk3* are viable (Newton et al. 2004). However, knockin mice expressing catalytically inactive RIPK3 D161N exhibited caspase-8-dependent embryonic lethality (Newton et al. 2014), raising concerns about the toxic effects of targeting RIPK3. Indeed, inhibitors of RIPK3 (GSK'840, GSK'843, and GSK'872) trigger RIPK3- and caspase-8-dependent apoptosis reminiscent of that seen in RIPK3 D161N mice (Kaiser et al. 2013; Mandal et al. 2014). Thus, further refinement of RIPK3-based therapies is needed.

Blockade of MLKL has been considered as a means of selectively inhibiting necroptosis. For example, necrosulfonamide (NSA), which is a compound that modifies Cys86 of human MLKL to block its oligomerization, has been suggested as a potential therapeutic for neurodegenerative diseases (Zhang et al. 2017b), but it has not been tested in clinical trials. The MLKL inhibitor compound 1 caused cell toxicity at high concentrations, and thus has not been used in clinical applications (Hildebrand et al. 2014). Recently, a new inhibitor (TC13172) targeting Cys86 of MLKL was demonstrated to block the translocation of MLKL to cell membranes in cell lines (Yan et al. 2017).

Inhibitors of caspase-8 have been proposed for patients with dysregulated cell death and/or inflammation. The pancaspase inhibitor Emricasan has antiapoptotic and anti-inflammatory effects, and has been explored for the treatment of liver disease (Frenette et al. 2019; Garcia-Tsao et al. 2019), renal disease, and diabetes (Kudelova et al. 2015). However, inhibition of caspase-8 might induce necroptosis in some cell types and thereby promote inflammation.

As a master regulator of immunity, NF-κB has been implicated in various autoimmune diseases (Herrington et al. 2016). Selective targeting of NF-κB activity presents another line of therapeutic modulation, but specificity is a major challenge. Commonly used anti-inflammatory agents, such as antirheumatic drugs, nonsteroidal anti-inflammatory drugs, and glucocorticoids have been shown to partly modulate NF-κB signaling at various levels (Yama-

moto and Gaynor 2001; Herrington et al. 2016). Specific inhibitors of NF-κB, such as caffeic acid phenethyl ester and carfilzomib, are now available for treatment of myeloma (Kane et al. 2003; Herndon et al. 2013), but remain to be evaluated for autoimmunity.

Mouse models have been exquisite tools for studying the pathomechanisms of diseases and for drug development. However, mice may respond differently from humans to therapies, and show distinct phenotypes from patients with monogenic disorders in orthologous genes. The characterization of PID provides critical molecular insights into key factors mediating TNFR1 signaling. Further studies on PID are required to explore genotype–phenotype correlations and the molecular mechanisms of disease in detail. These studies lay the groundwork for the development of targeted therapies for both rare and common immune and inflammatory diseases. Furthermore, patients with monogenic disorders affecting the TNFR1 pathway help to predict the therapeutic efficacy and side-effects of available therapies targeting TNFR1 signaling.

CONCLUDING REMARKS

TNFR1 signaling is a crucial "command center" controlling immunity, inflammation, and cell death. Dysregulation of these pathways may cause immunodeficiency and/or autoinflammation. Advances in genomic technologies have facilitated the identification of patients with life-threatening PID. The characterization of these patients has provided critical and unexpected insights into the essential role of human TNFR1 signaling in controlling inflammation. The identified candidate genes at the intersection of prosurvival and cell-death pathways have shown that modulation of the TNFR1 pathway can contribute to both severe immunodeficiency and chronic inflammation. Further mechanistic studies in mice and especially advanced human preclinical models will provide critical understanding of imbalanced inflammation and cell death in PID. This knowledge on rare monogenic diseases will help to optimize personalized treatments for children with devastating conditions, but will also prioritize new targets for drug development of common autoimmunity and autoinflammation.

ACKNOWLEDGMENTS

Together with patients suffering from inborn errors of immunity, we have a chance to investigate basic principles of human immunity and autoinflammation. We dedicate this work to all patients with inborn errors of immunity and their families, and thank them for their support of our translational studies. Furthermore, we are grateful to the interdisciplinary medical teams and all global collaboration partners, in particular, the international VEO-IBD consortium. We thank the Care-for-Rare Foundation and The Leona M. and Harry B. Helmsley Charitable Trust for financial support.

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Cold Spring Harb Perspect Biol published online December 16, 2019

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7. Acknowledgements

Words cannot express my gratitude for my supervisors, PD Dr. med. Dr. Daniel Kotlarz and Prof. Dr. med. Dr. sci. nat. Christoph Klein, who gave me the opportunity to learn from the excellent scientists in a well-equipped lab. They have taught me all the good qualities in order to become a scientific researcher, who ought to devote their time and effort to biomedical science so that the patients who are suffering from rare diseases can hope for a better future.

I'm also thankful to our patients. They are struggling to live a normal healthy life and feeling sad not able to play like other children. But I hope they and their families can take some solace from the fact that the scientific discoveries we make - while trying to make them better - will mean that fewer children in the future will have to suffer. They are the real super heroes.

I devote this thesis to my parents, whom I never felt far away even with 10,000 km between us, to my grandpa, who didn't talk much but was my strongest supporter, and to all the people who were with me during this long journey.