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Genetic Regulation of Cell Death: Insights from Autoinflammatory Diseases

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Abstract

Metazoans have evolved innate antimicrobial defenses that promote cellular survival and proliferation. Countering the inevitable molecular mechanisms by which microbes sabotage these pathways, multicellular organisms rely on an alternative, perhaps more ancient, strategy that is the immune equivalent of suicide bombing: Infection triggers cell death programs that summon localized or even systemic inflammation. The study of human genetics has now unveiled a level of complexity that refutes the naive view that cell death is merely a blunt instrument or an evolutionary afterthought. To the contrary, findings from patients with rare diseases teach us that cell death–induced inflammation is a sophisticated, tightly choreographed process. We herein review the emerging body of evidence describing a group of illnesses—inborn errors of cell death, which define many of the molecular building blocks and regulatory elements controlling cell death–induced inflammation in humans—and provide a possible road map to countering this process across the spectrum of rare and common illnesses.

1. INTRODUCTION

Monogenic systemic autoinflammatory diseases (SAIDs) compose a rapidly expanding and genetically heterogeneous group of inborn and acquired disorders caused by the hyperactivation of innate immunity (1, 2). The genetic architecture of SAIDs is complex and is linked to germline and somatic mutations in more than 60 genes (3). Although rare, these disorders have provided important insights into intricate mechanisms regulating innate immune responses. Notably, in recent years, a growing number of human genetic studies have underscored the contributions of cell death pathways, particularly of apoptosis and necroptosis, to the initiation and progression of several human SAIDs, which we recently proposed to term inborn errors of cell death (IECDs) (4).

Cell death is a fundamental biological process that plays an essential role in tissue homeostasis and pathological responses (5, 6). Molecularly characterized cell death pathways play an integral part during host defense against invading pathogens by effectively eliminating infected cells to halt pathogen replication and by alerting the immune system through the release of cellular contents known as damage-associated molecular patterns (DAMPs). Apoptosis is a nonlytic type of death wherein the cell condenses and breaks into small membranous bodies that are cleared by phagocytic cells. Although initially and currently considered to be immunologically silent, apoptosis can drive inflammation through the disruption of the body surface barrier or via cellular responses when tissue injury produces enough cell debris to overwhelm phagocytotic clearance (7–11). Necroptosis, which is mediated by the activation of the receptor-interacting protein kinase 3 (RIPK3), is a programmed form of lytic inflammatory cell death that results in plasma membrane rupture mediated by mixed lineage kinase domain–like protein (MLKL), a pore-forming pseudokinase, resulting in the release of DAMPs (12, 13). Pyroptosis, another example of lytic inflammatory cell death, is induced by inflammasome-mediated cleavage and oligomerization of gasdermin D (GSDMD), with subsequent plasma membrane rupture and the release of biologically active IL-1β and IL-18 (14). Finally, NETosis is a form of inflammatory cell death that begins when activated neutrophils extrude their decondensed chromatin NET-like fibers decorated with antimicrobial granules and other cytosolic proteins, called neutrophil extracellular traps (NETs) (15), which may perpetuate inflammatory responses (16, 17). An understanding of the multifaceted aspects of cell death and of the intersections among different pathways will shed light on the pathogenesis of diverse human conditions (18, 19).

In this article, we explore recent advances in our understanding of cell death by addressing how impaired regulation of apoptosis and necroptosis can trigger disease in individuals with genetic alterations in cell death machinery (**Table 1**). In addition to discussing the genetic and mechanistic findings underlying each disorder, we highlight commonalities and differences between human disorders and their murine counterparts. We conclude by reviewing the therapeutic potential of treatments that target apoptosis and/or necroptosis and the current major challenges in the development and application of these therapies. Finally, we comment that the contribution of pyroptosis has already been well-investigated in a specific group of SAIDs caused by the hyperactivation of inflammasomes (inflammasomopathies). We refer readers to comprehensive reviews of the contribution of pyroptosis as a molecular mechanism underlying SAIDs (20–24).

2. MECHANISTIC OVERVIEW OF CELL DEATH AND INFLAMMATION

The ability of innate immune cells to combat invading pathogens relies on the rapid activation of immune responses, such as those mediated by NF-κB (25). Tumor necrosis factor (TNF) stands out as one of the most critical humoral factors that initiate and amplify this response (6, 26)—not only by inducing inflammatory gene expression but also by activating cell death. The cellular

Table 1 Human disorders associated with dysregulation of cell death

Disease	Gene	Protein	Inheritance	OMIM disease number	Disease mechanism	Phenotype
HOIP deficiency	*RNF31*	HOIP	AR	620632	Loss of function (hypomorphic)	Severe immunodeficiency, autoinflammation, glycogen storage disease, lymphangiectasia
HOIL-1 deficiency	*RBCK1*	HOIL-1	AR	615895	Loss of function	Severe immunodeficiency, autoinflammation, glycogen storage disease
Sharpenia	*SHARPIN*	SHARPIN	AR	620795	Loss of function	Autoinflammation, mild immunodeficiency, glycogen storage disease
OTULIN deficiency/ORAS	*OTULIN*	OTULIN	AR, AD	617099	Loss of function	Autoinflammation, absence of immunodeficiency
OTULIN haploinsufficiency	*OTULIN*	OTULIN	AD, risk allele	619986	Loss of function	Skin abscesses, lung infections
RIPK1 deficiency	*RIPK1*	RIPK1	AR	618108	Loss of function	Severe immunodeficiency, gastrointestinal inflammation, arthritis
CRIA	*RIPK1*	RIPK1	AD	618852	Gain of function (resistance to caspase-mediated cleavage)	Fever, lymphadenopathy, splenomegaly
TBK1 deficiency	*TBK1*	TBK1	AR	620880	Loss of function	Systemic autoinflammation, CNS vasculitis, absence of immunodeficiency
TBK1 deficiency	*TBK1*	TBK1	AD	617900	Haploinsufficiency or dominant negative	Herpes simplex virus 1 encephalitis
TBK1-associated neurodegenerative diseases	*TBK1*	TBK1	Risk allele	616439	Hypomorphic	Amyotrophic lateral sclerosis/frontotemporal dementia
RAID	*RELA*	RelA (p65)	AD	618287	Haploinsufficiency or dominant negative	Mucocutaneous inflammation, autoimmunity, lymphoproliferation
NEMO deficiency	*IKBKG*	NEMO	XR	300291	Loss of function (hypomorphic)	Severe immunodeficiency, severe colitis, ectodermal dysplasia
Incontinentia pigmenti	*IKBKG*	NEMO	XD	308300	Loss of function; somatic in males	Genodermatosis with variable expressivity due to skewed X inactivation
NEMO-NDAS	*IKBKG*	NEMO	XR, XD	301081	Aberant splicing of exon 5	Systemic autoinflammation in males and females; skin rash, ectodermal dysplasia, dental abnormalities

(*Continued*)

Table 1 (*Continued*)

Disease	Gene	Protein	Inheritance	OMIM disease number	Disease mechanism	Phenotype
IκBα gain of function	*NFKBIA*	IκBα	AD	612132	Defects in IκBα degradation	Severe immunodeficiency, autoinflammation, ectodermal dysplasia
HA20	*TNFAIP3*	A20	AD	616744	Haploinsufficiency	Autoinflammation, mild immunodeficiency, susceptibility to B cell lymphoma
XIAP deficiency	*XIAP*	XIAP	XR	300635	Loss of function	Autoinflammation, susceptibility to HLH and B cell lymphoma
RIPK3 deficiency	*RIPK3*	RIPK3	Risk allele	NA	Loss of function	Herpes simplex virus 1 encephalitis, reduced penetrance
MLKL deficiency	*MLKL*	MLKL	Risk allele	NA	Loss of function	Neurodegeneration, reduced penetrance
MLKL-associated CRMO	*MLKL*	MLKL	Risk allele	NA	Hypermorphic	Sterile bone inflammation

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CNS, central nervous system; CRIA, cleavage-resistant RIPK1-induced autoinflammation; CRMO, chronic recurrent multifocal osteomyelitis; HA20, haploinsufficiency of A20; HLH, hemophagocytic lymphohistiocytosis; HOIL-1, heme-oxidized iron regulatory protein 2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; IκBα, inhibitor of NF-κB α; NA, not applicable; MLKL, mixed lineage kinase domain–like protein; NDAS, NEMO δ-exon 5-autoinflammatory syndrome; NEMO, NF-κB essential modulator; OMIM, Online Mendelian Inheritance in Man; OTULIN, ovarian tumor domain deubiquitylase with linear linkage specificity; ORAS, OTULIN-related autoinflammatory syndrome; RAID, *RELA*-associated inflammatory disease; RIPK, receptor-interacting protein kinase; SHARPIN, SHANK-associated RH domain-interacting protein; TANK, TRAF-associated NF-κB activator; TBK1, TANK-binding kinase 1; TRAF, tumor necrosis factor receptor–associated factor; XD, X-linked dominant; XR, X-linked recessive.

decision of survival or death in response to TNF is determined primarily by the assembly of two spatially distinct protein complexes, termed complex I and complex II. The formation of complex I [also called TNF receptor 1 (TNFR1) signaling complex (TNFR1-SC)] is initiated by the binding of TNF to monomeric TNFR1 receptors and the subsequent trimerization of the intracellular death domain (DD) of TNFR1; this trimerization creates a signaling scaffold by recruiting DD-containing molecules such as TRADD (TNFR1-associated DD protein) and RIPK1 (27) (**Figure 1**). Complex I is further stabilized through multiple ubiquitylation processes—e.g., Lys63-linked ubiquitylation (Lys63-Ub) by cIAP1 and cIAP2 (cellular inhibitor of apoptosis 1

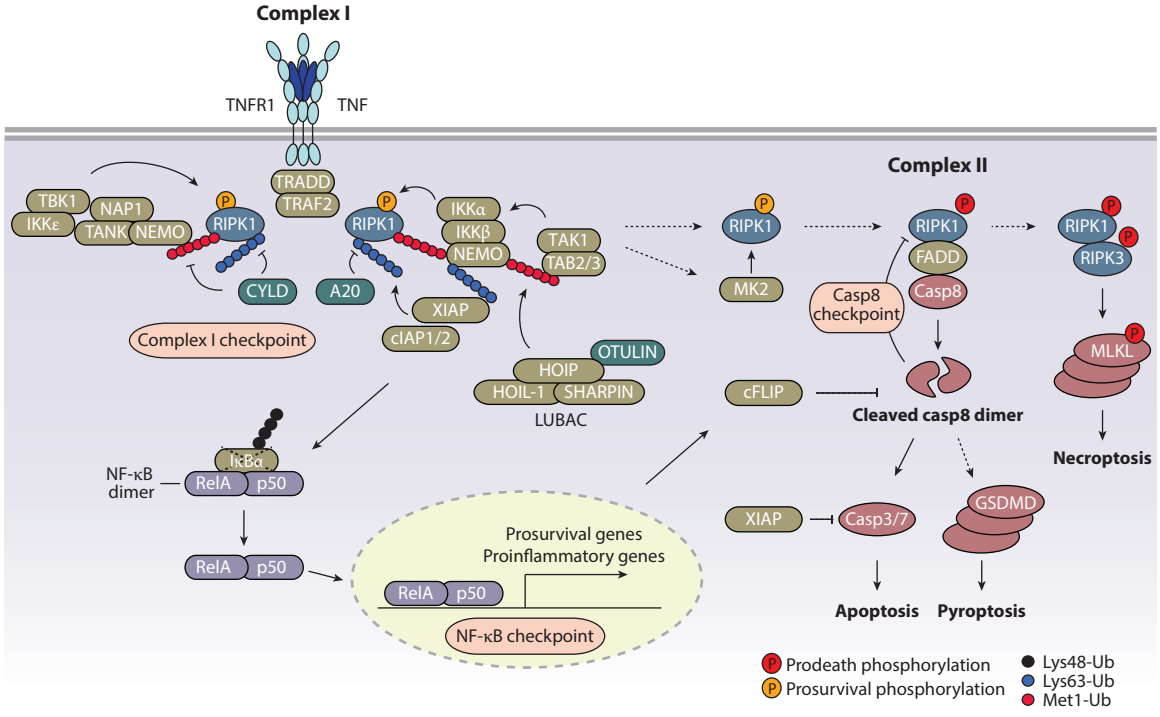


Figure 1

Overview of TNFR1 signaling and cell death checkpoints. The binding of TNF to its receptor TNFR1 induces the formation of a plasma membrane–bound receptor signaling complex (complex I), which activates NF-κB-mediated gene expression. A secondary cytosolic complex (complex II) is induced when complex I components dissociate from the receptor, initiating cell death. Several cell death checkpoints safeguard the induction of cell death: the complex I checkpoint, which maintains the integrity of complex I via post-translational modifications such as phosphorylation and ubiquitylation; the NF-κB checkpoint, which is based on NF-κB-mediated transcriptional upregulation of prosurvival genes; and the caspase-8 checkpoint, which cleaves and inactivates RIPK1 and ultimately prevents complex II formation. Abbreviations: casp, caspase; cFLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-like inhibitory protein; cIAP1/2, cellular inhibitor of apoptosis 1/2; CYLD, cylindromatosis; FADD, Fas-associated protein with death domain; GSDMD, gasdermin D; HOIL-1, heme-oxidized iron regulatory protein 2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; IκB, inhibitor of NF-κB α; IKK, IκB kinase; LUBAC, the linear ubiquitin chain assembly complex; Lys48-Ub, Lys48-linked ubiquitylation; Lys63-Ub, Lys63-linked ubiquitylation; Met1-Ub, linear ubiquitylation; MK2, mitogen-activated protein kinase–activated protein kinase 2; MLKL, mixed lineage kinase domain–like protein; NAP1, NAK (NF-κB-activating kinase)-associated protein 1; NEMO, NF-κB essential modulator; OTULIN, ovarian tumor domain deubiquitylase with linear linkage specificity; RIPK, receptor-interacting protein kinase; SHARPIN, SHANK-associated RH domain-interacting protein; TAB2/3, TAK1-binding protein 2/3; TAK1, TGF-β-activated kinase 1; TANK, TRAF-associated NF-κB activator; TBK1, TANK-binding kinase 1; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR1-associated death domain protein; TRAF, TNFR-associated factor; XIAP, X-linked inhibitor of apoptosis.

and 2) (28, 29), as well as linear ubiquitylation (Met1-Ub) by LUBAC (the linear ubiquitin chain assembly complex). Lys63-Ub and Met1-Ub stabilize complex I (30–32) by recruiting multiple kinases, including IKKα and IKKβ [IκB (inhibitor of NF-κB) kinase α and β] (33), via the adapter NEMO (NF-κB essential modulator) (34, 35); TAK1 (TGF-β-activated kinase 1) (36), via the adapters TAB2 and TAB3 (TAK1-binding proteins 2 and 3); and TBK1 {TANK [TRAF (TNFR-associated factor)-associated NF-κB activator]-binding kinase 1}, via Met1-Ub (37, 38); this ubiquitylation process is distinct from the proteasomal degradation of targets mediated by Lys48-linked ubiquitylation. These kinases eventually activate downstream transcriptional machinery of the canonical NF-κB pathway and upregulate the expression of hundreds of proinflammatory and prosurvival genes, resulting in immune activation and cell proliferation. The function of the ubiquitin chain–stabilized TNFR1 complex is regulated by multiple deubiquitylases (DUBs)—directly by A20 (for Lys63) and CYLD (cylindromatosis) (for Lys63 and Met1) and indirectly by OTULIN [the ovarian tumor (OTU) domain DUB with linear linkage specificity] (for Met1) (32, 39).

These signaling processes are safeguarded by multiple transcription-dependent and -independent checkpoints to provide optimal inflammatory responses and prevent excessive cell death (6, 40). First, TNF-mediated activation of NF-κB induces the expression of prosurvival genes such as *CFLAR*, which encodes cFLIP {cellular FLICE [FADD (Fas-associated protein with DD)-like IL-1β-converting enzyme]-like inhibitory protein} (41) (the NF-κB checkpoint). The long splicing isoform of cFLIP (cFLIP$_L$) is a catalytically inactive homolog of caspase-8; by generating the caspase-8/cFLIP$_L$ heterodimer, cFLIP$_L$ keeps both apoptosis and necroptosis induction in check (10, 11, 42, 43). Furthermore, multiple kinases regulate RIPK1 activity via phosphorylation. This regulation occurs within complex I and involves IKKα and IKKβ (44, 45), TAK1 (46, 47), MK2 (mitogen-activated protein kinase–activated protein kinase 2) (48–50), and TBK1 and IKKε (37, 38) at multiple regulatory phosphorylation sites on RIPK1, preventing RIPK1-mediated cell death (6, 40, 51) (the complex I checkpoint). These multiple layers of regulation indicate the critical importance of complex I as the signaling hub for immune activation and host defense.

When these prosurvival mechanisms that are active in complex I are compromised by genetic deficiency (52, 53), pharmacological stress (54, 55), or pathogens (56, 57), TNF stimulation can result in the formation of a cytosolic cell death–inducing complex II (**Figure 1**). The formation of complex II promotes caspase-8 activation via autoproteolytic processing that results in the activation of the executioner caspases, caspase-3 and caspase-7, and in subsequent apoptosis. When caspase-8 catalytic activity is absent, RIPK1 can recruit RIPK3. RIPK3 then becomes activated via autophosphorylation and subsequently induces necroptosis through phosphorylation-mediated oligomerization of MLKL. Of note, the cell death–inducing activity of RIPK1 is critically regulated by its prodeath autophosphorylation at Ser-166. Hence, RIPK1 plays a key role in regulating cell death signaling cascades. During this process, caspase-8-mediated proteolysis works as an additional checkpoint (the caspase-8 checkpoint) by cleaving RIPK1 (58–61) and thus preventing excessive cell death.

3. MONOGENIC DISORDERS MEDIATED BY CELL DEATH

Mendelian genetics began to investigate the role of cell death in immunity with the discovery of autoimmune lymphoproliferative syndrome (ALPS), which was reclassified recently as autoimmune lymphoproliferative immunodeficiencies (ALPIDs) (62). ALPS is a genetically heterogeneous disease that stems from loss-of-function (LOF) mutations in *FAS*, *FASLG* (which encodes the Fas ligand), and *FADD*; these mutations result in defective lymphocyte apoptosis and the progressive accumulation of autoreactive T and B cells (62). The genetics of ALPS is complex and involves

germline (dominant or recessive) and somatic mutations that result in perturbations in the FAS signaling pathway; this pathway is critical for the removal of autoreactive lymphocytes. The hallmark of the disease is an increased number of $CD3^+$ $TCR\alpha\beta^+$ $CD4^-$ $CD8^-$ (double-negative) T cells. Patients with ALPS present with lymphadenopathy, splenomegaly, hepatomegaly, and a heightened risk for B cell lymphomas. Therapeutic options for ALPS include various immunosuppressive therapies, while hematopoietic stem cell transplantation (HSCT) has been curative in refractory cases. To gain a detailed understanding of the genetics and the pathomechanisms of ALPS, we refer readers to recent comprehensive reviews (63–66).

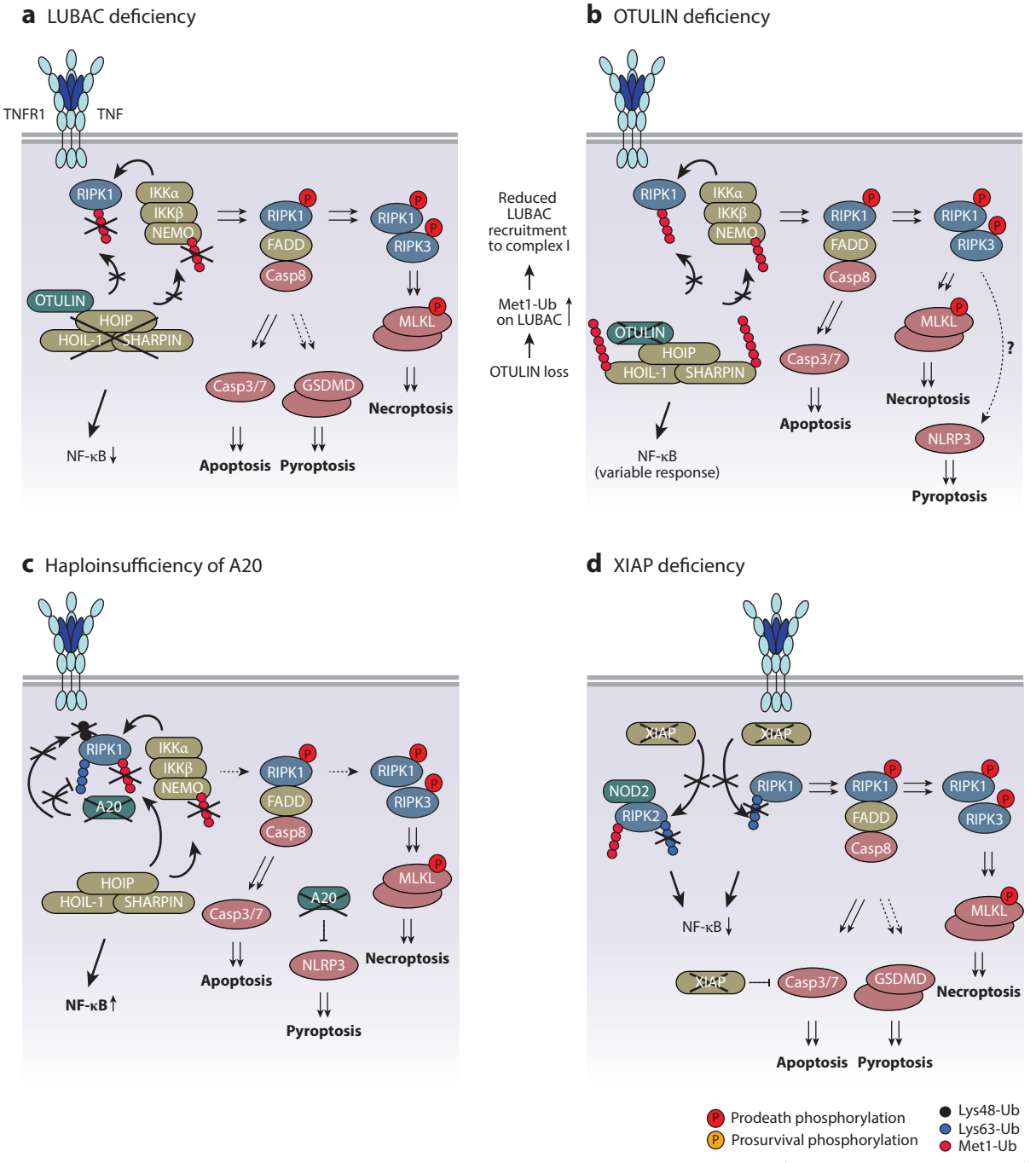
Parallel to the apoptosis deficiency observed in ALPS, the deregulation of cell death causes severe outcomes, as a growing body of human and mouse genetic evidence has begun to unveil. In this review, we focus on findings in humans, which we complement with results from animal models. We have attempted to categorize the disease classification based on the underlying molecular mechanisms, although the boundaries between the respective functional abnormalities are often blurred.

3.1. Diseases of Dysregulated Ubiquitylation

The role of ubiquitylation in the regulation of immune responses has been highlighted recently, first in animal model studies and later through the identification of patients with rare monogenic autoinflammatory diseases. These investigations have centered primarily on the members of the TNFR superfamily signaling pathway and revealed cell-specific dysregulation of ubiquitin-mediated protein modifications.

3.1.1. LUBAC deficiency. LUBAC is a trimeric protein complex of approximately 600 kDa and yet unknown stoichiometry, and it is composed of HOIL-1 (heme-oxidized iron regulatory protein 2 ubiquitin ligase-1), HOIP (HOIL-1-interacting protein), and SHARPIN (SHANK-associated RH domain-interacting protein) (67, 68). LUBAC is currently the only known E3 ubiquitin ligase that can conjugate Met1-Ub. HOIP has the main catalytic function for LUBAC's E3 ubiquitin ligase activity. Although HOIL-1 and SHARPIN had been considered scaffold proteins, recent publications suggest that HOIL-1 also has atypical E3 ubiquitin ligase activity for ester-linked monoubiquitylation of targets, including LUBAC itself (69) and polysaccharides, such as glycogen (70). Notably, HOIP, HOIL-1, and SHARPIN are all necessary for the stability of LUBAC, and the depletion of any of them destabilizes the complex and its Met1-Ub conjugation activity (30, 31, 71). Upon activation of TNFR1, LUBAC is recruited to TNFR1-SC (complex I) via cIAP1- and cIAP1-2-mediated Lys63-Ub to ubiquitylate multiple components of complex I, including NEMO and RIPK1. The loss of LUBAC causes the destabilization of complex I and the formation of the cytosolic complex II, which promotes cell death (**Figure 2*a***).

LUBAC deficiency with immune dysregulation is a rare and potentially lethal disorder that has been reported in eight patients: two with HOIP deficiency (72, 73), four with HOIL-1 deficiency (74), and two with SHARPIN deficiency (i.e., sharpenia) (4). Most patients presented with early-onset fever, splenomegaly, arthritis/arthropathy, and colitis. Skin inflammation was noted in half of the patients with HOIP and HOIL-1 deficiencies and has not been observed in human SHARPIN deficiency. Fibroblasts from LUBAC-deficient patients are sensitized to TNF-induced cell death primarily by apoptosis and via FADD/RIPK1/caspase-8-mediated complex II formation (4). The contribution of necroptosis seemingly is limited; however, additional investigation is warranted. In vivo dissection of colon biopsies from SHARPIN- and HOIP-deficient patients revealed a marked increase of phosphoactive RIPK1 (marked by autophosphorylation at Ser166) and evidence of pyroptosis by cleaved GSDMD; these findings could be consistent with the suggested role of LUBAC in inflammasome regulation (75, 76).



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Diseases of dysregulated ubiquitylation. (*a*) LUBAC deficiency impairs the linear ubiquitylation of complex I and leads to excessive cell death due to the increased formation of complex II. (*b*) Heightened sensitivity to TNF-induced cell death in OTULIN deficiency is attributable to excessive auto-linear ubiquitylation of LUBAC, hindering its recruitment to complex I. OTULIN-deficient cells show varying degrees of TNF-induced NF-κB activity. OTULIN deficiency also results in RIPK3-dependent but GSDMD-independent hyperactivation of NLRP3 through yet unidentified mechanisms. (*c*) Reduced Lys48-Ub-editing and Lys63-Ub-editing catalytic activity of A20 on RIPK1 enhances NF-κB induction. Additionally, A20 deficiency destabilizes linear ubiquitin chains in complex I, resulting in increased formation of complex II. This model is based on findings from A20 knock-in mouse studies and is yet to be confirmed in human haploinsufficiency of A20. The pathology associated with A20 deficiency is also attributed to the hyperactivation of the NLRP3 inflammasome. (*d*) XIAP deficiency impairs Lys63-Ub of RIPK2, disrupting NOD2-induced NF-κB activation, and allows unchecked cell death through the deregulation of caspase-3 and caspase-7, alongside defective Lys63-Ub of RIPK1. The loss of XIAP can also induce caspase-8-mediated GSDMD-dependent pyroptosis. Abbreviations: casp, caspase; FADD, Fas-associated protein with death domain; GSDMD, gasdermin D; HOIL-1, heme-oxidized iron regulatory protein 2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; IκB, inhibitor of NF-κB; IKK, IκB kinase; LUBAC, the linear ubiquitin chain assembly complex; Lys48-Ub, Lys48-linked ubiquitylation; Lys63-Ub, Lys63-linked ubiquitylation; Met1-Ub, linear ubiquitylation; MLKL, mixed lineage kinase domain–like protein; NEMO, NF-κB essential modulator; NLRP3, NLR family pyrin domain containing 3; NOD, nucleotide-binding oligomerization domain; OTULIN, ovarian tumor domain deubiquitylase with linear linkage specificity; RIPK, receptor-interacting protein kinase; SHARPIN, SHANK-associated RH domain-interacting protein; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; XIAP, X-linked inhibitor of apoptosis.

These findings in patients with rare diseases that demonstrate the contributions of cell death to inflammation are supported by extensive studies in animal models. A well-characterized model is the murine model for *Sharpin* deficiency, termed chronic proliferative dermatitis mice (*cpdm*) (77), which manifest severe inflammation affecting the skin, the joints, and internal organs (78). This inflammatory model is completely dependent on cell death mediated by *Tnf*, *Casp8*, *Fadd*, and the kinase activity of *Ripk1* (8, 9, 30, 79). Accordingly, skin inflammation was not rescued by the abrogation of necroptosis (i.e., in *cpdm* crossed with $Ripk3^{-/-}$ or $Mlkl^{-/-}$ mice), despite the amelioration of liver inflammation in *cpdm* crossed with $Ripk3^{-/-}$ mice (9). These genetic data highlight the critical role of TNF-induced, RIPK1 kinase activity–mediated cell death, primarily apoptosis, in the pathology of mouse skin inflammation. In contrast to *cpdm*, the complete deficiency of HOIP (encoded by *Rnf31*) and HOIL-1 (encoded by *Rbck1*) in mice leads to embryonic lethality at mid-gestation due to aberrant TNFR1-mediated endothelial cell death (52, 53, 80).

In addition to systemic inflammation, LUBAC-deficient patients manifest various degrees of immunodeficiency. Specifically, HOIP- and HOIL-1-deficient patients are susceptible to severe bacterial and viral infections (72–74) due to a reduced memory B cell population in the peripheral blood. In contrast, the single living patient with sharpenia did not present with overt episodes of severe infection (4). An in-depth characterization of the patient's adenoids revealed a specific reduction in the germinal center B cell population; this finding is consistent with that of the critical contribution of LUBAC-mediated Met1-Ub to CD40L-induced B cell activation in the secondary lymphoid organs in mice (81, 82). Furthermore, immunoglobulin maturation appeared to be affected, based on the somatic hypermutation rate of the patient's circulating class-switched memory B cells (4). The inflammatory features of LUBAC deficiency could be controlled with TNF inhibitors, whereas severe, refractory cases with immunodeficiency may require HSCT.

One of the most severe complications of LUBAC deficiency is the excessive deposition of glycogen in skeletal muscle, in the heart, and in the liver, leading to severe myopathy and heart failure (also known as amylopectinosis). This phenotype has also been described in a specific group of HOIL-1-deficient patients who did not have overt immune dysregulation (83–85). This metabolic phenotype has been attributed to the E3 ubiquitin ligase activity of HOIL-1, which directly ubiquitylates glucosaccharides in vitro (70). Importantly, HOIL-1-deficient patients

manifest more severe glycogen storage phenotypes than do HOIP-deficient or sharpenia patients; this observation further corroborates the role of HOIL-1 in mediating the ubiquitylation-dependent degradation of glycogen during metabolic homeostasis.

3.1.2. OTULIN-related human diseases. The synthesis of Met1-Ub chains by LUBAC is critical for complex I stabilization and is counteracted by specific DUBs that remove these ubiquitin chains from their substrates. OTULIN is a DUB that is highly selective for Met1-Ub chains (86). OTULIN also plays an important role in regulating proteasome assembly (87), the type I interferon pathway (39), and the NLRP3 (NLR family pyrin domain containing 3) inflammasome (88, 89).

The genetics of OTULIN-related diseases is becoming increasingly complex. Patients with OTULIN deficiency manifest early-onset recurrent fever, neutrophilic dermatitis, lipodystrophy, panniculitis (i.e., inflammation of subcutaneous tissues), colitis, arthritis/arthralgia, steatohepatitis, and failure to thrive. Immunodeficiency has not been noted. OTULIN deficiency is termed OTULIN-related autoinflammatory syndrome (ORAS), also known as otulipenia, and it results from either biallelic LOF variants (90–93) or heterozygous missense variants with dominant-negative (DN) effects (94, 95). Most mutations associated with ORAS are located within the large OTU domain (aa79–348) that is required for Met1-Ub hydrolysis. While protein instability or deficiency affects the binding of this domain to Met1-Ub and to LUBAC, the monoallelic mutations Cys129Ser (94) and Arg306Gln (95) abolish or reduce OTULIN's catalytic activity. Although patient-derived fibroblasts and peripheral blood mononuclear cells show an excessive accumulation of intracellular Met1-Ub chains, these cells exhibit varying degrees of NF-κB activation (90–95). In contrast, primary patient cells are sensitized to TNF-induced cell death predominantly via apoptosis (4, 91, 93–95). This sensitization has been attributed to the failure of LUBAC to be recruited to complex I, which is caused by excessive auto-ubiquitylation on LUBAC (**Figure 2*b***). Consistently, catalytic-inactive *Otulin* knock-in mice are embryonic lethal (39) because of the failure of LUBAC to control complex I stability, which leads to the subsequent formation of complex II and excessive cell death.

OTULIN haploinsufficiency has been identified in patients with *Staphylococcus aureus* skin infection and lung abscesses (96). Most patients experience disease onset during adolescence; however, only 30% of mutation carriers are affected. The low disease expressivity could be explained by the presence of neutralizing α-toxin IgG antibodies. Mechanistically, the reduction of OTULIN leads to the accumulation of caveolin-1 in dermal fibroblasts. Caveolin-1 regulates the expression of ADAM10 (a disintegrin and metalloprotease 10), which functions as the receptor for α-toxin. In *OTULIN*-haploinsufficient fibroblasts, stimulation with α-toxin stabilizes the expression of ADAM10 and makes cells less viable. Notably, a patient with *OTULIN* haploinsufficiency responded well to TNF inhibition; this response raises the hypothesis that *OTULIN* haploinsufficiency may also be driven by TNF-induced cell death (97). Interestingly, *OTULIN*-haploinsufficient alleles frequently appear in general population databases such as the Genome Aggregation Database (gnomAD). Further clinical, genetic, and mechanistic exploration is needed to fully elucidate the consequences of *OTULIN* haploinsufficiency in human skin immune homeostasis.

3.1.3. Haploinsufficiency of A20. A20 (encoded by *TNFAIP3*) is a master ubiquitin regulator of innate immune signaling, and its role in suppressing the NF-κB pathway has been well-characterized. A20 consists of an N-terminal OTU domain with DUB activity, followed by seven zinc finger (ZnF) domains. Among the ZnFs, the fourth domain (ZnF4) has E3 ubiquitin ligase activity for the Lys48-linked ubiquitin chains, and this activity is critical for the degradation of RIPK1 and termination of NF-κB signaling. These tandem DUB and E3 ubiquitin ligase domains

with opposing ubiquitin-editing functions work synergistically to downregulate NF-κB signaling. In addition to their ubiquitin-editing functions, ZnF4 and ZnF7 of A20 have ubiquitin-binding activity toward Lys63-linked and Met1-linked ubiquitin chains, respectively. A20 knockout mice die prematurely because of severe multiorgan inflammation (98). In contrast, transgenic knock-in mice with catalytically inactive point mutations for both DUB (C103A) and E3 ubiquitin ligase (C609A) activities develop normally and without a spontaneous phenotype (99–101). These results suggest that neither the DUB function nor the E3 ubiquitin ligase function of A20 is indispensable for its anti-inflammatory functions in vivo. Recent findings revealed that the ubiquitin-binding activity of A20 via ZnF7 (C764A/C767A) is critical for regulating inflammation in mice (102). Based on these animal models, the current hypothesis is that ZnF7 of A20 is indispensable for A20's recruitment to complex I and the stabilization of Met1-Ub chains; the latter is accomplished via the protection of these chains from degradation by the DUBs CYLD and OTULIN (32, 102). The absence of A20 or the abrogation of its Met1-Ub binding activity results in complex I destabilization and complex II formation (**Figure 2*c***). Therefore, A20 plays a critical role in suppressing cell death, as demonstrated in myeloid-specific A20 knockout mice, whose severe inflammatory phenotype is substantially delayed by RIPK3 deficiency or RIPK1 kinase inhibition (102). Additionally, A20 negatively regulates pyroptosis. This negative regulation has been explained by A20's role in suppressing NF-κB-dependent pro-IL-1β gene transcription (103, 104) and by the proteasomal degradation of NEK7 [NIMA (never in mitosis gene A)-related kinase 7], a critical activator of the NLRP3 inflammasome (105).

Because A20 functions as a master regulator of many different pathways, LOF variants in this gene have been associated with a plethora of autoimmune and autoinflammatory diseases and with cancer. Common, low-penetrance, noncoding single nucleotide polymorphisms in the *TNFAIP3* gene locus have been linked to systemic lupus erythematosus (SLE), Sjögren syndrome, psoriasis, rheumatoid arthritis, and systemic sclerosis. Rare, high-impact coding variants cause a severe autoinflammatory disease, termed HA20 (haploinsufficiency of A20) (105a). Patients with HA20 present with childhood-onset systemic inflammation, mucosal and skin lesions, and autoimmunity features. Immune profiling of these patients has identified the contribution of multiple cytokines, including NF-κB-driven cytokines and interferons. Most patients respond well to TNF inhibitors, although patients with severe, refractory disease may require combined therapies or HSCT. Milder hypomorphic coding polymorphisms in A20 might have been evolutionarily beneficial to human health by providing heightened immune responses against microbial pathogens. For example, the Denisovan variants (i.e., T108A and I207L inherited in *cis*), common in Southeast Asian and Oceanian populations, are associated with an enhanced NF-κB response in humans and mice (106). The contribution of cell death pathways to the pathogenesis of A20-associated diseases in humans needs further investigation.

3.1.4. X-linked inhibitor of apoptosis deficiency. X-linked inhibitor of apoptosis (XIAP) is an E3 ubiquitin ligase that plays a multifaceted role in the regulation of immune signaling and cell death signaling. XIAP consists of three baculovirus inhibitor of apoptosis protein repeat (BIR) domains (BIR1–3), a ubiquitin-associated (UBA) domain, and a C-terminal RING E3 ubiquitin ligase domain. The BIRs are necessary for XIAP's antiapoptotic function because they inhibit caspase-3, -7, and -9 during the expansion of adaptive immune cells in the setting of viral infection (107–110). Through the UBA domain, XIAP binds directly to Lys63-linked ubiquitin chains on NEMO and RIPK1 to activate NF-κB signaling (111) (**Figure 1**). XIAP also promotes NOD2-mediated NF-κB activation via Lys63-linked polyubiquitylation of RIPK2 and recruitment of LUBAC to the NOD2 signaling complex (112); these functions occur independently of RIPK2 kinase activity both in vitro and in vivo (113, 114). Furthermore, several groups have documented

the role of XIAP in RIPK1/RIPK3/caspase-8-dependent but MLKL-independent inflammatory cytokine production, NLRP3 inflammasome activation, and cell death (115–120) (**Figure 2*d***).

In males, LOF mutations in *XIAP*, mostly nonsense and frameshift variants, lead to X-linked lymphoproliferative syndrome, which manifests a broad spectrum of immune dysregulation, including recurrent infections, chronic inflammation, and a propensity for HLH (hemophagocytic lymphohistiocytosis) (121). XIAP-deficient patients have reductions in invariant natural killer T (iNKT) cells and mucosal-associated invariant T (MAIT) cells because of the cells' increased sensitivity to activation-induced cell death. These cell lineages highly express caspase-3 and -7, and their depletion is likely secondary to Epstein-Barr virus (EBV) infection, as patients who are not infected with EBV have normal iNKT and MAIT cell numbers (122). In addition, a substantial fraction of XIAP-deficient patients manifest gastrointestinal inflammation, and indeed, XIAP mutations have also been identified in cohorts of patients who have very early-onset inflammatory bowel disease (IBD) without HLH (110, 123). Gastrointestinal inflammation in XIAP deficiency is attributable to impaired NOD2 signaling, a major genetic contributor to the development of polygenic IBD (124). Recent studies in *Xiap*-deficient mice with spontaneous intestinal inflammation suggest that unchecked cell death driven by dysregulation in the intestinal microbiome contributes to this inflammation (125, 126). This finding is consistent with the observation of intestinal dysbiosis in patients with XIAP deficiency (127). Allogenic HSCT has been the curative treatment in patients with refractory IBD and HLH (127, 128), whereas recent case reports suggest the potential efficacy of therapies targeting cytokines downstream of the inflammasome (129, 130).

3.2. Diseases of Dysregulated RIPK1 Function

RIPK1 is a key molecule that governs the intricate balance between immune signaling and cell death. Numerous animal model studies have dissected the activation and regulation mechanisms of RIPK1. Aberrations in the RIPK1 signaling pathway have been implicated in many pathologies, ranging from inflammation to neurodegenerative diseases. The discovery of pathogenic variants in patients with autoinflammatory diseases has shed light on the fine-tuning mechanisms of RIPK1 in humans.

3.2.1. RIPK1-mediated human inflammatory conditions. RIPK1 is composed of an N-terminal kinase domain; an intermediate domain followed by a receptor-interacting protein homotypic interaction motif (RHIM) domain, which is critical for interactions with other RHIM-containing proteins; and a C-terminal DD. In humans, four proteins carry the RHIM domain and play a role in necroptosis: RIPK1, RIPK3, TIR (Toll/IL-1 receptor) domain containing adapter molecule 1, and Z-DNA binding protein 1 (ZBP1). The pivotal role of RIPK1 in regulating cell death pathways was initially characterized in murine models. *Ripk1*$^{-/-}$ mice on a C57BL/6 background are neonatally lethal because they experience severe multiorgan inflammation in utero (131, 132). This lethal inflammation is completely rescued by the removal of caspase-8 and the removal of RIPK3, which abolish apoptosis and necroptosis, respectively. Mice bearing a defective RHIM-mutant *Ripk1* are embryonic lethal and are instead rescued by either *Ripk3* or *Zbp1* deletion (133, 134). These findings highlight the complex functions of RIPK1 in fine-tuning cell death and inflammation.

Human diseases are linked to monoallelic gain-of-function (GOF) and biallelic LOF variants in the *RIPK1* gene; these diseases share deregulated cell death as the common pathomechanism. RIPK1 deficiency has been described in patients with severe viral, bacterial, and fungal infections due to combined T and B lymphocyte depletion (135–137). RIPK1 deficiency leads to the downregulation of the canonical NF-κB pathway (**Figure 3*a***). Concomitantly, these patients exhibit autoinflammatory manifestations, including gastrointestinal inflammation and arthritis. Two patients were treated with TNF blockade (infliximab) and exhibited a partial response

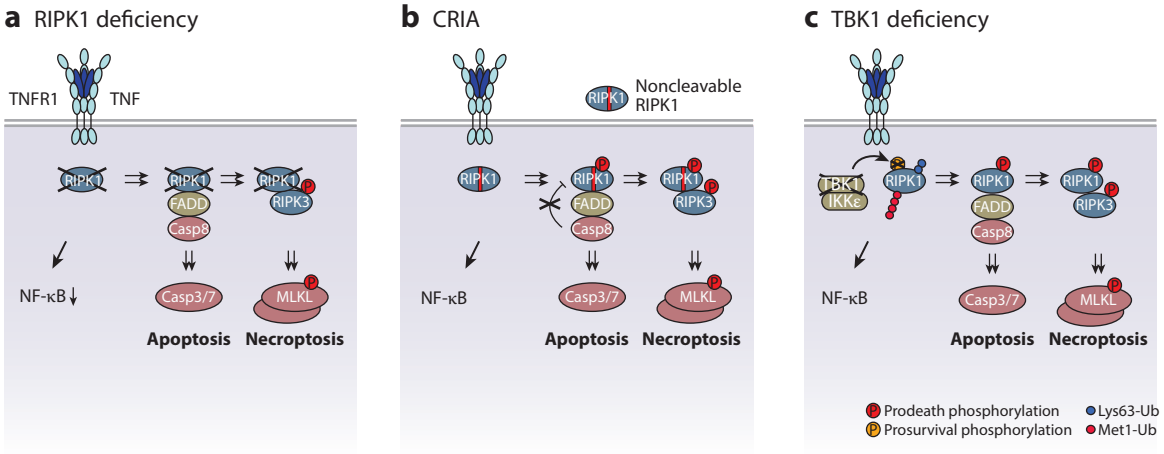


Figure 3

Diseases of dysregulated RIPK1 function. (*a*) RIPK1 deficiency attenuates NF-κB activation and leads to unchecked cell death. (*b*) CRIA results from caspase-mediated cleavage-resistant mutations in RIPK1, causing excessive apoptosis and necroptosis via the increased formation of complex II. In contrast to RIPK1 deficiency, no change in TNF-induced NF-κB activation is observed in CRIA. (*c*) TBK1 deficiency leads to heightened cell death due to decreased inhibitory phosphorylation of RIPK1. Abbreviations: casp, caspase; CRIA, cleavage-resistant RIPK1-induced autoinflammation; FADD, Fas-associated protein with death domain; IκB, inhibitor of NF-κB; IKK, IκB kinase; Lys63-Ub, Lys63-linked ubiquitylation; Met1-Ub, linear ubiquitylation; MLKL, mixed lineage kinase domain–like protein; RIPK, receptor-interacting protein kinase; TANK, TRAF-associated NF-κB activator; TBK1, TANK-binding kinase 1; TNF, tumor necrosis factor; TNFR, TNF receptor.

(136). HSCT could potentially be a curative therapy for patients diagnosed early (135, 138). In contrast, GOF mutations in *RIPK1* cause a disease termed cleavage-resistant RIPK1-induced autoinflammation (CRIA) (58, 59, 139). CRIA is caused by heterozygous missense variants that abolish the cleavage of human RIPK1 by caspase-6 and -8; this mechanism is critical for the attenuation of RIPK1 activation. Patients with cleavage-resistant RIPK1 mutations present with infantile- to childhood-onset recurrent fever and painful lymphadenopathy. Ex vivo studies using patient samples and *Ripk1*$^{-/-}$ mouse embryonic fibroblasts with reconstitution show that cells expressing CRIA mutants are more susceptible to apoptotic and necroptotic cell death; this cell death is associated with enhanced complex II formation (**Figure 3*b***). Patients with CRIA showed positive responses to treatment with an IL-6 inhibitor or IL-1 inhibitor (139).

To gain additional insights into how RIPK1 cleavage affects cell death–induced inflammation, several groups independently generated germline knock-in mouse models expressing cleavage-resistant *Ripk1* D325A, homologous to the *RIPK1* D324N/H/Y mutant in humans (58, 60, 61). Homozygous *Ripk1*$^{D325A/D325A}$ mice are not viable because of developmental defects associated with extensive cell death. Only the combined abrogation of apoptosis and necroptosis prevents embryonic lethality in this murine model. In vitro, cells from *Ripk1*$^{D325A/+}$ and *Ripk1*$^{D325A/D325A}$ mice succumb to TNF-induced cell death due to the enhanced stabilization of complex II. The introduction of a mutation abolishing RIPK1 catalytic activity (RIPK1-D138N) in *cis* led to a partial rescue of lethality in *Ripk1*$^{D325A/D325A}$ mice, although double-mutant knock-in mice (*Ripk1*$^{D138N,D325A/D138N,D325A}$) were runty and had multiorgan inflammation. When heterozygous *Ripk1* D325A mice were bred with *Casp8/Ripk3* double-knockout mice, LPS-induced hypercytokinemia was reversed, indicating that the the autoinflammation was due to increased cell death. Together, these findings suggest a critical role for RIPK1 cleavage in preventing excessive cell death by abrogating both kinase-dependent and -independent functions of RIPK1.

Finally, a new study reported biallelic missense mutations (K377E and R390G) in *RIPK1* in two siblings with early-onset gastrointestinal inflammation and recurrent infections (140). Notably, Lys63-Ub of RIPK1 on the residue Lys377 is critical for NF-κB activation and prevention of cell death, with its disruption leading to embryonic lethality in mice (141). Collectively, these human genetic diseases have provided valuable insights into the intricate mechanisms of RIPK1 regulation.

3.2.2. TBK1 mutations in human diseases. TBK1 is a multimeric serine-threonine kinase that, along with its closely related homolog IKKε (encoded by *IKBKE*), composes a unique family called noncanonical IKKs. TBK1 consists of five functional domains: the kinase domain, the ubiquitin-like domain, two coiled-coil domains (CCD1 and CCD2), and a C-terminal domain (CTD). TBK1 normally exists as a homodimer, and dimerization occurs through interactions between CCDs and possibly via additional intramolecular interactions. The homodimerization of TBK1 is required for its autophosphorylation at Ser172 within the activation loop of the kinase domain (142), leading to the activation of TBK1.

TBK1 has multilayered roles in the maintenance of cellular homeostasis and signal transduction pathways. TBK1 was identified initially as an activator kinase that mediates viral nucleic acid sensing via various pattern recognition receptors (PRRs), such as TLR3 (Toll-like receptor 3), RIG (retinoic acid–inducible gene)-I-like receptors (RLRs), and cGAS (cyclic GMP–AMP synthase). These PRRs assemble the specific adaptors TRIF, MAVS (mitochondrial antiviral-signaling protein), and STING (stimulator of interferon genes), all of which subsequently activate TBK1 through dimeric autophosphorylation (143). Activated TBK1 then phosphorylates the downstream substrates interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7) to induce type I interferon transcription. Recent reports have suggested that TBK1 is an additional kinase checkpoint for cell death at complex I (37, 38) (**Figure 1**). During TNF signaling, TBK1 is recruited to complex I in a manner that depends on LUBAC-mediated Met1-Ub. Genetic deletion or pharmacological inhibition of TBK1 sensitizes cells to TNF-induced, RIPK1-dependent apoptosis and/or necroptosis. TBK1 phosphorylates RIPK1 on multiple amino acid residues (38); one study suggests that TBK1 primarily phosphorylates RIPK1 on Thr189 to inhibit RIPK1-regulated cell death (37). TBK1 deficiency seemingly does not affect NF-κB; this observation corroborates the importance of the NF-κB-independent kinase checkpoint within complex I during TNF-induced cell death (38). Consistent with these findings, murine *Tbk1* deficiency causes severe multiorgan inflammation (144) or embryonic lethality (145); the latter depends on *Tnf*, on *Tnfr1*, and on the kinase activity of *Ripk1* (37, 145, 146).

The identification of pathogenic variants in patients with various phenotypes has further highlighted the complex roles of TBK1. Heterozygous missense variants in the kinase domain of *TBK1* (D50A and G159A) were found in patients susceptible to herpes simplex encephalitis (HSE), a rare but severe infectious condition (147). Functional studies show that G159A has a DN effect on polyinosinic-polycytidylic acid (poly I:C)–stimulated interferon gene expression, whereas D50A causes haploinsufficiency. Additionally, several association studies have suggested that *TBK1* plays a role in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), two major adult-onset neurodegenerative disorders with overlapping etiologies. Since 2015, multiple studies have reported the enrichment of heterozygous mutations in *TBK1* as the fourth-most-common cause of familial ALS/FTD (148). Thus far, 155 LOF *TBK1* variants have been associated with susceptibility to ALS/FTD (Human Gene Mutation Database). The penetrance of these variants is incomplete, as indicated by allele frequencies of heterozygous damaging *TBK1* variants in presumably healthy individuals included in public databases. This phenotype might be linked to an increase in RIPK1 kinase activity in aging brains (37), which may cooperate with other genetic risk factors.

In contrast, complete TBK1 deficiency in humans presents a very different phenotype (149). These patients manifest infantile-onset fever, variable degrees of dermatitis, polyarthritis, brain atrophy, growth retardation, and systemic vasculitis leading to cerebral infarction and seizures. Surprisingly, none of these patients have had a history of HSE, suggesting adequate antiviral protection. Two truncating (R440X and W619X) and one missense (Y212D) mutations have been reported, all in the homozygous status. When overexpressed, these mutants led to substantial attenuation of IRF3-target genes, suggesting functional deficiency. Interestingly, the response to extracellular dsRNA ligand (which targets endosomal TLR3) in patient fibroblasts was completely abolished, whereas the response to cytosolic dsRNA (which targets RLR) was appreciable, indicating the differential consequences of TBK1 loss in these RNA-sensing pathways. Reconstitution in patient fibroblasts showed that LOF missense mutants, such as HSE-susceptible G159A and phosphoinactive S172A, have a more disruptive effect on downstream phospho-IRF3–IFN-β-mediated viral suppression than does the complete loss of TBK1, suggesting a possible DN effect of these missense mutants on the partner kinase IKKε and thus accounting for the discordance in antiviral phenotype between TBK1-null versus TBK1-heterozygous patients. Meanwhile, TNF-induced NF-κB activation was not altered in these patients, reinforcing the NF-κB-independent role of TBK1 underlying the molecular etiology of the disease. Consistent with previous studies (37, 38), TBK1-deficient patient fibroblasts showed augmented susceptibility to TNF-induced necroptosis when concomitantly stimulated with a Smac-mimetic (SM) (**Figure 3*c***). The induction of apoptosis in fibroblasts seemingly was marginal during this TNF-plus-SM stimulation. In contrast to the effect on HSE susceptibility, the introduction of phosphoinactive S172A into patient cells had a partial rescue effect, which may coincide with the lack of autoinflammation in HSE-susceptible patients who have heterozygous *TBK1* mutations. Thus far, no obvious clinical phenotype, such as viral susceptibility or neurodegeneration, has been described among heterozygous family members of patients deficient in TBK1. The inflammatory features of TBK1 deficiency could be effectively suppressed with TNF inhibitors.

3.3. Diseases of NF-κB Signaling Deficiency

NF-κB is a group of prototypical transcriptional factors governing proper immune response. During the past three decades, our understanding of the NF-κB signaling pathway has been shaped by the close interplay between human genetics and molecular immunology, highlighting the role of NF-κB as the critical gatekeeper of cell death–induced inflammation.

3.3.1. NEMO-associated immune dysregulation/IκBα gain of function. NEMO (encoded by *IKBKG*) is critical for the activation of IKKα and IKKβ, the two catalytic subunits that phosphorylate the inhibitory protein IκBα. The phosphorylation of IκBα on Ser32 or Ser36 induces Lys48-polyubiquitylation of IκBα, leading to its proteasomal degradation and subsequent nuclear translocation of NF-κB dimers (typically RelA/p50 heterodimers) and resulting in the expression of prosurvival genes. Mechanistically, this kinase activity of the IKK complex is achieved via the recruitment of NEMO to complex I through NEMO's UBAN (ubiquitin-binding in ABIN and NEMO) domain and binding to Met1-Ub chains (33–35, 45). During this process, the phosphorylation of IKKβ by TAK1 and the subsequent autophosphorylation of IKKβ promote the activation of the IKK complex (150). Studies also have shown that the binding of NEMO by Met1 and Lys63 polyubiquitylation induces NEMO's liquid phase separation (151). In addition to the role of the IKKα/IKKβ complex in NF-κB-mediated gene transcription, this complex phosphorylates RIPK1 (44), including Ser25 in the kinase domain of RIPK1, and this phosphorylation serves as an NF-κB-independent cell death checkpoint within complex I to ensure the tight control of RIPK1-induced cell death (152, 153) (**Figure 4*a***).

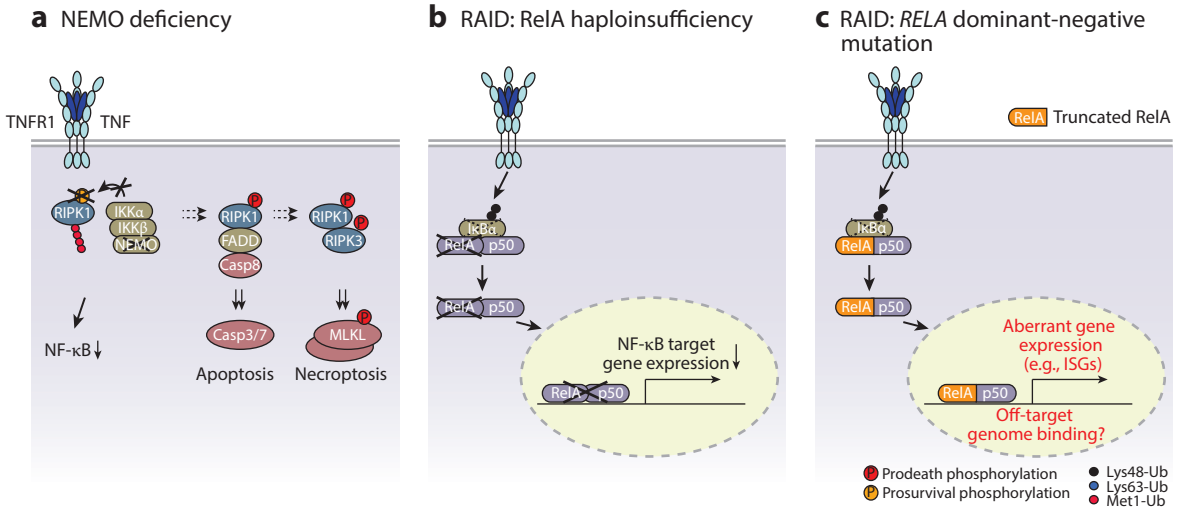


Figure 4

Diseases of NF-κB signaling deficiency. (*a*) NEMO deficiency attenuates NF-κB induction and increases cell death due to reduced inhibitory phosphorylation of RIPK1. In RAID, (*b*) RelA haploinsufficiency attenuates the transcription of NF-κB target genes, while (*c*) dominant-negative *RELA* mutations cause aberrant gene expression (e.g., of ISGs), possibly by creating cryptic binding sites on the genome. Abbreviations: casp, caspase; FADD, Fas-associated protein with death domain; IκB, inhibitor of NF-κB; IKK, IκB kinase; ISGs, type I interferon–stimulated genes; Lys48-Ub, Lys48-linked ubiquitylation; Lys63-Ub, Lys63-linked ubiquitylation; Met1-Ub, linear ubiquitylation; MLKL, mixed lineage kinase domain–like protein; NEMO, NF-κB essential modulator; RAID, *RELA*-associated inflammatory disease; RIPK, receptor-interacting protein kinase; TNF, tumor necrosis factor; TNFR1, TNF receptor 1.

Pathogenic mutations in NEMO underlie several clinically distinct human conditions, including anhidrotic ectodermal dysplasia (EDA), EDA with immunodeficiency (EDA-ID), and milder incontinentia pigmenti (IP). EDA is characterized by the abnormal development of ectodermal tissues, including teeth, skin, hair, and sweat glands, due to the attenuation of IKK-mediated NF-κB activation downstream of the ectodysplasin A receptor (EDAR). IP affects ectodermal tissues and is predominantly characterized by the presence of Blaschko linear hyperpigmentation on the trunk and extremities and by retinal abnormalities. Because *IKBKG*, which encodes NEMO, is on the X chromosome, NEMO's complete deficiency is not viable in males. Disease-causing variants in EDA and EDA-ID are primarily hypomorphic in male patients. Up to 80% of NEMO variants in the IP patient population, which includes heterozygous females, males with XXY chromosomal disorder, and a few males with somatic variants, are characterized by a recurrent deletion of exons 4–10, causing inactivation of the NF-κB pathway (154). Other types of mutations result in the absence of or defects in the NEMO protein. The molecular pathology of ectodermal features of NEMO deficiency is still unclear, although it has been hypothesized that the dysregulation of cell death during embryonic development of the skin may play a role (155). Heterozygous GOF mutations in IκBα (encoded by *NFKBIA*) cause an autosomal dominant form of EDA-ID (156). These mutations either directly abolish IκBα's phosphorylation at Ser32 or Ser36 or they translate a shorter IκBα protein that lacks these phosphorylation sites, preventing proper phosphorylation and degradation of this inhibitory protein.

In patients with EDA-ID, immune dysregulation has multiple components with overlapping features. Defects in NF-κB signaling cause immunodeficiency and manifest susceptibility to severe bacterial, mycobacterial, fungal, and viral infections because of a reduction in lymphocytes, especially in class-switched memory B cells and CD45RA$^-$ memory T cells (157, 158). In some

cases, a skewed T cell receptor repertoire consistent with oligoclonal expansion has been reported, accounting for autoimmunity. Furthermore, up to 25% of patients with EDA-ID present with inflammatory manifestations that predominantly affect the gut (159), which lead to intractable diarrhea and failure to thrive. This gastrointestinal phenotype is attributable to a tissue-intrinsic effect of NEMO deficiency rather than to lymphocyte-mediated autoimmunity and cannot be corrected by HSCT (160). Overall, the reported survival rate post-HSCT was 74%. Murine models have provided valuable insights into the underlying mechanisms of human NEMO deficiency. The conditional deletion of NEMO in the intestinal epithelium leads to severe spontaneous chronic inflammation (161). This inflammation is driven by aberration in epithelial cell–intrinsic TNFR1 signaling, consistent with the effects of TNF inhibitors in NEMO-deficient patients post-HSCT colitis (160). Furthermore, the gastrointestinal inflammation phenotype in mice is ameliorated by the combined deficiency of FADD and RIPK3 or by the genetic or pharmacological inhibition of RIPK1 kinase activity, suggesting the critical contribution of RIPK1 kinase–induced cell death (162, 163).

A novel genetic condition with in-frame skipping of *IKBKG* exon 5, NDAS (NEMO Δ-exon 5 autoinflammatory syndrome), was recently reported to cause distinct autoinflammatory manifestations in the skin and an elevated type I interferon signature (164). How this alternatively spliced NEMO protein affects complex I integrity and, subsequently, TNF-induced cell death activation remains an open question.

3.3.2. *RELA*-associated inflammatory disease. The NF-κB family consists of five subunits with overlapping yet distinct features (25). *RELA* (encoding p65), *RELB* (encoding RelB), and *REL* (encoding c-Rel) are translated as mature proteins with transcription activation domains at the C terminus. *NFKB1* (encoding p105) and *NFKB2* (encoding p100) are expressed as full-length precursors, while their N-terminal active forms, p50 and p52, are produced via proteolytic cleavage. Active NF-κB complexes consist of various combinations of these five monomers and can induce the expression of hundreds of genes, including those encoding not only inflammatory cytokines and chemokines but also cell death repressors, such as IAPs, A20, cFLIP, B cell lymphoma 2 (BCL2), and BCL-XL, underscoring the importance of NF-κB family genes as a transcriptional checkpoint for cell death (165). Signaling through the canonical NF-κB pathway mainly involves p50, p65, and c-Rel, whereas signaling through the noncanonical pathway primarily involves p52 and RelB (25, 165, 166). In mice, genetic knockout of *Rela*, but not of the other four NF-κB subunits, leads to embryonic lethality (167), suggesting that RelA has nonredundant functions during development.

Heterozygous LOF mutations in *RELA* have been described in patients with mucocutaneous ulcerations and intermittent fevers (168–171). Patient-derived fibroblasts showed impaired NF-κB target gene transcription and increased TNF-induced apoptosis (**Figure 4*b***). The attenuated induction of antiapoptotic genes, including those encoding cIAP1/2, suggests that there is a defect in the transcriptional cell death checkpoint. *RELA*-associated inflammatory disease (RAID) is phenocopied in *Rela*-haploinsufficient mice that have heightened sensitivity to TNF-dependent mucocutaneous inflammation in inducible models (168). The *RELA*-associated phenotype was expanded by the report of a patient with a de novo heterozygous nonsense variant in *RELA* that manifested autoimmunity due to lymphoproliferation, leading to refractory immune thrombocytopenic purpura, cytopenia, and splenomegaly in the absence of mucocutaneous ulcerations and IBD (172). Another recent report described patients diagnosed with SLE-like autoimmunity (173). Distinct from the findings in haploinsufficient cases, truncated RelA proteins were expressed in the cells of patients with lupus and were shown to exert a DN effect on downstream transcriptional machinery (**Figure 4*c***). Notably, patients with monoallelic DN mutations have an enrichment of

type I interferon–induced genes, consistent with the patients' clinical diagnosis of SLE. Treatment with anti-TNF biologics is effective in most patients who have both haploinsufficiency and DN mutations (170, 173).

3.4. Diseases Associated with Defects in Terminal Execution of Necroptosis

Necroptosis can instigate an uncontrolled inflammatory response. Human genetic studies have started to reveal that pathogenic variants in the terminal executioners of necroptosis, RIPK3 and MLKL, are linked to distinct phenotypes, necessitating further genetic and mechanistic investigations.

3.4.1. RIPK3 deficiency. RIPK3 is a serine-threonine kinase that regulates cell death outcomes, including apoptosis and necroptosis. When complex II formation is triggered, RIPK3 is integrated into complex II via interactions with RHIM domain–containing proteins, such as RIPK1, TRIF, and ZBP1. This interaction enhances proximity-induced autophosphorylation of RIPK3, which is mediated by the N-terminal kinase domain, leading to the phosphorylation and activation of MLKL and triggering necroptosis (12, 174). *Ripk3*$^{-/-}$ mice develop normally and manifest heightened susceptibility to many infections (175), suggesting that RIPK3-mediated cell death is important for the host immune response. Catalytically inactive knock-in *Ripk3*$^{D161N/D161N}$ mice show embryonic lethality driven by *Ripk1* and *Casp8*, suggesting that the kinase-independent scaffold function of RIPK3 also contributes to apoptosis induction (176, 177).

Biallelic LOF variants in *RIPK3* have been reported recently in a young patient who presented with recurrent HSE and no history of other viral infections or inflammatory features (178). The reported mutations, R422X and P493TfsTer9, cause a C-terminal truncation with an intact kinase domain. Mutant primary cells showed attenuated necroptosis by TNF and a TLR3 agonist in the presence of an SM and a pan-caspase inhibitor. Notably, these cells and cortical neurons differentiated from patient-derived induced pluripotent stem cells are more susceptible to HSV-1 infection ex vivo, corroborating the contribution of RIPK3-mediated cell death to host defense against HSV-1. Importantly, there are 7 and 25 homozygous carriers for the R422X and the P493TfsTer9 variants, respectively, among the 805,782 presumably healthy individuals from the gnomAD population database, suggesting the *RIPK3* gene mutation–environment (HSV-1 infection) interaction underlies this genetic susceptibility to HSE with incomplete penetrance.

3.4.2. MLKL deficiency. MLKL is the terminal effector of necroptosis. MLKL contains an N-terminal four-helix bundle (4HB), a two-helix brace region (BR), and a C-terminal pseudokinase domain (PD). In the absence of the catalytic activity of caspase-8, RIPK3 promotes the recruitment and phosphorylation of MLKL at Thr357 and Ser358. This phosphorylation induces a conformational change in MLKL that exposes the 4HB, leading to oligomerization and membrane pore formation (174). A recent report suggests that the tyrosine kinases collectively known as TAM (Tyro, Axl, and Mer) kinases also phosphorylate MLKL at Tyr376 within the PD to promote MLKL oligomerization (179). The human *MLKL* gene has two splicing isoforms; the alternative one lacks a large portion of the PD and is called *MLKL2* (180). *Mlkl*-deficient mice are viable and do not display any spontaneous immunological or behavioral abnormalities (174). In contrast, two missense changes in *Mlkl*—S82G, which abolishes the inhibitory phosphorylation site in 4HB (181), and D139V within the BR (182)—cause severe inflammatory consequences in the respective homozygous knock-in mice. Finally, a common missense GOF variant, S132P, confers resistance to the negative regulation of MLKL via phosphorylation when introduced in mice and alters inflammatory responses and pathogen clearance (183).

In humans, pathogenic variants in *MLKL* have been linked to different and distinct phenotypes. A rare homozygous small nucleotide deletion, rs561839347, that abolishes MLKL expression

(D369Efs*22) has been reported in two siblings with severe and progressive neurodegenerative disease (184). This finding is in line with the enrichment of another N-terminal truncating polymorphism, Q48X, in a Hong Kong Chinese cohort of patients with Alzheimer's disease (185). These data suggest that MLKL deficiency may contribute to neurodegeneration in humans, raising concerns about the long-term safety of pharmacological targeting of MLKL. However, considering the relatively high allele frequencies of these polymorphisms in the gnomAD database, the mechanisms by which MLKL deficiency contributes to neurodegeneration await further investigation. The common missense polymorphisms S132P and R146Q and an *MLKL2* isoform–specific polymorphism within the BR, G202V, are predicted to favor the activated protein conformation and were associated with chronic recurrent multifocal osteomyelitis, a sterile bone inflammatory disease, in a small cohort-based genetic study (182). Overall, we are beginning to understand the role of MLKL in human pathology, but further evidence is still needed.

4. CELL DEATH–TARGETING THERAPIES

The kinase-inactive mouse mutants *Ripk1*^{K45A} and *Ripk1*^{D138N}, which target the catalytic pocket for ATP binding and hydrolysis, are viable and exhibit normal growth, providing genetic evidence for the effectiveness of the suppression of RIPK1 kinase activity in mice (177, 186). Accordingly, RIPK1 kinase inhibition has held promise for the treatment of multiple inflammatory and neurodegenerative conditions, based on the observation of effective suppression of pathologies underlying multiple murine genetic and pharmacological disease models (187, 188).

Despite these promising preclinical data, RIPK1 inhibitors have encountered difficulties during clinical trials. GSK2982772 (GSK'772), a first-in-class RIPK1 inhibitor, has been tested in several phase 2 randomized, multicenter, placebo-controlled clinical trials in patients with ulcerative colitis, rheumatoid arthritis, and active plaque psoriasis (189–191) and has shown no efficacy compared with placebo. These unsatisfactory outcomes may underscore the importance of patient stratification based on molecular pathology, which may require robust preclinical experimental investigation. Recently, Chiou et al. (192) reported automated protocols for the immunodetection of intracellular core cell death regulators caspase-8, RIPK1, RIPK3, and MLKL in human colon biopsies from patients with IBD. This study identified a surprising heterogeneity in cell death pathway activation in IBD tissues. The authors also observed cytosolic caspase-8^{+} clusters in the epithelium of specimens wherein necroptotic rather than apoptotic pathway activation was confirmed by Western blotting. Although the mechanisms underlying the observed caspase-8^{+} clusters warrant further investigation, the application of this automated cell death immunodetection to samples from IECD patients is tempting as a human genetic proof of concept.

5. DISCUSSION

Recent advances in cell death research have revolutionized our view of cell death from a mere passive, damage-induced consequence to biochemically and chemically defined, active molecular cascades, providing a formal proof of concept for how cell death dysregulation leads to catastrophic inflammatory consequences. This understanding was initially achieved using sophisticated murine models wherein severe inflammatory or embryonic lethal phenotypes can be reversed by the deletion of cell death effectors or the genetic inactivation of critical drivers of cell death such as RIPK1. The discovery of Mendelian disorders of cell death–induced autoinflammation has revealed new insights into the molecular mechanisms critical for fine-tuning cell death pathways during innate immune responses. The currently identified genes are likely just the beginning; many other human immunological and nonimmunological genetic disorders could be linked to cell death dysregulation.

Research on IECDs has pointed to species-specific differences between patients and corresponding murine models. In our article on the concept of IECDs, we noted the absence of dermatitis in sharpenia patients compared with *Sharpin*-deficient mice as a striking example of these differences (4). Furthermore, whereas *Tbk1*$^{-/-}$ (145), *Ripk1*$^{-/-}$ (27), and *Rbck1*$^{-/-}$ (HOIL-1-null) (53, 80) mice show embryonic to neonatal lethality, patients with complete deficiencies in the corresponding genes generally survive childhood, albeit with various degrees of immune dysregulation (74, 135, 149). HOIL-1-deficient patients also manifest severe to life-threatening glycogen storage disease; this observation led to the discovery of nonprotein-linked ubiquitylation on glycogen by HOIL-1 (70). Furthermore, position- or domain-specific mutations identified by forward genetics–based Mendelian approaches have provided novel insights into mutant protein functions, which could have been overlooked in hypothesis-driven reverse genetics–based murine transgenesis (58, 59, 173). Also noteworthy is that some human IECDs are documented in heterozygous conditions, while the corresponding haploinsufficient murine models do not exhibit spontaneous phenotypes. Finally, unexpected susceptibility to infections in heterozygous carriers of mutations in cell death machinery genes, such as TBK1 (147) and OTULIN (96), underscores the significant contribution of environmental triggers to cell death–associated human immune pathologies.

How could these differences be explained? We and others have noted that, in the absence of secondary stimulations such as SM, human dermal fibroblasts (HDFs) from patients with IECDs are not susceptible to TNF-induced cell death in cases of deficiency in LUBAC, OTULIN, TBK1, and CRIA as opposed to murine dermal fibroblasts (MDFs) that have corresponding gene modifications (4, 91, 149). Furthermore, HDFs from many of these patients do not display cell death induction after TNF + SM + zVAD, conventional necroptosis-inducing stimuli, in contrast with the corresponding MDFs (4). These in vitro findings may indicate an apoptosis-prone potential of human cells compared with murine cells, which could be explained by the presence of caspase-10, a caspase-8 homolog, in the human genome but not in rodents. Additionally, the amino acid sequences of necroptotic axis RIPK3-MLKL are poorly conserved between humans and mice, wherein a human MLKL ortholog cannot complement MLKL deficiency in mouse cells (193, 194), which may indicate a difference in the threshold for necroptosis induction. The spatiotemporal regulation of cell death genes is still not well-understood, and cell death responses are likely under the dynamic influence of host–environment interactions (192).

The impact of the dysregulated cell death pathway on disease expressivity is notable. While patients with CRIA have a relatively milder phenotype, without mucocutaneous ulcerations, patients with *RELA* haploinsufficiency, HA20, or ORAS present with significant inflammation in their epithelial cells. These dissimilarities could be related to differences in cell-specific expression and in regulatory mechanisms of mutant protein functions. The contribution of environmental factors, such as exposure to infections, cannot be ignored either. People with OTULIN haploinsufficiency have an increased risk of developing skin and lung abscesses upon skin injury and exposure to *S. aureus*.

The possibility of complex interplay among various cell death pathways also should not be ignored because interactions involving apoptosis, necroptosis, and pyroptosis have been observed in the context of infections and cancer. The evidence for cross talk is further supported by animal model studies that highlight the functional redundancy of cell death pathways (195). For example, a recent study showed the role of GSDMD in the maintenance of the blood-brain barrier. GSDMD is activated via LPS-induced canonical and noncanonical inflammasomes, and its activation can damage brain endothelial cells and cause blood-brain barrier leakage (196). GSDMD can also be activated by caspase-8 to exert a similar effect on brain vascular integrity when combined with

TAK1 inhibition or genetic deletion (197, 198). Given these data, caspase-8 may play a central role in the integration of cell death pathways.

As described herein, recent attempts to pharmacologically target cell death for the treatment of polygenic inflammatory diseases are facing challenges, as opposed to cell death–inducing strategies for cancer treatment [e.g., BCL-2-targeting BH3 mimetics (14)], which have shown success. The discovery of human IECDs has not only provided proof-of-principle genetic evidence for the role of aberrant cell death activation underlying human immune pathologies but has also opened a path for the development of new therapies targeting molecules that regulate cell death.

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