Receptor-interacting protein kinase 1 (RIPK1) as a therapeutic target

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Abstract | Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is a key mediator of cell death and inflammation. The unique hydrophobic pocket in the allosteric regulatory domain of RIPK1 has enabled the development of highly selective small-molecule inhibitors of its kinase activity, which have demonstrated safety in preclinical models and clinical trials. Potential applications of these RIPK1 inhibitors for the treatment of monogenic and polygenic autoimmune, inflammatory, neurodegenerative, ischemic and acute conditions, such as sepsis, are emerging. This article reviews RIPK1 biology and disease-associated mutations in RIPK1 signalling pathways, highlighting clinical trials of RIPK1 inhibitors and potential strategies to mitigate development challenges.

Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is a master regulator of the cellular decision between pro-survival NF-κB signalling and death in response to a broad set of inflammatory and pro-death stimuli in human diseases3–5. RIPK1 kinase activation has been demonstrated in post-mortem human pathological samples of autoimmune and neurodegenerative conditions5–6, and inhibition of RIPK1 kinase activity has shown efficacy in a wide range of animal models of human diseases. As TNFR1-mediated RIPK1 activation is the most comprehensively characterized paradigm, RIPK1 inhibitors were originally considered to primarily offer a small-molecule alternative to anti-TNF antibody therapies for TNF-driven autoimmune conditions. However, as researchers continued to delve into the mechanisms governed by RIPK1, it has become apparent that RIPK1 inhibitors may offer key therapeutic options that anti-TNF therapies do not: first, RIPK1 inhibitors are safe in the central nervous system (CNS) as RIPK1 kinase does not signal through TNFR2 which has a protective role in the CNS5; second, RIPK1 participates in a broader set of pro-inflammatory activities than those restricted to TNF5; third, RIPK1 is regulated by a distinct set of signalling molecules that are genetically implicated in human autoimmune and autoinflammatory diseases, as discussed below, and thus patient stratification may be important in conducting clinical trials of RIPK1 inhibitors.

Necrostatin-1s (Nec-1s) was the first small-molecule inhibitor of RIPK1 kinase to be developed and has been widely used to investigate the role of RIPK1 in mechanistic studies and animal models of human diseases2,8–11. Broad therapeutic applications of RIPK1 inhibitors for the treatment of a wide range of human diseases are being investigated in clinical trials. The peripherally restricted GSK’772 is being developed for peripheral autoimmune diseases, including psoriasis, rheumatoid arthritis (RA) and ulcerative colitis12–14. The brain-penetrant DNL747 is in human clinical trial phase Ib/IIa for amyotrophic lateral sclerosis (ALS)15,16. These trials have laid the groundwork for advancing clinical applications of RIPK1 inhibitors.

The important role of RIPK1 in driving cell death and inflammation, the established safety of inhibiting RIPK1 kinase activity in humans and the ability to develop selective small-molecule kinase inhibitors of RIPK1 due to the presence of a unique kinase-regulating allosteric pocket are the major factors that have contributed to RIPK1’s prominence as a therapeutic target. In this Review, we outline the current understanding of RIPK1 biology in activating cell death and NF-κB signalling, systematically review monogenic and polygenic variants of known RIPK1 regulators and discuss how these mutations may contribute to disease pathology. The involvement of RIPK1 in sepsis and acute ischemic conditions is also discussed. We postulate that the improved understanding of genetic and mechanistic data may be beneficial in segmenting patients in clinical trials, particularly for neurodegenerative and inflammatory diseases. Finally, we summarize the current state of RIPK1 inhibitors in the clinic, including disease indications, small-molecule chemotypes, as well as RIPK1 target engagement and pharmacodynamic biomarkers.

Distinct kinase and scaffold functions of RIPK1

RIPK1 is a 76-kDa protein with an amino-terminal (N-terminal) kinase domain, a carboxy-terminal (C-terminal) death domain and an intermediate domain with a RHIM (receptor-interacting protein homotypic interacting motif) that can bind to other
TNF
(Tumour necrosis factor).
A pro-inflammatory cytokine typically secreted by macrophages/monocytes and microglia. TNF signals through two receptors: TNFR1, which mediates cell death and NF-κB signalling, and TNFR2, which mediates non-canonical NF-κB signalling.

Complex I
A transient complex associated with the intracellular domain of TNFR1 upon TNF stimulation that includes RIPK1 and many other regulators of NF-κB activation and cell death.

RIPK1-dependent apoptosis
(RDA). A form of apoptosis enacted by activated RIPK1 kinase that forms a transient intermediate cell death complex comprising highly ubiquitylated, activated RIPK1, FADD and Casp8, known as uRIPK1 complex, which then transitions to form complex Ila to mediate the activation of Casp8.

RIPK1 signalling pathway mediated by TNFR1
Activation of RIPK1 kinase mediated by TNFR1 signalling promotes most of the deleterious effects activated by TNF in human disease. In TNF-stimulated cells, the activation of RIPK1 is regulated in a transient multimeric complex associated with the intracellular domain of TNFR1, known as complex I (Fig. 1). The recruitment and activity of this proteins determines the balance between inhibited RIPK1 kinase activity, where NF-κB pro-survival signalling is activated, and activated RIPK1 kinase activity, which leads to inflammation and cell death. Mutations that impact the recruitment to complex I or activity of these proteins leads to aberrant RIPK1 kinase activity in disease, most commonly autoimmunity or inflammation (BOX 1).

RIPK1 and TRADD, another death domain-containing adaptor protein, are rapidly recruited into complex I by binding to the death domain of TNFR1. Activation of RIPK1 in complex I, as measured by the well-established phosphorylation of S166, can enact either apoptotic or necroptotic forms of cell death. In complex I, the activation of RIPK1 is determined by a code including complex ubiquitylation, phosphorylation and other modification events on RIPK1, which include those directly organized by TRADD and those mediated by proteins transcribed and translated downstream of the NF-κB pathway that are also recruited into the complex (for example, A20). This post-translational RIPK1 code, which may be both cell type-specific and stimulus-specific, modulates the extent of RIPK1 kinase activation, which in turn determines the mode of cell death. There is an extensive body of literature identifying cell type-specific roles for RIPK1 activity using conditional mouse models that we and others have summarized previously, and so do not cover in detail in this article. For example, in certain cell types, such as oligodendrocytes, TNF stimulation alone may promote the activation of RIPK1 and cell death; in other cell types, such as fibroblasts, sustained activation of RIPK1 kinase can only be achieved when TNF stimulation is combined with chemical inactivators of RIPK1 repressors, such as cIAP1/2 (inhibited by SM-164), TAK1, TBK1 or IKKs, that are both mediators of the NF-κB pathway and inhibitors of RIPK1 kinase activation.

Ubiquitylation of RIPK1 in complex I, which is organized by TRADD, is essential for suppressing the aberrant activation of RIPK1 kinase. TRADD recruits TRAF2 and the E3 ubiquitin ligases cIAP1 and cIAP2 into complex I to mediate RIPK1 K63 ubiquitylation. K63 ubiquitylation of RIPK1, in turn, mediates the recruitment and activation of TAK1 kinase through the polyubiquitin binding adaptors TAB2 and TAB3 (REVIEW). K63 ubiquitylation of complex I facilitates the recruitment of the LUBAC complex containing HOIP, HOIL1 and SHARPIN, which in turn performs M1 ubiquitylation of RIPK1 and TNFR1. M1 ubiquitylation of complex I is important for the recruitment of the trimeric IKK complex through the polyubiquitin-binding adaptor subunit IKKγ/NEMO, as well as other ubiquitin binding proteins such as A20 (binding and inhibitor of NF-κB-1) and the kinase TBK1 (REVIEW). The activation of RIPK1 is suppressed by direct inhibitory phosphorylation mediated by TAK1, IKKα/β and TBK1 (REVIEW); cIAP1 may also mediate K48 ubiquitylation of RIPK1 to promote its degradation by the proteasome. In addition, TNNAP3, which is transcriptionally induced by the NF-κB pathway, encodes the ubiquitin editing enzyme A20, which controls the activation of RIPK1 by modulating its ubiquitylation pattern.

TNF stimulation of fibroblasts derived from either wild-type mice or healthy patients does not lead to cell death owing to the suppression of RIPK1 activation in complex I by inhibitory ubiquitylation and phosphorylation. Stimulation with TNF in combination with factors that reduce the inhibition of RIPK1 leads to necroptosis, RIPK1-dependent apoptosis (RDA) and inflammation (BOX 1). In experimental paradigms, RIPK1 kinase activity and necroptosis can be activated by treatment with a combination of TNF, cycloheximide (CHX), which blocks the activation of NF-κB-mediated transcription and translation, and zVAD.fmk, which blocks the activation of caspases. Inhibition of caspases strongly sensitizes to RIPK1 activation, as the cleavage of RIPK1 by Casp8 provides an important inhibitory mechanism to block the overactivation of RIPK1 (REVIEW). RDA can be activated with a combination of TNF and the TAK1 inhibitor (5Z)-7-oxozenoal (known as 5Z7), or TNF and SM-164, which blocks inhibitory phosphorylation and ubiquitylation of RIPK1, respectively, and blocks activation of pro-survival NF-κB signalling. As detailed in this Review, these experimental manipulations mimic some aspects of human genetic deficiencies.
RIPK1 in immune and autoinflammatory diseases
Mutations in the genes that encode RIPK1 and multiple proteins that regulate RIPK1 signalling can lead to immune and autoinflammatory diseases. These clinically identified mutations highlight the important role of RIPK1 in regulating the innate immune response and provide mechanistic insights into the functional role of RIPK1 in disease. The spectrum of immune and auto-inflammatory diseases presents as a continuum between autoimmune disorders involving primarily the adaptive immune system and autoinflammatory conditions involving primarily the innate immune system, all of which can be found in diseases involving RIPK1.

**RIPK1 independent apoptosis**
A form of apoptosis enacted by activated (cleaved) caspase 8 that is independent of RIPK1 kinase activity.

**RIPK1 in TNFR1 signalling.** Complex I: stimulation of TNFR1 by TNF promotes the formation of an intracellular signalling complex associated with the death domain of trimerized TNFR1 that recruits two death domain-containing proteins: adaptor protein TRADD and receptor-interacting serine/threonine-protein kinase 1 (RIPK1). TRADD recruits E3 ubiquitin ligases cIAP1/2 and XIAP to perform K63 ubiquitylation of complex I, including RIPK1 K377, which in turn recruits the LUBAC complex, comprised of HOIP, HOIL1 and SHARPIN. LUBAC mediates linear (M1) ubiquitylation of RIPK1. Deubiquitinase CYLD and its adaptor protein SPATA2 modulate M1/K63 ubiquitylation of RIPK1. M1 deubiquitinase OTULIN activates LUBAC. K63 ubiquitin chains on RIPK1 recruit TAB2/3 and TAK1. M1 ubiquitin chains on RIPK1 recruit the NEMO– NF-κB kinase (IKK) complex, TBK1, A20, ABIN1 (A20 binding inhibitor of NF-κB-1) and OPTN. A20 in complex I suppresses the activation of RIPK1 kinase. NF-κB activation: activation of TAK1 and the IKKs promote NF-κB pathway activation to mediate transcription of both pro-inflammatory and pro-survival genes, including A20, which modulates the ubiquitylation of RIPK1 to control its activation, and cellular FLICE-like inhibitory protein (cFLIP), which modulates activation of caspase 8 (Casp8). RIPK1-dependent apoptosis (RDA): activation of TNFR1 under A20, ABIN1, cIAP1/2, NEMO, TBK1, IKK or TAK1-deficient conditions leads to RIPK1 kinase activation. Activated RIPK1 binds to FADD and Casp8 to form complex Ila and promote activation of caspases and apoptosis. Increased levels of A20 promote the activation of RIPK1 in complex Ila. Necroptosis: inhibition of Casp8-mediated cleavage of RIPK1 promotes RIPK1 dimerization via the C-terminal death domain, which leads to its activation and the subsequent formation of the necrosome (complex IIb) comprised of RIPK1, FADD, Casp8, RIPK3 and mixed-lineage kinase domain-like pseudokinase (MLKL), which in turn executes necroptosis. RIPK1-independent apoptosis: when the NF-κB pathway is inhibited, TNF stimulation can promote the formation of a cytosolic complex with FADD and Casp8 to mediate apoptosis independent of RIPK1. P, phosphate; RIPK1i, RIPK1 inhibitor; Ub, ubiquitin. Adapted from REF. 1, Springer Nature Limited.
RIPK1 mutations in humans
Rare mutations leading to loss of function (LoF) and gain of function (GoF) of RIPK1 have been identified in individuals with immunodeficiencies and autoinflammatory diseases (TABLE 2).

Biallelic loss-of-function RIPK1 mutations. Rare biallelic LoF mutations in RIPK1, including missense, nonsense and frameshift mutations, have been identified in patients with combined immunodeficiency and inflammatory bowel disease (IBD)44,45. Whereas Ripk1−/− mice die perinatally, patients with LoF mutations in RIPK1 exhibit paediatric onset of primary immunodeficiency characterized by an increased susceptibility to infections and early-onset IBD44,45. At the cellular level, the loss of RIPK1 in human skin fibroblasts impairs activation of the NF-κB pathway, MAPKs and Jun in response to TNF or poly(I:C) and increases the activation of necroptosis mediated by RIPK3 and mixed-lineage kinase domain-like pseudokinase (MLKL), but not apoptosis44,45. The production of pro-inflammatory cytokines, including TNF, IL-6 and IL-10, in response to LPS stimulation is severely impaired in peripheral blood mononuclear cells (PBMCs) from patients with RIPK1 LoF compared with control PBMCs44. Dysregulation of cytokine production and the host defence response towards the gut microbiome likely play a key role in promoting early-onset IBD and progressive polyarthritis in these patients.

Heterozygous non-cleavable RIPK1 mutations. Casp8 inactivates RIPK1 by cleaving human and mouse RIPK1 after residues D324 and D325, respectively, which separates the RIPK1 kinase domain from the intermediate and death domains46. Mice with a D325A knock-in mutation that prevents cleavage by Casp8 die embryonically, and can be rescued by cis-inactivation of RIPK1 by D138N mutation, the loss of TNFR1, or inactivating both necroptosis and apoptosis by double knockout of Ripk3 and Fadd, or Mlkl and FADD47,48. Rare variants of RIPK1, such as D324V and D324H, that block cleavage by Casp8 have been identified in individuals with an autosomal dominant autoinflammatory disease41,42. In contrast to the phenotype observed in patients with RIPK1 LoF mutations, patients with heterozygous non-cleavable RIPK1 mutations develop autoinflammatory disease characterized by recurrent fevers and lymphadenopathy. Marked increases in pro-inflammatory cytokines and chemokines, such as IL-6, TNF and interferon-γ (IFNγ), were found in sera from patients. Impaired cleavage of RIPK1 D324 variants by Casp8 hypersensitizes patient PBMCs to RIPK1 activation, including both apoptosis and necroptosis induced by TNF, which can be blocked by the RIPK1 inhibitor Nec-1s41,43.

Although PBMCs from patients with non-cleavable RIPK1 mutations are more susceptible to inflammatory stimulation, fibroblasts from one such patient showed resistance to necroptosis and ferroptosis and reduced expression of pro-inflammatory cytokines in response to stimuli43. Considerable changes in the gene expression patterns were found in these fibroblasts, including downregulated expression of RIPK1, RIPK3 and TNFR1, as well as elevated levels of the anti-oxidative glutathione and genes that offer resistance to ferroptosis. Such compensatory gene expression might be necessary to overcome the deleterious effects of non-cleavable RIPK1 and allow for patient survival, although these findings need to be expanded to additional patients.

Together, these data suggest that autoinflammatory disease caused by non-cleavable RIPK1 variants may represent a canonical human RIPK1 hyperactivating disease that can respond to RIPK1 inhibitor treatment.

Disease-associated mutations in regulators of RIPK1
Dysregulation of RIPK1 signalling is involved in a heterogeneous group of monogenic immune and autoinflammatory diseases that can present with either episodic or chronic symptoms (TABLE 2). As many of these disease-associated genes are also involved in regulating NF-κB signalling49, some amount of disease pathology can be attributed to altered NF-κB signalling. Interestingly, these genes, including TNFAIP3 (encoding A20), TNIP1 (encoding ABIN1), IKBKg (encoding NEMO), OTULIN and members of the LUBAC complex, are also direct regulators of RIPK1 activation (TABLE 1). Thus, exogenous triggers that lead to transient inflammation in healthy subjects may promote sustained inflammation and cell death involving different tissues and organs in individuals with aberrant RIPK1 regulation. Traditional treatment for autoimmune diseases has focused on managing immune hyperactivity by dampening non-specific inflammatory responses and immune cell proliferation. However, this approach renders patients vulnerable to opportunistic infections that can be life-threatening. Understanding the inflammatory
mechanisms regulated by RIPK1 may help to develop therapies that can specifically target the disease pathology in these rare diseases. Furthermore, understanding the contribution of RIPK1 in these rare diseases may also help to elucidate roles for RIPK1 in autoimmune and inflammatory diseases that are not genetically linked to RIPK1.

**A20 deficiency.** A20, encoded by the **TNFAIP3** gene, is an inducible ubiquitin-editing enzyme that restricts both Toll-like receptor (TLR) and TNF-induced inflammatory responses by regulating the ubiquitylation of key signalling proteins, including RIPK1, TRAF6 and NEMO. Mouse models with cell lineage-specific A20 deficiency phenocopy different human inflammatory diseases, suggesting an important role for A20 in restricting RIPK1 activation in multiple tissues (Table 3). A20 contains an N-terminal ovarian tumour (OTU) domain that can deubiquitylate K63/K48-linked polyubiquitin chains from its substrates, and C-terminal zinc finger domains that can bind and modulate linear (M1) ubiquitin chains. Multiple heterozygous LoF mutations in the **TNFAIP3** gene, including early nonsense and frameshift mutations, have been identified in a paediatric systemic inflammatory disease or systemic blood vessel inflammation similar to Behçet’s disease.

Table 1 | Mutations in regulators of NF-κB pathway lead to aberrant RIPK1 kinase activation in human diseases

| Gene (protein) | Impact of protein on RIPK1 | Disease associations | Refs |
|----------------|--------------------------|---------------------|------|
| TNF            | Activates RIPK1-dependent signalling through TNFR1 | RA, asthma, insulin resistance, AD | 93,167,193 |
| TNFRSF1A (TNFR1) | Receptor that initiates RIPK1-dependent signalling downstream of TNF | TRAPS, MS | 95,194 |
| RIPK1          | LoF: impairs NF-κB pro-survival pathways; GoF: loss of Casp8 cleavage site results in persistent activation of RIPK1 kinase activity | LoF: immunodeficiency; GoF: systemic autoinflammation | 41,42,44,45 |
| TRAF1/2        | Recruit cIAP1/2 to complex I to ubiquitylate RIPK1 | MS, RA | 195 |
| XIAP           | Regulates RIPK1 ubiquitylation in the necroosome | IBD, immunodeficiency | 198,199 |
| HOIL1          | LUBAC complex performs M1 ubiquitylation of complex I to activate pro-survival NF-κB signalling and suppress RIPK1 kinase activation | Systemic autoimmunity | 60 |
| HOIP           | Systemic autoimmunity | 70 |
| SHARPIN        | AD | 131 |
| TAK1           | Phosphorylates RIPK1 S321 and other sites to control RIPK1 kinase activation | Brain ageing, neuroinflammation | 18,310 |
| PEL1           | K63 ubiquitylation of RIPK1 to regulate cell death | SLE | 199 |
| OTULIN         | Reduces M1 ubiquitylation of LUBAC to activate LUBAC, which in turn inhibits RIPK1 kinase | OTULIPENIA/ORAS | 60,203 |
| TAB2           | Recruits TAK1 to complex I to phosphorylate RIPK1 and activate NF-κB | Congenital heart defects | 199 |
| OPTN           | Promotes K48 ubiquitylation of RIPK1 for its degradation | ALS/FTD | 4,200 |
| TNFAIP3 (A20)  | Primarily regulates M1 ubiquitylation of RIPK1 to control its activation | Systemic autoimmunity, Crohn’s disease, psoriasis, RA, allergy, atopic dermatitis, Behçet’s disease, MS, SLE | 50,52,53,54,94,98,201–208 |
| TNIP1 (ABIN1)  | Mediates A20 binding to complex I to regulate RIPK1 ubiquitylation and activation | Psoriasis, psoriatic arthritis, systemic sclerosis, SLE, ALS, schizophrenia, MS | 92,94,206,209–211 |
| TBK1           | Phosphorylates RIPK1 T189 and other sites to limit RIPK1 kinase activation | ALS/FTD | 18,200 |
| TBKBP1         | Adaptor protein that modulates TBK1 activity | MS | 212 |
| IKBKG (NEMO)   | Scaffold for IKK complex involved in inhibitory S25 phosphorylation of RIPK1 | Incontinentia pigmenti, anhidrotic ectodermal dysplasia with immune deficiency | 62,213–215 |
| IKBKA/IKKBK (IKK1/2) | Phosphorylates RIPK1 S25 to limit RIPK1 kinase activation | Cocoon syndrome, immunodeficiency | 210 |
| CASP8          | Cleaves RIPK1 to reduce RIPK1 activation | ALPS | 217 |

AD, Alzheimer disease; ALPS, autoimmune lymphoproliferative syndrome; ALS, amyotrophic lateral sclerosis; CASP8, caspase 8; FTD, frontotemporal dementia; GoF, gain of function; IBD, inflammatory bowel disease; IKK, inhibitor of κB kinase; LoF, loss of function; MS, multiple sclerosis; ORAS, OTULIN-related autoinflammatory syndrome; RA, rheumatoid arthritis; RIPK1, receptor-interacting serine/threonine-protein kinase 1; SLE, systemic lupus erythematosus; TRAPS, tumour necrosis factor receptor-associated periodic syndrome.
Table 2 | Monogenic human diseases involving RIPK1

| Gene (protein) | Mutation | Disease/clinical characteristics | Refs |
|---------------|----------|----------------------------------|------|
| RIPK1         | Biallelic LoF | Combined immunodeficiency and IBD | 44,45 |
| RIPK1         | Heterozygous, autosomal dominant non-cleavable mutants | Autoinflammation characterized by recurrent fevers and lymphadenopathy | 41,42 |
| TNFAIP3 (A20) | Haploinsufficiency | Early-onset systemic inflammation | 56,57,80,203 |
| NEMO          | X-linked dominant null allele (lethal in males) | Incontinentia pigmenti with skin inflammation, immune deficiency, blindness and mental retardation (female) | 214,215 |
| NEMO          | X-linked recessive partial LoF | Anhidrotic ectodermal dysplasia with immunodeficiency (male) | 213 |
| OTULIN        | Biallelic hypomorphic mutations | OTULIPENIA or ORAS is characterized by neonatal-onset fevers, skin rashes and neutrophilic dermatitis/panniculitis | 68,69 |
| HOIP          | Hypomorphic | Immunodeficiency and multi-organ autoinflammation | 69,70 |
| HOIL1         | Biallelic LoF | Immunodeficiency and multi-organ autoinflammation | 71 |

IBD, inflammatory bowel disease; LoF, loss of function; ORAS, OTULIN-related autoinflammatory syndrome; RIPK1, receptor-interacting serine/threonine-protein kinase 1.

OTULIN deficiency. OTULIN is the only known deubiquitylating enzyme that specifically removes M1 ubiquitin chains. Patients with biallelic hypomorphic mutations in OTULIN develop a severe form of autoinflammatory disease, known as OTULIPENIA or OTULIN-related autoinflammatory syndrome (ORAS). In OTULIN-deficient cells, levels of M1 ubiquitylation on multiple target proteins, such as NEMO, TNFR1 and RIPK1, are generally increased. As activity of the LUBAC activity complex is negatively regulated by M1 ubiquitylation, OTULIN-deficient cells have reduced LUBAC activity. Cells derived from patients with OTULIN deficiencies have a strong inflammatory signature including overproduction of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-18 and IL-10, in response to LPS, TNF or IL-1β. These OTULIN-deficient cells are also sensitized to TNF-induced cell death. Consistent with the important role of TNF in this disease, these patients responded well to treatment with anti-TNF antibodies (infliximab).

OTULIN−/− mice and Otulin<sup>C129A</sup> catalytically inactive knock-in mice die during embryonic development. Embryonic lethality of the Otulin<sup>C129A</sup> mice can be delayed by Ripk3<sup>101DIN/D101N</sup> or Tnfr1<sup>−/−</sup>, whereas combined loss of Ripk3 and Casp8 prolongs survival to the perinatal stage, suggesting that necroptosis is activated when OTULIN is not functional.

LUBAC deficiency. The LUBAC complex is composed of HOIP, HOIL1 and SHARPIN. Patients identified with LoF or hypomorphic mutations in HOIP or HOIL1 develop immunodeficiencies and autoinflammatory disease characterized by frequent viral and bacterial infections and multi-organ autoinflammation. These hypomorphic mutations in HOIP and biallelic inflammatory skin lesions in heterozygous females. The skin inflammation of Nemo<sup>−/−</sup> mice can be delayed by crossing them with Tnfr1<sup>−/−</sup>, suggesting that RIPK1 kinase may be involved in mediating inflammatory response in incontinentia pigmenti.

Mutant mice with specific loss of NEMO in intestinal epithelial cells (IECs) (Nemo<sup>−/−</sup> mice) show apoptosis of Paneth cells and colonicocytes, and microbiota-driven chronic colitis. The colitis of Nemo<sup>−/−</sup> mice was blocked by genetic or pharmacological inhibition of RIPK1 kinase activity, suggesting that the pathology in this mutant line may be driven more by RIPK1-dependent cell death and inflammatory mechanisms than failure to activate NF-κB.

Taken together, these studies suggest that RIPK1 kinase activity may play an important role in colitis and excessive inflammation in NEMO syndrome. Thus, RIPK1 kinase inhibitors may be beneficial for the treatment of colitis and other inflammatory issues in patients deficient in NEMO. However, as patients with NEMO deficiency syndrome have an inability to mount a successful immune response and are hypersensitive to infection, reminiscent of patients deficient in RIPK1, the inability to properly activate the NF-κB pathway may also contribute to the symptoms of NEMO deficiency syndrome.
LoF mutations in HOIL1 lead to a reduction in levels of the LUBAC complex driven by a loss of stability. LUBAC-deficient cells show reduced levels of IKK phosphorylation and compromised NF-kB activation. LUBAC is critical for suppressing RIPK1 activation, and, as a consequence, enhanced RIPK1 activity may be responsible for autoinflammatory symptoms in patients harbouring LUBAC mutations.

Mice deficient for the gene encoding the LUBAC regulatory subunit SHARPIN, also known as chronic proliferative dermatitis mice (cpdm) (Sharpin<sup>cpdm/cpdm</sup> mice), suffer from severe multi-organ inflammation, particularly in the skin with similarity to atopic dermatitis and psoriasis in humans<sup>76,77</sup>. Interestingly, the skin inflammation of cpdm mice can be blocked by reducing or knocking out TNF<sup>75</sup>, which points to a key role for this signalling node in the cpdm phenotype. Inhibition of RIPK1 by GNE684 or RIPK1-D138N protects against dermatitis in cpdm mice<sup>76,78,79</sup>.

**Polygenic diseases associated with RIPK1 regulators**

Polygenic human diseases, including several autoimmune and inflammatory diseases, are associated with mutations or risk variants in multiple genes, which can include regulators of RIPK1. When developing RIPK1 kinase inhibitors for the treatment of these diseases, patient stratification may be important to identify those with pathogenic activation of RIPK1.

**Inflammatory bowel disease.** Crohn’s disease and ulcerative colitis are the two primary forms of IBD that are both characterized by intestinal inflammation and epithelial cell loss. Genome-wide association studies (GWAS) of Crohn’s disease and ulcerative colitis have identified more than 200 loci that are above the thresholds of significance<sup>80</sup>. However, non-genetic factors likely play a major role in conferring susceptibility as the concordance rate in monozygotic twins is only 16%<sup>81</sup>. Whereas mice with enterocyte-specific A20 deletion are hypersensitive to experimental colitis and TNF-induced epithelial apoptosis<sup>82</sup>, excessive A20 can dimerize, which sensitizes IECs to undergo RIPK1-dependent apoptosis when stimulated by TNF<sup>79</sup> (Table 3). IECs with elevated A20 in the mucosa of patients with IBD are associated with Casp3 activation. Inhibition of RIPK1 has been shown to ameliorate pathology in both A20-deficient and elevated conditions<sup>79,80</sup>. As specific single-nucleotide polymorphisms (SNPs) in TNFAIP3 have been found to be correlated with primary therapeutic response to anti-TNF (infliximab) therapy<sup>81</sup>, changes in intestinal A20 expression may be used as a biomarker for RIPK1-dependent pathology to stratify patients with IBD for treatment with RIPK1 inhibitors.

GWAS have also identified ATG16L1 and other autophagy-related genes as risk factors for IBD<sup>82</sup>. ATG16L1 is a component in the macromolecular complex that lipitates LC3/ATG8 to promote the formation of the canonical double-membrane autophagosome. ATG16L1<sup>T300A</sup> mice, a risk allele associated with IBD susceptibility, introduces a caspase cleavage site that destabilizes the ATG16L1 protein product and reduces autophagy in the presence of TNF<sup>83,84</sup>. ATG16L1-deficient intestinal organoids show increased sensitivity to TNF-induced necroptosis, which can be effectively inhibited by Nec-1s<sup>156,218,219</sup>. Both ATG16L1 IEC conditional knockout mice and A20 IEC conditional knockout mice show increased severity of hypothermia induced by TNF, which can be blocked by the RIPK1 inhibitor GNE684<sup>85</sup>.

**Psoriasis.** GWAS identified more than 60 disease susceptibility regions, including the TNFAIP3, TNIP1 and RELA loci in psoriasis<sup>86</sup>. Reduced levels of TNFAIP3

### Table 3 | Cell lineage-specific A20 alterations in mouse models that phenocopy human inflammatory and immune diseases

| Mutant type | Lineage/model | Phenotype | Refs |
|-------------|---------------|-----------|------|
| A20<sup>-/-</sup> | Global knockout mouse | Spontaneous systemic inflammation, severe cachexia and premature lethality; survival prolonged with Ripk1<sup>T1510N/D138N</sup> or Ripk3<sup>-/-</sup> mice, but not with Mlkl<sup>1/-</sup> | 236,237,238 |
| A20<sup>-/-</sup> | Murine embryonic fibroblasts | Sensitized to RIPK1-dependent apoptosis and necroptosis | 35 |
| A20<sup>-/-</sup> | Keratinocyte-specific knockout mouse | Systemic inflammation and exacerbated disease in models of psoriasis and atopic dermatitis | 220 |
| A20<sup>IEC</sup> | Myeloid-specific knockout mouse | Spontaneous inflammatory joint arthritis; rescued by Ripk1<sup>T1510N/D138N</sup> mutation, Ripk3<sup>-/-</sup> or Mlkl<sup>-/-</sup> | 80,208 |
| A20<sup>IEC</sup> | IEC and myeloid-specific double-knockout mouse | Intestinal pathology through synergistic IEC and myeloid mechanisms to promote ileitis and severe colitis | 81 |
| A20<sup>-/-</sup> | Global C764A:C767A ZnF7 knock-in mouse | Spontaneous inflammatory arthritis with activated RIPK1 | 80 |
| A20<sup>IEC</sup> | IEC-specific knockout mouse | Hypersensitive to TNF-induced systemic inflammation; partially blocked by inhibition of RIPK1 (Ripk1<sup>T1510N/D138N</sup>) | 221 |
| A20<sup>IEC</sup> | IEC-specific overexpression mice | Hypersensitive to TNF-induced intestinal damage in RIPK1-dependent manner | 79 |

IEC, intestinal epithelial cell; MLKL, mixed-lineage kinase domain-like pseudokinase; RIPK1, receptor-interacting serine/threonine-protein kinase 1; TNF, tumour necrosis factor; ZnF7, zinc finger 7.
mRNA and A20 protein have been found in the skin of patients with psoriasis, which can be directly correlated to disease severity\(^9\). Furthermore, specific SNPs in TNFAIP3 have also been found to predict the response to anti-TNF therapies\(^8\), suggesting the predictive value of TNFAIP3 in identifying patients likely to respond to a RIPK1 inhibitor treatment for psoriasis.

TNIP1, encoding the ABIN1 protein, is one of the highest scoring non-MHC genes associated with various autoimmune and autoinflammatory diseases, including psoriasis, psoriatic arthritis, systemic sclerosis and systemic lupus erythematosus (SLE) across multiple GWAS\(^7\). ABIN1, a ubiquitin binding protein with a UBAN domain that binds preferentially to linear ubiquitin chains, interacts with A20 to negatively regulate the activation of MAPK and NF-κB- mediated gene transcription downstream of TNFR1 and TLRs\(^89-91\). ABIN1, which is recruited into the TNFR1 signalling complex by binding linear ubiquitin chains, in turn promotes the recruitment of A20 (Ref.\(^92\)). ABIN1-deficient cells and A20-deficient cells are both sensitized to RIPK1 activation and necroptosis. Tnip1\(^+/−\) mice die embryonically owing to liver necrosis that can be rescued by Ripk3\(^−/−\) or Ripk1\(^D138N/D138N\). TNIP1 heterozygosity sensitizes cells to a hyperactive antiviral response mediated by an NF-xB-dependent and RIPK1-independent mechanism that promotes the expression of pattern recognition receptors, such as TLR3, RIG-1 and MDA5 (Ref.\(^93\)). Inhibition of RIPK1 blocks the increased production of pro-inflammatory cytokines in Tnip1\(^−/−\) mice in response to viral stimuli. Thus, ABIN1 regulates the innate immune response through the NF-xB pathway, utilizing both RIPK1-dependent and independent signalling pathways. Inhibition of RIPK1 may reduce the exaggerated inflammatory response in patients with Tnip1 mutations without affecting their host defence responses.

**Rheumatoid arthritis.** Rheumatoid arthritis (RA) is a common inflammatory autoimmune disease characterized by chronic synovial inflammation, joint destruction and systemic complications. The HLA-DRB1 alleles are the primary genetic risk factor associated with RA. In addition, GWAS have identified ~100 non-HLA loci, including TNF, associated with RA. GWAS data also suggest the involvement of multiple immune cell types, including T cells, B cells, natural killer cells, dendritic cells and monocytes, as well as the pro-inflammatory cytokines produced by these cells, in mediating RA pathogenesis\(^94\). Interestingly, SNPs and reduced levels of TNFAIP3 have been associated with RA\(^95\). Inhibition of RIPK1 by GNE684 improved pathology in a rodent model of collagen-induced arthritis\(^96\).

**Multiple sclerosis.** Multiple sclerosis (MS) is an autoimmune disease of the CNS resulting in neurodegeneration that involves the loss of myelinating oligodendrocytes that normally insulate the axons of neurons. TNF is strongly linked with the aetiology of MS\(^97,98\). Oligodendrocytes can undergo necroptosis in the presence of TNF stimulation alone\(^99\). In lesions of brain samples from patients with MS or mouse models of MS, activation of RIPK1, RIPK3 and MLKL and formation of the necrosome were observed\(^99\). cFLIP is transcribed in response to NF-xB activation and can suppress the formation of apoptotic signalling complexes\(^100\). In the lesions from patients with MS, levels of cFLIP were found to be decreased, which may be the result of chronic neuroinflammation. Decreased cFLIP and reduced levels of activated Casp8 in patients with MS, and thus decreased Casp8 inactivating cleavage of RIPK1, likely explain the increased RIPK1 kinase activity and necroptosis seen in MS. Genetic (Ripk1\(^D138N/D138N\)) and pharmacological (Nec-1s) inhibition of RIPK1 kinase ameliorated disease pathology, improved animal behaviour, attenuated the production of pro-inflammatory cytokines and decreased recruitment of immune cells in two animal models of MS\(^3\). Another inhibitor of RIPK1 kinase developed by Takeda also showed efficacy in an animal model of MS\(^101\).

The contribution of peripheral immune cells and microglia to MS has been highlighted by a recent genetic association study using 47,429 MS cases and 68,374 controls\(^102\). This study identified 551 putative MS susceptibility genes that are highly expressed in cell types involved in adaptive immunity and innate immunity, including TNFAIP3, which encodes A20. As RIPK1 is highly expressed in macrophages and microglia in experimental autoimmune encephalomyelitis lesions, inhibition of RIPK1 may directly reduce inflammatory responses as RIPK1 activity is involved in mediating the inflammatory responses in these cells\(^103,104\). Thus, the beneficial effects of inhibiting RIPK1 may include modulation of both inflammation and cell death in susceptible oligodendrocytes, microglia and neurons. This raises the intriguing possibility that RIPK1 may act in both a cell-autonomous and a non-cell autonomous manner in MS. As the existing approved MS drugs all target adaptive immunity and do not directly address neuroinflammation that drives progression in MS, RIPK1 inhibition may represent a novel therapeutic approach that targets multiple aspects of disease pathology.

**Neutrophil and complement-mediated autoimmune disorders.** Systemic lupus erythematosus (SLE) is another heterogeneous autoimmune and inflammatory disorder characterized by excessive immune and inflammatory responses. SLE is a chronic condition that affects multiple organs and tissues. Multiple independent SNPs in the TNIP1 and TNFAIP3 genes are found to be associated with SLE\(^102-104\).

Assembly and activation of the necroptotic machinery, including RIPK1, has been implicated in NETosis, a programmed suicide of neutrophils in tissues involved in mounting normal immune responses against pathogens\(^105-107\). NETosis results in the formation of neutrophil extracellular traps (NETs); this leads to the release of autoantigens from neutrophils and contributes to autoimmune pathology\(^108\). In several diseases in which NETs drive toxicity, inhibiting RIPK1 and thereby blocking other components of the necroptosis pathway has shown efficacy in disease models, including autoimmune vasculitis, venous thrombosis and SLE\(^109-112\). More broadly, toxicity induced by neutrophil
extravasation and NET formation has been implicated in widespread diseases, ranging from autoimmune conditions to neurodegenerative diseases where an impaired blood–brain barrier results in aberrant NET formation in the brain parenchyma. The canonical and alternative complement pathways are well-characterized and essential contributors to the pathogenesis of diseases such as SLE. The activation of complement in disease results in complement-dependent cytotoxicity. Complement can activate RIPK1–RIPK3–MLKL-dependent necroptosis as a contributor of complement-dependent cytotoxicity, which can be attenuated by RIPK1 inhibitors. Furthermore, in some instances of autoimmunity where complement activation drives disease, the complement pathways can be activated by neutrophil NETosis, which is also regulated by RIPK1 activation. Taken together, these findings suggest that RIPK1 may be involved in the pathogenesis of neutrophil and complement-mediated autoimmune conditions.

**RIPK1 in chronic neurodegenerative diseases**

Given that RIPK1 activation and necroptosis have now been genetically and mechanistically linked with human neurodegenerative diseases, there has been a keen interest in developing CNS-penetrant RIPK1 inhibitors to alter disease progression for diseases such as ALS, frontotemporal dementia (FTD), AD and other ageing-related neurodegenerative diseases (FIG. 2). Beyond these indications where several independent groups and clinical data have confirmed a role for pathogenic RIPK1 kinase activation, limited data suggest that RIPK1 activation may also play a role in the pathology of Huntington disease and Duchenne muscular dystrophy; however, the mechanisms underpinning this have not yet been defined.

**ALS and FTD**

ALS is a progressive neurodegenerative disease that affects motor neurons in the brain and the spinal cord. FTD is a group of related neurodegenerative diseases that lead to the progressive degeneration of neurons in the temporal and frontal lobes of the brain, with consequences on social, emotional and language abilities. LoF mutations in two genes, OPTN and TBKI, lead to familial ALS/FTD. Loss of OPTN, a monogenic cause of ALS, resulted in activation of RIPK1 and necroptosis in the spinal cords of Optn–/– mice, which was rescued by genetic and pharmacological inactivation of RIPK1 kinase. OPTN–/– cells and the spinal cords in OPTN–/– mice show increased levels of total RIPK1 protein, due to decreased proteasomal degradation of RIPK1 protein. Furthermore, gain-of-function mutations have been found in p62 (encoded by SQSTM1), another causal ALS factor that associates with OPTN; p62 mutants have been found to interact with RIPK1 to shift the balance between necroptotic and apoptotic death.

**TBKI haploinsufficiency** is a monogenic cause of ALS/FTD. Mice lacking both copies of Tbk1 show profound RIPK1 activation, resulting in embryonic lethality that is rescued by genetic inactivation of RIPK1. Furthermore, TBK1 is responsible for directly inhibiting RIPK1 by phosphorylation at T189; thus, loss of TBK1 activity increases susceptibility to RIPK1 activation. Age-dependent reduction of TAK1 expression in human brains was shown to cooperate with heterozygous loss of TBKI1 to promote late-onset ALS/FTD-like pathology mediated by decreased RIPK1 inhibition.

The activation of RIPK1, RIPK3 and MLKL has been found in post-mortem spinal cord samples from patients with ALS. An ex vivo human co-culture system of astrocytes and motor neurons from patients harbouring a SOD1 familial ALS mutation showed necroptotic death of motor neurons, which could be rescued with Nec-1. In addition, a Sod1G93A mouse model of ALS treated orally with Nec-1 showed improvements in survival, motor activity and axonal pathology. Taken together, these observations suggest a potential approach to genetic stratification of patients in a disease where ~90% of patients exhibit idiopathic disease without known genetic identifiers.

**Alzheimer disease and ageing**

AD is a form of progressive dementia that leads to the irreversible loss of neurons involved in cognitive and executive functions. The neuropathology of AD includes the presence of amyloid plaques and neurofibrillary tangles. Chronically sustained neuroinflammation plays an important role in mediating neural dysfunction and neurodegeneration in AD. Similar to what is observed in the insoluble fractions of post-mortem human CNS samples from patients with MS and ALS, increased activation of RIPK1, RIPK3 and MLKL was also found in the insoluble fractions of sporadic AD post-mortem brains. A recent study reported the detection of all three activated components of the necroosome machinery, pRIPK1, pRIPK3 and pMLKL, in granulovacuolar degeneration lesions in degenerating neurons in AD and preclinical stages of AD pathology. Interestingly, the presence of activated necroosome components correlated with neuronal loss in AD-affected brain regions, such as the hippocampal CA1 region and the frontal cortex layer III, and Tau pathology, but not Aβ pathology.

RIPK1 may play an important role in driving neuroinflammation in AD. The activation of RIPK1 was shown to mediate the transcriptional induction of Csf7, which encodes an endosomal/lysosomal cathepsin inhibitor named cystatin F, in microglia of mouse models of AD. Csf7 is a biomarker for disease-associated microglia present in spatial proximity to Aβ plaques found in both post-mortem human AD brain samples and in an AD mouse model. Inhibition of RIPK1 suppresses the expression of a set of genes, such as Ch25H, which are altered in microglia in two animal models of AD, in the Sod1G93A model of ALS and in ageing microglia, which enhance the ability of microglia to uptake and degrade Aβ. Ch25H is one of the cholesterol metabolism-related genes associated with AD. Treatment with Nec-1 has been shown to be efficacious in reducing neuroinflammation and cognitive deficits in the APP/PS1 Aβ-driven mouse model of AD. These results suggest that RIPK1 may control a neuroinflammatory pathway involved in multiple neurodegenerative diseases and in ageing.
Similar to the loss of oligodendrocytes seen in MS, RIPK1 and necroptosis may also drive white matter loss, which has been found to be among the earliest brain pathological changes in AD, preceding the formation of neural fibrillary tangles and amyloid plaques. In addition, age-related changes in brain white matter — which include the reduction of myelinated fibres and myelin sheath degeneration co-localized with increased ubiquitin deposits — coupled with the presence of neuroinflammation may mediate the age-related cognitive decline seen in elderly individuals without AD. The neuroinflammation and oligodendrocyte degeneration present in normal ageing human brains suggests the possibility of modulating RIPK1 kinase to support cognitive function in ageing. In addition, ageing is associated with mitochondrial dysfunction and the subsequent production of reactive oxygen species (ROS). Cytosolic ROS can modulate cysteine residues on RIPK1, which promotes activation. Microglia and astrocytes: activation of RIPK1 leads to the production of pro-inflammatory cytokines in microglia. Activation of RIPK1 in Alzheimer disease (AD) leads to increased production of CST7 and CH2SH, both of which are associated with the disease-associated microglia phenotype. PD/lysosomal storage disorder (LSD)-associated genes, such as GBA and NPC1, also result in lysosomal dysfunction, which can promote RIPK1 activation, likely by promoting the accumulation of RIPK1. ALS-associated mutations in SOD1 are also known to promote mitochondrial dysfunction and ROS production, which may promote glial activation of RIPK1 in disease. MLKL, mixed-lineage kinase domain-like pseudokinase; P, phosphate; RDA, RIPK1-dependent apoptosis; RIPK1i, RIPK1 inhibitor.
RIPK1 at S321 may provide a biomarker for RIPK1 activation in ageing brains.

A non-synonymous variant in SHARPIN has been identified as a risk variant for late-onset AD. As SHARPIN-deficient cpdm mice develop TNF-dependent severe dermatitis and multi-organ inflammation that can be blocked upon inhibition of RIPK1 kinase, the reduction of SHARPIN activity might contribute to the risk of developing late-onset AD by reducing M1 ubiquitylation of RIPK1 to promote its activation.

**Parkinson disease and lysosomal storage disorders**

LRRK2, the most common monogenic cause of Parkinson disease (PD), which is also upregulated in sporadic disease, has been identified as a sensitizer to RIPK1-dependent apoptosis. Additionally, in vitro and in vivo models of these diseases suggest that RIPK1 can be activated by mitochondrial damage or lysosomal dysfunction. Induced pluripotent stem cell-derived dopaminergic neurons from patients harbouring OPA1 mutations that sensitize to mitochondrial fragmentation undergo necroptosis, which can be inhibited by RIPK1 inhibitors. Treatment with Nec-1 can also rescue in vivo loss of dopaminergic neurons in mice treated with MPTP, a rodent model of PD. As mitochondrial dysfunction is known to produce high levels of reactive oxygen species, it is possible that increased reactive oxygen species are directly responsible for RIPK1 activation in PD.

Consistent with RIPK1 activation downstream of lysosomal inhibition seen in AD models, lysosomal dysfunction caused by Niemann–Pick disease, type C1 and Gaucher’s disease also results in RIPK1-dependent activation of necroptosis.

**RIPK1 in sepsis and acute ischaemic insults**

**Sepsis**

Sepsis is a life-threatening condition caused by immune hyperreactivity to viral, bacterial and fungal infections. Sepsis is characterized by dysregulated production of pro-inflammatory cytokines (known as a cytokine storm), lymphopenia, coagulopathy, increased vascular permeability and eventual organ failure and death. Both genetic inhibition and pharmacological inhibition of RIPK1 in animal models have been shown to block TNF-induced sepsis. Kinase inhibition of RIPK1 leads to robust and highly reproducible protection against sepsis, including attenuation of hypothermia and complete rescue of lethality. These observations suggest that the kinase activity of RIPK1 is critical in propagating immune hyperreactivity following infection, which may happen in septic conditions associated with severe pathogen infection. The efficacy of RIPK1 inhibitors in sepsis is mechanistically attributable to the reduction of pro-inflammatory cytokines and circulating damage-associated molecular patterns, rescue of increased intestinal or vascular permeability and activation of the clotting cascade in the vascular endothelium compartment. Interestingly, the uncontrolled cytokine storm, which occurs in both patients and animal models, is thought to be driven by the interplay of inflammatory signalling and inflammatory cell death.

This deleterious cycle could be attenuated through RIPK1 inhibition, not only through a blockade of inflammatory response but also by inhibiting necrotic cell death; the RIPK1 inhibitor Nec-1 has been shown to protect against lung injury in a neonatal model of sepsis induced by bacteria-driven caecal slurry.

Patients with severe COVID-19 caused by the novel SARS-CoV-2 infection exhibit multiple hallmarks of sepsis with increased plasma pro-inflammatory cytokines, including IL-6, IFNγ, CCL2, IL-10, G-CSF and TNF, coagulation dysfunction and lymphopenia, which has been suggested to be a predictor for the severity of COVID-19. Activation of RIPK1 has been shown to promote the production of pro-inflammatory cytokines, including IL-6 and TNF, as seen in patients with non-cleavable RIPK1 mutations. RIPK1 has also been shown to promote the production of pro-inflammatory cytokines, including IL-6 and TNF, as seen in patients with non-cleavable RIPK1 mutations. RIPK1 kinase inhibition shows efficacy in middle cerebral artery occlusion models of stroke, with rapid activation of necroptosis in neurons and endothelial cells, which may happen in septic conditions associated with severe pathogen infection. The efficacy of RIPK1 inhibitors in sepsis is mechanistically attributable to the reduction of pro-inflammatory cytokines and circulating damage-associated molecular patterns, rescue of increased intestinal or vascular permeability and activation of the clotting cascade in the vascular endothelium compartment. Interestingly, the uncontrolled cytokine storm, which occurs in both patients and animal models, is thought to be driven by the interplay of inflammatory signalling and inflammatory cell death.

Widespread RIPK1 activation has been seen in ischaemia–reperfusion models of stroke, with rapid activation of necroptosis in neurons and endothelial cells, which may transition to apoptosis driven by the reduced TAK1 levels as the result of cerebral hypoperfusion. Although there are compelling in vivo data that RIPK1 inhibition is effective at rescuing pathological and behavioural attributes following acute cerebral insults, a RIPK1 inhibitor has yet to be advanced into clinical trials for these conditions, which is largely due to broad challenges seen in conducting clinical trials for acute cerebral insults. However, since a RIPK1 inhibitor may reduce cell death under both ischaemic and haemorrhagic stroke conditions, the ability to administer a RIPK1 inhibitor to stroke patients without differential diagnosis in an ambulance is an advantage that should be considered to reduce neuronal loss and preserve vascular function in the limited time window.
Other acute or ischaemic insults
Involvement of RIPK1 has also been suggested in mediating acute kidney injuries induced by cisplatin, gado- 
linium contrast, sepsis or ischaemia–reperfusion and hepatis induced by ethanol, LPS, acetaminophen or concanavalin A. Interestingly, a polymorphism in the TNF promoter at position 238 (known as the TNFA allele) leading to increased levels of TNF is associated with insulin resistance and is prevalent at higher rates in patients with nonalcoholic fatty liver disease. Similarly, patients with nonalcoholic steatohepatitis and mice fed high-fat diets also showed increased levels of TNF.

Clinical development of RIPK1 inhibitors
RIPK1 contains a unique hydrophobic pocket that allosterically regulates the activation of its kinase activity. All RIPK1 inhibitors described to date bind to this pocket. A phenotypic screen for small molecules that can block necroptosis led to the identification of the first specific inhibitors of RIPK1, 5-[(1H-indol-3-ylmethyl)-2-thiohydantoins and 5-[(1H-indol-3-ylmethyl) hydantoins known as Nec-1s. A subsequent family of benzoxazepinone RIPK1 inhibitors was published by GlaxoSmithKline (GSK), which included GSK 481 and the clinical candidate GSK 772, which was followed by the identification of a dihydroprazole chemotype of RIPK1 inhibitors including GSK 963 and GSK 547. Published and patented compounds generated by Takeda, Genentech and Rigel are all from the same benzoxazepinone family as the GSK 481 series.

Clinical trials
A timeline of clinical trials investigating RIPK1 inhibitors is summarized in Fig. 4. GSK 772 was the first RIPK1 kinase inhibitor to enter phase Ia clinical trials, and has subsequently progressed through phase Ia, Ib and II clinical trials. This peripherally restricted compound is currently being tested for peripheral autoimmune diseases. Following completion of the initial phase II clinical trials with GSK 772 in ulcerative colitis, RA and psoriasis, phase Ib trials in psoriasis were reinitiated at a much higher dose (960 mg versus 60 mg daily). GSK 772 has continued to show an excellent safety profile in a phase Ila clinical trial in mild to moderate plaque psoriasis, but additional data may be needed to demonstrate efficacy in this indication.

DNL104 (Denali Therapeutics) was the first brain-penetrant RIPK1 inhibitor advanced into a phase Ia clinical trial; although this programme was later discontinued owing to limited post-dosing liver toxicity deemed unrelated to RIPK1 inhibition, this study demonstrated the safety of inhibiting RIPK1 in the CNS. Denali Therapeutics subsequently initiated a phase Ia clinical trial for DNL747, another brain-penetrant RIPK1 inhibitor, followed by phase Ib/IIa trials of this compound in ALS and AD. As of June 2020, phase Ib/IIa clinical trials for DNL747 are ongoing for ALS; however, Denali announced that DNL788, a brain-penetrant back-up compound, will be entering phase Ia trials in early 2021. Due to the progressive, chronic nature of AD and that >95% of RIPK1 kinase inhibition may be required for this disease, DNL747 was deemed to have potential dose-limiting toxicities that were not seen preclinically with DNL788, although no toxicity has been seen in the clinic to date. Sanofi has partnered with Denali on RIPK1 inhibitors and completed a phase Ia clinical trial of the peripherally restricted DNL758 for peripheral autoimmune indications, including MS.
| Drug (company/institution)             | Chemotype            | Structure       | Indication and clinical trial ID                                                                 | Development phase | Refs                  |
|---------------------------------------|----------------------|-----------------|---------------------------------------------------------------------------------------------------|-------------------|-----------------------|
| GSK’481/GSK 772 (GSK)                 | Benzoxazepinone      | ![Structure Image](image1.png) | Ulcerative colitis NCT02903966 RA NCT02858492 Psoriasis NCT02776033 and NCT04316585          | Phase Ib/IIa      | [173,174,189]        |
| GSK’547/GSK 095 (GSK)                 | Dihydropyrazole      | ![Structure Image](image2.png) | Pancreatic ductal adenocarcinoma NCT03681951                                                    | Phase Ib/IIa      | [176]                 |
| DNL104 (Denali Therapeutics)          | Unpublished          | Unpublished     | NA                                                                                               | Phase Ia          | — terminated          |
| DNL747 (Denali Therapeutics)          | Unpublished          | Unpublished     | ALS NCT03757351 AD NCT03757325                                                                   | Phase Ib/IIa      | — terminated          |
| DNL758 (Denali Therapeutics)          | Unpublished          | Unpublished     | Peripheral autoimmune                                                                            | Phase Ia          |                       |
| R552 (Rigel)                          | Unpublished          | Unpublished     | Peripheral autoimmune                                                                            | Phase Ia          |                       |
| Necrostatin-1s (Harvard Medical School)| Indole-hydantoin     | ![Structure Image](image3.png) | The first RIPK1 inhibitor; used extensively in preclinical studies                                |                   | [11,14,26,222]       |
| Compound 22 (Takeda)                  | Benzoxazepinone      | ![Structure Image](image4.png) | NA                                                                                                | Preclinical       | [177]                 |
| GNE684 (Genentech)                    | Benzoxazepinone/ pyrido-oxazepinone | ![Structure Image](image5.png) | NA                                                                                                | Preclinical       | 29                    |
| GSK’963 (GSK)                         | Dihydropyrazole      | ![Structure Image](image6.png) | NA                                                                                                | Tool compound     | 175                   |
| Compound 8 (GSK)                      | Aminoisquinoline     | ![Structure Image](image7.png) | NA                                                                                                | Tool compound     | 222                   |
| RIPA-56 (National Institute of Biological Sciences Beijing) | N-benzykamide         | ![Structure Image](image8.png) | NA                                                                                                | Tool compound     | 223                   |
peripherally restricted based on the properties of this chemical family.\(^{188}\)

Given that RIPK1 kinase was suggested to promote macrophage-mediated adaptive immune tolerance in pancreatic cancer\(^{176}\) — a concept that was disputed later by another study\(^{30}\) — a combination trial of GSK’095, a clinical dihydropyrazole RIPK1 inhibitor candidate, and a checkpoint inhibitor was initiated for pancreatic ductal adenocarcinoma\(^{189}\). However, in late 2019 it was decided not to develop this asset further. To date, this remains the only RIPK1 combination therapy that has been advanced through investigational new drug (IND)-enabling studies into the clinic. Other combination therapies with RIPK1 inhibitors could be conceptualized based on our understanding of RIPK1 biology, on a disease-specific basis.

In summary, as of June 2020, RIPK1 inhibitors have entered human clinical trials for six indications: ALS and

| Drug (company/institution) | Chemotype | Structure | Indication and clinical trial ID | Development phase | Refs |
|---------------------------|-----------|-----------|---------------------------------|-------------------|------|
| 6E11 (Sorbonne Universités) | Flavenone | ![Flavenone structure](image1) | NA | Tool compound | 224 |
| PK68 (Soochow University) | Benzothioazole | ![Benzothioazole structure](image2) | NA | Tool compound | 225 |
| PN10 (Tufts University) | Indole-hydantoin | ![Indole-hydantoin structure](image3) | NA | Tool compound | 226 |
| Necrostatin-4 (Harvard Medical School) | Pyrrolonitrile | ![Pyrrolonitrile structure](image4) | NA | Tool compound | 9,272 |
| Necrostatin-5 (Shanghai Institute of Organic Chemistry) | Tricyclic hexahydro-benzothieno pyrimidines | ![Tricyclic hexahydro-benzothieno pyrimidines structure](image5) | NA | Tool compound | 227 |
| Necrostatin-7 (Shanghai Institute of Organic Chemistry) | Imino-N-thiazolylthiazolidinones | ![Imino-N-thiazolylthiazolidinones structure](image6) | NA | Tool compound | 228 |
| Necrostatin-21 (Shanghai Institute of Organic Chemistry) | Tetrahydro pyrazolocarbazoles | ![Tetrahydro pyrazolocarbazoles structure](image7) | NA | Tool compound | 229 |

AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; GSK, GlaxoSmithKline; NA, not applicable; RA, rheumatoid arthritis; RIPK1, receptor-interacting serine/threonine-protein kinase 1.
AD in neurodegeneration; psoriasis, ulcerative colitis and RA in autoimmune disease; and pancreatic ductal adenocarcinoma in oncology.

**Clinical biomarkers of RIPK1 activation**

Phosphorylation of S166 RIPK1 has been established as a biomarker of RIPK1 target engagement. In phase Ia clinical trials of DNL104, ex vivo assay-based phosphorylation of S166 RIPK1 in human PBMCs was used to assess the inhibition of RIPK1 kinase activity, suggesting clinical feasibility to determine the activation of RIPK1 in blood samples for target engagement studies. In the phase Ia study of GSK’772, target engagement was also measured in PBMCs ex vivo using a novel immunoassay based on a conformational change that occurred following inhibitor binding to RIPK1.

Production of several pro-inflammatory cytokines and chemokines regulated by RIPK1 kinase activity can also be used as biomarkers of RIPK1 activation. Of these rapidly inducible, secreted factors, GSK selected the chemokines macrophage inflammatory protein 1α (MIP1α; also known as CCL3) and MIP1β (also known as CCL4) as pharmacodynamic biomarkers downstream of RIPK1 activation. GSK’772 dose-dependently reduced MIP-1α and MIP1β production, which corresponded to their target engagement data. Denali presented data indicating that IL-1β, IL-6 and MCP1 (also known as CCL2) were also RIPK1 responsive, demonstrating that DNL747 dose-dependently reduced IL-1β in primary human cells.

Taken together, these studies demonstrate that RIPK1 preclinical biology has provided clinically validated biomarkers of RIPK1 activation in blood samples, which may also provide value as inclusion criteria to identify patients who will most benefit from RIPK1 inhibitor therapy.

**Outlook**

LoF or GoF mutations in the gene encoding RIPK1 itself, as well as in those encoding multiple regulators of the NF-κB pathway and RIPK1 activation, such as TAK1, NEMO, A20, ABIN1, OTULIN and the LUBAC complex, have been identified in human inflammatory and ageing-related diseases. In addition to these monogenic diseases, dysregulation of RIPK1, such as that which occurs on down-regulation or up-regulation of A20, may also be involved in mediating polygenic diseases involving RIPK1. The activation of RIPK1-mediated neuroinflammation may provide a common basis for the role of RIPK1 in human neurodegenerative diseases. Although a specific set of genetic associations between RIPK1 activation and neurodegenerative diseases has been discovered, the presence of activated RIPK1, RIPK3 and MLKL in post-mortem human pathological samples seen in MS, ALS and AD demonstrates the direct relevance of this pathway even in sporadic cases. Notably, the role of A20 and cFLIP, which are transcriptional targets of NF-κB, in suppressing the activation of RIPK1 suggests that defects in activation of the NF-κB pathway, in genetic and sporadic settings, may promote the activation of RIPK1 from dysregulated A20-mediated RIPK1 ubiquitylation modification as well as inactivation of Casp8 due to dysregulated cFLIP expression.

Phosphorylation of RIPK1 S166, as well as downstream events (such as pRIPK3 and pMLKL) and the increased production of specific pro-inflammatory cytokines (such as CCL2, MIP1α/CCL3, MIP1β/CCL4, IL-6 and TNF), can be used as biomarkers of RIPK1 activation. However, a brain-penetrant positron-emission tomography imaging probe for RIPK1 kinase activity would be particularly helpful in the development of RIPK1 inhibitors for the treatment of neurodegenerative diseases. As pathways downstream of RIPK1, including cell death, pro-inflammatory cytokines and NF-κB activation, can be modulated directly by disease pathogenesis, it is important to include biomarkers that specifically measure RIPK1 activation when considering a clinical study. It is also important to remember the cell type specificity of RIPK1 activation and the genetic context of the diseases, as sustained activation of RIPK1...
in specific cell types, such as fibroblasts, can only be achieved in certain settings.

Clinical trials of RIPK1 inhibitors in patients are still in their early days, and, to date, limited chemotypes of RIPK1 inhibitors with appropriate in vivo properties have been developed. Development of selective, potent and safe small-molecule inhibitors of RIPK1, biomarkers to reliably measure the clinical efficacy of RIPK1 kinase inhibition and patient stratification remain the key challenges facing future clinical development.

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Incontinentia pigmenti is an autosomal-dominant disorder characterized by localized patches of skin hyperpigmentation and non-specific developmental abnormalities. The condition is caused by mutations in the NEMO gene, which encodes the inhibitor of NF-κB (NEMO). These mutations lead to the activation of NF-κB and subsequent inflammation in various tissues and organs, including the skin, muscles, and nerves. The activation of NF-κB is mediated by the NF-κB signaling pathway, which is critical for the regulation of gene expression, inflammation, and cell survival. Mutations in the NEMO gene result in the loss of NF-κB inactivation and, consequently, in the activation of NF-κB and downstream pro-inflammatory pathways.

In the context of incontinentia pigmenti, mutations in the NEMO gene lead to the activation of NF-κB in various tissues, including the skin, muscles, and nerves. This activation results in the expression of pro-inflammatory genes and the induction of inflammation in these tissues. The inflammation can be characterized by the presence of granulomas, which are collections of cells that form in response to inflammation. These granulomas are composed of macrophages, lymphocytes, and other immune cells, and they can be found in various tissues and organs.

The loss of NF-κB inactivation in incontinentia pigmenti results in the activation of NF-κB and downstream pro-inflammatory pathways. This activation leads to the expression of pro-inflammatory genes and the induction of inflammation in various tissues and organs. The inflammation can be characterized by the presence of granulomas, which are collections of cells that form in response to inflammation. These granulomas are composed of macrophages, lymphocytes, and other immune cells, and they can be found in various tissues and organs.

In summary, the activation of NF-κB in incontinentia pigmenti results in the expression of pro-inflammatory genes and the induction of inflammation in various tissues and organs. The inflammation can be characterized by the presence of granulomas, which are collections of cells that form in response to inflammation. These granulomas are composed of macrophages, lymphocytes, and other immune cells, and they can be found in various tissues and organs.