Death by TNF: a road to inflammation

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Abstract

Tumour necrosis factor (TNF) is a central cytokine in inflammatory reactions, and biologics that neutralize TNF are among the most successful drugs for the treatment of chronic inflammatory and autoimmune pathologies. In recent years, it became clear that TNF drives inflammatory responses not only directly by inducing inflammatory gene expression but also indirectly by inducing cell death, instigating inflammatory immune reactions and disease development. Hence, inhibitors of cell death are being considered as a new therapy for TNF-dependent inflammatory diseases.

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Introduction

Cell death is increasingly recognized as a major driver of inflammatory disease. Compared with apoptosis, which is generally considered to be immunologically silent, lytic forms of cell death, such as necroptosis, pyroptosis and apoptosis-driven secondary necrosis, release intracellular factors, known as damage-associated molecular patterns, that activate immune receptors and induce inflammatory responses. In addition, the inflammatory signalling cascade may originate from and/or be amplified by loss of barrier function caused by epithelial cell death and the subsequent sensing of pathogen-associated molecular patterns from microorganisms that have breached the epithelial barrier. Therefore, cell demise, in its multiple modalities, acts as an initiator or amplifier of inflammation. Death is, however, not the default response in cells, and is usually suppressed unless certain cell death checkpoints are overridden. On the one hand, cell death-driven inflammation serves as a backup mechanism in microbial infection to ensure optimal antimicrobial responses when inflammatory gene activation has been hijacked by the pathogen. On the other hand, environmental factors and/or genetic predispositions can alter the tight regulation of the cell death processes, leading to unwanted or exacerbated inflammatory responses that may underlie various inflammatory diseases. Accumulating evidence suggests that blocking cell death can reverse the inflammatory pathology state in various mouse models of acute and chronic inflammatory diseases (reviewed in 1). Improving our understanding of the interplay between the various cell death modalities, their mode of execution, the molecular checkpoints that control them, and the physiological and pathological conditions that turn them off is therefore needed to identify new therapeutic targets. Moreover, such knowledge will help to define the disorders in which pharmacological cell death inhibitors may provide therapeutic advantage.

The inflammatory cytokine tumour necrosis factor (TNF) is central in orchestrating the inflammatory immune response. Hence, TNF-neutralizing therapies have been highly successful for the treatment of chronic inflammatory and autoimmune pathologies (Box 1). This Review briefly recalls the history and discovery of TNF as a target for therapy, and then focuses on more recent findings demonstrating that TNF indirectly promotes inflammation by inducing cell death. Consequently, direct inhibition of cell death is now being considered as a new therapeutic strategy for the treatment of TNF-mediated diseases, especially to treat patients who are non-responders or show adverse effects to anti-TNF treatment.

A short history of TNF

TNF was identified as a serum factor that could induce the haemorrhagic necrosis of tumours in patients following acute bacterial infections (Fig. 1). This anticancer activity had already been exploited nearly a century before by the New York surgeon William Coley, who described the treatment of patients with cancer with bacterial extracts termed ‘Coley’s mixed toxins’ 1,2. Later, lipopolysaccharide (LPS) was isolated from bacterial extracts and shown to induce some tumour regression in experimental cancer studies in mice 3. Carswell et al. later demonstrated that it was in fact not the LPS itself that killed the cancer cells but a necrotizing factor produced by the host macrophages in response to LPS. Hence, the necrotizing factor was named ‘tumour necrosis factor’ or ‘TNF’ (Fig. 1). In the years after, the genes encoding the human and mouse TNF and TNF receptors were purified, sequenced and cloned 4,5, and experimental studies with recombinant TNF were initiated to validate its antitumour potential for cancer treatment (reviewed in 6). However, the hope that TNF would be a powerful anticancer drug soon faded when it became clear that administration of the recombinant cytokine induces severe endotoxic shock. Indeed, and independently of these cancer studies, TNF was found to be identical to a previously identified protein named ‘cachectin’, which was responsible for endotoxin-induced wasting disease (cachexia) in mice 7. These findings also clearly demonstrated that TNF is a pleiotropic cytokine that must be tightly regulated.

Induction of cell death by TNF

The clinical success of anti-TNF biologics in treating inflammatory disorders has been attributed to their effectiveness in blocking TNF from binding to its cognate receptors TNF receptor 1 (TNFR1) and TNFR2. It was long thought that this blockade reduces inflammation
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by preventing TNFR1 from activating the mitogen-activated protein kinase (MAPK) pathway and the canonical nuclear factor-κB (NF-κB) pathway, which would otherwise collectively lead to the transcriptional upregulation of proinflammatory genes that underlie the inflammatory pathology (Fig. 2). While this initial belief is probably true, it is now clear that binding of TNF to TNFR1 also indirectly promotes and exacerbates inflammation by inducing cell death, in the form of apoptosis, necroptosis or pyroptosis. Indeed, dying cells release intracellular constituents that induce proinflammatory gene expression in neighbouring cells. In addition, epithelial cell death (in the skin or the intestine) may affect barrier integrity, inducing microbial tissue infiltration and inflammation (Fig. 2). Hence, genetic targeting of cell death was shown to reverse the inflammatory phenotype in various mouse models of TNF-induced inflammatory diseases (see later). Consequently, drugs that inhibit cell death, such as inhibitors of receptor-interacting serine/threonine protein kinase 1 (RIPK1), are currently under investigation as alternative therapies for TNF-driven human diseases (reviewed in 18,19).

Death is not the default response of cells to TNF. Protective brakes, or cell death checkpoints, normally actively repress TNF cytotoxicity to protect the organism from its potential detrimental consequences. Thus, while TNFR1 has the ability to trigger cell death, this response proceeds only when one of the cell death checkpoints is inactivated (Fig. 3). The survival versus death outcome of TNFR1 activation depends on the assembly of two distinct, but successive, protein complexes (Fig. 3) (reviewed in 18,19). The membrane-bound complex I forms within seconds of TNF sensing, and predominantly leads to inflammatory gene activation. Assembly of complex I starts with the binding of RIPK1 and TNFR1-associated death domain protein (TRADD) to the cytosolic portion of the receptor, allowing the subsequent recruitment of TNFR-associated factor 2 (TRAF2) and of the ubiquitin ligases cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2 and the linear ubiquitin chain assembly complex (LUBAC; which is composed of HOIL1, HOIP and SHARPIN). Together, these E3 ligases generate a dense network of ubiquitin chains that permits further recruitment of the kinases that activate the MAPK signalling pathway and the canonical NF-κB signalling pathway. More precisely, the K63-linked ubiquitin chains generated by cIAP1 and cIAP2 act as binding stations for the adaptor proteins TGFβ-activated kinase (TAK1) and TRADD, which recruit the upstream kinase TGFβ-activated kinase 1 (TAK1) for MAPK signalling. In addition, the MI-linked (linear) ubiquitin chains generated by LUBAC are recognized by the adaptor protein NF-κB essential modulator (NEMO), which brings the kinases inhibitor of NF-κB kinase-α (IKKα), IKKβ, TANK-binding kinase 1 (TBK1) and IKKe to the receptor complex. The close proximity of TAK1 and IKKα and IKKβ on the hybrid K63/MI-linked ubiquitin chains then permits activation of IKKα–IKKβ by TAK1, and the subsequent IKKα–IKKβ-dependent activation of the canonical NF-κB pathway (reviewed in 18,19) (Fig. 3). The ubiquitin network associated with complex I is negatively regulated by a subset of deubiquitylases, including A20, CYLD and OTULIN, which destabilize the signalling complex and restrict signalling to MAPKs and NF-κB (reviewed in 18,19).

How TNFR1 signalling further evolves to induce cell death is less clear, but it requires the assembly of a secondary cytosolic complex, termed complex II, which originates from the binding of FAS-associated death domain-containing protein (FADD) to the receptor-disassociated complex I components TRADD and/or RIPK1 (ref. 22). Complex II functions as a cytosolic platform for the binding and activation of caspase 8, which can process downstream effector caspases to induce apoptosis, or instead cleave gasdermin D (GSDMD) to induce pyroptosis, as recently reported 23–24. Complex II can further be defined as complex IIa or complex IIb to differentiate the complex that spontaneously assembles upon TNF sensing from the one that additionally forms upon RIPK1 enzymatic activation 25,26 (Fig. 3). So far, two cell death checkpoints have been found to inhibit apoptosis induction by these death complexes. The first one (‘IKK checkpoint’) occurs at the level of the receptor, within
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complex I, and consists of the phosphorylation-dependent inactivation of RIPK1 by IKKα–IKKβ and TBK1–IKKε, thereby preventing complex IIb assembly and subsequent RIPK1 kinase activity-dependent apoptosis induction27–29 (Fig. 3). The fact that single inhibition of IKKα–IKKβ or TBK1–IKKε complexes suffices to switch the TNF response from survival to RIPK1 kinase activity-dependent apoptosis and the observation that the combined inactivation of these kinases further increases RIPK1 cytotoxicity suggest that IKKα–IKKβ and TBK1–IKKε inhibit distinct pools of RIPK1 in TNFR1 complex I. S25 of RIPK1 was identified as a substrate of IKKα–IKKβ, TBK1–IKKε and protein phosphatase 1 regulatory subunit 3G (PPP1R3G)27,28,30, and mimicking phosphorylation on that residue was demonstrated to inhibit RIPK1 activity and cytotoxicity31. However, preventing S25 phosphorylation of RIPK1 is not sufficient to activate RIPK1 by TNF, indicating that IKKα–IKKβ and TBK1–IKKε additionally regulate RIPK1 cytotoxicity independently of S25, possibly by phosphorylating RIPK1 on other residues or, alternatively, by phosphorylating other targets. The second cell death checkpoint (‘NF-xB checkpoint’) occurs downstream in the pathway, in the nucleus, and relies on the NF-xB-dependent transcriptional and translational upregulation of prosurvival proteins, such as FLICE-like inhibitory protein (FLIP; also known as CFLAR), which counteract caspase 8 activation in complex IIa and protect the cells from RIPK1 kinase activity-independent apoptosis25,32. Since IKKα and IKKβ are upstream kinases in the canonical NF-xB pathway, they control two successive checkpoints downstream of TNFR1, which respectively protect the cells from RIPK1 kinase activity-dependent apoptosis (IKK checkpoint) and RIPK1 kinase activity-independent apoptosis (NF-xB checkpoint) (Fig. 3). By contrast, TBK1 and IKKε repress RIPK1 activation only in complex I, and their inactivation consequently only switches the TNF response from survival to RIPK1 kinase activity-dependent cell death, without disturbing NF-xB28.

Activation of the kinases that control the two aforementioned cell death checkpoints is highly dependent on the ubiquitin network associated with complex I. Consequently, conditions that affect ubiquitination of complex I, such as inhibition of cIAP1, cIAP2 and LUBAC33–40, and also mutations in the RIPK1 ubiquitin acceptor site K377 (K376 in mouse RIPK1)41–43 or deficiencies of A20 and OTULIN44–49, indirectly perturb these cell death checkpoints and activate TNF cytotoxicity. Of note, the inhibitory effect of ubiquitin on RIPK1 death signalling...
Fig. 3 | Signalling by TNFR1 and overview of the three characterized cell death checkpoints in the TNFR1 pathway. a, Sensing of tumour necrosis factor (TNF) by TNF receptor 1 (TNFR1) leads to the formation of a primary membrane-bound receptor signalling complex (complex I) that activates the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signalling pathways, leading to proinflammatory gene expression. A secondary, potentially cytotoxic, cytosolic complex (complex II) originates from the dissociation of complex I components from the receptor, and from their association with FAS-associated death domain-containing protein (FADD) and caspase 8. Three cell death checkpoints actively repress TNF cytotoxicity. First, the inhibitor of nuclear factor-κB kinase (IKK) checkpoint consists of the inhibition of receptor-interacting serine/threonine protein kinase 1 (RIPK1) kinase activity through phosphorylation mediated by complexes of IKKα and IKKβ and TANK-binding kinase 1 (TBK1) and IKKε. Second, the NF-κB checkpoint, which consists of the NF-κB-dependent transcriptional upregulation of prosurvival genes (including the gene encoding FLICE-like inhibitory protein (FLIP)). Third, the caspase 8 checkpoint, which consists of RIPK1 inactivation by caspase 8-mediated cleavage. b, Inhibition of the IKK checkpoint leads to activation of RIPK1 in complex I, and the subsequent kinase-dependent assembly of complex IIb, which drives apoptosis or pyroptosis depending on the cellular context. Of note, conditions that affect proper IKKα–IKKβ activation will additionally inactivate the NF-κB checkpoint. c, Conditions leading to inactivation of the NF-κB checkpoint, such as the in vitro use of the translation inhibitor cycloheximide, activate complex IIa and result in RIPK1 kinase activity-independent apoptosis. d, Inhibition of the caspase 8 checkpoint induces RIPK1 cytotoxicity by the kinase-dependent assembly of complex IIb and the necrosome. TNF sensing in caspase 8–inhibited conditions will result only in necroptosis induction. Additional checkpoints may exist. cIAP, cellular inhibitor of apoptosis protein; GSDMD, gasdermin D; LUBAC, linear ubiquitin chain assembly complex; NAPI, NAK-associated protein 1; NEMO, NF-κB essential modulator; TAB, TGFβ-activated kinase 1-binding protein; TAK1, TGFβ-activated kinase 1; TANK, TRAF family member-associated NF-κB activator; TRADD, TNFR1-associated death domain protein; TRAF2, TNFR-associated factor 2; Ub, ubiquitin.
can be dissociated from its role in inducing NF-κB-mediated gene transcription71–74. Interestingly, although the two described cell death checkpoints are in place to restrain caspase 8 processing, a non-lethal pool of caspase 8 is still activated by TNF sensing, and functions as a third checkpoint in the pathway (the ‘caspase 8 checkpoint’), which prevents RIPK1 kinase activity-dependent apoptosis and necroptosis by cleaving RIPK1 (refs.50–54) (Fig. 3). What restrains this pool of activated caspase 8 from inducing cell death is currently unclear, but suggests the existence of additional protective mechanisms. Accordingly, inactivation of caspase 8 switches the TNF response to RIPK1 kinase activity-dependent necroptosis, which additionally requires recruitment of the kinase RIPK3 and of the potential pore-forming pseudo-kinase mixed lineage kinase domain-like protein (MLKL) to complex II, now called the ‘necosome’. Association between RIPK3 and RIPK1 occurs via their RIP homotypic interaction motifs, and appears to be sufficient to activate RIP3 within the necosome. The phosphorylation of MLKL by RIPK3 then induces a conformational change in MLKL resulting in its oligomerization and translocation from the cytosol to the plasma membrane, where it induces cell death via unknown mechanisms. The enzymatic activity of RIPK1 is dispensable for complex I and complex IIa assembly, but is required for the formation of complex IIb and the necosome. Depending on the cellular context, the catalytic activity of RIPK1 can therefore promote apoptosis, caspase 8-mediated pyroptosis or necroptosis downstream of TNFR1 (refs.55–58) (Fig. 3).

All three cell death checkpoints described so far were shown to be essential to prevent TNF-dependent embryonic lethality or severe inflammatory pathology in mice. Moreover, mutations that affect these checkpoints have been identified as the cause of autoinflammatory diseases in humans, further providing clinical relevance of these cell death checkpoints (see later). Of note, additional molecular mechanisms restraining TNF cytotoxicity have been reported, including the phosphorylation by RIPK1 of the MAPK-activated kinase MK2 (refs.59–61) or by the kinase Unc-51-like autophagy activating kinase 1 (ULK1)62, as well as the poly(ADP-ribosylation) of complex IIb by tankyrase 1 (ref.63). In contrast to the three cell death checkpoints described above, inactivation of these additional protective mechanisms does not switch the TNFR1 response from survival to death. It increases TNF cytotoxicity only in conditions of a previously compromised checkpoint, indicating that they do not regulate the most critical brake in the pathway but rather control additional layers of regulation, limiting the extent of cell death.

**TNF-induced cell death in pathogen defence**

Host–pathogen interactions are a major selective pressure acting on both organisms. While the host must adapt to survive infection by pathogens, pathogens must in turn develop mechanisms to avoid elimination by the host’s immune defences. This continuous pressure selects for multiple, layered and interconnected defence mechanisms in the host. Similarly, the pathogen has developed sophisticated strategies to evade host immunity by hijacking inflammatory signalling pathways or by blocking other antimicrobial defence mechanisms. The different TNFR1 cell death checkpoints appear to have evolved as a response of the host to this microbial hijacking. Indeed, TNF signalling aims to establish an inflammatory response, primarily by promoting inflammatory gene activation by the MAPK and NF-κB signalling pathways. Remarkably, the kinases that activate these signalling pathways are also the ones putting a break on TNF cytotoxicity. Consequently, when the pathogen tries to suppress inflammatory gene activation in the host by delivering virulence or effector factors that affect proper activation of these kinases, the cell will switch its response to induce cell death, thereby activating an alternative pathway to alert the immune system though the release of damage-associated molecular patterns. This is nicely illustrated in the context of infection by the mammalian pathogenic species of the Gram-negative genus *Yersinia*, which injects an acetyltransferase, named ‘Yop/P’, capable of inhibiting the catalytic activity of TAK1, IKKα and IKKβ in an attempt to escape host defences by preventing MAPK- and NF-κB-dependent expression of proinflammatory mediators64–66. As a consequence of this hijacking, RIPK1 is no longer blocked by MK2 and IKKα–IKKβ phosphorylation, and TNFR1-mediated and Toll-like receptor 4-mediated RIPK1 kinase activity-dependent and caspase 8-dependent apoptosis and/or pyroptosis are induced, releasing damage-associated molecular patterns to promote optimal antibacterial immunity.

Cell death, in its multiple forms, is thus recognized as a host defence mechanism for the elimination of pathogens, stripping them of their replication niche and simultaneously alerting the immune system to kick in. As a consequence, microorganisms have developed multiple strategies to interfere with the different cell death pathways to avoid their eradication by the host (reviewed in67). However, host cells have, in turn, developed strategies to also cope with this by activating backup mechanisms. In this context, the TNFR1 cell death checkpoint that controls RIPK1 cleavage by caspase 8 appears to have evolved to ensure activation of necroptosis as a backup cell death mode when the apoptotic pathway is blocked by pathogenic caspase 8 inhibitors, such as the poxvirus-encoded serpin CrmA68 or the viral FLICE-inhibitory protein (vFLIP) identified in herpesviruses and in the human poxvirus *Molluscum contagiosum virus*69. As a response, several pathogens also express proteins that specifically target necroptosis by inhibiting RIPK1, RIPK3, MLKL or the effects downstream of MLKL70.

Cell death by TNF is, however, not always a beneficial response for the host. At least in some specific context, excessive activation of TNF-mediated cell death is indeed reported to drive, rather than prevent, microorganism pathogenicity and lethality, as seen upon infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), *Mycobacterium tuberculosis* and *Bacillus anthracis*71–74.

**TNF-induced cell death in inflammatory disease**

Although TNF-induced cell death can help to mount proper immune responses during microbial infection, it can also turn into a highly detrimental process at the origin of various (sterile) inflammatory diseases when aberrantly induced as a result of environmental factors and/or genetic mutations. It is now clear that TNF contributes to the pathogenesis of inflammatory disease not only by inducing expression of inflammatory mediators but also by triggering cell death. For instance, TNF induces a lethal septic shock in mice that is caused by RIPK1 kinase activity-dependent cell death, as genetic or pharmacological inhibition of RIPK1 enzymatic activity-dependent apoptosis and/or pyroptosis are induced, releasing damage-associated molecular patterns to promote optimal antibacterial immunity. In this model, the triggering event was first believed to be necroptosis, but later studies suggested additional contribution of RIPK1 kinase activity-dependent apoptosis and pyroptosis. Indeed, caspase 8 heterozygosity was reported to partially rescue hypothermia, and GSDMD loss was reported to limit lethality75–77. The reason why TNF induces RIPK1 kinase activity-dependent cell death in vivo while most cells do not succumb to single TNF stimulation in vitro is not fully understood, but indicates that the in vivo inflammatory context somehow affects RIPK1 cell death checkpoints. It is tempting to speculate that the cytotoxicity originates from the co-sensing of multiple cytokines, which are provided by the inflammatory context. Indeed, a subclass
of TNF family ligands, which includes CD40, TNF-like weak inducer of apoptosis (TWEAK) and lymphoxygen-β, activates the non-canonical NF-kB pathway upon binding of the ligands to their cognate receptors. Activation of this pathway involves the ligand-dependent degradation of a pool of TRAF2–TRAF3 and cIAP1–cIAP2, resulting in stabilization of NF-kB-inducing kinase (NIK), and finally in the activation of IKKα by NIK-mediated phosphorylation. While single stimulation of cells with TNF or one of these additional ligands is mostly not toxic to cells, their combination may instead result in TNFR1-induced RIPK1 kinase activity-dependent and RIPK1 kinase activity-independent apoptosis or pyroptosis due to partial cIAP1 and cIAP2 degradation, affecting proper ubiquitylation of complex I, which consequently indirectly inactivates two of the aforementioned cell death checkpoints in the TNFR1 pathway. In line with this idea, it is interesting to note that TWEAK and CD40L are upregulated in inflammatory bowel disease (IBD) and rheumatoid arthritis, two TNF-driven human diseases for which RIPK1 inhibitors may be promising.

Of note, binding of TNF to TNFR2 also activates the non-canonical NF-kB pathway. Consequently, co-sensing of TNF by TNFR1 and TNFR2 also has the potential to switch the TNFR1 response from survival to RIPK1 kinase activity-dependent death. It is therefore possible that part of the discrepancy between the in vitro cytotoxic response and the in vivo cytotoxic response to TNF originates from difference in TNFR2 expression levels, or in the expression of membrane-bound TNF versus soluble TNF, as the latter is relatively poor at activating TNFR2. In addition to ligands of the TNF family, the co-sensing of TNF and interferon-γ was also recently reported to induce RIPK1 kinase activity-dependent cell death (apoptosis, pyroptosis and necroptosis) by activating the JAK–STAT1–IRF1 axis. However, it remains unclear whether, and how, activation of this pathway affects the known cell death checkpoints downstream of TNFR1. Interestingly, the combination of neutralizing antibodies to TNF and interferon-γ was shown to protect mice from death during SARS-CoV-2 infection, which may support the reported causative role of pyroptosis in hyperinflammation during severe COVID-19.

Mutations that either directly or indirectly inactivate some of the cell death checkpoints within the TNFR1 pathway are also sufficient to cause mouse and human inflammatory diseases, as highlighted by some examples in the non-exhaustive list of studies mentioned below (Tables 1 and 2). The in vivo inflammatory consequence of inactivating the caspase 8 checkpoint that prevents RIPK1-dependent cytotoxicity was initially revealed by genetic studies in mice that lack caspase 8 or FADD. Genetic full-body deletion of Casp8 or Fadd in mice results in embryonic lethality, which can be rescued by birth deletion of Ripk1 and to adulthood by deletion of Ripk3 or Mlkl. Also, specific deletion of Casp8 or Fadd in the intestinal epithelium leads to the development of a severe intestinal pathology that is TNF dependent and rescued by Ripk3 or Mlkl deficiency or by inhibition of RIPK1 kinase activity, providing evidence that intestinal inflammation results from necroptosis of FADD- or caspase 8-deficient intestinal epithelial cells (IECs). High levels of RIPK3 and increased necroptosis could be confirmed in the intestine of patients with Crohn’s disease, and mutations in Casp8 have been identified in patients who develop autoimmune lymphoproliferative syndrome and also in patients with severe forms of very early onset IBD. In IBD, aberrant cell death leads to impairment of the epithelial barrier and invasion of the underlying tissues by the microbiota, promoting inflammation. Deficiency of the adaptor protein myeloid differentiation primary response protein 88 (MyD88) and antibiotic treatment were shown to prevent colon inflammation in IEC-specific FADD-deficient mice, demonstrating that bacterially mediated Toll-like receptor activation by intestinal bacteria is essential for disease pathogenesis. Follow-up studies in mice revealed that FADD prevents intestinal inflammation not only by inhibiting necroptosis but also by inhibiting caspase 8–GSDMD-mediated pyroptosis of epithelial cells. How FADD simultaneously promotes and inhibits caspase 8 to respectively inhibit necroptosis but promote pyroptosis is currently unclear. Inducible deletion of Casp8 in the endothelium of 6-week-old mice causes fetal haemorrhagic lesions exclusively within the small intestine driven by microbial commensals and TNF. This phenotype is prevented in mice that lack MLKL, confirming that the haemorrhage is caused by unregulated necroptosis in the small intestine. Deficiency of FADD or caspase 8 in keratinocytes causes keratinocyte necroptosis and severe skin inflammation in mice, which is prevented by Ripk3 loss and is partly dependent on TNF. Since Ripk3 also contributes to TNF-induced caspase 8 activation, additional studies in MLKL-deficient mice will be required to formally demonstrate that keratinocyte necroptosis drives the inflammatory skin phenotype in these mice. Keratinocyte-specific Ripk1 deficiency also causes keratinocyte necroptosis and skin inflammation, which is only partially rescued in a TNFR1-deficient background, but is completely prevented by Ripk3 or Mlkl deficiency. As genetic targeting of RIPK1 kinase activity does not lead to any overt phenotype, these results identify RIPK1 scaffold function as an inhibitor of RIPK3–MLKL-dependent necroptosis in keratinocytes.

More recent studies specifically targeted the caspase 8 checkpoint by the generation of mice expressing a caspase 8 cleavage-resistant variant of RIPK1 (Ripk1 D325A). The mutation induces embryonic lethality in mice, which is prevented by loss of Ripk1 kinase activity, loss of TNFR1 or loss of both MLK (or Ripk3) and FADD (or caspase 8), but not by loss of MLK or Ripk3 alone, confirming combined induction of apoptosis and necroptosis. Importantly, patients with pathogenic mutations in Ripk1 that prevent caspase 8 cleavage were also identified, and were shown to experience early-onset autoinflammatory disease, so-called cleavage-resistant RIPK1-induced autoinflammatory syndrome, which is caused by hypersensitivity of patients’ cells to RIPK1 activation, apoptosis and necroptosis.

By serving as anchoring sites for the kinases IKKα, IKKβ, IKKε and Tbk1, the ubiquitin chains conjugated to TNFR1 complex I by cIAP1, cIAP2 and LUBAC indirectly control the two checkpoints that counteract caspase 8-dependent cell death, either in an RIPK1 kinase activity-dependent manner or in an RIPK1 kinase activity-independent manner (Fig. 3). Consequently, mutations that affect proper regulation of ubiquitin signalling can trigger aberrant TNF-mediated cell death and result in inflammatory disorders. Furthermore, studies demonstrated that deletion of cIAP1 and cIAP2 in adult mice causes inflammation and lethality by the release of a brake on caspase 8-dependent cell death. Deficiency in ubiquitin ligase X-linked inhibitor of apoptosis protein (XIAP) is the cause of X-linked lymphoproliferative syndrome 2, a severe inflammatory disease. With use of gene-targeted mice, XIAP was shown to prevent TNF- and Ripk3-dependent cell death by regulating ubiquitylation of Ripk3, which might explain the hyperinflammation in patients with X-linked lymphoproliferative syndrome 2. The notion that linear ubiquitin chains protect against cell death-driven inflammation is supported by the phenotypes of Lubac- and Otulinn-deficient mice. Mutation in Sharpin, in so-called Cpdm mice, causes chronic proliferative dermatitis characterized by inflammatory...
Table 1 | A selection of studies in mouse models demonstrating that inflammation results from unrestrained cell death

| Genotype | Phenotype | Expected inactivated CDC | Rescue background | Refs. |
|----------|-----------|--------------------------|------------------|-------|
| Casp8<sup>−/−</sup> | Embryonic lethality | Caspase 8 checkpoint | Ripk3<sup>+/+</sup>, Mlkl<sup>−/−</sup> | 28,35,37 |
| Casp8<sup>IEC-KO</sup> | Severe intestinal pathology; enterocyte necroptosis | | Ripk3<sup>+/+</sup>, Mlkl<sup>−/−</sup>, Tnf<sup>−/−</sup> (colon) | 24,31,34 |
| Cdh5<sup>CreER</sup> Casp8<sup>IEC-KO</sup> | Fatal necroptotic haemorrhage in the small intestine | | Mlkl<sup>−/−</sup>, LPS-Rs administration | 87 |
| Casp8<sup>−/−</sup> | Severe skin inflammation; keratinocyte necroptosis | | Ripk3<sup>−/−</sup> (partial rescue) | 34,38 |
| Fadd<sup>−/−</sup> | Embryonic lethality | Caspase 8 checkpoint | Ripk1<sup>−/−</sup>, Mlkl<sup>−/−</sup> | 30,31 |
| Fadd<sup>IEC-KO</sup> | Severe intestinal pathology; enterocyte necroptosis and pyroptosis | | Ripk3<sup>−/−</sup>, Mlkl<sup>−/−</sup> (colon) | 24,33 |
| Fadd<sup>−/−</sup> | Severe skin inflammation; keratinocyte necroptosis | | Ripk3<sup>−/−</sup> (partial rescue) | 99 |
| Ripk1<sup>F<sup>252</sup>A/D<sup>325</sup>A</sup> | Embryonic lethality | Caspase 8 checkpoint | Tnf<sup>−/−</sup>, Ripk3<sup>−/−</sup>, Casp8<sup>−/−</sup> | 21,22,24 |
| Clay2<sup>−/−</sup> Clay1<sup>−/−</sup> | Embryonic lethality | IKK checkpoint, NF-κB checkpoint | Tnf<sup>−/−</sup>, Ripk3<sup>−/−</sup>, Casp8<sup>−/−</sup> | 32,33,34 |
| CreER<sup>CreER</sup> Clay1<sup>−/−</sup> Clay2<sup>−/−</sup> | Lethal upon tamoxifen injection | | Tnf<sup>−/−</sup>, Ripk3<sup>−/−</sup>, Casp8<sup>−/−</sup> | 100 |
| Xiap<sup>−/−</sup> | Ileal inflammation | Unknown | Tnf<sup>−/−</sup>, Ripk3<sup>−/−</sup>, Casp8<sup>−/−</sup> | 109 |
| Sharp<sup>SkinH<sup>SkinH</sup>/xdm</sup> | Chronic proliferative dermatitis (inflammatory skin lesions, multi-organ inflammation) | IKK checkpoint | Tnf<sup>−/−</sup>, Ripk1<sup>−/−</sup>, Ripk3<sup>−/−</sup>, Casp8<sup>−/−</sup> | 38,39,77,112–115 |
| Hoip<sup>−/−</sup> | Embryonic lethality | IKK checkpoint, NF-κB checkpoint | Tnf<sup>−/−</sup>, Ripk1<sup>−/−</sup>, Ripk3<sup>−/−</sup>, Casp8<sup>−/−</sup> | 38,40 |
| Hoip<sup>IEC-KO</sup> | Severe skin inflammation; keratinocyte necroptosis | | Tnf<sup>−/−</sup>, Ripk1<sup>−/−</sup>, Ripk3<sup>−/−</sup> (partial), Casp8<sup>−/−</sup> | 37 |
| Hoil1<sup>−/−</sup> | Embryonic lethality | IKK checkpoint, NF-κB checkpoint | Tnf<sup>−/−</sup>, Ripk1<sup>−/−</sup>, Ripk3<sup>−/−</sup> | 38,40 |
| Hoil1<sup>IEC-KO</sup> | Severe skin inflammation; keratinocyte necroptosis | | Tnf<sup>−/−</sup>, Ripk1<sup>−/−</sup> (partial rescue), Casp8<sup>−/−</sup> | 37 |
### Table 1 (continued) | A selection of studies in mouse models demonstrating that inflammation results from unrestrained cell death

| Genotype | Phenotype | Expected inactivated CDC | Rescue background | Refs. |
|----------|-----------|---------------------------|-------------------|-------|
| **Otulin**<sup>C129A</sup> <br>**Otulin**<sup>L272P</sup> | Embryonic lethality | IKK checkpoint <br>NF-κB checkpoint | **Tnfr1**<sup>−/−</sup> (partial rescue) <br>**Ripk3**<sup>−/−</sup>**Casp8**<sup>−/−</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> (partial rescue) | 46,117 |
| **Otulin**<sup>−/−</sup> | Severe skin inflammation; keratinocyte necroptosis | **Tnfr1**<sup>−/−</sup> <br>**Tnfr1**<sup>−/−</sup>**Ripk3**<sup>−/−</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> (partial rescue) <br>**Mlkl**<sup>−/−</sup> (partial rescue) <br>Fadd/Mlk<sup>ΔD0</sup> | 117,118 |
| **Ikbkg**<sup>−/−</sup> | Embryonic lethality | IKK checkpoint <br>NF-κB checkpoint | **Tnfr1**<sup>−/−</sup> <br>**Tnfr1**<sup>−/−</sup>**Ripk3**<sup>−/−</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> (partial rescue) | 117,118,154 |
| **Ikbkg**<sup>−/−</sup> | Severe skin inflammation; keratinocyte cell death | **Tnfr1**<sup>−/−</sup> <br>**Tnfr1**<sup>−/−</sup>**Ripk3**<sup>−/−</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> (partial rescue) <br>**Fadd**<sup>−/−</sup> | 150,151 |
| **Ikbkb**<sup>−/−</sup> | Severe intestinal pathology; enterocyte apoptosis | **Tnfr1**<sup>−/−</sup> <br>**Tnfr1**<sup>−/−</sup>**Ripk3**<sup>−/−</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> (partial rescue) | 153,154 |
| **Ikkb**<sup>−/−</sup> | Embryonic lethality | IKK checkpoint <br>NF-κB checkpoint | **Tnfr1**<sup>−/−</sup> <br>**Tnfr1**<sup>−/−</sup>**Ripk3**<sup>−/−</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> (partial rescue) | 150,153 |
| **Ikkb**<sup>−/−</sup> | Severe skin inflammation; keratinocyte cell death | **Tnfr1**<sup>−/−</sup> <br>**Tnfr1**<sup>−/−</sup>**Ripk3**<sup>−/−</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> (partial rescue) <br>**Fadd**<sup>−/−</sup> | 150,153 |
| **Ikka**<sup>−/−</sup>**Ikkb**<sup>−/−</sup> | Embryonic lethality | IKK checkpoint <br>NF-κB checkpoint | **Mlk3**<sup>−/−</sup>**Fadd**<sup>−/−</sup> <br>**Ripk3**<sup>−/−</sup> <br>**Tnfr1**<sup>−/−</sup> | 42,43,150,151 |
| **Ripk1**<sup>ΔK376R/ΔK376R</sup> | Embryonic lethality (C57BL/6 background) | IKK checkpoint | **Mlk3**<sup>−/−</sup>**Fadd**<sup>−/−</sup> <br>**Ripk3**<sup>−/−</sup> <br>**Ripk1**<sup>ΔK376R/ΔK376R</sup> | 29,155,156 |
| **Tbk1**<sup>−/−</sup> | Embryonic lethality (C57BL/6 background) | IKK checkpoint | **Ripk1**<sup>ΔD138N/ΔD138N</sup> <br>**Ripk1**<sup>ΔD138N/ΔD138N</sup> | 29,155 |
| **Rela**<sup>−/−</sup> | Viable (129 background), spontaneous inflammation in multiple tissues | IKK checkpoint | **Ripk1**<sup>ΔD138N/ΔD138N</sup> | 156 |
| **CreERT2**<sup>Cflar</sup> | Viable (129 background), spontaneous inflammation in multiple tissues | IKK checkpoint | **Ripk1**<sup>ΔD138N/ΔD138N</sup> | 94,160,161 |
| **CreERT2**<sup>Cflar</sup> | Severe skin inflammation; keratinocyte apoptosis | IKK checkpoint | **Ripk1**<sup>ΔD138N/ΔD138N</sup> | 94,162 |
| **Ripk1**<sup>ΔK376R/ΔK376R</sup> | Severe skin inflammation; keratinocyte necroptosis | IKK checkpoint | **Ripk1**<sup>ΔK376R/ΔK376R</sup> | 101,102 |

Cflar, gene encoding FLICE-like inhibitory protein (FLIP). CDC, cell death checkpoint. CreERT<sup>2</sup>, tamoxifen-inducible Cre expression. E-KO, epidermis-specific knockout. IEC-KO, intestinal epithelial cell-specific knockout. Ikbkg, gene encoding nuclear factor-κB essential modulator (NEMO). Ikk, inhibitor of nuclear factor-κB kinase. LPS-Rs, lipopolysaccharide from Rhodobacter sphaeroides, which acts as an inhibitor of Toll-like receptor 4 signalling. NF-κB, nuclear factor-κB. TNF, tumour necrosis factor.
Table 2 | Autoinflammatory diseases caused by mutations in genes encoding essential TNF signalling proteins

| Gene symbol | Protein name | Disease mechanism | Patient phenotype | OMIM entry | Refs. |
|-------------|--------------|------------------|------------------|------------|-------|
| CASP8       | Caspase 8    | Homozygous, loss of function | Autoimmune lymphoproliferative syndrome; very early onset IBD | 607271 | 95,125 |
| IKBKG       | NEMO         | Loss of function | Incontinentia pigmenti in heterozygous females (lethal in males); immunodeficiency; EDA-ID | 308300 | 127 |
| IKBKG       | NEMO         | Carboxy-terminal deletions in NEMO | Inflammatory skin and intestinal disease; ectodermal dysplasia with anhidrosis and immunodeficiency | NA | 129 |
| IKBKG       | NEMO         | NEMO lacking the domain encoded by exon 5 | Severe autoinflammatory syndrome; NDAS | 301081 | 146,149 |
| OTULIN      | OTULIN       | Homozygous, loss of function | Early-onset recurrent fever; neutrophilic dermatitis/panniculitis, joint swelling: ORAS | 617099 | 122–125 |
| OTULIN      | OTULIN       | Compound heterozygous variants | Atypical late-onset ORAS | NA | 126 |
| RBCK1       | HOIL1        | Homozygous, loss of function | Severe multi-organ autoinflammation, immunodeficiency, invasive bacterial infections, muscular amylopectinosis | 615895 | 120-121 |
| RELA        | RelA         | Haploinsufficiency | Fever, abdominal pain, mucocutaneous lesions | 618287 | 130 |
| RIPK1       | RIPK1        | Homozygous, loss of function | Recurrent infections, early-onset IBD, progressive polyarthritis, immunodeficiency | 618108 | 195,196 |
| RIPK1       | RIPK1        | Heterozygous mutation of the RIPK1 caspase 8 cleavage site | Early-onset periodic fever syndrome and lymphadenopathy; CRIA | 618852 | 52,53,103 |
| RNF31       | HOIP         | Homozygous, loss of function | Severe multi-organ autoinflammation, immunodeficiency | NA | 111-121 |
| TBK1        | TBK1         | Homozygous, loss of function | Chronic and systemic autoinflammation | NA | 107 |
| TNFAIP3     | A20          | Haploinsufficiency | Early-onset severe multiorgan autoinflammatory syndrome; HA20 | 616744 | 137,148 |
| XIAP        | XIAP         | Loss of function | Pathogen-associated hyperinflammation, fever, severe IBD; XLP2 | 300635 | 127 |

Details of the genetic disorders can be found in OMIM. CRIA, cleavage-resistant receptor-interacting serine/threonine protein kinase 1 (RIPK1)-induced autoinflammatory syndrome; EDA-ID, anhidrotic ectodermal dysplasia with immune deficiency; HA20, haploinsufficiency of A20; IBD, inflammatory bowel disease; NA, not available; ORAS, OTULIN-related autoinflammatory syndrome; NDAS, nuclear factor-κB essential modulator (NEMO) deleted exon 5-autoinflammatory syndrome; TBK1, TANK-binding kinase 1; XIAP, X-linked inhibitor of apoptosis protein; XLP2, X-linked lymphoproliferative syndrome 2.

Skin lesions, multi-organ inflammation and immune system dysregulation, which is fully caused by TNF-mediated RIPK1 kinase activity-dependent cell death [80,103-105]. Cpdn mice lacking RIPK3 or MLKL show a delayed onset of the dermatitis and only a partial amelioration of the multi-organ pathology, indicating a contribution of necroptosis to the phenotype. However, epidermal deletion of FADD together with deficiency in RIPK3 completely prevented keratinocyte death and skin inflammation, demonstrating that FADD-mediated apoptosis of keratinocytes is the driver of skin inflammation in Cpdn mice [80,103-105]. Importantly, genetic ablation of MyD88 or depletion of the microbiota can be treated in most patients with cytokine inhibitors, including infliximab and golimumab. Patients with carboxy-terminal deletions in NEMO, which impair interactions with A20, also develop an autoinflammatory phenotype that resembles HA20 (refs.139).

The role of MI-linked ubiquitylation in preventing cell death-driven inflammation is further demonstrated by mutations affecting the protein A20. The anti-inflammatory properties of A20 are commonly attributed to its ability to suppress inflammatory NF-κB signalling, but gene-targeting studies in mice have demonstrated that A20 primarily suppresses inflammation by preventing cell death 44,47,146–149. In the TNFR1 pathway, A20 represses RIPK1 kinase activity-dependent and RIPK1 kinase activity-independent cell death induction by binding and stabilizing the MI-linked ubiquitin chains associated with TNFR1 complex 1 (refs.44,45,46,147–149). Single-nucleotide polymorphisms in TNFAIP3, the gene encoding A20, have been linked to many inflammatory and autoimmune diseases 136. Importantly, rare heterozygous loss-of-function variants have been shown to cause a severe autoinflammatory syndrome, named ‘HA20’ (haploinsufficiency of A20) 137,138, which can be treated in most patients with cytokine inhibitors, including infliximab 137,138. Patients with carboxy-terminal deletions in NEMO, which impair interactions with A20, also develop an autoinflammatory phenotype that resembles HA20 (refs.139).
Binding of NEMO to the M1-linked ubiquitin chains associated with complex I permits the recruitment and activation of the kinases IKKa, IKKB, IKKe and TBK1 to TNFR1 complex I. While these kinases prevent RIPK1 kinase activity-dependent apoptosis and pyroptosis by phosphorylaying RIPK1, IKKa–IKKB additionally repress RIPK1 kinase activity-independent apoptosis through the NF-kB-dependent expression of prosurvival molecules, including FLIP (Fig. 3). Disruption of the genes encoding NEMO (Ikkg), IKKa (Ikka; also known as Chuk) and/or IKKB (Ikb) also known as Ikkb) in mice results in early lethality with massive cellular death in several organs, such as the liver, the skin and, in the case of mice lacking IKKa and IKKB, the nervous system[160–163]. Moreover, specific loss of NEMO in keratinocytes causes severe and lethal skin inflammation in mice that requires TNF[146]. In humans, NEMO deficiency causes embryonic lethality in males and incontinentia pigmenti in heterozygous females, a genetic disease characterized by development of skin lesions among other symptoms[157]. Also patients with NEMO deleted exon 5-autoinflammatory syndrome have recently been described. In contrast to patients with loss of function NEMO mutations who exhibit immunodeficiency, patients with the NEMO spliced mutant develop a severe autoinflammatory disease involving uveitis, panniculitis and hepatitis[158,159]. TNF also causes skin inflammation in mice with epidermis-specific knockout of Ikb or both Rela and Rel (which encode NF-kB subunits) by inducing RIPK1 kinase activity-dependent necroptosis of keratinocytes[160,161]. In humans, a heterozygous mutation in RELA, causing RelA haploinsufficiency, induces chronic mucocutaneous ulceration, which can be suppressed by anti-TNF therapy[162]. Fibroblasts from such patients have impaired NF-kB activation and exhibit increased cell death in response to TNF. Similarly, Rela heterozygous mice show impaired NF-kB activation, develop cutaneous ulceration from TNF exposure and exhibit severe gastrointestinal inflammation upon exposure to dextran sodium sulfate, which is suppressed by TNF inhibition[150]. NEMO deficiency in IECs triggers intestinal pathology caused by TNF-induced apoptosis[150]. Inhibition of RIPK1 kinase activity or combined deficiency of FADD and RIPK3 prevents IEC death and colitis development in these mice, suggesting that RIPK1 inhibitors could be useful for the treatment of colitis in patients with NEMO mutations and possibly in IBD[151]. However, it remains puzzling that inactivation of the kinase activity of RIPK1 is sufficient to fully prevent pathology in these mice. Indeed, with a defect in NF-kB activation, the IECs should still be sensitized to RIPK1 kinase activity-independent apoptosis.

According to the specific role of TBK1 in repressing RIPK1 kinase activity in the TNFRI pathway, biallelic loss of Tbk1 is embryonic lethal, and viability is rescued by TNF deficiency or by complementation with kinase-inactive RIPK1 (refs. 153–155). Interestingly, loss of Tbk1 in mice with a 129 genetic background was reported to be viable, but was shown to cause inflammation in multiple tissues[156]. Transferring this allele onto the C57BL/6 background, however, also resulted in embryonic lethality[156]. In agreement, biallelic loss-of-function mutations in Tbk1 cause an early-onset autoinflammatory syndrome in humans that was shown to depend on TNF and RIPK1 kinase activity-dependent cell death. Hence, autoinflammation in these patients is suppressed with anti-TNF therapy[157].

FLIP plays a central role in NF-kB-dependent cell survival, as shown by the phenotypes of FLIP-deficient mice. Genetic deletion of Cflar (which encodes FLIP) results in embryonic lethality, due to a defect in the vascular development of the yolk sac[158], and combined deletion of Fadd and Ripk3 is required for prevention of the lethal phenotype of FLIP-deficient mice[159]. IEC-specific FLIP-deficient mice develop severe colitis due to IEC apoptosis and necroptosis, which can be suppressed by loss of TNFR1 or by treatment with neutralizing anti-TNF antibodies[160,161]. Postnatal deletion of Cflar in keratinocytes induces severe skin inflammation in mice due to TNF-dependent keratinocyte apoptosis[162]. Interestingly, loss of FLIP expression in skin epidermis could be shown in patients with different skin diseases associated with epidermal cell apoptosis[163].

Finally, full-body Ripk1 ablation causes postnatal lethality which is rescued by caspase 8 and RIPK3 deficiency, demonstrating a key function for RIPK1 in inhibiting cell death and subsequent inflammation[164,165]. RIPK1 deficiency in IECs in mice induces a severe pathology caused by TNF-mediated IEC apoptosis[160,161]. Whereas RIPK1 contributes to the NF-kB-dependent checkpoint by serving as a ubiquitylated substrate for NEMO recruitment, in vitro studies suggest a more prominent anti-apoptotic role of RIPK1 through another, and yet to be discovered, additional cell death checkpoint in the TNF pathway[166]. In agreement, patients with RIPK1 deficiency experience inflammatory diseases, including IBD[160,161].

**Perspective: cell death-blocking drugs**

Although experimental studies in mice genetically altered to lack (or express mutant versions of) essential proteins of the apoptotic, necroptotic and pyroptotic apparatus have provided formal proof of the concept that aberrant cell death could instigate inflammatory disease development, functional validation using specific inhibitors will be required to establish the importance of proinflammatory cell death for the pathogenesis of human diseases. RIPK1 and RIPK3 inhibitors, as well as GSDMD inhibitors, are currently under investigation as potential therapies for human inflammatory diseases. Such inhibitors may become an alternative treatment for patients with autoimmune diseases, especially for those patients who do not respond to or show adverse effects of anti-TNF treatment. Indeed, one third of patients with rheumatoid arthritis will discontinue treatment with an anti-TNF drug in the first year of therapy, mostly because of inefficacy or adverse events[170].

RIPK1 has a unique hydrophobic pocket that allosterically regulates its kinase activity, which enabled the development of small-molecule kinase inhibitors that dock into that pocket[171]. Some of these RIPK1-targeting compounds have entered clinical trials for the treatment...
of inflammatory disorders, such as ulcerative colitis, psoriasis and rheumatoid arthritis (reviewed in 16). Also blood–brain barrier-permeant RIPK1 inhibitors have entered clinical trials for the treatment of neurodegenerative diseases including amyotrophic lateral sclerosis, Alzheimer disease and multiple sclerosis (reviewed in 17). However, the first results from two such trials using the RIPK1 inhibitor GS2982772 did not show clinical efficacy in a small group of patients with ulcerative colitis or rheumatoid arthritis 18,19. One explanation for this could be the lack of proper patient stratification, highlighting the need to stratify patients on the basis of detection of specific markers. In this respect, antibodies targeting RIPK1 phosphorylation at S166 and phospho-Y176 MLKL may become useful. However, the requirement of RIPK1 enzymatic activity for TNF-induced cell death may also be different between mice and humans, raising the important question of the exact function of RIPK1 kinase activity, as no lethal substrate apart from RIPK1 has been identified so far. RIPK3 kinase inhibitors are also being considered for the treatment of inflammatory diseases, but so far no such inhibitors have been selected for therapeutic development, mainly because of the surprising observation that such compounds may assemble a caspase 8–FADD–RIPK1 complex that induces apoptotic cell death 20.

As described earlier herein, TNF can trigger caspase 8-dependent GSDMD cleavage 17,18,21. Since the discovery of GSDMD as a central mediator of pyroptosis 17,18,21, GSDMD inhibition has been proposed as a novel therapeutic strategy to prevent inflammatory pathology in different diseases (reviewed in 19). Disulfiram (Antabuse), a US Food and Drug Administration (FDA)-approved drug used to treat alcohol addiction, was shown to inhibit pyroptosis and inflammatory cytokine secretion in human and mouse cells, and in mouse models of LPS-induced septic shock 22 and multiple sclerosis 23,24. Nercosulfonamide was shown to be efficacious in sepsis 25, and dimethyl fumarate was reported to suppress pathology in mouse models of familial Mediterranean fever, sepsis and multiple sclerosis 26,27. All three currently available GSDMD inhibitors (disulfiram, nercosulfonamide and dimethyl fumarate) covalently modify reactive cysteines and hence are not specific, and specific small-molecular inhibitors of GSDMD will need to be discovered. As secondary necrosis–pyroptosis can also be induced via caspase 3-mediated cleavage of GSDME 23,28, and via caspase 8-induced cleavage of GSDMC 23, other GSDMD inhibitors need to be considered.

Future research should also investigate whether ninjurin 1 inhibition could be beneficial in suppressing TNF-mediated inflammation. Indeed, a recent study revealed that plasma membrane rupture, a common feature of pyroptotic and necrototic cell death, but also happening during secondary necrosis of apoptotic cells that are not engulfed and removed in a timely manner, is actively regulated and mediated by ninjurin 1 (ref. 20). Proof-of-principle studies have already demonstrated that an antibody targeting the amino-terminal extracellular region of ninjurin 1, which is shown to be critical for its cytotoxicity, impairs plasma membrane rupture in pyroptotic macrophages 29.

Finally, preclinical studies in mice have also made clear that the different cell death signalling pathways do not operate in isolation but are highly interconnected whereby intervention in one module may be unable to confer protection but instead may engage a backup cell death pathway. This intimate crosstalk between cell death pathways may ultimately compromise the use of single inhibitory drugs and may require multiple agents to simultaneously inhibit multiple cell death modules or to target central signalling hubs.

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This review article discusses the role of RIPK1 in cell death and inflammation. It highlights several key points:

1. **RIPK1 in Cell Death Regulation**: RIPK1 is a crucial regulator of cell death, with roles in both apoptosis and necroptosis. The dephosphorylation and activation of RIPK1 by PPP1R3G/PP1gamma regulates necroptosis.

2. **RIPK1 and Caspase-8 Interaction**: Caspase-8 promotes TNF-mediated apoptosis, and its activity is regulated by RIPK1.

3. **IAPs and RIPK1**: IAPs (Inhibitors of Apoptosis) limit the activation of RIPK1 and RIPK3, modulating cell death pathways.

4. **Ubiquitination of RIPK1**: Ubiquitination of RIPK1 on lysine 377 inhibits TNF-induced apoptosis, playing a key role in regulating cell death.

5. **NF-kappaB Checkpoint**: The NF-kappaB-dependent checkpoint balances apoptosis and necroptosis, ensuring cell survival.

6. **TNF-Related Inflammation**: TNF-α induces inflammation through TNFR1-dependent cell death, which is regulated by caspase-8.

7. **Autoinflammatory Disease**: Autoinflammatory diseases caused by mutation in RIPK1 in specific tissues or organs are discussed.

The article further explores the role of RIPK1 in various diseases, including SARS-CoV-2 infection, autoinflammatory diseases, and tumor cell sensitization to TNFalpha.

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