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Review article

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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.

Box 1

Anti-TNF biologics are among the most successful drugs in history

Following the discovery that tumour necrosis factor (TNF) has strong proinflammatory activities, attention turned to the development of biologics that neutralize TNF's activity for the treatment of inflammatory diseases. This turned out to be highly successful.

TNF was the first cytokine to be validated as a therapeutic target for rheumatoid arthritis. TNF inhibition using a neutralizing antibody was shown to block the synthesis of several other important proinflammatory cytokines in cell cultures and mice, which led to the pivotal concept that TNF is at the apex of an inflammatory cascade of cytokines in rheumatoid arthritis[195–197]. Soon after, a small-scale clinical study using anti-TNF antibodies was initiated in patients with rheumatoid arthritis, and demonstrated marked clinical improvement in most patients[198]. Subsequent clinical trials confirmed high efficacy of TNF neutralization in the treatment of rheumatoid arthritis[199–201], which paved the way for testing use of TNF inhibitors in other inflammatory autoimmune diseases.

Five distinct antibody- or receptor-based TNF-neutralizing drugs have been approved over the years for treating rheumatoid arthritis, psoriatic arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, psoriasis, Crohn's disease and ulcerative colitis (reviewed in[202]). The chimeric antibody infliximab (sold under the brand name Remicade) and the TNF receptor 2 (TNFR2)–Fc fusion protein etanercept (Enbrel) were the first two TNF biologics to be approved, in 1998, followed by the first fully human antibody, adalimumab (Humira), in 2002. Certolizumab pegol (Cimzia), a pegylated Fab fragment, was approved in 2008, and another fully human antibody, golimumab (Simponi), was approved in 2009 (reviewed in[203]). From 2015 on, several of these TNF inhibitors had lost their market exclusivities, allowing biosimilar alternatives to enter the market. Together, these TNF-neutralizing therapies are among the most successful protein-based drugs in history, with global sales estimated at US $30 billion annually.

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[2] (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'[3,4]. Later, lipopolysaccharide (LPS) was isolated from bacterial extracts and shown to induce some tumour regression in experimental cancer studies in mice[5]. 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'[2]. In the years after, the genes encoding the human and mouse TNF and TNF receptors were purified, sequenced and cloned[6–13], and experimental studies with recombinant TNF were initiated to validate its antitumour potential for cancer treatment (reviewed in[14]). 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[15] (Fig. 1). 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

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[1,16,17]).

Death is not the default response of cells to TNF. Protectives 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 1-binding protein 2 (TAB2) and TAB3, which recruit the upstream kinase TGFβ-activated kinase 1 (TAK1) for MAPK signalling. In addition, the M1-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 IKKε to the receptor complex. The close proximity of TAK1 and IKKα and IKKβ on the hybrid K63/M1-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[20]).

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-dissociated complex I components TRADD and/or RIPK1 (ref.[21]). 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 trigger pyroptosis, as recently reported[22–24]. 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

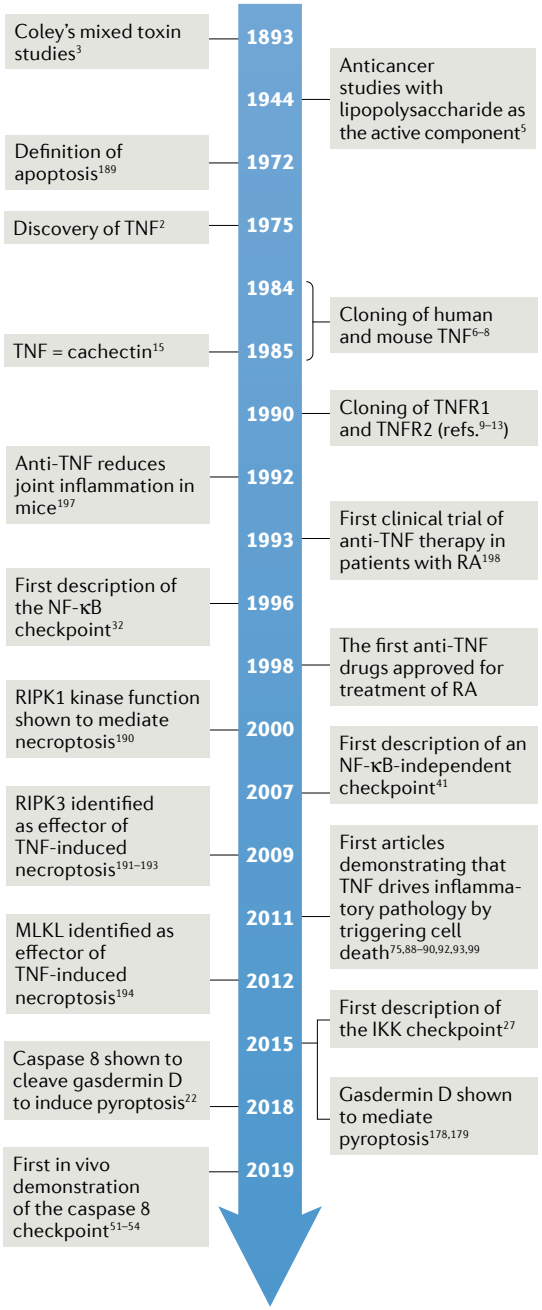

Fig. 1 | Timeline of key events in the history of TNF and TNF-induced cell death. IKK, inhibitor of nuclear factor-κB kinase; MLKL, mixed lineage kinase domain-like protein; NF-κB, nuclear factor-κB; RA, rheumatoid arthritis; RIPK, receptor-interacting serine/threonine protein kinase; TNF, tumour necrosis factor; TNFR, tumour necrosis factor receptor.

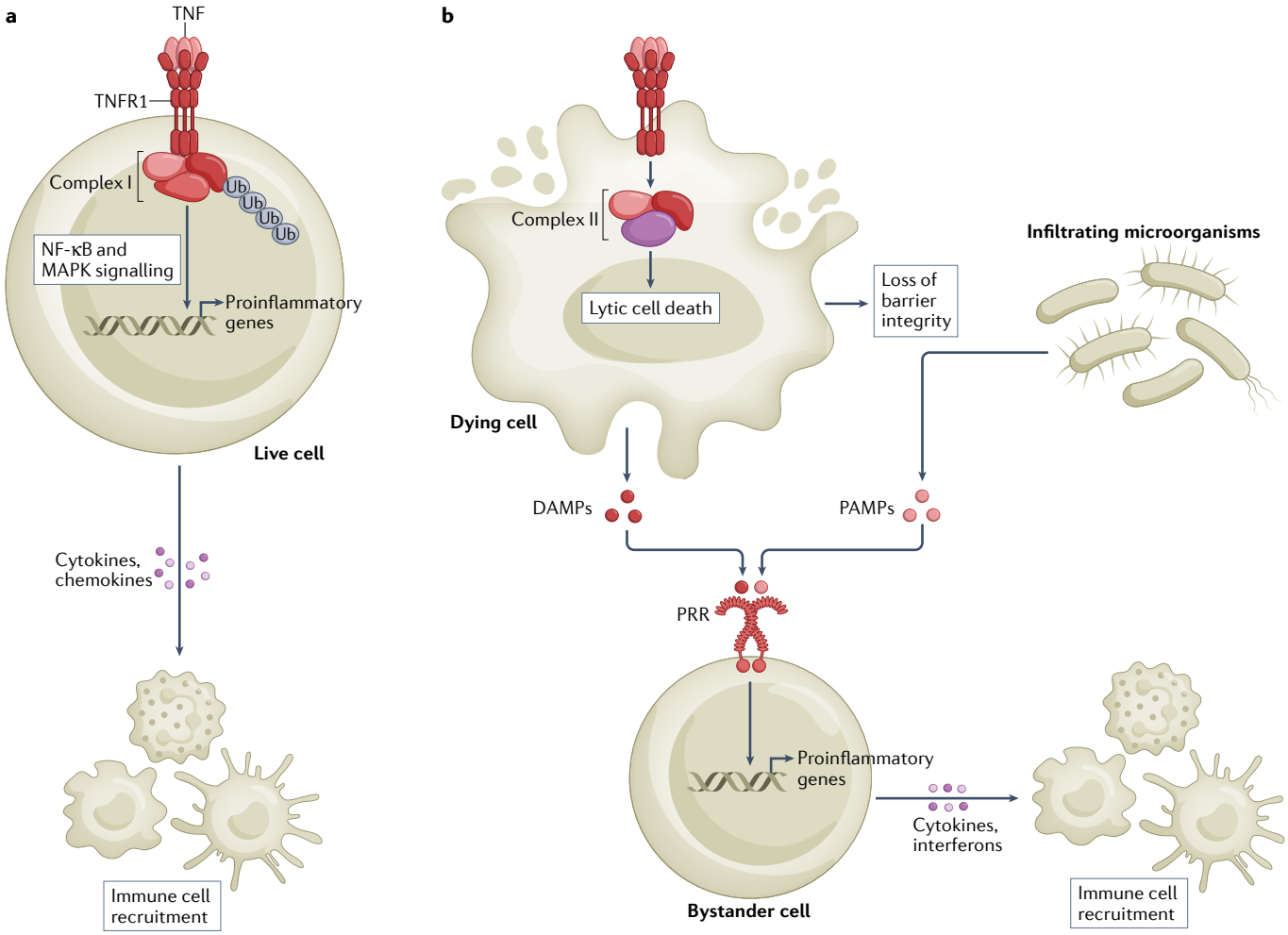

Fig. 2 | Inflammatory signalling by TNFR1. a, Binding of tumour necrosis factor (TNF) to TNF receptor 1 (TNFR1) directly promotes inflammation by activating the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways, which collectively lead to the transcriptional upregulation of genes encoding proinflammatory mediators, such as cytokines and chemokines. **b**, TNFR1 activation also indirectly promotes inflammation by triggering cell death. Lytic forms of cell death, such as apoptosis-driven secondary necrosis, pyroptosis and necroptosis, release damage-associated molecular patterns (DAMPs) that activate proinflammatory gene expression in bystander cells. In addition, the inflammatory response may originate from and/or be amplified by loss of barrier function caused by epithelial cell death (lytic and non-lytic) and the subsequent sensing of pathogen-associated molecular patterns (PAMPs) from microorganisms that have breached the epithelial barrier. PRR, pattern recognition receptor.

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 induction[27–29] (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 cytotoxicity[31]. 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-κB checkpoint') occurs downstream in the pathway, in the nucleus, and relies on the NF-κB-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 apoptosis[25,32]. Since IKKα and IKKβ are upstream kinases in the canonical NF-κB 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-κB 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-κB[28].

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 ubiquitylation of complex I, such as inhibition of cIAP1, cIAP2 and LUBAC[33–40], and also mutations in the RIPK1 ubiquitin acceptor site K377 (K376 in mouse RIPK1)[41–43] or deficiencies of A20 and OTULIN[44–49], indirectly perturb these cell death checkpoints and activate TNF cytotoxicity. Of note, the inhibitory effect of ubiquitin on RIPK1 death signalling

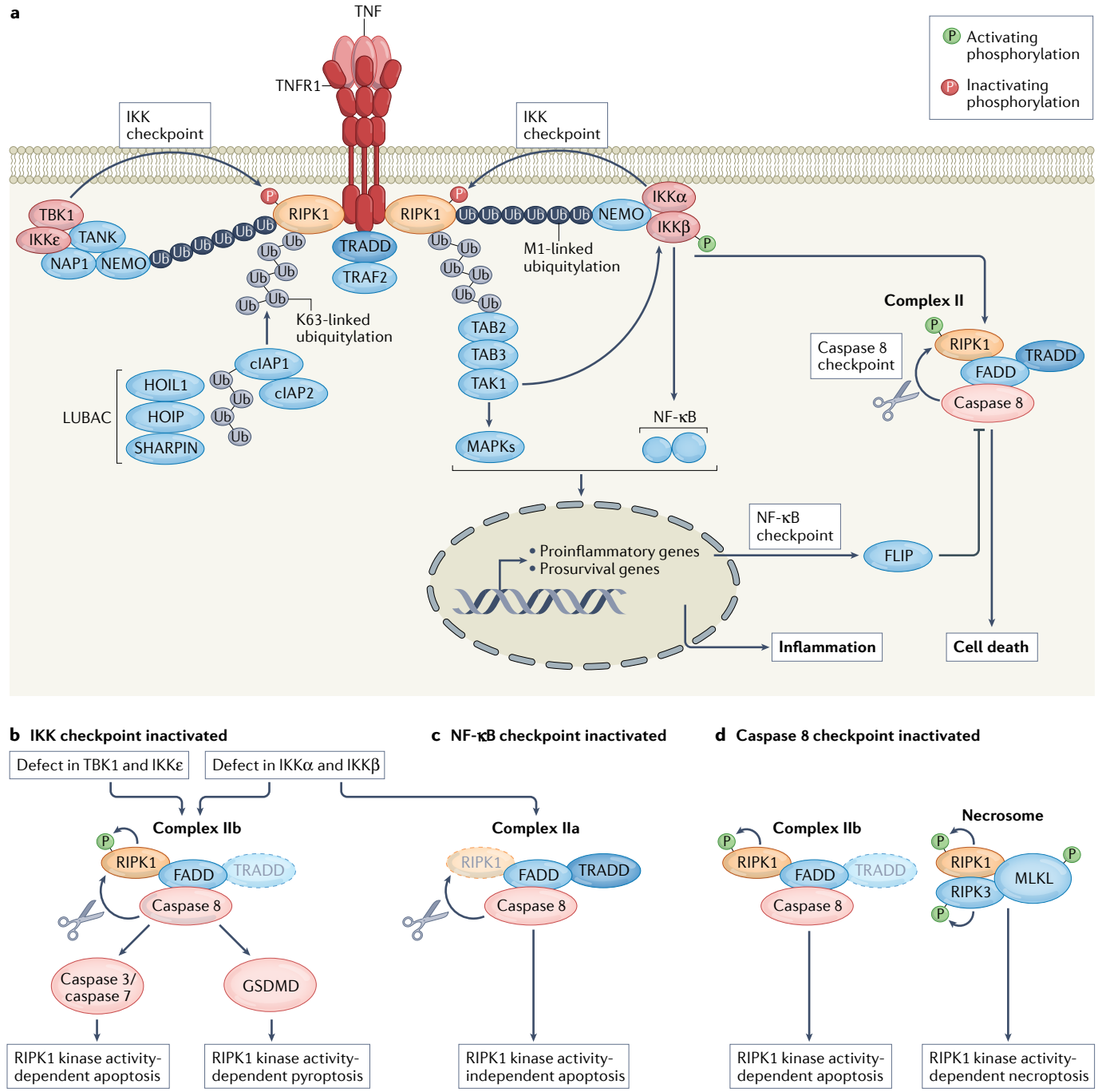

Fig. 3 | Signalling by TNFR1 and overview of the three characterized cell death checkpoints in the TNFR1 pathway. a, Sensing of tumour necrosis factor (TNF) by TNF receptor 1 (TNFR1) leads to the formation of a primary membrane-bound receptor signalling complex (complex I) that activates the mitogen-activated 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; NAP1, 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 transcription[18,41]. 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 'necrosome'. Association between RIPK3 and RIPK1 occurs via their RIP homotypic interaction motifs, and appears to be sufficient to activate RIPK3 within the necrosome. 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 necrosome. Depending on the cellular context, the catalytic activity of RIPK1 can therefore promote apoptosis, caspase 8-mediated pyroptosis or necroptosis downstream of TNFR1 (refs.[19,23]) (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 of RIPK1 by the MAPK-activated kinase MK2 (refs.[55–57]) or by the kinase Unc-51-like autophagy activating kinase 1 (ULK1)[58], as well as the poly(ADP-ribosyl)ation of complex II by tankyrase 1 (ref.[59]). 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 acyltransferase, named 'YopJ/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 mediators[60–63]. 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[22,23,31,56,64–66].

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 in[67]). 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 CrmA[68] or the viral FLICE-inhibitory protein (vFLIP) identified in herpesviruses and in the human poxvirus Molluscum contagiosum virus[69,70]. As a response, several pathogens also express proteins that specifically target necroptosis by inhibiting RIPK1, RIPK3, MLKL or the effects downstream of MLKL[67].

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 fully protects the mice from the cytokine storm, hypothermia and morbidity induced by TNF[75–77]. 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 lethality[23,78]. 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 lymphotoxin-β, activates the non-canonical NF-κB 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-κB-inducing kinase (NIK), and finally in the activation of IKKα by NIK-mediated phosphorylation[79]. 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[55,80]. In line with this idea, it is interesting to note that TWEAK and CD40L are upregulated in inflammatory bowel disease (IBD) and rheumatoid arthritis[81,82], 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-κB 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[71]. 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[71], which may support the reported causative role of pyroptosis in hyperinflammation during severe COVID-19 (refs.[83–85]).

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[86,87], which can be rescued to birth by deletion of *Ripk1* and to adulthood by deletion of *Ripk3* or *Mlkl*[88–91]. 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)[24,92–94]. High levels of RIPK3 and increased necroptosis could be confirmed in the intestine of patients with Crohn's disease[93], 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[95,96]. 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[92]. 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[24]. 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 fatal 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 unrestrained necroptosis in the small intestine[97]. 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[94,98,99]. Since RIPK3 also contributes to TNF-induced caspase 8 activation[100], 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[101,102]. 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 MLKL (or RIPK3) and FADD (or caspase 8), but not by loss of MLKL or RIPK3 alone, confirming combined induction of apoptosis and necroptosis[51,52,54]. 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[52,53,103].

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 (reviewed in[104,105]). This is, for instance, the case upon deletion of cIAP1 and cIAP2, which results in embryonic lethality caused by TNFR1 signalling[35]. Further 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[106]. Deficiency in ubiquitin ligase X-linked inhibitor of apoptosis protein (XIAP) is the cause of X-linked lymphoproliferative syndrome 2, a severe inflammatory disease[107]. With use of gene-targeted mice, XIAP was shown to prevent TNF- and RIPK3-dependent cell death by regulating ubiquitylation of RIPK1, which might explain the hyperinflammation in patients with X-linked lymphoproliferative syndrome 2 (refs.[108–110]).

The notion that linear ubiquitin chains protect against cell death-driven inflammation is supported by the phenotypes of LUBAC- and OTULIN-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$^{-/-}$	Embryonic lethality	Caspase 8 checkpoint	*Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$	88,89,91
Casp8$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte necroptosis		*Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$ *Tnfr1*$^{IEC\text{-}KO}$ (colon) *Ripk1*$^{D138N/D138N}$	24,93,94
Cdh5–CreER^{T2} *Casp8*$^{fl/fl}$	Fatal necroptotic haemorrhage in the small intestine		*Mlkl*$^{-/-}$ *Tnf*$^{-/-}$ LPS-Rs administration	97
Casp8$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Ripk3*$^{-/-}$ *Tnf*$^{-/-}$ (partial rescue)	94,98
Fadd$^{-/-}$	Embryonic lethality	Caspase 8 checkpoint	*Ripk1*$^{-/-}$ *Mlkl*$^{-/-}$	90,91
Fadd$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte necroptosis and pyroptosis		*Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$ (colon) *Tnf*$^{-/-}$ (colon) *Tnfr1*$^{IEC\text{-}KO}$ (colon) *Ripk1*$^{D138N/D138N}$ *Mlkl*$^{-/-}$*Gsdmd*$^{-/-}$	24,92
Fadd$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Ripk3*$^{-/-}$ *Tnf*$^{-/-}$ (partial rescue) *Tnfr1*$^{-/-}$ (partial rescue)	99
Ripk1$^{D325A/D325A}$	Embryonic lethality	Caspase 8 checkpoint	*Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ *Ripk1*$^{D138N/D138N}$	51,52,54
Ciap2$^{-/-}$*Ciap1*$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ *Mlkl*$^{-/-}$*Casp8*$^{-/-}$	35,106
CreER^{T2}*Ciap1*$^{-/-}$*Ciap2*$^{fl/fl}$	Lethal upon tamoxifen injection		*Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$*Casp8*$^{-/-}$	106
Xiap1$^{-/-}$	Ileal inflammation	Unknown	*Tnf*$^{-/-}$ *Tnfr1*$^{-/-}$ *Tnfr2*$^{-/-}$ *Ripk3*$^{-/-}$	109
Sharpin$^{cpdm/cpdm}$	Chronic proliferative dermatitis (inflammatory skin lesions, multi-organ inflammation)	IKK checkpoint	*Tnf*$^{-/-}$ *Ripk1*$^{K45A/K45A}$ *Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$*Casp8*$^{+/-}$ *Tnfr1*$^{E\text{-}KO}$ (skin) *Fadd*$^{E\text{-}KO}$*Ripk3*$^{-/-}$ (skin) *Tradd*$^{E\text{-}KO}$*Ripk3*$^{-/-}$ (skin) *Mlkl*$^{-/-}$*Casp8*$^{-/-}$ (skin)	38,39,77,112–115
Hoip$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ (partial rescue) *Mlkl*$^{-/-}$*Casp8*$^{-/-}$	38,40
Hoip$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ (partial rescue) *Mlkl*$^{-/-}$*Casp8*$^{-/-}$ *Tnfr1*$^{-/-}$*Mlkl*$^{-/-}$	37
Hoil1$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ (partial rescue) *Mlkl*$^{-/-}$*Casp8*$^{-/-}$ *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ (partial) *Ripk3*$^{-/-}$*Casp8*$^{-/-}$*Ripk1*$^{-/-}$	38
Hoil1$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ (partial rescue) *Ripk3*$^{-/-}$*Casp8*$^{-/-}$	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^{C129A} *Otulin*^{L272P}	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ (partial rescue) *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ *Ripk1*$^{D138N/D138N}$ (partial rescue)	49,117
Otulin$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ *Tnfr1*$^{E\text{-}KO}$ *Ripk1*$^{D138N/D138N}$ *Ripk3*$^{-/-}$ (partial rescue) *Mlkl*$^{-/-}$ (partial rescue) *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ *Fadd/Mlkl*$^{E\text{-}KO}$	117,118
Ikbkg$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	-	142
Ikbkg$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte cell death		*Tnfr1*$^{-/-}$	146
Ikbkg$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte apoptosis		*Tnfr1*$^{-/-}$ *Tnfr1*$^{E\text{-}KO}$ *Fadd*$^{IEC\text{-}KO}$*Ripk3*$^{-/-}$ *Ripk3*$^{-/-}$ (partial rescue) *Ripk1*$^{D138N/D138N}$	153,154
Ikkb$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	–	140
Ikkb$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte cell death		*Tnfr1*$^{-/-}$ *Tnfr1*$^{E\text{-}KO}$ *Ripk3*$^{-/-}$ (partial rescue) *Ripk3*$^{E\text{-}KO}$ (partial rescue) *Mlkl*$^{-/-}$ (partial rescue) *Fadd*$^{E\text{-}KO}$*Ripk3*$^{-/-}$ *Ripk1*$^{D138N/D138N}$	150,151
Ikka$^{-/-}$*Ikkb*$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	–	141
Ikka/Ikkb$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte apoptosis			153
Ripk1$^{K376R/K376R}$	Embryonic lethality	IKK checkpoint	*Mlkl*$^{-/-}$*Fadd*$^{-/-}$ *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ *Tnfr1*$^{-/-}$ (partial rescue) *Tnfr1*$^{-/-}$*Ripk3*$^{-/-}$	42,43
Tbk1$^{-/-}$	Embryonic lethality (C57BL/6 background)	IKK checkpoint	*Ripk1*$^{D138N/+}$	29,155
	Viable (129 background), spontaneous inflammation in multiple tissues		*Ripk1*$^{D138N/D138N}$ *Ripk3*$^{-/-}$ (partial rescue)	156
Rela$^{+/-}$	Cutaneous ulceration from TNF exposure; severe dextran sodium sulfate-induced colitis	NF-κB checkpoint	Anti-TNF antibodies	152
Cflar$^{-/-}$	Embryonic lethality	NF-κB checkpoint	*Ripk3*$^{-/-}$*Fadd*$^{-/-}$	158,159
Cflar$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte death		*Tnfr1*$^{-/-}$ (partial rescue)	94,160,161
CreER^{T2}*Cflar*$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte apoptosis		Anti-TNF antibodies (partial rescue)	94,162
Ripk1$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte apoptosis	NF-κB checkpoint Unknown checkpoint	*Tnfr1*$^{-/-}$ (partial rescue) *Casp8*$^{-/-}$ (colon) *Fadd*$^{IEC\text{-}KO}$ (partial rescue) *Fadd*$^{IEC\text{-}KO}$ *Ripk3*$^{-/-}$	101,166
Ripk1$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ (partial rescue) *Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$	101,102

Cflar, gene encoding FLICE-like inhibitory protein (FLIP); CDC, cell death checkpoint; CreER^{T2}, 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	95,96
IKBKG	NEMO	Loss of function	Incontinentia pigmenti in heterozygous females (lethal in males); immunodeficiency; EDA-ID	308300	147
IKBKG	NEMO	Carboxy-terminal deletions in NEMO	Inflammatory skin and intestinal disease; ectodermal dysplasia with anhidrosis and immunodeficiency	NA	139
IKBKG	NEMO	NEMO lacking the domain encoded by exon 5	Severe autoinflammatory syndrome; NDAS	301081	148,149
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
RELA	RelA	Haploinsufficiency	Fever, abdominal pain, mucocutaneous lesions	618287	152
RIPK1	RIPK1	Homozygous, loss of function	Recurrent infections, early-onset IBD, progressive polyarthritis, immunodeficiency	618108	168,169
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	119,121
TBK1	TBK1	Homozygous, loss of function	Chronic and systemic autoinflammation	NA	157
TNFAIP3	A20	Haploinsufficiency	Early-onset severe multiorgan autoinflammatory syndrome; HA20	616744	137,138
XIAP	XIAP	Loss of function	Pathogen-associated hyperinflammation, fever, severe IBD; XLP2	300635	107

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[39,77,111–115]. *Cpdm* 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 *Cpdm* mice[39,115]. Importantly, genetic ablation of MyD88 or depletion of the microbiota by antibiotics rescued the skin inflammation in *Cpdm* mice, demonstrating that the death of keratinocytes affects barrier integrity and induces inflammatory skin disease through the sensing of pathogen-associated molecular patterns from microorganisms that have breached the barrier[116]. Mutations in *Hoip* (also known as *Rnf31*) or *Hoil1* (also known as *Rbck1*) lead to embryonic lethality in mice which is partially dependent on TNFR1 and RIPK1 enzymatic activity but is prevented by co-deletion of *Casp8* or *Mlkl*, but not *Ripk3* (refs.[38,40]). Also knock-in mice that express catalytically inactive OTULIN (C129A) or a hypomorphic L272P mutation die at midgestation as a result of cell death mediated by TNFR1 and RIPK1 kinase activity, and these mice can be rescued by the combined loss of caspase 8 and RIPK3 expression[49,117]. Studies in tissue-specific LUBAC- and OTULIN-deficient mice further confirmed TNF- and RIPK1 kinase activity-mediated cell death as a driver of inflammatory pathology[37,117,118]. Homozygous loss-of-function mutations in *HOIP*, *HOIL1* and *OTULIN* have been identified in humans. These mutations are rare and cause a neonatal potentially fatal multi-organ autoinflammatory condition[119–125]. Most patients with OTULIN-related autoinflammatory syndrome (ORAS; also known as otulipenia) are successfully treated with TNF-blocking agents, identifying TNF as the main driver of the autoinflammatory condition[122–124]. Recently, two new compound heterozygous variants in OTULIN were identified in a patient who developed a fulminant atypical late-onset ORAS, which differs clinically from classical ORAS, but is also triggered by perturbed TNF signalling[126].

The role of M1-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[45,47,48,127–133]. In the TNFR1 pathway, A20 represses RIPK1 kinase activity-dependent and RIPK1 kinase activity-independent cell death induction by binding and stabilizing the M1-linked ubiquitin chains associated with TNFR1 complex I (refs.[44,45,48,134]). Single-nucleotide polymorphisms in *TNFAIP3*, the gene encoding A20, have been linked to many inflammatory and autoimmune diseases[135,136]. 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 IKKα, IKKβ, IKKε and TBK1 to TNFR1 complex I. While these kinases prevent RIPK1 kinase activity-dependent apoptosis and pyroptosis by phosphorylating RIPK1, IKKα–IKKβ additionally repress RIPK1 kinase activity-independent apoptosis through the NF-κB-dependent expression of prosurvival molecules, including FLIP (Fig. 3). Disruption of the genes encoding NEMO (*Ikbkg*), IKKα (*Ikka*; also known as *Chuk*) and/or IKKβ (*Ikkb*; also known as *Ikbkb*) 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 IKKα and IKKβ, the nervous system[140–145]. 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[147]. 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[148,149]. TNF also causes skin inflammation in mice with epidermis-specific knockout of *Ikkb* or both *Rela* and *Rel* (which encode NF-κB subunits) by inducing RIPK1 kinase activity-dependent necroptosis of keratinocytes[150,151]. In humans, a heterozygous mutation in *RELA*, causing RelA haploinsufficiency, induces chronic mucocutaneous ulceration, which can be suppressed by anti-TNF therapy[152]. Fibroblasts from such patients have impaired NF-κB activation and exhibit increased cell death in response to TNF. Similarly, *Rela* heterozygous mice show impaired NF-κB activation, develop cutaneous ulceration from TNF exposure and exhibit severe gastrointestinal inflammation upon exposure to dextran sodium sulfate, which is suppressed by TNF inhibition[152]. NEMO deficiency in IECs triggers intestinal pathology caused by TNF-induced apoptosis[153]. 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[154]. 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-κB 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 TNFR1 pathway, biallelic loss of *Tbk1* is embryonic lethal, and viability is rescued by TNF deficiency or by complementation with kinase-inactive RIPK1 (refs.[28,29,155]). 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-κB-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[94,160,161]. Postnatal deletion of *Cflar* in keratinocytes induces severe skin inflammation in mice due to TNF-dependent keratinocyte apoptosis[94,162]. Interestingly, loss of FLIP expression in skin epidermis could be shown in patients with different skin diseases associated with epidermal cell apoptosis[162].

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[163–165]. RIPK1 deficiency in IECs in mice induces a severe pathology caused by TNF-mediated IEC apoptosis[101,166]. Whereas RIPK1 contributes to the NF-κB-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[167]. In agreement, patients with RIPK1 deficiency experience inflammatory diseases, including IBD[168,169].

Glossary

Necroptosis
A programmed form of necrosis whose execution relies on the activation of receptor-interacting serine/threonine protein kinase 3 (RIPK3) and subsequent phosphorylation of mixed lineage kinase domain-like protein (MLKL) by RIPK3, ultimately leading to the translocation of phosphorylated MLKL to the plasma membrane, where it either directly or indirectly causes plasma membrane rupture.

Pyroptosis
A highly inflammatory form of programmed necrosis, usually caused by microbial infection, that relies on the proteolytic activation of the pore-forming molecule gasdermin D by caspase 1 and caspase 11 (or caspase 8). It is classically activated downstream of the inflammasome pathways and is associated with the release of biologically active IL-1β and IL-18.

Secondary necrosis
A lytic, or necrotic, form of cell death that occurs when apoptotic cells are not efficiently removed by efferocytosis. It involves proteolytic activation of the pore-forming molecule gasdermin E by the effector caspase 3.

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], and similar efficacy profiles have been shown in patients with IBD and psoriasis[171,172].

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[173,174]. 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 GSK2982772 did not show clinical efficacy in a small group of patients with ulcerative colitis or rheumatoid arthritis[175,176]. 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 phosphorylated 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[177].

As described earlier herein, TNF can trigger caspase 8-dependent GSDMD cleavage[22–24]. Since the discovery of GSDMD as a central mediator of pyroptosis[178,179], GSDMD inhibition has been proposed as a novel therapeutic strategy to prevent inflammatory pathology in different diseases (reviewed in[180]). 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[181] and multiple sclerosis[182]. Necrosulfonamide was shown to be efficacious in sepsis[183], and dimethyl fumarate was reported to suppress pathology in mouse models of familial Mediterranean fever, sepsis and multiple sclerosis[184]. All three currently available GSDMD inhibitors (disulfiram, necrosulfonamide 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[185,186], and via caspase 8-induced cleavage of GSDMC[187], other GSDM 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 necroptotic 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.[188]). 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[188].

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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