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Death by TNF: A Road to Inflammation

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Abstract

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

Introduction

Cell death is increasingly recognized as a major driver of inflammatory disease. Next to 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 danger-associated molecular patterns (DAMPs), that activate immune receptors and induce inflammatory responses. In addition, the inflammatory signaling 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 (PAMPs) from microbes that have breached the 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 anti-microbial 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 pathologies. Accumulating evidence suggests that blocking cell death can revert 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 pathologies in which pharmacological cell death inhibitors may provide therapeutic advantage.

The inflammatory cytokine Tumor 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. This review briefly reminds us of 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 hemorrhagic necrosis of tumors in patients following acute bacterial infections [2]. This anti-cancer activity had already been exploited nearly a century before by the New York surgeon William Coley that described a therapy to treat cancer patients with bacterial extracts termed 'Coley's mixed toxins' [3,4]. Later, lipopolysaccharide (LPS) was isolated from bacterial extracts and shown to induce some tumor 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 'tumor 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 anti-tumor potential for cancer treatment (reviewed in [14]). However, the hope that TNF would be a powerful anti-cancer drug soon faded away when it became clear that administration of the recombinant cytokine induces severe endotoxic shock. Indeed, and independently from these cancer studies, TNF was found to be identical to cachectin, a protein responsible for endotoxin-induced wasting disease (cachexia) in mice [15]. These findings also clearly demonstrated that TNF is a pleiotropic cytokine that must be tightly regulated (**Figure 1 – Timeline**).

Anti-TNF biologicals as most successful drugs in history

TNF's anti-cancer effects turned out to be secondary to its strong pro-inflammatory activities, which shifted the attention to the development of biologicals 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 (RA). TNF inhibition using a neutralizing antibody was shown to block the synthesis of several other important pro-inflammatory 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 RA [16-18]. Soon after, a small-scale clinical study using anti-TNF antibodies was initiated in RA patients demonstrating marked clinical improvement in the majority of patients [19]. Subsequent clinical trials confirmed high efficacy of TNF neutralization in the treatment of RA [20-22], which paved the way for testing TNF inhibitors also in other inflammatory autoimmune diseases (**Figure 1 – Timeline**).

Five distinct antibody- or receptor-based TNF neutralizing drugs have been approved over the years for treating RA, psoriatic arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, psoriasis, Crohn's disease and ulcerative colitis (reviewed in [23]). 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 biologicals 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), in 2009 (reviewed in [24]). 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.

Origin of the protective effect of anti-TNF biologics: inhibition of inflammatory gene activation or inhibition of cell death?

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) and canonical Nuclear factor kappa-κB (NF-κB) pathways, which would otherwise collectively lead to the transcriptional up-regulation of pro-inflammatory genes that underlie the inflammatory

pathology (**Figure 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 pro-inflammatory gene expression in neighboring cells. In addition, epithelial cell death (in the skin or the intestine) may affect barrier integrity inducing microbial tissue infiltration and inflammation (**Figure 2**). Hence, genetic targeting of cell death was shown to revert the inflammatory phenotype in various mouse models of TNF-induced inflammatory diseases (see below). Consequently, drugs that inhibit cell death, such as Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) kinase inhibitors, are currently under investigation as alternative therapies for TNF-driven human pathologies (reviewed in [1,25,26]).

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 only proceeds when one of the cell death checkpoints is inactivated (**Figure 3**). The survival/death outcome of TNFR1 activation depends on the assembly of two distinct, but successive, protein complexes (**Figure 3**) (reviewed in [27,28]). 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 (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-1/2 (cIAP1/2) and linear ubiquitin chain assembly complex (LUBAC) (composed of Heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1), HOIL-1-interacting protein (HOIP) and SHANK-associated RH domain interactor (SHARPIN)). Together, these E3 ligases generate a dense network of ubiquitin chains that permits further recruitment of the kinases that activate the MAPKs and canonical NF-κB signaling pathways. More precisely, the K63-ubiquitin chains generated by cIAP1/2 act as binding stations for the adaptor proteins transforming growth factor-β-activated kinase 1-binding protein 2/3 (TAB2/3) that recruit the upstream kinase transforming growth factor-β-activated kinase 1 (TAK1) for MAPK signaling. On the other hand, the M1(linear)-ubiquitin chains generated by LUBAC are recognized by the adaptor protein NF-κB essential modulator (NEMO) that brings the kinases inhibitor of NF-κB kinase

α/β (IKKα/β) and TANK-binding kinase 1 (TBK1)/IKKε to the receptor complex. The close proximity between TAK1 and IKKα/β on the hybrid K63/M1-ubiquitin chains then permits activation of IKKα/β by TAK1, and the subsequent IKKα/β-dependent activation of the canonical NF-κB pathway (reviewed in [27,28])(**Figure 3**). The ubiquitin network associated to Complex I is negatively regulated by a subset of deubiquitylases (DUBs), including A20, cylindromatosis (CYLD) and OTU deubiquitilase with linear linkage specificity (OTULIN), which destabilize the signaling complex and restrict signaling to MAPKs and NF-κB (reviewed in [29]).

How TNFR1 signaling further evolves to induce cell death is less clear, but requires assembly of a secondary cytosolic complex, termed Complex II, which originates from the binding of FAS-associating death domain-containing protein (FADD) to the receptor-dissociated Complex I components TRADD and/or RIPK1 [30]. 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 [31-33]. Complex II can further be defined as IIa or IIb to differentiate the one that spontaneously assembles upon TNF sensing from the one that additionally forms upon RIPK1 enzymatic activation [34,35] (**Figure 3**). So far, two cell death checkpoints were found to inhibit apoptosis induction by these death complexes. The first one (*'IKK checkpoint'*) takes place at the level of the receptor, within Complex I, and consists in the phosphorylation-dependent inactivation of RIPK1 by IKKα/β and TBK1/IKKε, thereby preventing Complex IIb assembly and subsequent RIPK1 kinase-dependent apoptosis induction [36-38] (**Figure 3**). The fact that single inhibition of IKKα/β or TBK1/IKKε suffices to switch the TNF response from survival to RIPK1 kinase-dependent apoptosis, and the observation that the combined inactivation of these kinases further increases RIPK1 cytotoxicity, suggest that IKKα/β and TBK1/IKKε inhibit distinct pools of RIPK1 in TNFR1 Complex I. Serine 25 (S25) of RIPK1 was identified as a substrate of IKKα/β, TBK1/IKKε and of the phosphatase Protein phosphatase 1 regulatory subunit 3 (GPPP1R3G)/PP1γ [36,37,39], and mimicking phosphorylation on that residue was demonstrated to inhibit RIPK1 activity and cytotoxicity [40]. However, preventing S25 phosphorylation of RIPK1 is not sufficient to activate RIPK1 by TNF, indicating that IKKα/β 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/translational upregulation of pro-survival proteins, such as

cFLIP, which counteract caspase-8 activation in Complex IIa and protect the cells from RIPK1 kinase-independent apoptosis [34,41]. Since IKKα/β 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-dependent (*'IKK checkpoint'*) and -independent (*'NF-κB checkpoint'*) apoptosis (**Figure 3**). In contrast, TBK1/IKKε only repress RIPK1 activation in Complex I and their inactivation consequently only switches the TNF response from survival to RIPK1 kinase-dependent cell death, without disturbing NF-κB [37].

Activation of the kinases that control the two above-mentioned cell death checkpoints is highly dependent on the ubiquitin network associated to complex I. Consequently, conditions that affect ubiquitylation of Complex I, such as cIAP1/2 and LUBAC inhibition [42-49], but also mutations in RIPK1 ubiquitin acceptor site K377 (K376 in mouse RIPK1) [50-52] or A20 and OTULIN deficiencies [53-58], indirectly perturb these cell death checkpoints and activate TNF cytotoxicity. Of note, the inhibitory effect of ubiquitin on RIPK1 death-signaling can be dissociated from its role in inducing NF-κB-mediated gene transcription [50,27]. Interestingly, while 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 (*'casp-8 checkpoint'*) that prevents RIPK1 kinase-dependent apoptosis and necroptosis by cleaving RIPK1 [59-63] (**Figure 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-dependent necroptosis, which additionally requires recruitment of the kinase RIPK3 and of the potential pore-forming pseudo-kinase Mixed lineage kinase domain like (MLKL) to complex II, now called the necrosome. Association between RIPK3 and RIPK1 occurs via their RIP homotypic interaction motifs (RHIM), and appears 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 yet 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 of 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 [28,32] (**Figure 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 to these cell death checkpoints (see below). Of note, additional molecular mechanisms restraining TNF cytotoxicity have been reported, including the phosphorylation of RIPK1 by the MAPK-activated kinase MK2 [64-66] or by the kinase Unc-51 like autophagy activating kinase 1 (ULK1) [67], as well as the poly-ADP-ribosylation (PARylation) of Complex II by Tankyrase-1 [68]. 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 only increases TNF cytotoxicity 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.

Physiological relevance of TNF toxicity: cell death in pathogen defense

Host-pathogen interactions represent 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 hosts' immune defenses. This continuous pressure selects for multiple, layered and interconnected defense mechanisms in the host. Similarly, the pathogen has developed sophisticated strategies to evade host immunity by hijacking inflammatory signaling pathways or by blocking other antimicrobial defense mechanisms. The different TNFR1 cell death checkpoints appear to have evolved as a response of the host to this microbial hijacking. Indeed, TNF signaling aims to establish an inflammatory response, primarily by promoting inflammatory gene activation by the MAPK and NF-κB signaling pathways. Remarkably, the kinases that activate these signaling 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/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 DAMPs. This is nicely illustrated in the context of infection by the mammalian pathogenic

species of the Gram-negative *Yersinia* genus that injects an acyltransferase, named YopJ/P, capable of inhibiting the catalytic activity of TAK1 and IKKα/β in an attempt to escape host defenses by preventing MAPK- and NF-κB-dependent expression of pro-inflammatory mediators [69-72]. As a consequence of this hijacking, RIPK1 is no longer blocked by MK2 and IKKα/β, and TNFR1- and TLR4-mediated RIPK1 kinase-dependent and caspase-8-dependent apoptosis and/or pyroptosis are induced, releasing DAMPs to promote optimal antibacterial immunity [31,32,40,65,73-75].

Cell death, in its multiple forms, is thus recognized as a host defense mechanism for the elimination of pathogens, stripping them of their replication niche and simultaneously alerting the immune system to kick in. As a consequence, microbes have developed multiple strategies to interfere with the different cell death pathways to avoid their eradication by the host (reviewed in [76]). Host cells, however, have on their 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 cytokine response modifier A (CrmA) [77] or the viral FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (vFLIP) identified in herpesviruses and in the human poxvirus *Molluscum contagiosum virus* (MCV) [78,79]. As a response, several pathogens also express proteins that specifically target necroptosis by inhibiting RIPK1, RIPK3, MLKL, or effects downstream of MLKL [76].

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, microbe pathogenicity and lethality, as seen upon infection with SARS-CoV-2, *Mycobacterium tuberculosis,* and *Bacillus anthracis* [80-83].

Pathological consequences of TNF toxicity: cell death-induced inflammation in the pathogenesis of inflammatory disease

While TNF-induced cell death can help mounting proper immune responses during microbial infection, it can also turn into a highly detrimental process at the origin of various (sterile) inflammatory pathologies when aberrantly induced as a result of environmental factors

and/or genetic mutations. It is now clear that TNF not only contributes to the pathogenesis of inflammatory disease 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-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 [84-86]. In this model, the triggering event was first believed to be necroptosis, but later studies suggested additional contribution of RIPK1 kinase-mediated caspase-8-dependent apoptosis and pyroptosis. Indeed, caspase-8 heterozygosity was reported to partially rescue hypothermia, and GSDMD loss to limit lethality [32,87]. The reason why TNF induces RIPK1 kinase-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 (LTβ), activates the non-canonical NF-κB pathway upon binding to their cognate receptors. Activation of this pathway involves the ligand-dependent degradation of a pool of TRAF2/3 and cIAP1/2, resulting in stabilization of NIK, and finally in the activation of IKKα by NIK-mediated phosphorylation [88]. 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-dependent and -independent apoptosis/pyroptosis due to partial cIAP1/2 degradation, affecting proper ubiquitylation of Complex I, which consequently indirectly inactivates two of the above-mentioned cell death checkpoint in the TNFR1 pathway [64,89]. In line with this idea, it is interesting to notice that TWEAK and CD40L are upregulated in inflammatory bowel disease (IBD) and RA [90,91], two TNF-driven human pathologies for which RIPK1 kinase inhibitors may turn very 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-dependent death. It is therefore possible that part of the discrepancy between the *in vitro* and *in vivo* cytotoxic response to TNF originates from difference in TNFR2 expression levels, or in the expression of membrane-bound vs 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 (IFNγ) was also recently reported to

induce RIPK1 kinase-dependent cell death (apoptosis, pyroptosis and necroptosis) by activating the JAK/STAT1/IRF1 axis [80]. It however remains unclear if, and how, activation of this pathway affects the known cell death checkpoints downstream of TNFR1. Interestingly, the combination of neutralizing antibodies against TNF and IFN-γ were shown to protect mice from mortality during SARS-CoV-2 infection [80], which is in line with the reported causative role of pyroptosis in hyperinflammation during severe COVID-19 [92-94].

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 pathologies, as highlighted by some examples in the non-exhaustive list of studies mentioned below (**Table 1 and 2**). The *in vivo* inflammatory consequence of inactivating the caspase-8 checkpoint that prevents RIPK1 kinase-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 [95,96], which can be rescued by deletion of *Ripk1, Ripk3* or *Mlkl* [97-100]. Also, specific deletion of caspase-8 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) [33,101-103]. High levels of RIPK3 and increased necroptosis could be confirmed in the intestine of patients with Crohn's disease [102], and mutations in the *CASP8* gene have been identified in patients that develop autoimmune lymphoproliferative syndrome (ALPS) but also in patients with severe forms of very early onset IBD [104,105]. In IBD, aberrant cell death leads to impairment of the epithelial barrier and invasion of microbiota to the underlying tissues promoting inflammation. Deficiency of the adaptor protein Myeloid differentiation primary response 88 (MyD88) and antibiotic treatment were shown to prevent colon inflammation in IEC-specific FADD-deficient mice, demonstrating that bacteria-mediated Toll-like receptor (TLR) activation by intestinal bacteria is essential for disease pathogenesis [101]. Follow-up studies in mice revealed that FADD not only prevents intestinal inflammation by inhibiting necroptosis, but also by inhibiting caspase-8-GSDMD-mediated pyroptosis of epithelial cells [33]. How FADD simultaneously promotes and inhibits caspase-8 to respectively inhibit necroptosis but promote pyroptosis is currently unclear. Inducible deletion of caspase-8 in the endothelium of 6 weeks-old mice causes fatal hemorrhagic lesions exclusively within the small

intestine driven by microbial commensals and TNF. This phenotype is prevented in mice that lack MLKL, confirming that the hemorrhage is caused by unrestrained necroptosis in the small intestine [106]. Deficiency of FADD or caspase-8 in keratinocytes causes keratinocyte necroptosis and severe skin inflammation in mice, which is prevented by RIPK3 loss and partly dependent on TNF [103,107,108]. Since RIPK3 also contributes to TNF-induced caspase-8 activation [109], 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 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 as an inhibitor of RIPK3-MLKL-dependent necroptosis in keratinocytes [110,111].

More recent studies specifically targeted the caspase-8-dependent cell death 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 [60,61,63]. Importantly, patients with pathogenic mutations in *RIPK1* that prevent caspase-8 cleavage were also identified, and shown to suffer from early-onset auto-inflammatory disease, so-called 'cleavage-resistant RIPK1-induced autoinflammatory syndrome' or CRIA, which is caused by hypersensitivity of patients' cells to RIPK1 activation, apoptosis and necroptosis [61,62,112].

By serving as anchoring sites for the kinases IKKα/β and IKKε/TBK1, the ubiquitin chains conjugated to the TNFR1 Complex I by cIAP1/2 and LUBAC indirectly control the two checkpoints that counteract caspase-8-dependent cell death, either in a RIPK1 kinase-dependent or -independent manner (**Figure 3**). Consequently, mutations that affect proper regulation of ubiquitin signaling can trigger aberrant TNF-mediated cell death and result in inflammatory pathologies (reviewed in [113,114]). This is for instance the case upon deletion of cIAP1/2, which results in embryonic lethality caused by TNFR1 signaling [44]. Further studies demonstrated that deletion of cIAP1/2 in adult mice causes inflammation and lethality by the release of a brake on caspase-8-dependent cell death [115]. Deficiency in ubiquitin ligase X-linked inhibitor of apoptosis protein (XIAP) is the cause for X-linked lymphoproliferative syndrome 2

(XLP2), a severe inflammatory disease [116]. Using 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 XLP2 [117-119].

The notion that linear ubiquitin chains protect against cell death-driven inflammation is supported by the phenotypes of the LUBAC- and OTULIN-deficient mice. Mutation in the *Sharpin* gene, in so-called *Cpdm* mice, causes chronic proliferative dermatitis (cpdm) characterized by inflammatory skin lesions, multi-organ inflammation and immune system dysregulation, that is fully caused by TNF-mediated RIPK1 kinase-dependent cell death [48,86,120-124]. *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 [48,124]. 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 pathology through the sensing of PAMPs from microbes that have breached the barrier [125]. Mutations in *HOIP* or *HOIL-1* lead to embryonic lethality in mice which is partially dependent on TNFR1 and RIPK1 kinase activity but prevented by co-deletion of caspase-8 and MLKL, but not RIPK3 [47,49]. 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 [58,126]. Studies in tissue-specific LUBAC- and OTULIN-deficient mice further confirmed TNF- and RIPK1 kinase-mediated cell death as a driver of inflammatory pathology [46,126,127]. Homozygous loss-of-function mutations in *HOIP*, *HOIL-1* and *OTULIN* have been identified in humans. These mutations are rare and cause a neonatal potentially fatal multi-organ auto-inflammatory condition [128-134]. Most patients with OTULIN-related auto-inflammatory syndrome (ORAS, also known as otulipenia) are successfully treated with TNF blocking agents, identifying TNF as the main driver of the auto-inflammatory condition [131-133]. Recently, two new compound heterozygous variants in OTULIN have been identified in a patient that developed a fulminant atypical late-onset ORAS, that differs clinically from classical ORAS, but is also triggered by perturbed TNF signaling [135].

The role of M1-ubiquitylation in preventing cell death driven-inflammation is further demonstrated with mutations affecting the protein A20. The anti-inflammatory properties of A20 are commonly attributed to its ability to suppress inflammatory NF-κB signaling, but gene targeting studies in mice have demonstrated that A20 primarily suppresses inflammation by preventing cell death [54,56,57,136-142]. In the TNFR1 pathway, A20 represses RIPK1 kinase-dependent and -independent cell death induction by binding and stabilizing the M1-linked ubiquitin chains associated to TNFR1 Complex I [53,54,57,143]. Single nucleotide polymorphisms in *TNFAIP3*, the gene coding for A20, have been linked to many inflammatory and autoimmune diseases [144,145]. Importantly, rare heterozygous loss-of-function variants have been shown to cause a severe auto-inflammatory syndrome, named HA20 (haploinsufficiency of A20) [146,147], which can be treated in most patients with cytokine inhibitors including infliximab [146,147]. Patients with C-terminal deletions in NEMO, impairing interactions with A20, also develop an auto-inflammatory phenotype which resembles HA20 [148].

Binding of NEMO to the M1-ubiquitin chains associated to Complex I permits the recruitment and activation of the kinases IKKα/β and IKKε/TBK1 to the TNFR1 complex I. While these kinases prevent RIPK1 kinase-dependent apoptosis/pyroptosis by phosphorylating RIPK1, IKKα/β additionally repress RIPK1 kinase-independent apoptosis through the NF-κB-dependent expression of pro-survival molecules, including cFLIP (**Figure 3**). The genetic disruption of NEMO, IKKα, IKKβ, or IKKα/β in mice results in early lethality with massive cellular death in several organs, such as the liver, the skin, and, in the case of *Ikkα/β*$^{-/-}$ mice, the nervous system [149-154]. Moreover, specific deletion of NEMO in keratinocytes causes severe and lethal skin inflammation in mice that requires TNF [155]. 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 [156]. Also patients with NEMO-deleted exon 5–autoinflammatory syndrome (NEMO-NDAS) 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 [157,158]. TNF also causes skin inflammation in mice with epidermis-specific knockout of IKKβ or both NF-κB subunits RelA and c-Rel by inducing RIPK1-dependent necroptosis of keratinocytes [159,160]. In humans, a heterozygous mutation in *RELA*, causing RelA haploinsufficiency, causes chronic mucocutaneous ulceration which can be suppressed by anti-TNF therapy [161]. 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 [161]. NEMO deficiency in intestinal epithelial cells triggers intestinal pathology caused by TNF-induced apoptosis [162]. Inhibition of RIPK1 kinase activity or combined deficiency of FADD and RIPK3 prevents epithelial cell 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 [163]. It remains however puzzling that RIPK1 kinase inactivation is sufficient to fully prevent pathology in these mice. Indeed, with a defect in NF-κB activation, the epithelial cells should still be sensitized to RIPK1 kinase-independent apoptosis.

According to its specific role 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 [37,38,164]. Interestingly, loss of TBK1 in mice with a 129 genetic background was reported to be viable, but shown to cause inflammation in multiple tissues [165]. Transferring this allele onto the C57BL/6 background, however, also resulted in embryonic lethality [165]. 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-dependent cell death. Hence, auto-inflammation in these patients is suppressed with anti-TNF therapy [166].

cFLIP plays a central role in NF-κB-dependent cell survival, as shown by the phenotypes of cFLIP deficient mice. Genetic deletion of cFLIP results in embryonic lethality, due to a defect in the vascular development of the yolk sac [167], and combined deletion of FADD and RIPK3 is required for preventing the lethal phenotype of cFLIP deficient mice [168]. IEC-specific cFLIP deficient mice develop severe colitis due to IEC apoptosis and necroptosis, which can be suppressed by deletion of TNFR1 or by treatment with neutralizing anti-TNF antibodies [103,169,170]. Postnatal deletion of cFLIP in keratinocytes induces severe skin inflammation in mice due to TNF-dependent keratinocyte apoptosis [103,171]. Interestingly, loss of cFLIP expression in skin epidermis could be shown in patients with different skin diseases associated with epidermal cell apoptosis [171].

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 [172-174]. RIPK1 deficiency in intestinal epithelial cells in mice induces a severe pathology caused by TNF-mediated IEC apoptosis [110,175]. While 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 [176]. In agreement, patients with RIPK1 deficiency suffer from inflammatory diseases including IBD [177,178].

Perspective : blocking cell death as a new therapeutic approach

Although experimental studies in mice, genetically altered to lack (or express mutant versions of) essential proteins of the apoptotic/necroptotic/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 kinase inhibitors, as well as GSDMD inhibitors, are currently under investigation as potential therapies for human inflammatory pathologies. Such inhibitors may become an alternative treatment for autoimmune patients, especially for those patients that do not respond or show adverse effects to anti-TNF treatment. Indeed, one-third of RA patients will discontinue treatment with an anti-TNF drug in the first year of therapy, mostly because of inefficacy or adverse events [179], and similar efficacy profiles have been shown in patients with IBD and psoriasis [180,181].

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 [182,183]. 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 [25]). Also blood–brain barrier-permeable RIPK1 inhibitors have entered clinical trials for the treatment of neurodegenerative diseases including amyotrophic lateral sclerosis

(ALS), Alzheimer's disease and multiple sclerosis (reviewed in [26]). However, first results from two of such trials using the RIPK1 inhibitor GSK2982772 did not show clinical efficacy in a small group of patients with ulcerative colitis or with RA [184,185]. One explanation for this could be the lack of proper patient stratification, highlighting the need to stratify patients based on detection of necroptosis markers. In this respect, pS166-RIPK1 and p-MLKL antibodies may become useful. However, the requirement of RIPK1 enzymatic activity for TNF-induced cell death may also be different between mouse and human, 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 Casp8-FADD-RIPK1 complex inducing apoptotic cell death [186].

As described above, TNF can trigger caspase-8-dependent GSDMD cleavage [31-33]. Since the discovery of GSDMD as a central mediator of pyroptosis [187,188], GSDMD inhibition has been proposed as a novel therapeutic strategy to prevent inflammatory pathology in different diseases (reviewed in [189]). Disulfiram (Antabuse), an 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 [190] and multiple sclerosis (MS) [191]. Necrosulfonamide (NSA) was shown to be efficacious in sepsis [192], and dimethyl fumarate (DMF), the FDA-approved drug Tecfidera used for the treatment of MS, was reported to suppress pathology in mouse models of familial Mediterranean fever, sepsis and MS [193]. All three currently available GSDMD inhibitors (Disulfiram, NSA and DSF) covalently modify reactive cysteines and hence are not specific, and more specific small-molecular inhibitors of GSDMD will need to be discovered. Since pyroptosis can also be induced via caspase-3-mediated cleavage of GSDME [194,195], and via caspase-8-induced cleavage of GSDMC [196], also other GSDM inhibitors need to be considered.

Future research should also investigate whether Ninjurin-1 inhibition could be beneficial in suppressing 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 [197]. Proof-of-principle studies already demonstrated that an antibody targeting the N-terminal extracellular region of Ninjurin-1 that is shown to be critical for its cytotoxicity, impairs plasma membrane rupture in pyroptotic macrophages [197].

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

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Tables

Table 1 : summary of a selection of studies in mouse models demonstrating that inflammation results from unrestrained cell death.

Genotype	Phenotype	Expected inactivated CDC	Rescue background	Reference
Casp8$^{-/-}$	Embryonic lethality	Casp-8 checkpoint	*Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$	9,97,98,100
Casp8$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte necroptosis		*Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$ *Tnfr1*$^{IEC\text{-}KO}$ (colon) *Ripk1*$^{D138N/D138N}$	33,102,103
Cdh5-CreER^{T2} *casp8*$^{fl/fl}$	fatal necroptotic haemorrhage within the small intestine		*Mlkl*$^{-/-}$ *Tnf*$^{-/-}$ LPS-Rs administration	106
Casp8$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Ripk3*$^{-/-}$ *Tnf*$^{-/-}$ (partial)	103,107
Fadd$^{-/-}$	Embryonic lethality	Casp-8 checkpoint	*Ripk1*$^{-/-}$ *Mlkl*$^{-/-}$	99,100
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*$^{-/-}$	33,101
Fadd$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Ripk3*$^{-/-}$ *Tnf*$^{-/-}$ (partial) *Tnfr1*$^{-/-}$ (partial)	108
Ripk1$^{D325A/D325A}$	Embryonic lethality	Casp-8 checkpoint	*Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$ *Mlkl*$^{-/-}$ *Fadd*$^{-/-}$ *Ripk3*$^{-/-}$ *Fadd*$^{-/-}$ *Ripk1*$^{D138N/D138N}$	60,61,63
clap2$^{-/-}$*clap1*$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$	44,115
CreER^{T2}*clap1*$^{-/-}$ *clap2*$^{fl/fl}$	Lethal upon TAM injection		*Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$	105
Xiap1$^{-/-}$	Ileal inflammation	unknown	*Tnf*$^{-/-}$ *Tnfr1*$^{-/-}$ *Tnfr2*$^{-/-}$ *Ripk3*$^{-/-}$	118
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)	47,48,86,121-124

			Tradd$^{E\text{-}KO}$ *Ripk3*$^{-/-}$ (skin) *Mlkl*$^{-/-}$*Casp8*$^{-/-}$ (skin)	
Hoip$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ (partial) *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$	47,49
Hoip$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ (partial) *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$ *Tnfr1*$^{-/-}$ *Mlkl*$^{-/-}$	46
Hoil1$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ (partial) *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ (partial) *Ripk3*$^{-/-}$*Casp8*$^{-/-}$*Ripk1*$^{-/-}$	47
Hoil1$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ (partial) *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$	46
Otulin^{C129A} *Otulin*^{L272P}	Embryonic lethality	IKK checkpoint NF-κB checkpoint	*Tnfr1*$^{-/-}$ (partial) *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$ *Ripk1*$^{D138N/D138N}$ (partial)	58,126
Otulin$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ *Tnfr1*$^{E\text{-}KO}$ *Ripk1*$^{D138N/D138N}$ *Ripk3*$^{-/-}$ (partial) *Mlkl*$^{-/-}$ (partial) *Ripk3*$^{-/-}$ *Fadd*$^{-/-}$ *Fadd/Mlkl*$^{E\text{-}KO}$	126,127
Nemo$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint		151
Nemo$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte cell death		*Tnfr1*$^{-/-}$	155
Nemo$^{IEC\text{-}KO}$	severe intestinal pathology; enterocyte apoptosis		*Tnfr1*$^{-/-}$ *Tnfr1*$^{E\text{-}KO}$ *Fadd*$^{IEC\text{-}KO}$ *Ripk3*$^{-/-}$ *Ripk3*$^{-/-}$ (partial) *Ripk1*$^{D138N/D138N}$	162,163
Ikkb$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint		149
Ikkb$^{E\text{-}KO}$	Severe skin inflammation; keratinocyte cell death		*Tnfr1*$^{-/-}$ *Tnfr1*$^{E\text{-}KO}$ *Ripk3*$^{-/-}$ (partial) *Ripk3* $^{E\text{-}KO}$ (partial) *Mlkl*$^{-/-}$ (partial) *Fadd*$^{E\text{-}KO}$ *Ripk3*$^{-/-}$ *Ripk1*$^{D138N/D138N}$	159,160
Ikka$^{-/-}$*Ikkb*$^{-/-}$	Embryonic lethality	IKK checkpoint NF-κB checkpoint		150
Ikka/Ikkb$^{IEC\text{-}KO}$	Severe intestinal pathology; enterocyte apoptosis			162
Ripk1$^{K376R/K376R}$	Embryonic lethality	IKK checkpoint	*Mlkl*$^{-/-}$ *Fadd*$^{-/-}$ *Ripk3*$^{-/-}$ *Fadd*$^{-/-}$ *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$ *Tnfr1*$^{-/-}$ (partial) *Tnfr1*$^{-/-}$*Ripk3*$^{-/-}$	51,52

Tbk1$^{-/-}$	Embryonic lethality (C57BL/6 background) Viable (129 background), spontaneous inflammation in multiple tissues	IKK checkpoint	*Ripk1*$^{D138N/+}$ *Ripk1*$^{D138N/D138N}$ *Ripk3*$^{-/-}$ (partial)	38,164 165
RelA$^{+/-}$	cutaneous ulceration from TNF exposure; severe dextran sodium sulfate–induced colitis	NF-κB checkpoint	Anti-TNF antibodies	161
cFlip$^{-/-}$	Embryonic lethality	NF-κB checkpoint	*Ripk3*$^{-/-}$ *Fadd*$^{-/-}$	167,168
cFlip^{IEC-KO}	Severe intestinal pathology; enterocyte death		*Tnfr1*$^{-/-}$ (partial)	103,169,170
CreER^{T2}*cFlip*$^{E-KO}$	Severe skin inflammation; keratinocyte apoptosis		Anti-TNF antibodies (partial)	103,171
Ripk1^{IEC-KO}	severe intestinal pathology; enterocyte apoptosis	NF-κB checkpoint Unknown checkpoint	*Tnfr1*$^{-/-}$ (partial) *Casp8*$^{-/-}$ (colon) *Fadd*$^{IEC-KO}$ (partial) *Fadd*$^{IEC-KO}$ *Ripk3*$^{-/-}$	110,175
Ripk1^{E-KO}	Severe skin inflammation; keratinocyte necroptosis		*Tnfr1*$^{-/-}$ (partial) *Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$	110,111

CDC, cell death checkpoint; CreERT2, Tamoxifen-inducible Cre expression; E-KO, epidermis-specific knockout; IEC-KO, intestinal epithelial cell-specific knockout; LPS-Rs, LPS from Rhodobacter spheroides which acts as an inhibitor of TLR4 signaling; TAM, tamoxifen

Table 2 - auto-inflammatory diseases caused by mutations in genes coding for essential TNF signalling proteins

Gene name	Protein name	Disease mechanism	Patient phenotype	OMIM	Reference
CASP8	Caspase-8	Homozygous, loss-of-function	autoimmune lymphoproliferative syndrome (ALPS) Very early onset IBD	607271	[104,105]
IKBKG	NEMO	Loss-of-function	Incontinentia Pigmenti in heterozygous females (lethal in males) immunodeficiency; EDA-ID	308300	[156]
IKBKG	NEMO	C-terminal deletions in NEMO	Inflammatory skin and intestinal disease, ectodermal dysplasia with anhidrosis and immunodeficiency	NA	[148]
IKBKG	NEMO	NEMO lacking the domain encoded by exon 5	Severe autoinflammatory syndrome; NEMO-NDAS	301081	[157,158]
OTULIN	OTULIN	Homozygous, loss-of-function	Early-onset recurrent fever, neutrophilic dermatitis/panniculitis, joint swelling; ORAS or otulipenia	617099	[131-134]
OTULIN	OTULIN	Compound heterozygous variants	Atypical late-onset ORAS	NA	[135]
RBCK1	HOIL-1	Homozygous, loss-of-function	Severe multiorgan autoinflammation, immunodeficiency, invasive bacterial infections, muscular amylopectinosis	615895	[129]
RELA	RelA	Haploinsufficiency	Fever, abdominal pain, mucocutaneous lesions	618287	[161]
RIPK1	RIPK1	Homozygous, loss-of-function	Recurrent infections, early-onset IBD, progressive polyarthritis, immunodeficiency	618108	[177,178]
RIPK1	RIPK1	Heterozygous mutation of the RIPK1 caspase-8 cleavage site	Early-onset periodic fever syndrome and lymphadenopathy; CRIA	618852	[61,62,112]
RNF31	HOIP	Homozygous, loss-of-function	Severe multiorgan autoinflammation, immunodeficiency	NA	[128,130]
TBK1	TBK1	Homozygous, loss-of-function	chronic and systemic autoinflammation	NA	[166]
TNFAIP3	A20	Haploinsufficiency	Early-onset severe multiorgan autoinflammatory syndrome; HA20	616744	[146,147]
XIAP	XIAP	Loss-of-function	pathogen-associated hyperinflammation, fever, severe IBD; XLP2	300635	[116]

CRIA, cleavage-resistant 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; NEMO-NDAS, NEMO-deleted exon 5–autoinflammatory syndrome; OMIM, online mendelian inheritance in man; XLP2, X-linked lymphoproliferative syndrome 2

Figure legends

Figure 1 – Timeline

Figure 2 – Inflammatory signaling by TNFR1. (A) Binding of TNF to TNFR1 directly promotes inflammation by activating the NF-κB and MAPKs signaling pathway, 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 Danger Associated Molecular Patters (DAMPs) that activate inflammatory gene activation 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 and the subsequent sensing of PAMPs from microbes that have breached the barrier.

Figure 3 – Signaling by TNFR1 and overview of the three characterized cell death checkpoints in the TNFR1 pathway. (A) Sensing of TNF by TNFR1 leads to the formation of a primary membrane-bound receptor signaling complex (Complex I) that activates the MAPKs and NF-κB signaling pathways, leading to inflammatory gene expression. A secondary, potentially cytotoxic, cytosolic complex (Complex II) originates from dissociation of Complex I components from the receptor, and from their association with FADD and caspase-8. Three cell death checkpoints actively repress TNF cytotoxicity: the '*IKK checkpoint*' consists in the inhibition of RIPK1 kinase activity through IKKα/β- and TBK1/IKKε-mediated phosphorylation, the '*NF-κB checkpoint*' in the NF-κB-dependent transcriptional upregulation of pro-survival genes (including cFLIP), and the '*caspase-8 checkpoint*' in RIPK1 inactivation by caspase-8-meditated 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/β activation will additionally inactivate the '*NF-κB checkpoint*'. (C) Conditions leading to inactivation of the '*NF-kB checkpoint*', such as the *in vitro* use of the translation inhibitor cycloheximide (CHX), activates Complex IIa and results in RIPK1 kinase-independent apoptosis. (D) Inhibition of the '*caspase-8 checkpoint*' induces RIPK1 cytotoxicity by the

kinase-dependent assembly of Complex IIb and of the necrosome. TNF sensing in caspase-8-inhibited conditions will only result in necroptosis induction. Additional checkpoint(s) may exist.

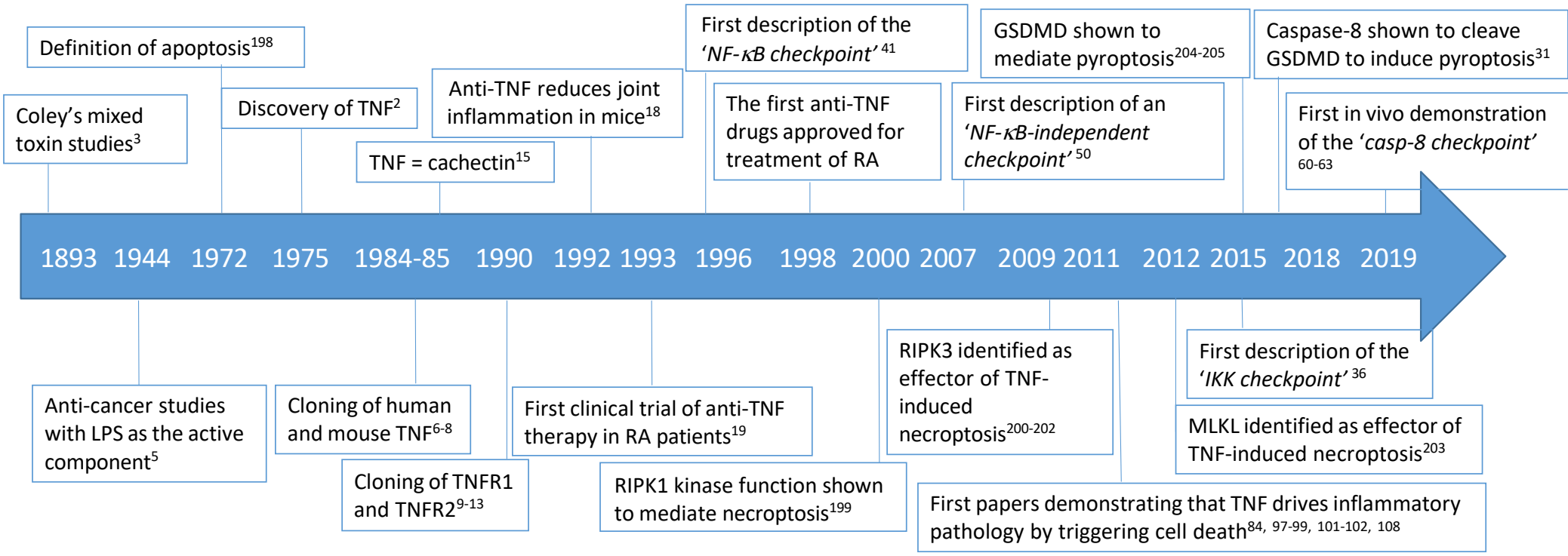
Coley's mixed toxin studies[3]
Definition of apoptosis[198]
Discovery of TNF[2]
TNF = cachectin[15]
Anti-TNF reduces joint inflammation in mice[18]
First description of the 'NF-κB checkpoint' [41]
The first anti-TNF drugs approved for treatment of RA
First description of an 'NF-κB-independent checkpoint' [50]
GSDMD shown to mediate pyroptosis[204-205]
Caspase-8 shown to cleave GSDMD to induce pyroptosis[31]
First in vivo demonstration of the 'casp-8 checkpoint' [60-63]
1893 1944 1972 1975 1984-85 1990 1992 1993 1996 1998 2000 2007 2009 2011 2012 2015 2018 2019
Anti-cancer studies with LPS as the active component[5]
Cloning of human and mouse TNF[6-8]
Cloning of TNFR1 and TNFR2[9-13]
First clinical trial of anti-TNF therapy in RA patients[19]
RIPK1 kinase function shown to mediate necroptosis[199]
RIPK3 identified as effector of TNF-induced necroptosis[200-202]
First papers demonstrating that TNF drives inflammatory pathology by triggering cell death[84, 97-99, 101-102, 108]
First description of the 'IKK checkpoint' [36]
MLKL identified as effector of TNF-induced necroptosis[203]

Figure 1

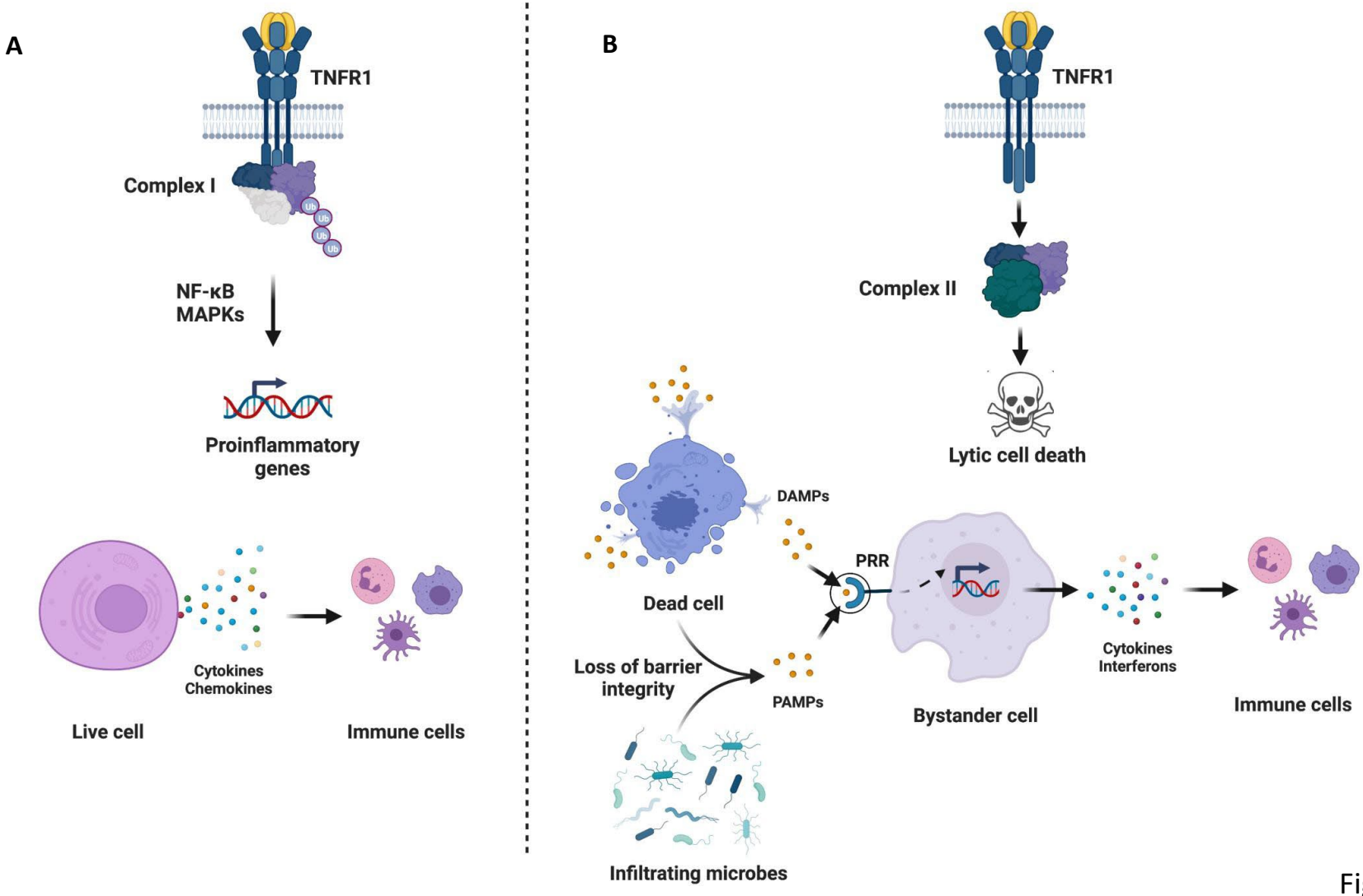
A
TNFR1
Complex I
Ub
Ub
Ub
NF-κB
MAPKs
Proinflammatory
genes
Cytokines
Chemokines
Live cell
Immune cells
B
TNFR1
Complex II
Lytic cell death
DAMPs
PRR
Dead cell
Loss of barrier
integrity
PAMPs
Bystander cell
Cytokines
Interferons
Immune cells
Infiltrating microbes

Figure 2

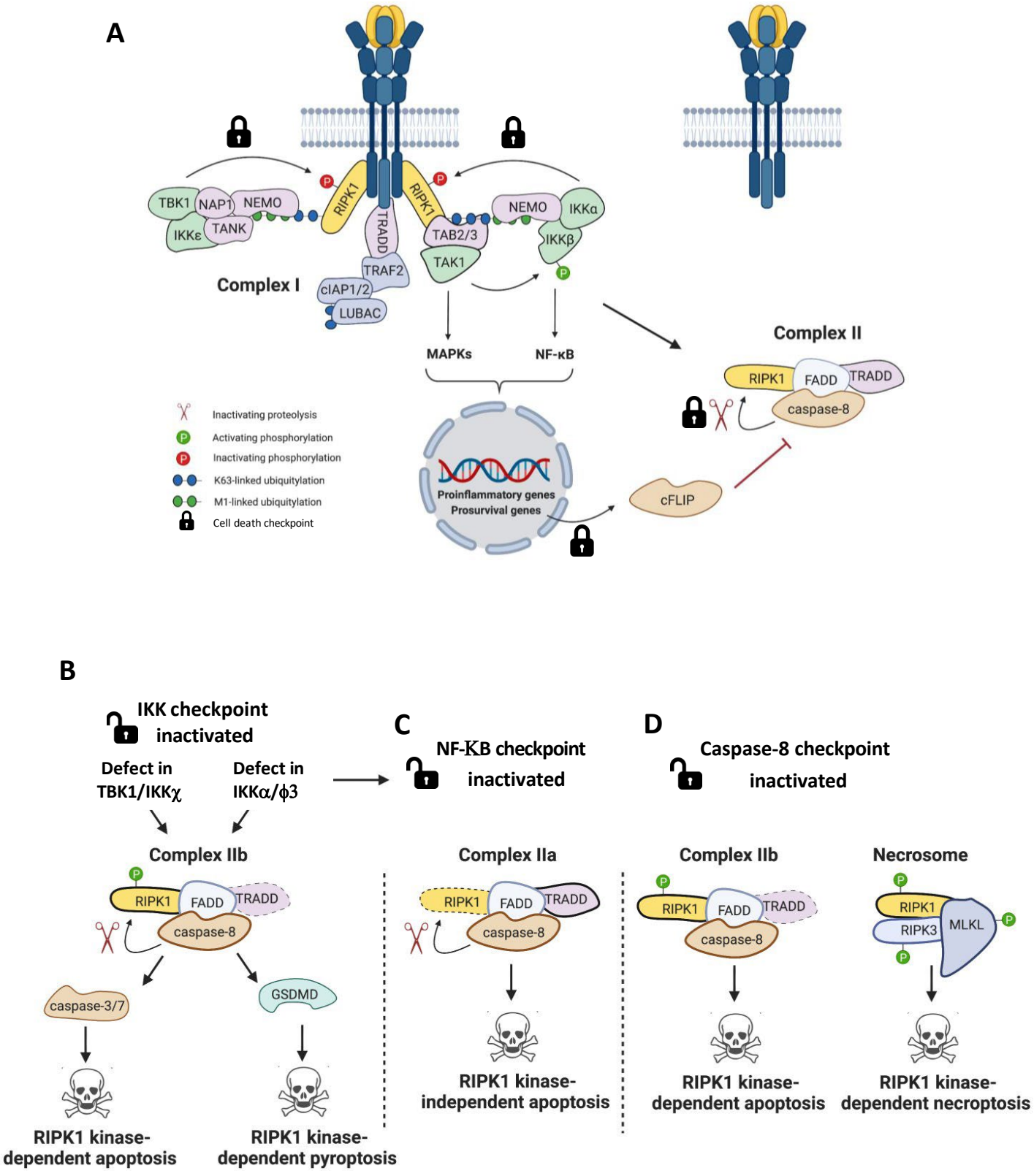
A
TBK1
NAP1
NEMO
IKKε
TANK
RIPK1
TRADD
TRAF2
cIAP1/2
LUBAC
Complex I
RIPK1
TAB2/3
TAK1
NEMO
IKKα
IKKβ
MAPKs
NF-κB
Proinflammatory genes
Prosurvival genes
cFLIP
Complex II
RIPK1
FADD
TRADD
caspase-8
Inactivating proteolysis
Activating phosphorylation
Inactivating phosphorylation
K63-linked ubiquitylation
M1-linked ubiquitylation
Cell death checkpoint
B
IKK checkpoint inactivated
Defect in TBK1/IKKχ
Defect in IKKα/ϕ3
Complex IIb
RIPK1
FADD
TRADD
caspase-8
caspase-3/7
GSDMD
RIPK1 kinase-dependent apoptosis
RIPK1 kinase-dependent pyroptosis
C
NF-ΚB checkpoint inactivated
Complex IIa
RIPK1
FADD
TRADD
caspase-8
RIPK1 kinase-independent apoptosis
D
Caspase-8 checkpoint inactivated
Complex IIb
RIPK1
FADD
TRADD
caspase-8
RIPK1 kinase-dependent apoptosis
Necrosome
RIPK1
RIPK3
MLKL
RIPK1 kinase-dependent necroptosis

Figure 3