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1 # Cell Death Checkpoints in the TNF Pathway

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ABSTRACT

Tumor Necrosis Factor (TNF) plays a central role in orchestrating mammalian inflammatory responses. It promotes inflammation either directly by inducing inflammatory gene expression, or indirectly by triggering cell death. TNF-mediated cell death-driven inflammation can be beneficial during infection by providing cell-extrinsic signals that help mounting proper immune responses. Uncontrolled cell death by TNF is instead highly detrimental and believed to cause several human autoimmune diseases. Death is not the default response to TNF sensing. Molecular brakes, or cell death checkpoints, actively repress TNF cytotoxicity to protect the organism from its detrimental consequences. These checkpoints therefore constitute essential safeguards against inflammatory diseases. Recent advances in the field revealed the existence of several new and unexpected brakes against TNF cytotoxicity and pathogenicity.

MAIN TEXT

TNF-INDUCED CELL DEATH: A FINE LINE BETWEEN PHYSIOLOGY AND PATHOLOGY

Inflammation, the first response of the immune system to invading pathogens and injured tissue, is a beneficial response that protects the body from these insults. Dysregulation of inflammatory signaling pathways by environmental factors and/or genetic predispositions can however turn inflammation into a maladaptive response that causes damage to the body, as seen in various acute and chronic inflammatory pathologies (such as sepsis, psoriasis, arthritis, asthma and inflammatory bowel disease) [1]. Tumor Necrosis Factor (TNF) is a master pro-inflammatory cytokine, whose pharmacological inhibition proved to be beneficial in the treatment of various inflammatory disorders [2]. In mice and humans, TNF directly promotes inflammation by activating the MAPK and NF-κB signaling pathways, which collectively lead to the transcriptional upregulation of various pro-inflammatory genes. TNF also indirectly promotes and exacerbates inflammation by triggering cell death (apoptosis, **necroptosis** and **pyroptosis**), which induces the production of pro-inflammatory mediators by activating innate immune receptors on neighboring cells. Indeed, lytic forms of cell death, including apoptosis-driven **secondary necrosis**, necroptosis, and pyroptosis, release intracellular constituents, known as **Damage-Associated Molecular Patterns (DAMPs)**, that bind and activate **Pattern Recognition Receptors (PRRs)** [3]. Furthermore, by causing the death of epithelial cells, TNF may affect barrier integrity and additionally initiate inflammatory signaling through PRR-mediated sensing of **Pathogen-Associated Molecular Patterns (PAMPs)** from microbes that have breached the epithelial barriers [3].

TNF-induced cell death has been shown to be beneficial in the context of microbial infection, helping to mount proper immune responses by eliminating infected cells and by alerting the environment through the release of DAMPs [3]. Nevertheless, this response needs tight regulation since excessive TNF cytotoxicity can also be the cause of multiple (sterile) inflammatory diseases. Therefore, cell demise is not the standard outcome of TNF sensing. Molecular brakes, known as cell death checkpoints, actively counteract TNF cytotoxicity. Cell demise only occurs when one of these protective brakes is inactivated in the pathway [1]. This situation can occur as a consequence of the action of microbial effector proteins that attempt to interfere with host immune signaling, or alternatively, due to environmental factors and/or genetic mutations [1]. The cell death checkpoints present in the TNF pathway therefore constitute safeguards for physiological inflammation and prevent development of inflammatory pathologies, as evidenced by the phenotypes of mice and humans deficient in their constituents. This review provides an update on the cell death checkpoints in the TNF pathway characterized so far, and briefly discusses the physiological and pathological consequences

54 of their inactivation. Recent findings revealed unexpected crosstalk with other signaling pathways,
55 such as autophagy and the JAK-STAT pathway, widening the spectrum of patients with pathologies
56 that may benefit from anti-TNF therapies.

57

58 TNFR1 SIGNALING: TWO COMPLEXES THAT REGULATE LIFE AND 59 DEATH

60 In mice and humans, TNF binds both TNFR1 and TNFR2, but most of its biological activity has been
61 associated to TNFR1, the only of the two receptors with cytotoxic potential [4]. The outcome of
62 TNFR1 activation depends on the assembly of two successive complexes [5] (**Figure 1**). **Complex I**
63 forms within seconds of TNF sensing. It is a receptor-bound complex that predominantly signals to
64 MAPK- and NF-κB-dependent gene activation (Reviewed in [6,7]). The binding of TRADD and
65 RIPK1 to the cytosolic portion of TNFR1 serves as an initial step in Complex I assembly. The
66 subsequent recruitment of TRAF2/5 and of the ubiquitin ligases cIAP1/2 and LUBAC (composed
67 HOIL-1, HOIP and SHARPIN) then leads to the generation of a dense network of ubiquitin chains
68 around Complex I [8,9]. These ubiquitin chains stabilize the complex and permit further recruitment
69 of additional proteins, including the kinases that activate the MAPK- and NF-κB signaling pathways.
70 Specifically, cIAP1/2 conjugate various types of ubiquitin chains, including K63-linked chains, to
71 RIPK1 and other Complex I components [9–16]. These ubiquitin chains serve as scaffolds for the
72 recruitment and activation of the TAK1 complex, which consists of the catalytic subunit TAK1 and of
73 the adaptor proteins TAB2/3 that specifically bind to these K63-linked chains. Upon recruitment,
74 TAK1 auto-phosphorylates and activates downstream MAPK signaling pathways. The enzymatic
75 activity of cIAP1/2 is additionally needed for the recruitment of LUBAC, which further decorates
76 Complex I components with linear (M1)-linked ubiquitin chains, generating, in some cases, hybrid
77 K63/M1-linked chains [17]. The adaptor protein NEMO (also known as IKKγ) specifically binds to
78 M1-ubiquitin chains, and brings along kinases IKKα/IKKβ and TBK1/IKKε (via the adaptor proteins
79 TANK/NAP1) [18]. The close proximity between TAK1 and IKKα/IKKβ on the hybrid K63/M1-
80 ubiquitin chains allows phosphorylation of IKKα/IKKβ by TAK1, and the subsequent IKKα/IKKβ-
81 dependent activation of the canonical NF-κB pathway. In brief, the phosphorylation of IκBα by IKKβ
82 leads to its K48-ubiquitination and subsequent proteasomal degradation. Once released from its
83 inhibitor, the NF-κB dimer RELA (p65)/p50 migrates to the nucleus to induce TNF-dependent gene
84 expression [19]. Since K63- and M1-ubiquitination are pivotal in relaying the TNF signal, the ubiquitin
85 network associated to Complex I is subject to tight regulation. A subset of deubiquitinases (DUBs)
86 negatively regulates the ubiquitin network associated to Complex I and thereby controls the dynamics
87 of MAPK and NF-κB signaling (Reviewed in [20]). Among them, the protein A20 was proposed to

88 dock on the M1-ubiquitin chains in Complex I and to protect them from CYLD-mediated degradation
89 [21–23]. In contrast, OTULIN indirectly impacts on the number of M1-ubiquitin chains associated to
90 Complex I by repressing LUBAC auto-ubiquitination and regulating its activity outside of the complex
91 [21,24,25].

92 Shortly after their initial binding to TNFR1, RIPK1 and TRADD dissociate from the receptor. As a
93 consequence, the receptor-dissociated complex, now referred to as **Complex II**, translocates to the
94 cytosol where it recruits FADD and the protease CASPASE-8 (CASP8) [5,26,27] (**Figure 1**). This
95 contrasts with the recruitment of FADD and CASP8 to the receptor-associated **Death Inducing**
96 **Signaling Complex (DISC)** that forms upon sensing of other ligands of the TNF superfamily, such as
97 TRAIL and FasL. Of note, Complex II is decorated with M1-ubiquitin chains [28,29], which may have
98 been inherited from Complex I or, alternatively, conjugated after its dissociation from the receptor.
99 With the recruitment of CASP8, Complex II gains cytotoxic potential (**Figure 2**). Indeed, activation
100 of CASP8 within the complex may allow processing of downstream effector caspases to induce
101 apoptosis, or alternatively, cleavage of GSDMD to trigger pyroptosis, as recently reported [30–32].
102 Furthermore, activation of RIPK1 within Complex II may also allow its association with RIPK3 to
103 form the **Necrosome** and to trigger necroptosis. Indeed, the interaction between RIPK1 and RIPK3
104 appears sufficient to activate RIPK3, and the phosphorylation of MLKL by RIPK3 can then induce a
105 conformational change in MLKL, resulting in its oligomerization and translocation to the plasma
106 membrane for necroptosis induction [33]. Nevertheless, despite formation of Complex II, cell death is
107 not the default response of most cells to TNF sensing. Several cell death checkpoints are in place to
108 limit the cytotoxic potential of Complex II and prevent unnecessary damage to the organism.
109 Moreover, the molecular mechanisms of these safeguards are intricately connected to the proper
110 functioning of the TNF signaling pathway. It therefore appears that Complex II assembly has evolved
111 as a backup mechanism to ensure rapid induction of multiple cellular suicide routes when the cell is
112 facing a serious threat.

113

114 CELL DEATH CHECKPOINTS: PUTTING THE BRAKES ON COMPLEX II

115 The NF-κB checkpoint: Limiting CASP8 activity

116 The first identified cell death checkpoint is transcription/translation-dependent and consists in the NF-
117 κB-dependent upregulation of pro-survival proteins (**Figure 1**). This checkpoint is shared with other
118 death ligands of the TNF family, including TRAIL and FasL [34,35]. The existence of this checkpoint
119 is nicely illustrated by the wide in vitro use of the protein translation inhibitor cycloheximide (CHX)
120 to induce CASP8-dependent apoptosis in murine and human cells stimulated with these TNF-related
121 ligands. Among the NF-κB responsive genes, the one encoding cFLIP plays a major role in preventing

122 cell death induction (Reviewed in [36]). cFLIP is a proteolytically inactive CASP8 homolog that forms
123 heterodimers with CASP8, thereby preventing trans-CASP8 auto-processing and full-blown CASP8
124 activation [37]. Because cFLIP is a protein with a short half-life, blocking its TNF-mediated NF-κB-
125 dependent upregulation quickly results in reduced cFLIP levels, allowing full-blown activation of
126 CASP8 within Complex IIa (or within the DISC following TRAIL or FasL stimulation) and apoptosis
127 induction [5,26,38–43] (**Figure 2**). Notably, although RIPK1 is a constituent of Complex II, specific
128 inactivation of this cell death checkpoint in cultured fibroblasts and epithelial cells (obtained for
129 example by CHX pre-treatment [26], expression of a degradation-resistant IκBα mutant [44] or RELA
130 (p65) deficiency [45] and cFLIP deficiency [26]) does not lead to its enzymatic activation, and the cell
131 death observed in these conditions is consequently referred to as RIPK1 kinase-independent apoptosis.
132 A defect in any of the upstream components of the TNF pathway required for NF-κB signaling (such
133 as in cIAP1/2, TAK1, IKK, NEMO and LUBAC) will also disturb this cell death checkpoint, and
134 consequently trigger RIPK1 kinase-independent apoptosis. However, because these proteins may
135 additionally contribute to the IKK checkpoint (that represses RIPK1 cytotoxicity) (see below), their
136 deletion will also cause RIPK1 kinase-dependent apoptosis, which becomes the predominant form of
137 cell death in such conditions.
138

139 **The unconventional autophagy checkpoint: Removing active CASP8**

140 Even though cFLIP greatly limits CASP8 activation within Complex II, it does not fully abrogate its
141 proteolytic activity. This is illustrated by the CASP8-dependent cleavage of the Complex II
142 components cFLIP and RIPK1 upon stimulation of murine and human cells with TNF [5,26,46,47].
143 An unconventional LC3-independent selective autophagy pathway was recently found to serve as a
144 second cell death checkpoint in the TNF pathway by promoting the lysosomal degradation of active
145 CASP8 contained within Complex II in murine and human fibroblasts [48] (**Figure 1**) (**BOX 1**).
146 Results from structure-function analysis and immunoprecipitation experiments suggested that the M1-
147 ubiquitin chains conjugated to RIPK1 in Complex II serve as a cargo recognition signal for the
148 selective autophagy receptor TAX1BP1, which bridges Complex II to the autophagy machinery
149 through direct interaction with FIP200 [48]. The subsequent recruitment of additional autophagy
150 proteins, including the lipid scramblase ATG9A, would then promote the in situ autophagosome
151 formation around Complex II [48]. Once encapsulated and awaiting lysosomal destruction, active
152 CASP8 would be sequestered away from the cytosol, no longer able to access apoptotic substrates. In
153 contrast to the NF-κB checkpoint, this unconventional autophagy checkpoint is not shared with other
154 death ligands of the TNF family since it specifically involves the turnover of a cytosolic CASP8-
155 activating complex [48]. Defects in this detoxification process result in the cytosolic accumulation of
156 active CASP8, which eventually results in full-blown activation of CASP8, switching the TNF

157 response from survival to RIPK1 kinase-independent apoptosis (**Figure 2**). Such scenario is observed
158 upon deficiency in the ATG proteins involved in this unconventional LC3-independent process, but
159 also upon deficiency in M1-ubiquitination or in RIPK1 [48]. The latter conditions block the lysosomal
160 destruction of Complex II by removing TAX1BP1's recognition signal within the cargo [48]. The
161 identification of this new detoxification process clarifies the previously reported NF-κB-independent
162 pro-survival role of RIPK1 [49] and reveals an additional function of LUBAC in counteracting TNF-
163 mediated RIPK1 kinase-independent apoptosis. Together, these results demonstrate the cytotoxic
164 potential of the Complex II that forms upon TNF sensing alone and identify Complex II as a new cargo
165 of the recently discovered unconventional autophagy pathway.
166

167 **The IKK checkpoint: Repressing RIPK1 enzymatic activity in Complex I**

168 Besides having a pro-survival scaffolding role in Complex I and II, RIPK1 can also turn into a killer
169 kinase that promotes Complex II assembly following its enzymatic activation in Complex I [50]. The
170 Complex II that assembles upon sensing of TNF alone has accordingly been renamed IIa to
171 differentiate it from IIb that forms in conditions triggering RIPK1 kinase activation. It however
172 remains unclear if these two complexes are truly distinct or if they instead represent one unique
173 complex, in which case the catalytic activity of RIPK1 would be required for its stabilization or
174 amplification. Noteworthy, it remains unknown how activated RIPK1 kills, as to our knowledge, no
175 lethal substrate of RIPK1 has been identified. To prevent Complex IIb formation and the induction of
176 RIPK1 kinase-dependent apoptosis, a third cell death checkpoint represses RIPK1 enzymatic activity
177 in Complex I. This additional checkpoint consists in the phosphorylation-dependent inactivation of
178 RIPK1 by IKKα/β [45] and TBK1/IKKε [18,51,52] (**Figure 1**). The inhibitory functions of IKKα/β
179 and TBK1/IKKε on RIPK1 are non-redundant, as the individual inactivation of each set of kinases
180 suffices to switch the TNF response from survival to RIPK1 kinase-dependent apoptosis [18,45,52].
181 In addition, their combined inhibition in murine fibroblasts further increases RIPK1 cytotoxicity,
182 which may indicate that IKKα/β and TBK1/IKKε repress distinct pools of RIPK1 in Complex I. RIPK1
183 was found to be phosphorylated on many residues (Reviewed in [50]), and Ser^{25} was identified as a
184 common substrate of IKKα/β [53], TBK1/IKKε [18] and of the phosphatase complex PPP1R3G/PP1γ
185 [54]. Moreover, phosphorylation of Ser^{25} inhibits RIPK1 enzymatic activity, but preventing this
186 phosphorylation event is insufficient to induce RIPK1 cytotoxicity following TNF sensing [53].
187 Therefore, the inhibitory roles of IKKα/β and TBK1/IKKε on RIPK1 extend beyond Ser^{25}
188 phosphorylation. A recent study reported that the PIAS1-dependent decoration of RIPK1 with SUMO
189 chains in Complex I promotes RIPK1 kinase activity and cytotoxicity in murine fibroblasts and
190 hepatocytes [55], a process that was counteracted by the deSUMOylating enzyme SENP1 [55]. It is
191 interesting to speculate that IKKα/β and TBK1/IKKε may also negatively regulate RIPK1

SUMOylation by PIAS1, as this would explain why preventing RIPK1 phosphorylation on Ser^{25} is insufficient to switch the TNF response to RIPK1 kinase-dependent cell death. However, the mechanism by which SUMOylation of RIPK1 leads to its activation awaits characterization.

Apart from the direct inactivation of IKKα/β or TBK1/IKKε, tempering with their ubiquitination-dependent recruitment to Complex I (for instance by cIAP1/2 or LUBAC deficiencies [8,9], mutation of the RIPK1 ubiquitin acceptor site Lys^{377} (Lys^{376} in mice) [56–60], NEMO [61], A20 [22] or OTULIN [62] deficiencies) or activation within Complex I (for instance by TAK1 inhibition [44]) indirectly perturbs the IKK checkpoints and switches the TNF response from survival to RIPK1 kinase-dependent apoptosis [51]. In contrast to TBK1/IKKε, IKKα/β function as upstream kinases in the NF-κB pathway [18,52]. Consequently, inhibition of IKKα/β not only results in RIPK1 kinase-dependent apoptosis by disrupting the IKK checkpoint, but also in RIPK1 kinase-independent apoptosis by additionally affecting the NF-κB checkpoint (**Figure 2**). Such a scenario is consequently also observed upon deficiency in any component of the TNF pathway required for proper IKKα/β activation (such as in cIAP1/2, TAK1, IKK, NEMO). Of note, through the generation of M1-ubiquitin chains, LUBAC contributes to the NF-κB, unconventional autophagy and IKK checkpoints. Its inactivation therefore also results in a combination of RIPK1 kinase-dependent and -independent apoptosis.

The non-receptor Tyrosine kinases checkpoint: Repressing RIPK1 enzymatic activity in Complex I and II

It was recently reported that inhibition of the non-receptor Tyrosine kinases JAK1 or SRC also switches the TNF response to RIPK1 kinase-dependent apoptosis in murine fibroblasts and BMDMs [63] (**Figure 1**). The inhibitory function of these kinases on RIPK1 is non-redundant. Indeed, their individual inactivation suffices to activate RIPK1 cytotoxicity, and their combined inhibition further sensitizes cells to RIPK1 kinase-dependent apoptosis [63]. Both JAK1 and SRC phosphorylate the same essential RIPK1 residue Tyr^{384} (Tyr^{383} in mice), but while JAK1 phosphorylates RIPK1 in Complex I, SRC does so inside Complex II, indicating that different pools of RIPK1 are differentially regulated in a spatial and temporal fashion [63] (**Figure 1**). How JAK1 and SRC are respectively recruited to Complex I and II is unknown, but may involve direct interaction with RIPK1 [63]. It is suggested that the activation of RIPK1 cytotoxicity in JAK1- and SRC-inhibited conditions is caused, at least partially, by impaired recruitment and activation of MK2, another direct kinase of RIPK1 [64–67]. This causal link is however unclear and requires further investigation. Indeed, MK2 deficiency in murine fibroblasts and BMDMs was previously shown not to switch the TNF response to death but only to increase cytotoxicity when one of the checkpoints was already inactivated, therefore acting as a secondary protective mechanism against cell death (**BOX 2**). Nevertheless, the fact that RIPK1$^{Y383F/Y383F}$-expressing cells are still sensitized to TNF-induced cell death in the presence of BV6

227 (a cIAP1/2 antagonist) suggests that the regulation of RIPK1 cytotoxicity by JAK1 and SRC is
228 independent of IKKα/β and TBK1/IKKε [63]. Indeed, the latter kinases require cIAP1/2-dependent
229 ubiquitin chains to be recruited to Complex I [18,45,52]. In summary, this discovery highlights the
230 unexpected involvement of other immune signaling pathways in regulating life/death decisions
231 downstream of TNF.
232
233 **The CASP8 checkpoint: Cleaving the culprits**
234 The goal of the cell death checkpoints described earlier is to prevent lethal activation of CASP8 within
235 Complex II. Paradoxically, complete inhibition of CASP8 switches the cellular response to TNF from
236 survival to death in murine fibroblasts [68]. Indeed, the CASP8-dependent cleavage of RIPK1 at Asp^{324}
237 (Asp^{325} in mice) in Complex II serves as an additional checkpoint in the TNF pathway that prevents
238 RIPK1 kinase-dependent apoptosis and necroptosis [47,69–72] (**Figure 1**). By cleaving RIPK1,
239 CASP8 limits the cytosolic accumulation of kinase-active RIPK1 that can either promote Complex IIb
240 assembly or instead associate with RIPK3 to form the Necrosome. Accordingly, inactivation of this
241 checkpoint by CASP8 inhibition in murine fibroblasts and primary human fibroblasts and PBMCs
242 solely switches the TNF response to RIPK1 kinase-dependent necroptosis and not apoptosis (**Figure
243 2**) [47,69–72]. How RIPK1 enzymatic activity promotes CASP8 processing and RIPK3 recruitment
244 and activation remain outstanding questions in the field. As mentioned, to our knowledge, no lethal
245 substrates of RIPK1 have been identified. Of note, the early detection of RIPK1 fragments suggests
246 that RIPK1 cleavage by CASP8 occurs prior to encapsulation of Complex II into an autophagosome,
247 indicating that the unconventional autophagy checkpoint and the CASP8 checkpoint co-exist [5,48].
248 This may suggest that an M1-ubiquitinated fragment of RIPK1 remains inside Complex II to serve as
249 a marker for lysosomal degradation. Accordingly, the domain structure of RIPK1 implies that cleavage
250 of RIPK1 at Asp^{312} would create a fragment that lacks the kinase domain but still contains both the
251 Dead Domain (DD) and the predominant M1-ubiquitin acceptor sites Lys^{377}/Lys^{627} (Lys^{376}/Lys^{612} in
252 mice) [57,73,74]. We posit that this M1-ubiquitinated fragment likely stays connected to Complex II
253 via the DD-interaction with FADD. Alternatively, it is also possible that CASP8 only cleaves some of
254 the RIPK1 molecules in Complex II, allowing full-length ubiquitinated RIPK1 to drive detoxification.
255 In addition to RIPK1, the CASP8-mediated cleavage of the DUB CYLD has also been proposed to act
256 as a brake on TNF-induced necroptosis [75,76]. The finding that CYLD increases the enzymatic
257 activity of RIPK1 in Complex II [76] led to the hypothesis that its cleavage by CASP8 would prevent
258 RIPK1 kinase-dependent necroptosis induction. In line with this hypothesis, in vitro expression of a
259 cleavage-resistant CYLD mutant in murine fibroblasts was shown to switch the TNF response from
260 survival to necroptosis regardless of CASP8 activity [75]. However, in contrast to the $Ripk1^{D325A/D325A}$
261 (uncleavable) mice that die during embryogenesis, those expressing a cleavage-resistant CYLD mutant

262 develop normally [69–72], thereby questioning the relevance of this cleavage event as a cell death
263 checkpoint. Similarly, proteolytic processing of RIPK3 by CASP8 is proposed to counteract
264 necroptosis induction by promoting Necrosome disassembly [77], but the physiological importance of
265 this cleavage awaits demonstration. In conclusion, while CASP8 can promote apoptosis it
266 paradoxically also prevents RIPK1 kinase-dependent cell death by cleaving Complex II components.
267

268 MULTIPLE CHECKPOINTS: THE BRIGHT AND DARK SIDES

269 To date, a minimum of five non-redundant cell death checkpoints have been characterized downstream
270 of TNFR1 in mice and humans. These molecular brakes on TNF cytotoxicity act at different stages of
271 the TNF signaling pathway and are consequently localized within different cellular compartments
272 [1,48,63]. Several lines of evidence indicate that more checkpoints remain to be discovered and
273 characterized. For instance, deficiency in additional components of the pathway, such as ABIN1, also
274 switches the TNF response to death in murine fibroblasts [78], but it is currently unclear if the resulting
275 cellular demise is caused by a defect in a known or yet-to-be characterized cell death checkpoint.
276 One can legitimately wonder why so many non-redundant cell death checkpoints exist in the TNF
277 pathway. The answer to this question may be found in the beneficial effect of cell death in the fight
278 against microbial infection. The constant arms race between pathogens and their hosts has forced
279 microbes to develop strategies that ensure persistence. A plethora of microbial effector proteins allow
280 pathogens to temper with host immune signaling pathways that promote their elimination. In turn, cell
281 death appears to have evolved as a backup response from the host to ensure proper immune responses
282 when inflammatory signaling has been hijacked by the microbe (**BOX 3**) [1]. Indeed, cell death not
283 only facilitates the elimination of persistent intracellular pathogens, but also provides cell-extrinsic
284 signals (DAMPs) to activate immune receptors expressed by non-infected neighboring cells [3]. By
285 orchestrating the inflammatory response, TNF serves as a major weapon to combat infections. Indeed,
286 anti-TNF therapeutics increase the risk of infections with a multitude of pathogens [79], and it is
287 becoming clear that the anti-microbial role of TNF does not solely originate from the direct induction
288 of pro-inflammatory signaling pathways but also from its cytotoxic properties [80]. The induction of
289 inflammatory signaling pathways, the lysosomal clearance of pathogens by xenophagy, or the
290 induction of apoptosis are strategies to clear intracellular pathogens and are intricately connected with
291 the cell death checkpoints in the TNF pathway. When certain virulence factors or effector proteins try
292 to ensure microbial persistence, they switch off one of the checkpoints and render infected cells
293 sensitive to TNF-induced cell death (**BOX 3**) [1]. Therefore, the checkpoints that prevent TNF
294 cytotoxicity can be considered as fuse sensors that detect pathogenic interference with these strategies.
295 In light of this, we can speculate that the existence of so many checkpoints reflects the various ways
296 microbes attempt to hijack normal immune signaling [81].

297 However, this multi-sensing system comes with a price. Indeed, by multiplying these non-redundant
298 brakes on TNF cytotoxicity, it augments the risk of mutations affecting one of them, resulting in
299 unwanted cell death induction with severe consequences to the organism. Indeed, the constitutive
300 inactivation of the direct regulators of each of the above-mentioned cell death checkpoints was shown
301 to cause embryonic lethality in mice, except for *Jak1*$^{-/-}$ and *Src*$^{-/-}$ mice that respectively die perinatally
302 or survive to adulthood (**Table 1**) [1]. These lethalities are associated with excessive cell death that
303 was, in many cases, demonstrated to be driven by TNF, since additional TNFR1 deletion rescued the
304 embryonic lethality (**Table 1**) [1]. Depending on the cell death modality regulated by the checkpoint,
305 the additional cross with RIPK1 kinase-dead mice or with mice deficient in the downstream death
306 effector proteins (FADD, CASP8, RIPK3 and MLKL) also rescued the death of embryos, thereby
307 demonstrating the causal link (**Table 1**). Notably, because deficiency in FADD or CASP8 causes
308 embryonic lethality due to massive necroptosis induction [82–84], the in vivo implication of CASP8-
309 dependent apoptosis needs to be evaluated in mice that harbor compound deficiency in RIPK3 or
310 MLKL. For instance, LUBAC contributes to three checkpoints that prevent both RIPK1 kinase-
311 independent apoptosis (unconventional autophagy and NF-κB checkpoints) and RIPK1 kinase-
312 dependent cell death (IKK checkpoint). Mice deficient in the LUBAC components HOIL-1 and HOIP
313 succumb at embryonic stage E10.5 [85,86]. The embryonic lethality of these mice is rescued up to
314 E15.5/E16.5 in the *Tnfr1*$^{-/-}$ background, but only to E14.5 in mice expressing kinase inactive RIPK1.
315 Knowing that the *Hoil-1*$^{-/-}$ *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$ and *Hoip*$^{-/-}$ *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$ are viable, these results clearly
316 demonstate that the embryonic lethality of the HOIL-1- or HOIP-deficient mice is caused by a
317 combination of TNF-induced RIPK1 kinase-dependent and –independent cell death up to embryonic
318 stage E15.5/E16.5. Of note, the embryonic lethality resulting from deficiency in RELA/p65, a
319 component of the NF-κB checkpoint that counteracts RIPK1 kinase-independent apoptosis, is
320 surprisingly rescued by crossing the mice with the RIPK1 kinase-dead mice [87,88], highlighting
321 possible differences in the cell death modalities that this checkpoint regulates in vitro and in vivo. This
322 discrepancy in the cell death modality resulting from the in vitro and in vivo inactivation of RELA is
323 intriguing. In some cells, chronic inactivation of the canonical NF-κB pathway may affect basal
324 expression of NF-κB responsive genes (such as cIAP1/2) that contribute to the IKK checkpoint (that
325 represses RIPK1 cytotoxicity), and may explain the contribution of RIPK1 kinase-dependent cell death
326 to the lethality of RELA-deficient mice. This would not happen following acute inhibition of
327 transcription/translation. Accordingly, acute inactivation of the NF-κB checkpoint by D-
328 Galactosamine, which inhibits transcription, was shown to cause lethality in mice solely due to the
329 induction of TNF-mediated RIPK1 kinase-independent apoptosis [45].

330 In addition to their whole body inactivation, the tissue-specific targeting of each of these cell death
331 checkpoints was shown to cause a multitude of TNF-mediated cell death-driven inflammatory

332 pathologies in mice [1]. For example, inactivation of the checkpoints in murine keratinocytes causes
333 the spontaneous development of inflammatory skin lesions (**Table 2**). Importantly, inactivating
334 mutations in genes encoding components of these cell death checkpoints have also been reported to
335 cause inflammatory diseases in human patients. For instance, a heterozygous mutation in *RELA*,
336 affecting the NF-κB checkpoint, causes chronic mucocutaneous ulceration that can be suppressed by
337 anti-TNF therapy [89]. Patients with loss-of-function mutations in *RIPK1*, affecting the
338 unconventional autophagy checkpoint, suffer from combined immunodeficiency and inflammatory
339 bowel disease [90,91]. Moreover, biallelic loss-of-function mutations in *TBK1*, affecting the IKK
340 checkpoint, cause an early-onset inflammatory syndrome (characterized by arthritis, vasculitis and
341 neurocognitive disability) that relies on RIPK1 kinase-dependent cell death, and is successfully treated
342 with anti-TNF therapy [92]. Also, inactivating mutations in *NEMO*, affecting both the NF-κB and IKK
343 checkpoints, cause embryonic lethality in males and *Incontinentia Pigmenti* in heterozygous females,
344 a genetic ectodermal dysplasia affecting the skin, hair, teeth, microvasculature, and central nervous
345 system [93]. As a last example, patients with mutations in *RIPK1* that prevent its CASP8-mediated
346 cleavage, affecting the CASP8 checkpoint, suffer from early-onset auto-inflammatory disease
347 (characterized by lymphadenopathy, chronic gastro-intestinal inflammation and fevers) attributed to
348 hypersensitivity towards RIPK1 kinase-dependent apoptosis and necroptosis [71,72,94]. In summary,
349 these studies demonstrate that the acute inactivation of the cell death checkpoints can be beneficial in
350 the context of infection, while their constitutive inactivation causes inflammatory pathologies.
351

352 CONCLUDING REMARKS

353 It is becoming increasingly clear that multiple TNF-driven (sterile) inflammatory pathologies are
354 caused by uncontrolled inflammation resulting from excessive cell death induction [1]. The recent
355 characterization of new brakes on TNF cytotoxicity (non-receptor Tyrosine kinase and unconventional
356 autophagy checkpoints) has warranted an updated view on how these checkpoints are organized. We
357 propose to categorize them based on their mode of action. The first class of cell death checkpoints
358 (NF-κB, IKK and non-receptor Tyrosine kinase checkpoints) consists of mechanisms that are required
359 to directly limit the activation of CASP8 and prevent the amplification of the lethal signal that
360 originates from the cytotoxic Complex II. The second class (unconventional autophagy and CASP8
361 checkpoints) is comprised of mechanisms that eliminate Complex II (or the complexes that emerge
362 from it) regardless of the actions of the first class. The identification of such protective system still
363 raises several questions (**Outstanding Questions Box**). For instance, how can deficiency in one
364 checkpoint suffice to trigger cell death when all the other brakes are still in place? In other words, how
365 can cFLIP deficiency induce apoptosis when Complex IIa is still targeted for lysosomal degradation?
366 The same question holds for the conditions leading to Complex IIb or Necrosome assembly. Indeed,

367 RIPK1 is also reported to be M1-ubiquitinated in these complexes [28,29] and may therefore also serve
368 as a recognition signal for their lysosomal degradation by the unconventional autophagy checkpoint.
369 Moreover, because M1-ubiquitin chains are implicated in a plethora of inflammatory signaling
370 pathways, it is tempting to speculate that the unconventional autophagy checkpoint could additionally
371 detoxify cytotoxic complexes that form downstream of other innate immune receptors with cytotoxic
372 potential, such as ZBP1 or **inflammasomes**. Finally, the identification of new (components of) cell
373 death checkpoints in the TNF pathway widens the spectrum of patients with pathologies that may
374 benefit from anti-TNF therapies. Indeed, thanks to the molecular understanding of these cell death
375 checkpoints, patients with mutations in the genes encoding RELA, RIPK1, TBK1, OTULIN or
376 LUBAC are now successfully treated with TNF blocking agents [24,25,71,89,92,94–98]. This list may
377 extend in the future to patients with mutations in ATG components of the unconventional autophagy
378 checkpoint or in JAK1 and SRC, which remains to be investigated. Moreover, the development and
379 use of cell death inhibitors (Reviewed in [99]) may also become a future alternative for the treatment
380 of the described inflammatory or immunodeficiency-associated diseases, especially for nonresponsive
381 patients or patients who show adverse effects to anti-TNF treatments.

382

383 # GLOSSARY

384 - **Cell death checkpoint**: Molecular mechanism to inhibit cell death induction.
385 - **Necroptosis**: Regulated form of lytic cell death relying on the RIPK3 kinase and on the
386 pseudokinase MLKL. Necroptosis is triggered when activated RIPK3 phosphorylates MLKL
387 to induce its oligomerization and translocation to the plasma membrane where it promotes pore
388 formation.
389 - **Pyroptosis**: Regulated form of lytic cell death executed by the pore-forming molecule
390 GSDMD. Activation of GSDMD requires proteolytic processing by the inflammasome-
391 associated CASP1 and CASP11 (CASP4/5 in humans) or by CASP8.
392 - **Secondary necrosis**: Lytic phase of an apoptotic cell in the absence of phagocytosis; involves
393 the proteolytic activation of the pore-forming molecule GSDME by the effector CASP3.
394 - **Pattern Recognition Receptors (PRRs)**: Membrane-bound or cytosolic sensors that detect
395 invariant molecular motifs present on microbes or released/exposed by distressed or dying
396 cells.

397 - **Pathogen-Associated Molecular Patterns (PAMPs)**: Class of invariant molecular motifs
398 found in structural components that comprise the cell surface of various microbes, but not host
399 cells.
400 - **Damage-Associated Molecular Patterns (DAMPs)**: Class of molecular motifs acting as
401 endogenous danger signals. In healthy cells, DAMPs normally stay in the intracellular
402 environment, but can be exposed or released when the cell is distressed or dying.
403 - **TNFR1 Complex I**: Also known as TNFR1 Signaling Complex (TNFR1-SC). Primary
404 receptor-bound signaling complex forming at the plasma membrane within seconds of TNF
405 sensing; promotes the activation of the MAPK and NF-κB signaling pathways.
406 - **TNFR1 Complex II:** Secondary cytosolic FADD- and CASP8-containing complex
407 assembling within minutes of TNF sensing; originates from dissociation of Complex I from the
408 receptor.
409 - **TNFR1 Complex IIa**: Apoptosis-inducing Complex II resulting from inhibition of the NF-κB,
410 or unconventional autophagy checkpoint.
411 - **TNFR1 Complex IIb**: Cytosolic apoptosis-inducing complex of similar composition as
412 Complex IIa but whose assembly relies on RIPK1 kinase activity. It is unclear if Complex IIa
413 and IIb are distinct complexes or if they represent one unique complex.
414 - **Necrosome**: Cytosolic necroptosis-inducing complex produced when CASP8 fails to cleave
415 RIPK1 within TNFR1 Complex II. The formation of the Necrosome depends on the stable (RIP
416 Homotypic Interaction Motif) RHIM-dependent interaction between kinase-active RIPK1 and
417 RIPK3, further allowing MLKL recruitment and activation.
418 - **Death Inducing Signaling Complex (DISC)**: Receptor-associated CASP8-activating complex
419 forming at the plasma membrane following cell stimulation with members of the TNF
420 superfamily of ligands, such as TRAIL and FasL.
421 **-Inflammasome:** CASP1-activating platform that promotes the proteolytic activation of IL-1β
422 and IL-18; and induction of pyroptosis by GSDMD cleavage.
423

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728

729

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737

TEXT BOXES

BOX 1: Unconventional MC3-independent selective autophagy

Autophagy is the process in which a double membrane vesicle called the autophagosome isolates part of the cytosol and later fuses with a lysosome for the proteolytic degradation of the engulfed cargo (reviewed in [104]). Autophagy can promote the non-specific degradation and recycling of bulk cargo to liberate nutrients during starvation, but can also be a highly specific catabolic process in nutrient-replete conditions, promoting the removal of defective organelles, protein aggregates, or cytosolic bacteria. During selective autophagy, autophagy receptors shuttle specific cargoes to growing phagophores by simultaneously binding the ubiquitin chains conjugated to the cargo and the ATG8/LC3-moeities present on the inner phagophore leaflet [104]. Recent experimental evidence has demonstrated the existence of an unconventional pathway that does not require the LC3-conjugation machinery, but instead promotes the selective autophagy-dependent degradation of cargo via the direct binding between the autophagy receptor and FIP200, a component of the autophagy initiation complex [49,105–109].

BOX 2: Secondary protective mechanisms

In addition to the checkpoints that prevent cell death induction, several additional molecular mechanisms have been reported to limit the extent of TNF-induced cell death in conditions of a previously compromised checkpoint. Here are a few examples of these secondary protective mechanisms. First, the K48-ubiquitination of RIPK1 by cIAP1 promotes the proteasomal degradation of kinase-active RIPK1, thereby limiting RIPK1 kinase-dependent cell death downstream of TNF in murine fibroblasts and BMDMs [100]. In addition, the cIAP1/2-independent ubiquitination of RIPK1 by MIB2 was reported to limit activation of RIPK1 and consequently Complex IIb-dependent cell death induction in murine and human epithelial cells. The authors hypothesized that steric hindrance caused by the ubiquitin chains conjugated to RIPK1 by MIB2 would interfere with oligomerization and trans-activation of RIPK1 and with FADD binding [101]. Apart from RIPK1, cFLIP was also reported to be a substrate of MIB2 and LUBAC, and the ubiquitination of cFLIP by these E3 ligases was shown to increase its stability and to limit TNF-induced cell death in conditions affecting the NF-κB-dependent upregulation of cFLIP in human and murine cells [38,102]. Furthermore, the Poly-ADP-Ribosylation (PARylation) of RIPK3 by TANKYRASE-1 was shown to serve as a signal for the K48-ubiquitination of the Necrosome by the E3 ligase RNF146, thereby limiting necroptosis induction in murine fibroblasts and BMDMs [103]. Apart from proteasomal degradation, canonical LC3-dependent selective autophagy was also reported to reduce necroptosis by promoting the lysosomal turnover of the RHIM-containing proteins RIPK1 and RIPK3 in BMDMs [104]. Additionally, in murine fibroblasts the autophagy-initiating kinase ULK1 was found to reduce RIPK1 kinase-dependent cell

772 death by phosphorylating cytosolic RIPK1 at Ser^{357} (Ser^{356} in mice) [105]. This phosphorylation event
773 does not affect activation of RIPK1 in Complex I but reduces the amount of kinase-active RIPK1 inside
774 Complex IIb. This suggests that phosphorylation of RIPK1 at Ser^{357} impairs the cytosolic transfer of
775 active RIPK1 from Complex I to Complex IIb and/or reduces trans-phosphorylation and activation of
776 RIPK1 within Complex IIb. Moreover, in murine fibroblasts and BMDMs cytosolic phosphorylation
777 of RIPK1 at Ser^{320} and Ser^{335} (Ser^{321} and Ser^{336} in mice) by MK2, a kinase activated downstream of
778 TAK1 and p38, was shown to reduce RIPK1 activation in Complex I and/or to limit Complex IIb
779 assembly [64–67].

780 **BOX 3: Cell death checkpoints – Fuse sensors of microbial invasion**

781 Microbes have developed sophisticated strategies to evade host immune defenses by hijacking
782 inflammatory signaling pathways. For example, pathogenic *Yersinia sp.* deliver the virulence factor
783 YopJ/P into mammalian cells to subvert inflammatory gene activation. YopJ/P is an acyltransferase
784 that inhibits the catalytic activity of TAK1 and IKKα/β, and consequently prevents the MAPK- and
785 NF-κB-dependent expression of pro-inflammatory mediators [106]. Because of this hijacking, the
786 IKKα/β-dependent cell death checkpoint that normally maintains RIPK1 in pro-survival mode is
787 inactivated, as well as the secondary protective mechanism consisting in RIPK1 repression by MK2.
788 Consequently, infected human PMBCs and murine BMDMs become sensitized to RIPK1 kinase-
789 dependent CASP8-mediated apoptosis and/or pyroptosis [30,32,50,66,67,107–109], and the resulting
790 cellular demise was demonstrated to be beneficial in mice by promoting optimal anti-bacterial
791 immunity. Due to its central role in orchestrating anti-viral immunity, TBK1 is the target of many
792 virally encoded virulence factors [110–113]. The action of these viral effector proteins is therefore
793 expected to similarly trigger RIPK1 kinase-dependent apoptosis/pyroptosis by inactivating the
794 IKKε/TBK1-dependent cell death checkpoint. While microbes do not encode ubiquitin themselves, a
795 plethora of pathogens have evolved effector proteins that affect the host ubiquitination systems in an
796 attempt to counteract immune signaling [114]. For example, *Legionella pneumophila*, the causative
797 agent of human Legionnaire's disease, encodes RavD which specifically hydrolyzes M1-ubiquitin
798 chains, and is consequently also expected to inhibit multiple cell death checkpoints in the TNF pathway
799 [115]. The elimination of intracellular pathogens by xenophagy, a selective form of autophagy,
800 constitutes another arm of the host immune defense. Accordingly, numerous pathogens have developed
801 strategies to prevent lysosomal destruction by inhibiting the core autophagy machinery. This is the
802 case for *Listeria monocytogenes* which expresses two membrane-disrupting phospholipases (PlcA/B)
803 that antagonize the formation of autophagosomal membranes in human epithelial cells and BMDMs
804 [116,117]. It is therefore expected that PlcA/B switch the TNF response to death by inhibiting the
805 unconventional autophagy checkpoint. Finally, because apoptosis is an ancient form of cell death that
806 evolved to combat invading pathogens [118], many microbes encode effector proteins that inhibit

807 apoptotic caspases [119]. For example, Vaccinia Virus (VACV) encodes the virulence factor
808 B13R/Spi2 that acts as a potent CASP8 inhibitor [120]. However, by inhibiting the CASP8 checkpoint
809 B13R/Spi2 switches the TNF response to necroptosis in various murine cell types, which was
810 demonstrated to be critical in the control of VACV infections in mice [27,121]. Recently, the
811 enteropathogenic bacteria *Shigella sp.* and *Escherichia coli* were shown to in turn encode proteases
812 that can specifically cleave RIPK1 in human epithelial cells and in mice as a strategy to prevent
813 necroptosis induction [122,123].

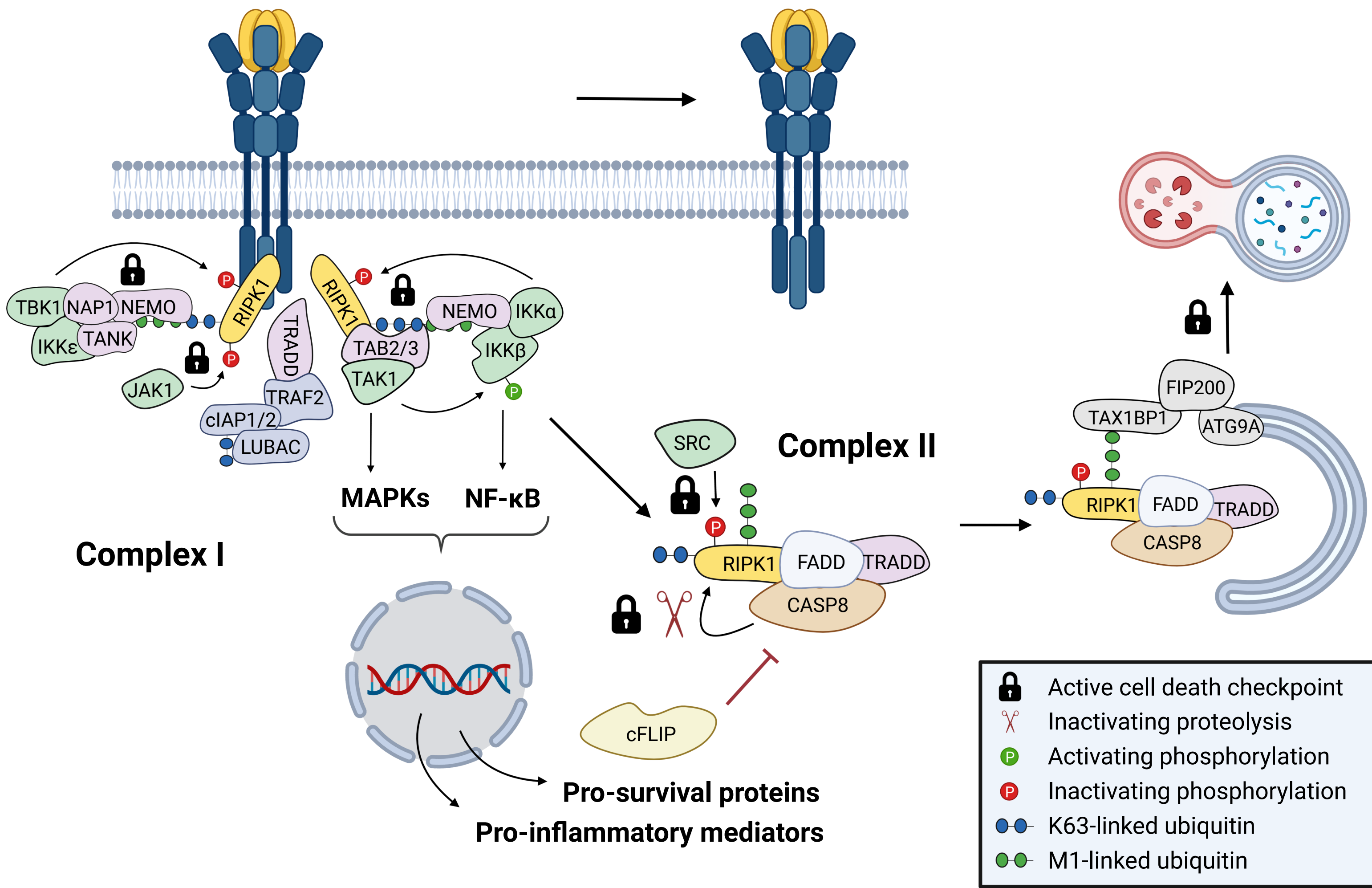
FIGURE LEGENDS

Figure 1. Normal cellular response of TNFR1 activation by TNF.

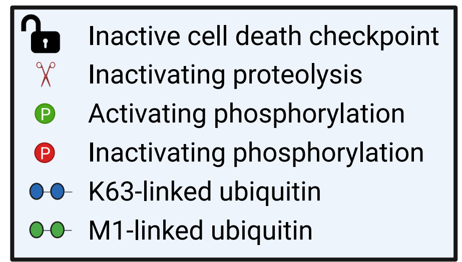
In most murine and human cell types, including macrophages and fibroblasts, sensing of TNF by TNFR1 does not trigger cell death but results in gene activation. TNFR1 Complex I forms at the plasma membrane within seconds following TNF sensing. It serves as a recruitment and activation platform for the kinases that promote the activation of the NF-κB and MAPK signaling pathways resulting in the production of pro-inflammatory mediators and pro-survival proteins [6,7]. Later, Complex I destabilizes and detaches from the receptor to allow the formation of the CASP8-containing TNFR1 Complex II in the cytosol [5]. The cytotoxic potential of Complex II is kept in check by several molecular mechanisms referred to as cell death checkpoints [1,48,63], which includes its lysosomal degradation by an unconventional selective autophagy pathway [48]. The cell death checkpoints that prevent lethal activity of Complex II are indicated in the figure as locks. For clarity the figure depicts only a selected subset of core constituents of the cell death checkpoints and the central components that are present in Complex I/II.

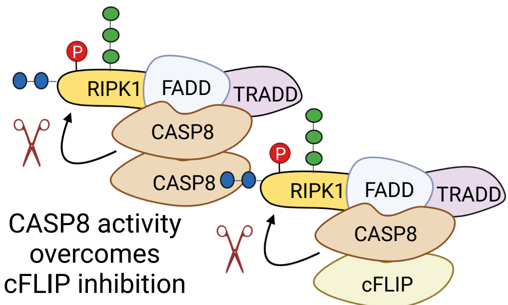
Figure 2. TNF-induced cell death caused by inactivation of cell death checkpoints downstream of TNFR1.

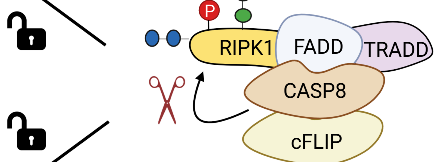
TNF cytotoxicity in mice and humans requires inactivation of one of the cell death checkpoints present downstream of TNFR1. The inactivation of the NF-κB checkpoint quickly results in the reduction of cFLIP amounts, allowing the lethal activation of CASP8 in Complex II and the formation of the cytotoxic Complex IIa that induces RIPK1 kinase-independent apoptosis [5,26]. Similarly, interfering with the unconventional autophagy checkpoint results in the cytosolic accumulation of Complex IIa that overcomes inhibition by cFLIP and causes CASP8-mediated RIPK1 kinase-independent apoptosis [48]. Instead, inhibition of the IKK or non-receptor Tyrosine kinase checkpoint leads to the activation of RIPK1 enzymatic activity, formation of Complex IIb and induction of RIPK1 kinase-dependent apoptosis (or pyroptosis) [18,45,52,63]. Complex IIb therefore enables active CASP8 to overcome repression by cFLIP. Finally, preventing cleavage of RIPK1 by CASP8 in Complex II leads to the cytosolic accumulation of kinase-active RIPK1 that promotes Necrosome assembly and MLKL-dependent necroptosis induction [47,69–72]. For clarity the figure depicts only a selected subset of the central components of the cytotoxic complexes that originate from Complex II in conditions of checkpoint inhibition and their downstream effectors.



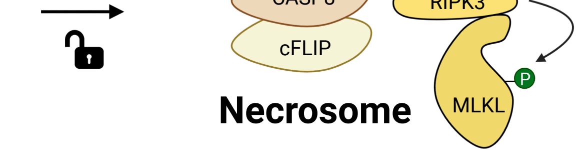
TBK1
NAP1
NEMO
IKKε
TANK
RIPK1
TRADD
JAK1
TRAF2
cIAP1/2
LUBAC
RIPK1
NEMO
IKKα
TAB2/3
IKKβ
TAK1
MAPKs
NF-κB
Complex I
Complex II
SRC
RIPK1
FADD
TRADD
CASP8
cFLIP
Pro-survival proteins
Pro-inflammatory mediators
FIP200
TAX1BP1
ATG9A
RIPK1
FADD
TRADD
CASP8
Active cell death checkpoint
Inactivating proteolysis
Activating phosphorylation
Inactivating phosphorylation
K63-linked ubiquitin
M1-linked ubiquitin

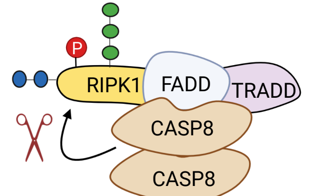

Inactive cell death checkpoint
Inactivating proteolysis
Activating phosphorylation
Inactivating phosphorylation
K63-linked ubiquitin
M1-linked ubiquitin


P
RIPK1
FADD
TRADD
CASP8
CASP8
RIPK1
FADD
TRADD
CASP8
cFLIP
CASP8 activity
overcomes
cFLIP inhibition
Complex IIa

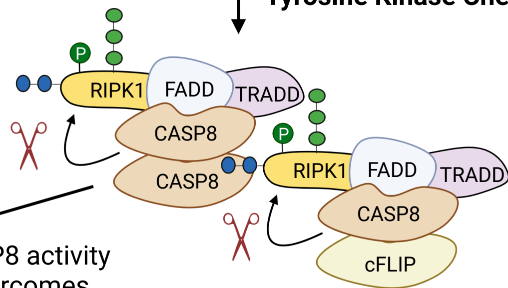

Unconventional
Autophagy
Checkpoint
P
RIPK1
FADD
TRADD
CASP8
cFLIP

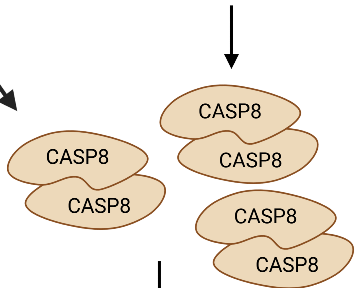
NF-κB
Checkpoint
Complex II


CASP8
Checkpoint
TRADD
FADD
P
RIPK1
P
RIPK3
CASP8
cFLIP
P
MLKL
Necrosome


P
RIPK1
FADD
TRADD
CASP8
CASP8
Complex IIa

IKK Checkpoint
Tyrosine Kinase Checkpoint


P
RIPK1
FADD
TRADD
CASP8
CASP8
P
RIPK1
FADD
TRADD
CASP8
cFLIP
CASP8 activity
overcomes
cFLIP inhibition
Complex IIb


CASP8
CASP8
CASP8
CASP8
CASP8
CASP8
Apoptosis
(Pyroptosis)

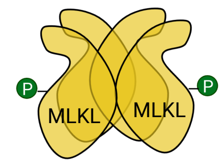

P
MLKL
MLKL
P
Necroptosis

Table 1: Phenotypes of mice constitutively deficient in direct regulators of TNF cell death checkpoints

Checkpoint	*Gene* (Protein)	Genotype	Phenotype	Rescue	Ref.
NF-κB	*Rela* (p65)	*Rela*$^{-/-}$	Embryonically lethal (E14.5), liver apoptosis		[124]
				Tnfr1$^{-/-}$	[125]
				Fadd$^{-/-}$ *Mlkl*$^{-/-}$, *Fadd*$^{-/-}$ *Ripk3*$^{-/-}$, *Ripk1*$^{K45A/K45A}$ (kinase-dead)	[87]
	Cflar (cFLIP)	*Cflar*$^{-/-}$	Embryonically lethal (E10.5), keratinocyte apoptosis, defective heart development		[126]
				Fadd$^{-/-}$ *Ripk3*$^{-/-}$	[39]
Unconventional autophagy	*Atg9a* (ATG9A)	*Atg9a*$^{-/-}$	Embryonically lethal (E14.5), liver apoptosis		[127,128]
				Tnfr1$^{-/-}$	[48]
	Rb1cc1 (FIP200)	*Rb1cc1*$^{-/-}$	Embryonically lethal (E14.5), liver apoptosis, defective heart development		[129]
				Tnfr1$^{-/-}$	[130]
IKK	*Ikkb* (IKKβ)	*Ikkb*$^{-/-}$	Embryonically lethal (E13.5), liver apoptosis		[131,132]
				Tnfr1$^{-/-}$	[133]
	Tbk1 (TBK1)	*Tbk1*$^{-/-}$	Embryonically lethal (E13.5), liver apoptosis	*Tnfr1*$^{-/-}$	[134,135]
				Ripk1$^{D183N/D183N}$ (kinase-dead), *Ripk3*$^{-/-}$ (partial)	[52]
Tyrosine Kinase	*Jak1* (JAK1)	*Jak1*$^{-/-}$	Perinatal lethality, defective thymocyte production	ND	[136]
	Src (SRC)	*Src*$^{-/-}$	Viable, osteopetrosis	ND	[137,138]
	Ripk1 (RIPK1)	*Ripk1*$^{Y383F/Y383F}$	Viable, systemic inflammation, emergency hematopoiesis	*Tnfr1*$^{-/-}$, *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$, Nec1s treatment	[63]
CASP8	*Casp8* (CASP8)	*Casp8*$^{-/-}$	Embryonically lethal (E10.5), defective heart development, abdominal hemorrhage, keratinocyte apoptosis		[139–141]
				Ripk3$^{-/-}$	[82,83,142]
				Ripk3$^{K51A/K51A}$ (kinase-dead)	[143]
				Ripk3$^{D161N/D161N}$ (kinase-dead)	[144]
				Ripk1$^{-/-}$	[145]
	Casp8 (CASP8)	*Casp8*$^{C326A/C326A}$ (catalytically inactive)	Embryonically lethal (E10.5), defective heart development	*Mlkl*$^{-/-}$	[69,146]
	Ripk1 (RIPK1)	*Ripk1*$^{D325A/D325A}$ (uncleavable)	Embryonically lethal (E10.5), defective heart development	*Ripk3*$^{-/-}$ (partial)	[69–71]
				Fadd$^{-/-}$ *Ripk3*$^{-/-}$	[69,70]
				Ripk1$^{D138N/D138N}$ (kinase-dead), *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$	[69,71]
				Fadd$^{-/-}$ *Mlkl*$^{-/-}$, *Tnfr1*$^{-/-}$	[69]

ND: Not Determined

Table 2: Mouse inflammatory skin diseases caused by conditional deficiency in direct regulators of TNF cell death checkpoints

Checkpoint	*Gene* (Protein)	Genotype	Phenotype	Rescue	Refs
NF-κB	*Rela* (p65) / *Rel* (c-Rel)	*RelA*$^{EKO(K14)}$ *c-Rel*$^{fl/fl}$	Inflammatory skin lesions	RIPK1$^{D138N/D138N}$ (kinase-dead; partial), *Mlkl*$^{-/-}$ (partial), *Tnfr1*$^{EKO(K14)}$	[147]
	Cflar (cFLIP)	*Cflar*$^{EKO(K14)}$	Embryonically lethal (E10.5)		[40]
	Cflar (cFLIP)	*Cflar*$^{EKO(K14-ERT)}$	Inflammatory skin lesions	Anti-TNF treatment	[40]
	Cflar (cFLIP)	*Cflar*^{ERT}	Inflammatory skin lesions		[41]
Unconventional autophagy	*Atg9a* (ATG9A)	*Atg9a*$^{EKO(K5)}$	Inflammatory skin lesions	*Tnfr1*$^{-/-}$	[48]
	Rb1cc1 (FIP200)	*Rb1cc1*$^{EKO(MMTV)}$	Inflammatory skin lesions - Skin tumorigenesis		[148]
IKK	*Ikkb* (IKKβ)	*Ikkb*$^{EKO(K14)}$	Inflammatory skin lesions	*Tnfr1*$^{-/-}$	[149]
				Tnfr1$^{EKO(K14)}$, *Fadd*$^{EKO(K14)}$ *Ripk3*$^{-/-}$, *Ripk3*$^{-/-}$ (partial), *Ripk3*$^{EKO(K14)}$ (partial), *Mlkl*$^{-/-}$ (partial), *Ripk1*$^{D138N/D138N}$ (partial)	[147]
	Tbk1 (TBK1)	ND	ND		
Tyrosine Kinase	*Jak1* (JAK1)	ND	ND		
	Src (SRC)	*Src*$^{-/-}$	No reported skin phenotype in constitutive KO		[137,138]
	Ripk1 (RIPK1)	*Ripk1*$^{Y383F/Y383F}$	No reported skin phenotype in constitutive KI		[63]
CASP8	*Casp8* (CASP8)	*Casp8*$^{EKO(K5)}$	Inflammatory skin lesions	*Tnf*$^{-/-}$ (partial), *Tnfr1*$^{-/-}$ (partial)	[150]
		Casp8^{ERT}	Inflammatory skin lesions	*Ripk3*$^{-/-}$, anti-TNF treatment (partial)	[41]

ND: Not Determined; EKO: Epidermis-specific KO; ERT: CreER-Tamoxifen-inducable