The FLIP Side of Life

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Abstract

The anti-apoptotic protein c-FLIP, a catalytically inactive homolog of caspase-8, is an important regulator of death receptor signaling. Death receptors constitute a subgroup of the tumor necrosis factor receptor (TNFR) superfamily, which includes TNFR1, Fas, DR4, and DR5. When activated by their respective ligands, TNF, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL), these receptors cause caspase-8–mediated apoptosis. If caspase activity is blocked, however, then these receptors promote death by necroptosis (programmed necrosis), which requires the kinases receptor-interacting kinase 1 (RIPK1) and RIPK3, as well as Mixed Lineage Kinase Like protein MLKL. Necroptosis has become the subject of intense research because it promotes inflammation, and inhibiting this pathway can limit extensive tissue damage and even lethality in inflammatory syndromes. A study now reports on the role of c-FLIP in vivo from experiments with a range of conditional knockout

mice and demonstrates that c-FLIP plays a critical role in inhibiting both apoptotic as well as necroptotic cell death within the whole mouse.

The death receptor–induced apoptotic pathway (also called the extrinsic apoptotic pathway) is initiated by so-called "death ligands," most commonly Fas ligand (FasL), tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL). Ligand-induced activation of pre-assembled death receptor trimers drives the formation of an intracellular death-inducing signaling complex (DISC), which contains receptor-interacting serine-threonine kinase 1 (RIPK1), Fas-associated protein with death domain (FADD), and caspase-8 (Fig. 1A). FADD-like interleukin-1β–converting enzyme (FLICE) inhibitory protein (c-FLIP) is a catalytically inactive homolog of caspase-8 that inhibits the activity of the DISC (1, 2). Knockout or mutation of the genes encoding death ligands or their receptors do not cause developmental abnormalities and result in only relatively mild phenotypes, with the exception of the lymphadenopathy and autoimmunity seen in FasL- or Fas-deficient mice (3). In striking contrast, loss of FADD or caspase-8 causes embryonic lethality at around embryonic day 10 (E10). Why this should be the case is not immediately obvious. The simple assumption is that loss of the ligands should be equivalent to loss of the intracellular effectors; however, studies show that the embryonic lethality of *Fadd*- and *caspase-8*–deficient mice can be rescued by crossing the mice with *Ripk3* knockout mice. Because RIPK3 is required for necroptosis (4), these results suggest that in the absence of these apoptotic effectors in vivo, death receptor signaling activates the alternative necroptotic pathway, which is responsible for the embryonic lethality of the *Fadd* and *caspase-8* knockout mice.

These data fit readily into a TNF receptor (TNFR) signaling pathway model (Fig. 1) in which stimulation of TNFR1 by TNF drives the nuclear factor κB (NF-κB)-mediated transcription of the gene encoding c-FLIP (*c-Flip*), which is usually sufficient to limit caspase-8 activity so that the apoptotic pathway is not activated. In the absence of active caspase-8, which cleaves and inactivates RIPK1 and RIPK3, the amounts of these kinases increase, ultimately leading to their auto-activation and the triggering of necroptosis. The phenotype of the complete *c-Flip* knockout mice is intriguing, because these animals, like *caspase-8*– and *Fadd*-deficient mice, also die at E10. It is assumed that the *c-Flip*-deficient embryos die at E10 because of unregulated caspase-8 activity, which leads to uncontrolled apoptosis. It is, however, also possible that c-FLIP$_L$ acts to inhibit necroptosis as well (5, 6). Furthermore, it has been suggested that heterodimers of caspase-8 and c-FLIP, but not caspase-8 homodimers, prevent the stable association of FADD, RIPK1, and RIPK3, and thus prevent necroptotic death (7).

Because of the lack of definitive markers of necroptosis and because the apoptotic and necroptotic pathways are so intertwined, it is not easy to determine to what extent the excessive cell death caused by loss of c-FLIP can be attributed to deregulated necroptosis or apoptosis. Although the lethality of *c-Flip* knockout embryos can be rescued by the additional loss of both *Fadd* and *Ripk3*, it cannot be achieved by loss of either *Fadd* or *Ripk3* alone (8). The latest work from Piao *et al*. adds support to the idea that c-FLIP may inhibit a necroptotic pathway in vivo, in addition to regulating death receptor–mediated apoptosis. Because constitutive loss of *c-Flip* in all tissues causes embryonic lethality, Piao *et al*. generated conditional *c-Flip* knockout mice. The *c-Flip*$^{F/F}$*;Villin-Cre* mice, which lack *c-Flip* only in their intestinal epithelial cells

(IECs), were born at the expected Mendelian ratios, but all died within one day of birth. Both apoptotic as well as necrotic cells were already prominent in the intestines of E18.5 embryos, revealing that the defect in these mice began in utero. Remarkably, even loss of one *Tnfr1* allele substantially delayed the premature death of the mice deficient in *c-Flip* in their IECs. This demonstrates that TNF is a critical, but not the only, factor responsible for the early death of these mice (9).

Piao *et al*. also deleted *c-Flip* specifically from hepatocytes using *Albumin-Cre* transgenic mice. Consistent with a previous report (10), *c-Flip*$^{F/F}$*;Alb-Cre* mice were viable, but showed abnormally increased sensitivity to Fas-mediated hepatocyte killing. However, because *Alb-Cre* proved inefficient at deleting the floxed *c-Flip* gene from hepatocytes, Piao *et al*. also generated *c-Flip*$^{F/F}$*;Alfp-Cre* mice, with which they achieved more efficient deletion of the floxed *c-Flip* alleles. None of the *c-Flip*$^{F/F}$*;Alfp-Cre* mice survived beyond 3 weeks of age, with most dying within two days after birth. As with the IECs in *c-Flip*$^{F/F}$*;Villin-Cre* mice, many hepatocytes displayed caspase activity and an apoptotic phenotype, whereas other hepatocytes displayed a more necrotic morphology. In contrast to the studies with the *c-Flip*$^{F/F}$*;Villin-Cre* mice, loss of *Tnfr1* was unable to delay the early death of the *c-Flip*$^{F/F}$*;Alfp-Cre* mice. Finally, the authors showed that acute (inducible) loss of *c-Flip* in *c-Flip*$^{F/F}$;*Mx1-Cre* mice injected with poly I:C caused massive killing of hepatocytes and fatal hepatitis within 72 hours. Pretreatment of these mice with a cocktail of three neutralizing antibodies against TNF, TRAIL, and FasL substantially diminished hepato-toxicity.

Collectively, these results show that c-FLIP is essential for inhibiting death receptor–induced killing of diverse cell types (IECs and hepatocytes) within the whole animal. The results also demonstrate that selective loss of *c-Flip* from either IECs or hepatocytes is lethal. In the case of *c-Flip*–deficient IECs, the lethality is driven predominantly by TNFR1 signaling, whereas with *c-FLIP*–deficient hepatocytes, fatal hepatitis was mediated by at least three death ligands: TNF, FasL, and TRAIL. The phenotype of the mice lacking *c-Flip* selectively in IECs is very reminiscent of that of animals lacking the inhibitor of κb kinase (IKK) regulatory subunit NEMO in the same cells ($NEMO^{IEC}$ mice); these mice also develop severe colitis throughout the large intestine within 3 weeks of birth. The colitis in $NEMO^{IEC}$ mice is also alleviated by loss of *Tnfr1* (11, 12). This is consistent with the notion that TNF-dependent TNFR1 signaling requires NEMO to activate NF-κB to drive sufficient c-FLIP production to block TNF- and TNFR1-induced cell death (13). As might be expected from the considerable overlap in phenotypes caused by complete knockout of *c-Flip*, *Fadd*, and *caspase-8*, specific loss of *c-Flip* in IECs causes similar defects to those seen as a result of IEC-restricted loss of *Fadd* ($FADD^{IEC}$) or *caspase-8* ($Caspase\text{-}8^{IEC}$), with early onset colitis that can be ameliorated by loss of TNF (14, 15). Severe colitis in $FADD^{IEC}$ mice is also prevented by deficiency in *Ripk3 (14)*This indicates that FADD inhibits RIPK3-dependent necroptosis, most likely through activation of caspase-8, to maintain epithelial integrity in the gut and thereby prevents chronic intestinal inflammation.

The observation by Piao *et al*. that the intestinal epithelia of $c\text{-}Flip^{F/F}$*;Villin-Cre* mice were already disturbed in utero suggests that TNF is already produced within this tissue before the gut is exposed to microbiota. The question then is where does TNF,

or (judging from the studies with acute loss of *c-Flip* in hepatocytes) potentially a cocktail of TNF, FasL and TRAIL, come from? One possibility is that death receptors and their ligands exert normal developmental functions that are distinct from their ability to induce cell death. Indeed, TNF kills only a limited number of cell lines (and even more rarely non-transformed cells) and in the context of the gut, TNF is required for Peyer's patch formation (16). In the absence of *c-Flip*, developmentally regulated TNF (and possibly also TRAIL and FasL) would result in an increase in cell death, which could provoke inflammation and thereby further increase the production of TNF (and possibly related ligands) that on a *c-Flip*-deficient background would drive even more cell death, in a vicious feed-forward cycle (Fig. 1B).

Extrapolating from the phenotype of mice lacking combinations of inhibitor of apoptosis proteins (IAPs), this vicious cycle might also be the reason for the embryonic death of *c-Flip* knockout mice at E10 (17). Moulin *et al*. demonstrated that *Ciap1*$^{-/-}$*Ciap2*$^{-/-}$ and *Ciap1*$^{-/-}$*Xiap*$^{-/-}$ mice die at around E10, the developmental stage at which the animals deficient in *c-Flip*, *Fadd*, or *caspase-8* also die. As in the case of loss of *c-Flip*, *Fadd*, and *caspase-8*, the embryonic lethality of *Ciap1*$^{-/-}$*Ciap2*$^{-/-}$ mice is prevented by concomitant loss of *Ripk3*. Thus, it is highly likely that cIAP1 and cIAP2 are regulating the same signaling events that are also controlled by c-FLIP, FADD, and caspase-8. Furthermore, Moulin *et al*. showed that the embryonic lethality of *Ciap1*$^{-/-}$*Ciap2*$^{-/-}$ mice is prevented by loss of *Tnfr1*. Collectively, these results suggest that at around E10 (and possibly other stages), TNF (and perhaps other death receptor ligands) exert a critical developmental function, most likely through activation of NF-κB family transcription factors. This death receptor signaling must be tightly controlled, because if it goes awry (as a result of the loss of c-FLIP, FADD,

caspase-8, or IAPs) embryonic or even adult mice can succumb to a lethal inflammatory cascade with extensive cell death.

As an alternative to this "out of control" developmental TNF model, it is possible that loss or blockade of the critical intracellular signal transducers of the death receptor pathway (for example, c-FLIP, caspase-8, and FADD) somehow results in abnormally increased production of TNF, and possibly of related ligands. Such a mechanism for FADD- or caspase-8–dependent limitation of TNF production might be similar to the process by which caspase-8 limits cytokine production dependent on the transcription factor interferon regulatory factor 3 (IRF3) (18). Because excess production of TNF (and possibly related cytokines) can, in a sensitized background (for example, as a result of loss of caspase-8 or FADD) rapidly lead to further amplification of TNF production by promoting necroptotic death, the end result will probably be identical: death of the embryo. It will be very difficult to distinguish between these two possibilities, but it is clear that there is a real need to develop better markers of necroptotic death, rather than use the painstaking transmission electron microscopy approach adopted by Piao *et al*., to help resolve these questions. Regardless of how inflammation is initiated or propagated, the accumulating data suggest that inhibiting both the apoptosis and necroptosis signaling pathways could be helpful in the treatment of diseases that are caused or exacerbated by inflammation.

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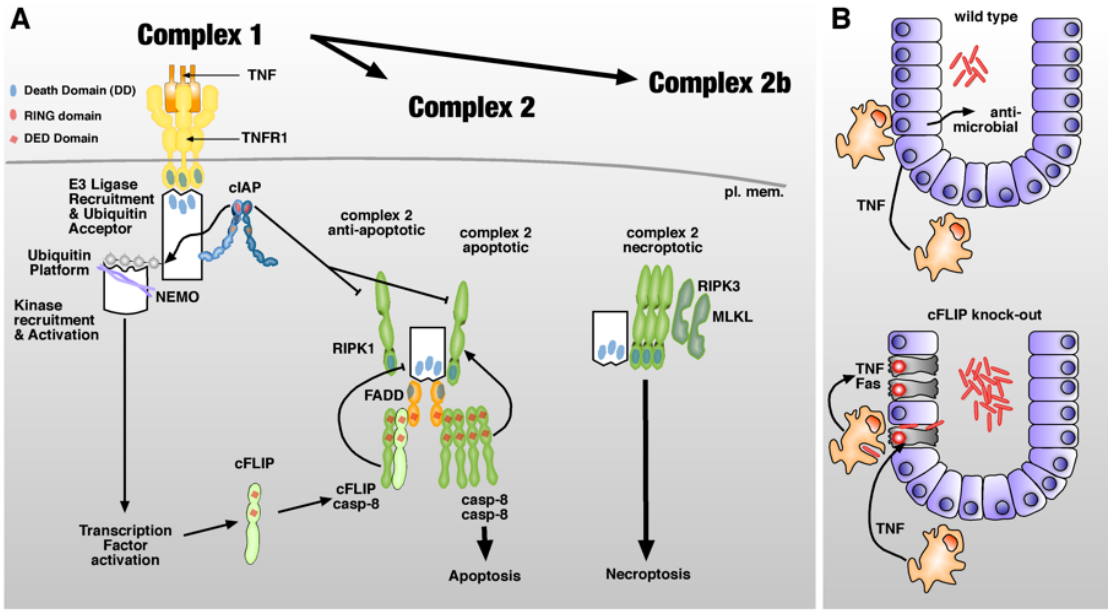

Figure 1

Figure 1. (**A**) TNF- and TNFR1-induced cell death pathways. Similar pathways are activated by related death ligands, such as Fas and TRAIL. (**B**) The mechanism by which intestinal epithelial cell death provokes rapidly amplifying inflammation in the gut in *c-Flip* knockout mice.