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The roles of FADD in extrinsic apoptosis and necroptosis

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Fas-associated protein with death domain (FADD), an adaptor that bridges death receptor signaling to the caspase cascade, is indispensible for the induction of extrinsic apoptotic cell death. Interest in the non-apoptotic function of FADD has greatly increased due to evidence that FADD-deficient mice or dominant-negative FADD transgenic mice result in embryonic lethality and an immune defect without showing apoptotic features. Numerous studies have suggested that FADD regulates cell cycle progression, proliferation, and autophagy, affecting these phenomena. Recently, programmed necrosis, also called necroptosis, was shown to be a key mechanism that induces embryonic lethality and an immune defect. Supporting these findings, FADD was shown to be involved in various necroptosis models. In this review, we summarize the mechanism of extrinsic apoptosis and necroptosis, and discuss the *in vivo* and *in vitro* roles of FADD in necroptosis induced by various stimuli. [BMB Reports 2012; 45(9): 496-508]

INTRODUCTION

Fas-associated protein with death domain (FADD) is a critical adaptor protein for death receptor (DR)-mediated apoptosis. FADD is composed of two domains called the death domain (DD) and death effector domain (DED). The DD of FADD binds to the DD of the death receptor and FADD recruits procaspase-8 through the DED-DED interaction, forming a death-inducing signaling complex (DISC), where procaspase-8 is activated by self-cleavage. Active caspase-8 cleaves downstream effector caspases such as caspase-3, -6, and -7, inducing apoptosis.

Interestingly, FADD deficiency results in embryonic lethality, displaying a defect in immune homeostasis and immune cell proliferation despite the defect in inducing apoptosis. In addition, FADD is also implicated in non-apoptotic functions such as cell cycle progression, proliferation, autophagy, inflammation and innate immunity (1, 2). Particularly, FADD phosphorylation at Ser194 (pFADD) by several kinases is associated with its nuclear localization and cell cycle regulation (3-8). Although FADD and pFADD are often overexpressed in various tumors, their functions in cancer development or chemotherapy- sensitivity are still controversial (9-14).

Recent strong evidence from *in vivo* mice studies suggested negative roles of FADD in RIP1- and RIP3-dependent necroptosis (15-18). DR-mediated caspase-8 activation requires FADD, and leads to the cleavage of RIP1, RIP3, and CYLD, preventing necroptosis (19-22). Thus, FADD deficiency is thought to inhibit caspase-8 and subsequent apoptosis, but activate necroptosis. Since necroptosis can be initiated by various stimuli in a variety of cell types independently of DRs, the exact mechanisms and functions of FADD require further investigation. This review focuses on the recent discoveries about the roles of FADD in apoptosis and necroptosis in various models, and we refer the reader to two comprehensive reviews of the diverse functions of FADD (1, 2).

EXTRINSIC APOPTOSIS

Molecular mechanism of extrinsic apoptosis

Extrinsic apoptosis, which is triggered by the extracellular signals that activate the death receptor family, is distinguished from intrinsic apoptosis, which is induced by intracellular signals such as DNA damage, oxidative stress, and nutrient deprivation (23). Extrinsic apoptosis is initiated by the binding of specific ligands such as tumor necrosis factor α (TNFα), Fas ligand (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to their corresponding receptors called 'death receptors' (DRs) (24). DR is a member of the TNF receptor superfamily and specifically contains a conserved cytosolic death domain (DD) (25). The eight kinds of DRs have different amino acid sequences that determine ligand specificity, and they can be divided into two groups according to the cytosolic adaptor protein that makes a distinct complex (24, 26, 27).

The first group includes CD95/Fas, DR4/TRAIL-R1, and DR5/TRAIL-R2, all of which recruit death-inducing signaling complex (DISC) composed of FADD and procaspase-8 (28). Fas and DR4/5 are activated by the ligation of the specific ligands FasL and TRAIL, respectively, and bind to the DD of FADD, a pivotal adaptor protein, through the DD domain. Then, DED of FADD binds to DED of procaspase-8 and -10 to construct the DISC. The

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DISC facilitates auto-proteolytic cleavage of procaspase-8 and -10, which confers their enzymatic activity and release (2). Activated caspase-8 and -10 lead to proteolytic stimulation of downstream effector caspase-3, -6, and -7, which can cleave intracellular substrates such as lamin A, poly (ADP-ribose) polymerase (PARP), and inhibitor of caspase-activated DNase (ICAD) to induce apoptotic circumstances including cell shrinkage, nuclear fragmentation, apoptotic DNA fragmentation, and ultimately, cell death (24). The series of events described above is sufficient to induce apoptotic cell death in certain cell types called type I cells, such as lymphocytes and thymocytes, but is insufficient in type II cells, including hepatocytes and pancreatic β cells, because of the relatively low levels of DISC in spite of comparable levels of the DISC components. (29-32). Type II cells require a mitochondria-dependent pathway to amplify a DR-mediated apoptotic signaling. Activated caspase-8 cleaves pro-apoptotic protein BID as well as effector caspases to generate truncated BID (tBID), which binds to pro-apoptotic proteins BAX and BAK, resulting in the leakage of the mitochondrial membrane and the release of cytochrome c and SMAC/DIABLO (33, 34). Released cytochrome c forms an apoptosome with procaspase-9 and Apaf-1 to cleave and activate caspase-9, which in turn stimulates caspase-3, -6, and -7 (24). SMAC/DIABLO, meanwhile, facilitates apoptosis by suppressing inhibitors of apoptosis proteins (IAPs) (35, 36).

The second group of DRs including TNFR1, DR3, DR6, and EDAR recruit TRADD for an adaptor protein that links DRs to TNF receptor-associated factors 2,5 (TRAF2,5), receptor-interacting protein kinase (RIP1 or RIPK1), and cellular inhibitor of apoptosis (cIAPs), forming a signaling complex called 'complex I' (24, 37). Upon the ligation of DR with their specific ligands, complex I is assembled close to the plasma membrane to stimulate mitogen-activated protein kinase/c-Jun N-terminal kinases (MAPK/JNK) involved in cell survival, proliferation or apoptosis (38-40). Complex I also stimulates nuclear factor kappa (NF-κB) pathway, facilitating cell survival and inflammatory signal (38). Complex I-mediated NF-κB stimulation is caused by cIAP-induced K63-linked polyubiquitination of RIP1 and linear ubiquitin chain assembly complex (LUBAC)-mediated linear ubiquitination of RIP1, providing a scaffold for the recruitment of TGF-beta-activated kinase 1 (TAK1) binding protein 2 and 3 (TAB2 and 3), which finally activates TAK1 (41-43). IKKγ/NEMO also binds to the polyubiquitin chain of RIP1, bringing the whole IKK complex. Then, active TAK1 phosphorylates and stimulates IKKβ, resulting in the phosphorylation and subsequent degradation of IκB, which sequesters NFκB in the cytosol (42). Subsequently, unconstrained NFκB enters the nucleus to turn on the transcription of its target gene encoding anti-apoptotic, pro-survival, and inflammatory factors. The second group of DRs can also form a cytosolic complex II. RIP1 deubiquitinating enzymes such as cylindromatosis (CYLD) remove K63-linked polyubiquitination, leading to internalization of receptor complex (38, 44). It is unclear whether the deubiquitination function of A20, ubiquitin-specific protease 21 (USP21) and cezanne (OTUB7B) are also able to induce complex II formation (45-48). The conformation changes of complex I after receptor internalization result in two kinds of cytosolic complex II (i.e., TRADD-dependent complex IIA and RIP1-dependent complex IIB), both of which can initiate apoptosis (38, 44). TRADD recruits FADD and caspase-8, forming complex IIA, where caspase-8 is activated and apoptosis is initiated (49, 50). Complex IIB is composed of RIP1 and FADD-caspase-8 and is negatively regulated by cIAPs since polyubiquitinated RIP1 cannot be incorporated into complex IIB. Therefore, IAP antagonist, including smac mimetics that induce the proteasomal degradation of IAPs, can promote complex II formation and subsequent apoptosis (51, 52).

Regulation of extrinsic apoptosis

Several regulatory machineries are involved in the DR-mediated extrinsic apoptosis pathway. Cellular FLICE-like inhibitory proteins (cFLIPs) are crucial regulators of DR signaling (See ref. 53 for Review). All of the cFLIP isoforms, including cFLIP long ($cFLIP_L$), cFLIP short ($cFLIP_S$), and cFLIP raji ($cFLIP_R$), contain two DED domains and bind to FADD via DED-DED interaction (53). All cFLIPs prevent DISC formation and consequent apoptosis by competing with caspase-8 for binding to FADD (54). The role of $cFLIP_L$ on apoptosis, however, is still controversial. $cFLIP_L$ also contains a caspase-8-like domain and forms a heterodimer with caspase-8, resulting in partial caspase-8 autoprocessing that is sufficient to generate the p43/41 and the p12 fragments (55, 56). The levels of cFLIP are regulated by numerous pathways. For instance, JNK activated by TNFα can phosphorylate and stimulate the E3 ubiquitin ligase, Itch, inducing polyubiquitination and proteasomal degradation of cFLIP (57). Moreover, the phosphatidylinositol 3-kinase/Akt pathway can upregulate cFLIP expression (58).

Various post-translational modifications (PTMs) are also implicated in the regulation of DR-mediated extrinsic apoptosis. DRs can be directly modified by several PTMs (59, 60). For example, palmitoylation of Fas/CD95 facilitates the formation of high molecular weight DISC, which includes FADD and caspase-8, resulting in caspase-8 cleavage and apoptotic cell death (60). O-glycosylation of TRAIL-R1/2 results in improved receptor clustering and subsequent DISC formation, causing better sensitivity to TRAIL (61). Furthermore, cFLIP is also regulated by various PTMs including nitrosylation, ubiquitylation, and phosphorylation (62-66). For example, phosphorylation of cFLIP isoform by protein kinase C (PKC) does not affect its interaction with the DISC. On the other hand, phosphorylation of $cFLIP_S$ induces stabilization of $cFLIP_S$ by reducing polyubiquitination, enhancing its anti-apoptotic function (62). S-receptor kinase (Srk) phosphorylates caspase-8, prevents procaspase-8 cleavage, and impairs DRs Fas-mediated apoptosis (67). Caspase-8/10-associated RING proteins (CARPs) suppress caspase-8 and -10 via ubiquitin-mediated degradation. Therefore, down-regulation of CARPs enhances DR-mediated apoptosis in human lung cancer cells (68). CARP2 also mediates K48-linked polyubiquitination and degra-

dation of RIP1, suppressing TNFα-induced NFκB activation (69). In addition to the molecules described above, many proteins involved in DR signaling such as IAPs, effector caspases, and the Bcl-2 family (e.g., Bax, Bid, and Bcl-2) are also modulated by mono- or poly-ubiquitination (70, 71). Although FADD is phosphorylated by several kinases, it is not clear whether phosphorylation influences FADD activity toward extrinsic apoptosis (3-8). Rather, it seems to be involved in cell cycle regulation (3-8). Recently, we determined that FADD turnover is mediated by MKRN1-mediated ubiquitination, affecting DR-induced apoptosis *in vitro* and *in vivo* (72).

PROGRAMMED NECROSIS (NECROPTOSIS)

Introduction to necroptosis

For a long time, necrosis has been regarded as an uncontrolled cell death. However, in the last 20 years, there has been a lot of evidence indicating that necrosis is a tightly regulated mechanism. The idea that necrosis is regulated is based on the finding that TNFα can trigger both apoptotic cell death and necrotic cell death (73). Several studies have shown that Fas or TNFR activation leads to necrotic cell death upon caspase inhibition in various cell lines including L929 mouse fibroblasts, mouse embryonic fibroblasts (MEFs), and Jurkat T cells (19, 74, 75). In addition, the serine-threonine kinase RIP1 has been identified as an essential mediator of caspase-independent necrosis (19, 74). There is an accumulation of evidence in support of regulated mechanisms and the identification of a chemical inhibitor, necrostatin-1 (nec-1), that specifically inhibits RIP1 kinase activity, leading to 'programmed necrosis' or 'necroptosis' (19, 45, 76, 77). Interestingly, the kinase activity of RIP1 (RIPK1) is indispensible for inducing necroptosis, but it is dispensable for NFκB activation (49, 78, 79). Recently, RIP3 (RIPK3, a member of RIP kinase family) was reported to be an indispensible factor for necroptosis (78-80). DRs including Fas (19), TNFR1/2 (77), and TRAIL-R1/2 (73) are well-known to induce necroptosis. Pathogen-associated molecular patterns (PAMPs) including lipopolysaccharide (LPS) and double-stranded RNA (dsRNA) also can induce necrotic cell death (80-83). Toll-like receptors (TLRs) activated by LPS, a component of gram-negative cell walls, poly(I:C) and viral dsRNA also trigger necroptosis in various cell types such as MEF, T cells, macrophage, and L929 cells (80-83). In addition to PAMPs, damaged-associated molecular patterns (DAMPs) including N-formylated peptides and mitochondrial DNA, which are released with necrotic cell death, trigger necroptosis. High-mobility group box 1 (HMGB1) induces septic shock by interacting with receptor for advanced glycation end-product (RAGE) or TLR4 (84).

Mechanism of TNF-induced necroptosis

Mechanisms of necroptosis are extensively studied using a TNFα-induced necroptosis model. TNFα-induced necroptosis is initiated by the binding of ligand to TNFR1. Activation of TNFR1 recruits complex I, leading to a prosurvival pathway such as the NF-kB pathway (41, 85). Similar to apoptosis induction, necroptosis also requires the removal of the K63-linked polyubiquitin chain of RIP1, which provides a docking site for TAK/TAB and IKK complexes and induces the NF-kB pathway (42, 43, 85). CYLD is required for necroptosis induction by deubiquitinating RIP1, whereas the involvements of other deubiquitinating enzymes that trigger RIP1 deubiquitination such as A20, USP21 and cezanne in necroptosis induction are currently unknown (46-48, 86). Inhibition of cIAP function, which is critical for RIP1 polyubiquitination, by genetic deletion or pharmacological methods, has been reported to sensitize cells to necroptotic cell death by preventing RIP1 K63-linked ubiquitination (49, 78, 87-90). RIP1 deubiquitination leads to the formation of complex II, which is composed of TRADD, FADD, RIP1 and caspase-8 (91). Recently, RIP3 was identified as an essential mediator of necroptosis using screening methods (78-80). Most importantly, RIP3-expressing cells have an ability to undergo necroptosis, whereas RIP3-deficient cells do not (78). Furthermore, RIP3-deficient MEFs and cells depleted of RIP3 showed resistance to necrotic cell death (78-80). Mechanistically, RIP3 is recruited to a signaling complex containing RIP1, FADD, and caspase-8 in response to necrotic stimuli (78, 79). Phosphorylation of RIP1 at Ser 161 and RIP3 at Ser 199 occur after necrotic stimuli, and these phosphorylations are considered essential for necrosome assembly and activation (78). RIP1 can be phosphorylated by RIP3 or RIP1 itself, whereas RIP3 is known to be phosphorylated by itself. However, it remains unclear whether other kinases are involved in RIP1/RIP3 phosphorylation and necrosome activation (44, 79). The crucial role of RIP1 kinase activity on necroptosis has been implicated in many studies (19, 49), and is supported by the critical roles of necrostain-1, which specifically inhibits RIP1 kinase activity and prevents necrosome assembly and necroptosis (19, 78, 79, 92, 93).

Regulation of necroptosis by caspase-8 activity

In 1998, it was reported that the pharmacological inhibition of caspase activity sensitizes TNF-mediated necrotic cell death in L929 cells (94). From this finding, the concept of apoptosis blocking necrosis by caspase activity has been accepted as an established theory. Indeed, zVAD-fmk, a pan-caspase inhibitor, has been widely used to induce necroptosis in a variety of cell lines as well as mice models (93, 94). Among the caspases, caspase-8 is responsible for the transition from apoptosis to necroptosis (93). Caspase-8-deficient Jurkat cell lines underwent necroptosis instead of apoptosis upon Fas and TNFR stimulation. Recent *in vivo* work showed that caspase-8-deficient mice have significant necroptotic death, leading to embryonic lethality, also supporting the roles of caspase-8 in necroptosis suppression. T cell- or intestinal epithelial cell-specific deletion of caspase-8 in mice also exhibited severe necroptotic features, inducing immunodeficiency or terminal ileitis, respectively (95, 96).

The critical roles of caspase-8 on necroptosis are known to induce the cleavage of RIP1 and RIP3 (20, 21). Therefore, cells treated with caspase inhibitor or a deficient in caspase-8 increase

the RIP1-RIP3 complex formation as well as necroptosis (22, 78, 79). In addition, CYLD was recently identified as a target of caspase-8 and a critical mediator. Caspase-8-mediated CYLD cleavage at Asp215 prevents necroptosis, whereas expression of mutant CYLD (D215A), which is resistant to caspase-8-mediated cleavage, into CYLD -/- MEF enhances necrosome formation and necroptosis (22).

Execution of necroptosis

Mitochondrial reactive oxygen species (ROS) has a pivotal role in necrotic cell death in certain cell types (97). TNF stimulation is known to activate JNK-dependent ROS production (98). Ferritin degradation by JNK activation increases the labile iron pool, thereby enhancing ROS production (98). RIP1-deficient MEFs failed to elevate the labile iron pool and induce cell death upon TNF stimulation, suggesting a critical role for RIP1 on TNF-induced ROS production and necrosis (99). However, the detailed molecular mechanism by which RIP1 affects ROS production is unclear.

Activation of necrosome complex also promotes ROS production by directly activating metabolic pathways. RIP3 physically interacts with and activates several key metabolic enzymes including GLUL, GLUD1 and PYGL to induce ROS production and necroptosis upon TNFα activation (80). GLUL and GLUD1, which convert glutamate to α-ketoglutarate, induce glutamate depletion, causing glutaminolysis. An increase in glutaminolysis results in ammonia accumulation, leading to ROS production to detoxify the ammonia in mitochondria (100). PYGL activation triggers the breakdown of glycogen to glucose-1-phosphate (G1P), which can be converted to its isomer, glucose-6-phosphate (G6P). Methyglyoxal generated by G6P covalently binds to many proteins, producing ROS by activating NAD(P)H oxidase (101). In addition to these mechanisms, induction of a respiratory burst by these enzymes contributes to over-production of ROS and necroptosis (102).

ATP depletion has also been implicated as an executor of necroptosis. During apoptosis, caspase cleaves and inactivates poly (ADP-ribose) polymerase-1 (PARP-1), preventing ATP depletion. Inhibition of protein translation and proteasomal degradation upon apoptosis activation also suppresses ATP depletion (103-105). In contrast, ROS-mediated DNA damage activates PARP-1, inducing the depletion of ATP and NAD during TNF-mediated necroptosis (106).

Lysosomal membrane permeabilization (LMP) has also been shown to be an executor of necroptosis. Over-produced ROS attacks polyunsaturated fatty acids, and generates toxic aldehydes (4-hydroxynonenal). This toxic material modifies many cellular proteins and destabilizes the lipid components of membranes (107). 4-hydroxynonenal-mediated modification leads to mitochondrial malfunction, including blockage of oxidative phosphorylation and ATP synthesis, inner membrane permeabilization, mitochondrial transmembrane potential dissipation and reduction of the $Ca2+$ buffering system (108). In addition to ROS-mediated LMP, treatment with TNF in L929 cells triggers SMases activation, which converts ceramide to sphingosine, a known LMP inducer (109, 110). However, the detailed molecular mechanism underlying necroptosis remains to be elucidated.

Recently, two direct target of RIP3 kinase involving necroptosis execution have been identified. Phosphorylation of mixed lineage kinase domain like protein (MLKL) by RIP3 is shown to be crucial for TNF- induced necroptosis (111). PGAM5 is recruited to the RIP1-RIP3 necrosome complex and is phosphorylated by RIP3. PGAM5-RIP1-RIP3 complex also recruits and activates Drp1 by inducing dephosphorylation. Drp1 dephosphorylation eventually leads to mitochondrial fragmentation and induces necroptosis (112).

THE ROLES OF FADD ON APOPTOSIS AND NECROPTOSIS *IN VITRO* AND *IN VIVO*

The roles of FADD on DR-mediated apoptosis and necroptosis

DR activation is the best known pathway for activation of necroptosis as well as apoptosis. Being an essential adaptor protein that links DRs with caspase-8, FADD is considered to be critical for death receptor-induced apoptosis. As FADD is a direct and essential adaptor for Fas and TRAIL-R, FADD deficiency abolished Fas- or TRAIL-R-induced apoptosis and necroptosis (Fig. 1) (19). Supporting this, FADD-deficient mouse embryonic fibroblasts and thymocytes were resistant to Fas-induced apoptosis (113, 114). Furthermore, FADD has been shown to be a critical factor for TRAIL-induced apoptosis using FADD-deficient Jurkat

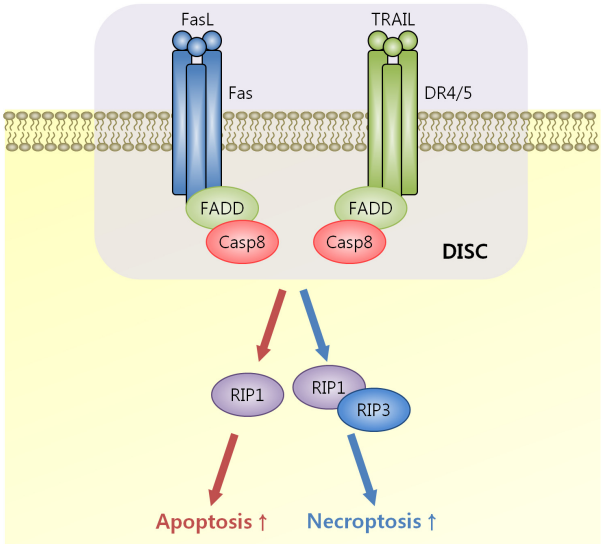

Fig. 1. The role of FADD in Fas and TRAIL signaling. (A) FADD is required for both apoptosis and necroptosis induced upon Fas and TRAIL-R activation. FADD is an essential adaptor protein that links death receptor for FasL or TRAIL to caspase-8, thereby inducing DISC formation. These complexes have an ability to induce both apoptosis and necroptosis.

T cells (115-117). In case of TNF-induced cell death, in which TRADD, RIP1 and TRAF2/5 act as adaptors, the roles of FADD are controversial because FADD is not a direct adaptor (Fig. 2A). Indeed, FADD was detected in TNF-induced complex II and necrosome complex in numerous studies (Fig. 2B) (22, 78, 79, 118, 119). Since FADD is required for full activation of caspase-8, both FADD and caspase-8 are required for apoptosis (Fig. 2C). Consistent with this idea, TNF-induced apoptosis was abrogated by FADD deficiency (19, 113). In contrast, several studies also suggest FADD is indispensable for TNF-induced apoptosis (120). Since caspase-8 suppresses necroptosis and leads to apoptosis, inhibition of caspase-8 is a prerequisite for converting signaling from apoptosis to necroptosis in most cell types. (Fig. 2C and 2D) (121). Similar to TNF-induced apoptosis, several studies suggest FADD is also required for TNF-induced necroptosis (Fig. 2E). FADD KO MEF exhibited resistance to TNF-induced necroptosis (118). On the other hand, many reports have suggested negative roles of FADD in TNF-induced necroptosis. A study using L929 cells with FADD knockdown by shRNA showed FADD depletion induced necroptosis (Fig. 2F) (90). We also recently reported that siRNA- or shRNA-mediated knockdown of FADD in L929 and HT-29 cells resulted in an increase in RIP1-RIP3 necrosome formation and necroptosis in the presence of caspase inhibitor, whereas FADD overexpression in L929 cells delayed RIP1-RIP3 necrosome formation and necroptosis (Fig. 2G versus 2D) (72). Since FADD is required for the activation of caspase-8, which is able to suppress necroptosis, FADD-deficient cells facilitate necroptotic cell death by abrogating caspase-8 function (Fig. 2F). However, the evidence that FADD also prevents necroptosis in the presence of caspase inhibitor suggests that FADD might regulate necroptosis in a caspase-8-independent manner (Fig. 2G). Indeed, since FADD can interact with RIP1 and RIP3, FADD might directly regulate the RIP1-RIP3 interaction (79) (unpublished data). The involvement of FADD in necroptosis induced by other DRs such as TWEAK is currently unknown.

The role of FADD in necroptosis induced by other signals

In addition to DRs, various signals are capable of inducing necroptosis (See ref. 122 for review) . Toll-like receptor signaling activated by LPS, poly(I:C), and viral dsRNAs is well known to induce necroptosis although exact mechanisms are unclear (122). Toll/IL-1 receptor domain-containing adapter inducing IFN-β (TRIF) has been suggested as a key molecules for TLR3- and TLR4-induced necroptosis as it forms a complex with RIP3 (Fig.

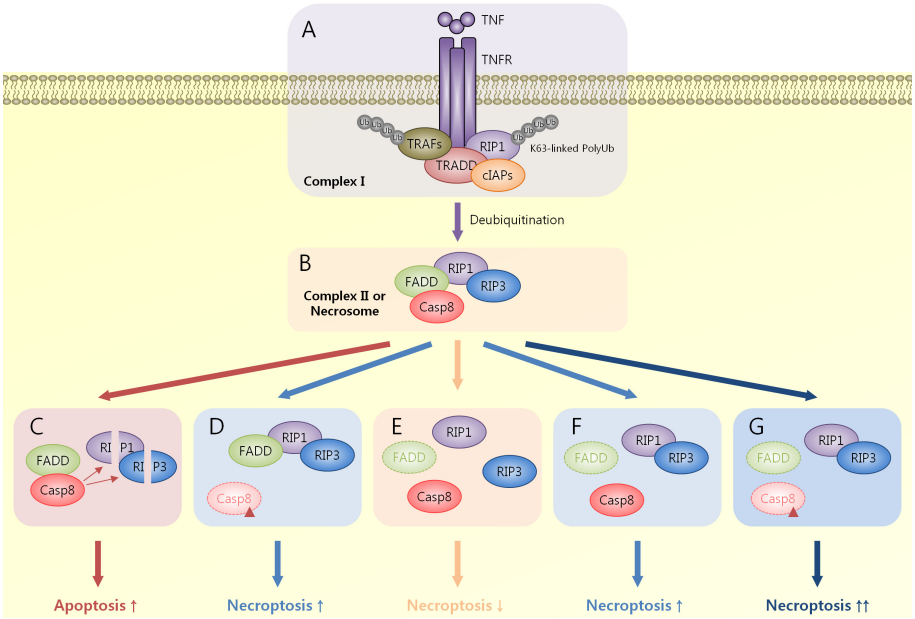

Fig. 2. Proposed model for FADD regulation of apoptosis and necroptosis upon TNFR activation. (A) TNFR activation recruits several adaptor proteins including TRADD, RIP1, and TRAF2/5, but not FADD, and forms a complex I to activate the NF-kB pathway. (B) Upon RIP1 deubiquitination, RIP1 forms cytosolic complex II by recruiting FADD, caspase-8, and RIP3. (C) Both FADD and caspase-8 are required to mediate apoptosis by activating effector caspases and suppress necroptosis by inducing proteolytic cleavage of RIP1, RIP3, and CYLD. (D) In the absence of caspase-8 or in the presence of caspase inhibitor, RIP1, RIP3, and CYLD are protected and can form a necrosome complex and induce necroptosis. (E) One model suggests that FADD deficiency abolishes the formation of RIP1 and RIP3 complex, thereby suppressing necroptosis. (F) The other model, which is in conflict with the above-mentioned model (E), suggests that caspase-8 activity might be limited in the absence of FADD, and thus RIP1 and RIP3 are able to induce necroptosis. (G) In the presence of caspase-8 inhibitor, FADD depletion can enhance necrosome formation and necroptosis. FADD might interfere with the association between RIP1 and RIP3 in a caspase-8-indepdendent way.

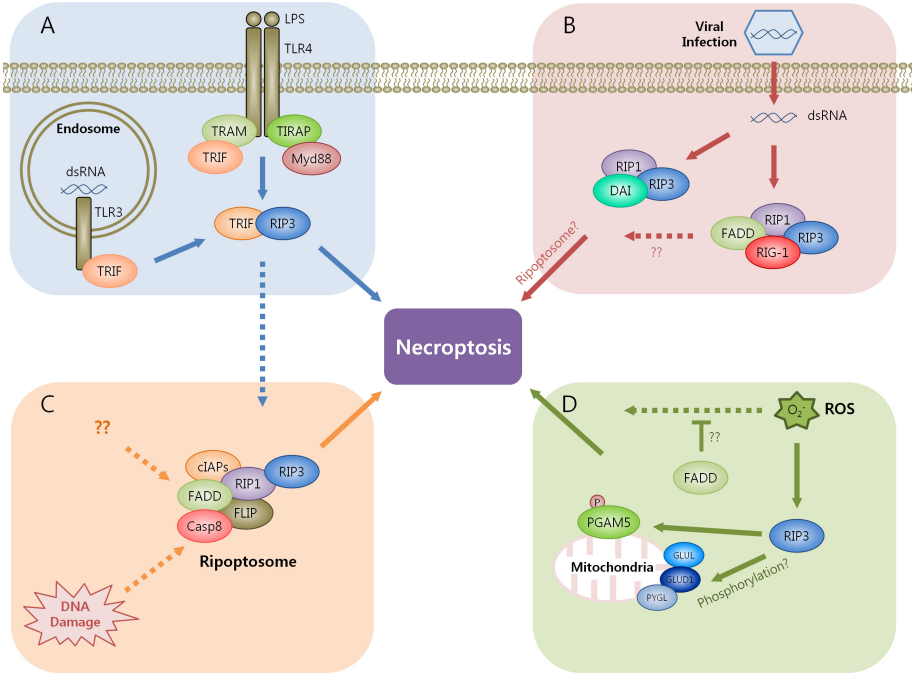

Fig. 3. Possible roles of FADD on necroptosis in response to various stimuli. (A) TLR3 or TLR4 activation leads to the interaction between TRIF and RIP3, inducing RIP3 activation and necroptosis. FADD-deficient cells were sensitive to TLR-mediated necroptosis, indicating that FADD negatively regulates TLR-mediated necroptosis. However, whether FADD directly regulates TRIF and RIP3 is currently unknown. Also, RIP1 interacts with TRIF to induce cell survival and RIP3 to induce necroptosis, suggesting an intricate function of RIP1. (B) Upon viral infection, dsRNA is recognized by RIG-1 and DAI. FADD forms a complex with RIP1, TRADD, and RIG-1 upon viral infection. Although this complex seems to have a potential role in inducing necroptosis, it has not been studied in a necroptosis model. DAI recruits RIP1 and RIP3, mediating necroptosis. (C) RIP1, FADD, caspase-8, FLIP and IAPs form a ripoptosome complex upon DNA damage or IAP depletion independently of death receptor pathway, and mediates apoptosis and necroptosis. Ripoptosome is also thought to be involved in necroptosis, occurring in the cytosol independently of DR activation such as TLR signaling. (D) ROS is an important executor mechanism for necroptosis. Recently, RIP3 was found to directly activate several proteins to induce mitochondrial ROS generation. Since FADD-deficient cells seem to be more sensitive to H_2O_2-induced necroptosis, FADD might interfere with ROS-mediated necroptosis directly or indirectly.

3A) (123). TRIF is also known to interact with RIP1 to induce cell survival (124). Since RIP1 has a role in cell survival and necroptotic cell death, the role of RIP1 in TLR-induced necroptosis is not still clear (123). FADD-deficient Jurkat T cells underwent necrosis, while wild-type Jurkat T cells underwent apoptosis in response to dsRNA, suggesting FADD might have a negative role in TLR-induced necroptosis (Fig. 3A) (83). However, whether FADD directly regulates TRIF-RIP3 complex has not been studied yet. Activation of RIG-1 upon viral infection also triggers the formation of signaling complex containing RIP1, FADD, and TRADD, which are essential for dsRNA signaling (122) (Fig. 3B). But, it remains to be determined if the RIG-1-FADD pathway induces necroptosis. The cytosolic sensor DNA-dependent activator of IFN-regulatory factors (DAI) also recognizes viral dsRNA and recruits RIP1 and RIP3 to initiates necroptosis or NF-kB pathway (125, 126). Recently, a 2-MDa intracellular death-inducing complex called a 'ripoptosome' was identified (127, 128). This complex contains RIP1, FADD, caspase-8, cFLIP, cIAP1/2, and XIAP. Ripoptosome can be formed and induce both apoptosis and necroptosis upon genotoxic stress or TLR3 activation independent of DR activation (Fig. 3C) (127, 128). Since DAI is also associated with RIP1 and RIP3, ripoptosome might be involved in DAI-induced necroptosis (125). IAP antagonists enhance ripoptosome formation, suggesting that cIAP1/2 and XIAP prevent ripoptosome function. However, it remains to be studied whether FADD controls apoptosis and necroptosis as a member of the ripoptosome.

For a long time, oxidative stress and reactive oxygen species (ROS) were thought to induce necrosis. Indeed, TNF-induced necroptosis activates mitochondria to generate massive ROS, which is regarded as an executioner of necroptosis. RIP3 is known to directly bind to and phosphorylate many enzymes (related to ROS) (80, 112) (Fig. 3D). FADD-deficient MEF has been shown to be hypersensitive to H_2O_2-induced necroptosis (18, 129), but the regulatory mechanism remains unclear (Fig. 3D). To summarize, although FADD is indispensible for DR-induced apoptosis, the roles of FADD and its mechanism on signal-mediated necroptosis is still controversial and requires fur-

ther research in detail.

The roles of FADD in an *in vivo* mice model

Although FADD seems to have contradictory roles in necroptosis after induction by various stimuli in cell-based models, the *in vivo* role of FADD in necroptosis under various conditions has been shown to be consistent. Despite the defect in inducing apoptosis, genetic deletion of FADD has shown embryonic lethality with a defect in T cell proliferation (113, 114). In addition, ectopic expression of the dominant-negative form of FADD or T cell-specific depletion of FADD suppresses T cell development (130-132), in part, due to defect in immune signaling and cell proliferation (2, 133, 134). Interestingly, necroptosis was recently suggested to be a main cause of embryonic lethality in FADD-deficient mice. FADD KO mice showed increased necrosis with upregulation of RIP1, and lethality of FADD KO was successfully rescued by RIP1 deletion (18). In addition, necroptosis was also involved in the T cell defect in functional FADD deficiency although the roles of RIP1 and RIP3 are controversial. The earlier study using T cell-specific deletion of FADD resulted in necroptosis independently of RIP1 or RIP3 (135), whereas the later study using T cell-specific expression of the dominant-negative form of FADD (FADDdd) resulted in RIP3-dependent necroptosis, and this was rescued by RIP3 deletion (16). Autophagy is also increased in T cell-specific FADD depletion, leading to RIP1-dependent necroptosis (136). Furthermore, epidermal keratinocyte-specific deletion of FADD resulted in RIP3-dependent necroptosis, which induced skin inflammation (15). FADD has also been shown to suppress epithelial cell necroptosis using an intestinal epithelial cell-specific knockout mouse model (17). Since FADD is involved in necroptosis, which is induced by a variety of stimuli, the specific mechanism by which FADD regulates necroptosis *in vivo* requires further studies.

Proposed mechanisms by which FADD regulates necroptosis in a caspase-8-dependent and -independent manner

Caspase-8 has critical roles in regulating between apoptosis and necroptosis. In normal condition, necroptosis is suppressed by caspase-8-mediated cleavage and inhibition of RIP1 and RIP3. Thus, most necroptosis models require caspase inhibition by a viral protein or chemical inhibitor. Many *in vivo* experiments using caspase-8-deficient mice support these ideas. Caspase-8-deficient mice also die between E10.5 and E11.5, despite the absence of apoptosis (119, 137). Embryonic lethality of these mice, which showed massive necroptosis, was rescued by RIP3 deletion (119, 137). Furthermore, caspase-8-deficient T cells displayed necroptosis cell death, which was revered by RIPK3- deficiency (95, 138). In addition, caspase-8 is shown to negatively regulate epithelial cell necroptosis and terminal ileitis using conditional caspase-8-deficient mice (96). As a critical role of FADD in caspase-8 activation, FADD deficiency is thought to lead to necroptosis by inhibiting caspase-8 function. Supporting this idea, FADD deficiency abrogated caspase-8 activity and resulted in necroptosis (90). However, FADD's functions on various stimuli-induced necroptosis are contradictory; it seems to be dependent of stimuli. Since many studies have analyzed the roles of FADD in necroptosis under caspase inhibition using zVAD-fmk, and pan-caspase inhibitor, the presence and absence of FADD will not affect caspase-8 activity. Interestingly, FADD-depleted cells formed a more RIP1/RIP3 necrosome complex upon TNF activation in the presence of caspase inhibitor (72). Since FADD can bind to RIP1 and RIP3, FADD might directly modulate the interaction between RIP1 and RIP3 (72, 79). Thus, we cannot exclude the idea that FADD might have caspase-8-independent role as well as caspase-8-dependent role in necroptosis. The exact mechanism by which FADD regulates necroptosis in response to various stimuli requires further investigation.

CONCLUDING REMARKS

Beyond the essential function of FADD on the induction of DR-mediated apoptosis, FADD has been implicated in numerous signaling pathways, including those for cell cycle regulation, immune signaling, and autophagy as well as physiological outcome inducing inflammation, cell proliferation, embryonic and immune cell development and tumorigenesis (2). Recent studies have used various mouse model, highlighting the important roles of FADD on necroptosis affecting embryonic development, immune function, intestinal and skin inflammation (15-18). Nevertheless, the exact molecular mechanism of FADD-dependent necroptosis regulation has been poorly studies and is still controversial. In this review, we have discussed the roles of FADD on necroptosis triggered by a variety of signals based on the existing research on FADD's function in necroptosis. FADD seems to be essential for the Fas- or TRAIL receptor-activated necroptosis, whereas it seems to act negatively to TNF-induced necroptosis. Upon various stimuli, FADD forms a signaling complex with RIP1 and RIP3, both of which are important to necroptosis induction. However, whether FADD affects necroptosis positively or negatively upon a range of stimuli has not been extensively explored. Taken together, as an important regulator for development, tumorigenesis and immune function, the studies on FADD *in vitro* and *in vivo* will advance the perspective for developing therapeutic methods for cancer and immune disease.

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