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# In mouse embryonic fibroblasts, neither caspase-8 nor cellular FLICE-inhibitory protein (FLIP) is necessary for TNF to activate NF-κB, but caspase-8 is required for TNF to cause cell death, and induction of FLIP by NF-κB is required to prevent it

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**Binding of TNF to TNF receptor-1 can give a pro-survival signal through activation of p65/RelA NF-κB, but also signals cell death. To determine the roles of FLICE-inhibitory protein (FLIP) and caspase-8 in TNF-induced activation of NF-κB and apoptosis, we used mouse embryonic fibroblasts derived from FLIP and caspase-8 gene-deleted mice, and treated them with TNF and a smac-mimetic compound that causes degradation of cellular inhibitor of apoptosis proteins (cIAPs). In cells treated with smac mimetic, TNF and Fas Ligand caused wild-type and FLIP$^{-/-}$ MEFs to die, whereas caspase-8$^{-/-}$ MEFs survived, indicating that caspase-8 is necessary for death of MEFs triggered by these ligands when IAPs are degraded. By contrast, neither caspase-8 nor FLIP was required for TNF to activate p65/RelA NF-κB, because IκB was degraded, p65 translocated to the nucleus, and an NF-κB reporter gene activated normally in caspase-8$^{-/-}$ or FLIP$^{-/-}$ MEFs. Reconstitution of FLIP$^{-/-}$ MEFs with the FLIP isoforms FLIP-L, FLIP-R, or FLIP-p43 protected these cells from dying when treated with TNF or FasL, whether or not cIAPs were depleted. These results show that in MEFs, caspase-8 is necessary for TNF- and FasL-induced death, and FLIP is needed to prevent it, but neither caspase-8 nor FLIP is required for TNF to activate NF-κB.**
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Ligation of members of the TNF receptor superfamily typically triggers activation of transcription factors such as NF-κB, as well as proteins that can lead to cell death, such as RIPK1 and caspase-8.[1,2] The regulatory pathways that culminate in a pro-survival or apoptosis signal depend on the specific receptor that is ligated and the actions of proteins that transduce and modulate the signals flowing from it. For example, cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2) and TNF receptor-associated factors (TRAF2 and TRAF3) are needed to prevent apoptosis of cells exposed to TNF, and favor activation of canonical (p65/RelA) NF-κB *versus* non-canonical (p100) NF-κB2.[3–5]

Although TNF alone does not kill wild-type (WT) MEFs, it does cause apoptosis of MEFs in which genes for TRAF2 or p65/RelA NF-κB are deleted.[6] TNF can also kill MEFs that are mutant for cIAP1, and those in which cIAPs are depleted owing to treatment with a synthetic IAP antagonist 'smac-mimetic' compound.[7] These experiments indicate that pathways requiring cIAPs, TRAF2, and p65/RelA are normally able to block TNF-induced pro-apoptotic signals. Because treatment of cells with the translational inhibitor cycloheximide also sensitizes MEFs to killing by TNF, these experiments suggest that NF-κB drives the expression of cell death-inhibitory genes.[8]

One of the NF-κB-regulated genes that is proposed to inhibit TNF-induced apoptosis is FLICE-inhibitory protein (FLIP). FLIP is structurally related to caspase-8. Like caspase-8, FLIP's N-terminus contains two death effector domains (DEDs), and its C-terminus has a caspase-like domain that is proteolytically inactive.[9]

Transcripts from the murine FLIP gene can undergo alternative splicing to produce two different forms, FLIP-L and FLIP-R. In humans, a second short isoform, FLIP-S, is also produced that, like FLIP-R, harbors two DEDs but does not contain the caspase-8-like domain that is present in the FLIP-L form.

Like FLIP, expression of cIAP genes, in particular cIAP2, is regulated by NF-κB.[10] Although it has been proposed that FLIP and cIAPs are the long sought after key NF-κB-dependent genes that allow survival of TNF-treated cells, it has more recently been proposed that cIAPs, FLIP, and

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even caspase-8 might instead act upstream from NF-$\kappa$B to cause its activation, rather than acting downstream from NF-$\kappa$B. For example, whereas activation of caspase-8 can cause apoptosis of many cell types, in others, such as T-lineage cells and hematopoietic progenitor cells, caspase-8 has been reported to act upstream, and was required for activation of NF-$\kappa$B or for cellular proliferation.[11–14] In human Jurkat T cells, FLIP has also been reported to activate NF-$\kappa$B, and thereby to inhibit caspase-8-induced cell death.[15–17]

It has been reported that FLIP and caspase-8 are sometimes necessary for activation of NF-$\kappa$B, or are capable of activating NF-$\kappa$B when overexpressed.[13,14] For example, Hu *et al*.[13] found that overexpression of FLIP or caspase-8 caused potent activation of an NF-$\kappa$B reporter in 293HEK cells, and Chaudhary *et al*.[14] found that overexpression of caspase-8 or FLIP in 293T or MCF7 cells activated NF-$\kappa$B reporters. Interestingly, both groups found that, although catalytically inactive forms of caspase-8 were still able to induce NF-$\kappa$B, and neither viral nor synthetic caspase inhibitors could block this, these inhibitors were able to block NF-$\kappa$B induction by overexpression of FLIP, suggesting that proteolysis of FLIP by caspase-8 is not required for it to activate NF-$\kappa$B, but might be necessary for caspase-8 to somehow activate FLIP.

In human lymphocytic and dendritic cells, cleavage of FLIP-L at ASP198 by pro-caspase-8 yields the N-terminal FLIP-p22 cleavage product that has been proposed to bind to the I$\kappa$B kinase-regulatory complex and trigger activation of NF-$\kappa$B.[18] On the other hand, in human epithelial (293HEK) and lymphocytic cells, FLIP-L has been reported to be cleaved at ASP376 by mature caspase-8 to produce FLIP-p43, which can directly interact with TRAF2, to also promote activation of NF-$\kappa$B.[17] Despite some discrepancies, these reports suggest that both caspase-8 and FLIP are required for activation of NF-$\kappa$B in human epithelial, lymphocytic, and dendritic cells in response to certain stimuli, such as treatment with LPS or anti-CD3/CD28.

In murine T lymphocytes, Salmena *et al*.[19] and Ch′en *et al*.[20] showed that deletion of caspase-8 in T-lineage cells caused immunodeficiency, revealing a requirement for caspase-8 in T-cell homeostasis and T-cell-mediated immunity *in vivo*. However, in B lymphocytes, Beisner *et al*.[21] found that, although caspase-8 deficiency prevented proliferation in response to LPS, activation of NF-$\kappa$B was unaffected.

To examine the roles of and requirements for FLIP and caspase-8 in TNF-triggered responses in a cell type that could be readily manipulated genetically and biochemically, and, in particular, to determine whether cIAPs, TRAF2, FLIP, and caspase-8 act before or after activation of NF-$\kappa$B, we chose to study them in mouse embryonic fibroblast (MEF) lines derived from WT and gene-deleted mice. These experiments showed that in TNF-treated MEFs, FLIP is not required for induction of canonical RelA/p65 NF-$\kappa$B, but is essential to prevent cell death, because FLIP $^{-/-}$ MEFs activated NF-$\kappa$B normally, but unlike WT MEFs, died when exposed to TNF. While caspase-8 was not needed for TNF to activate NF-$\kappa$B, it was required for death of MEFs treated with Fas Ligand or TNF when IAPs were depleted by a smac-mimetic compound.

## Results

**NF-$\kappa$B activation is not impaired in caspase-8$^{-/-}$ and FLIP$^{-/-}$ MEFs.** Several groups have reported that caspase-8 and FLIP can cause activation of NF-$\kappa$B in lymphocytes and 293HEK cells.[15,17,18] Moreover, over-expression of FLIP-L was sufficient to activate the NF-$\kappa$B signaling pathway in the absence of added cytokine.[17] To determine whether caspase-8 and FLIP are required for TNF to activate NF-$\kappa$B in MEFs, we used immunofluorescence to follow translocation of p65/RelA NF-$\kappa$B to the nucleus after treating WT, caspase-8$^{-/-}$, and FLIP$^{-/-}$ MEFs with TNF.

As seen in Figure 1a, the majority of p65/RelA resides within the cytoplasm of untreated WT cells, but after 20-min

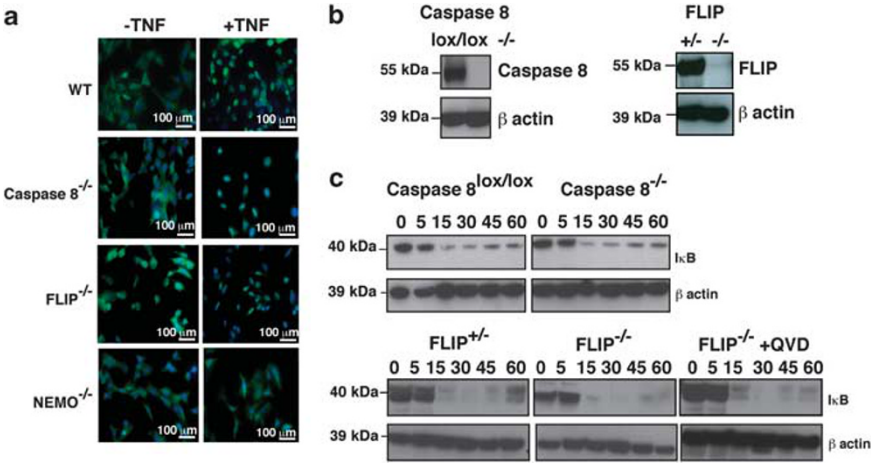


**Figure 1** TNF-induced nuclear translocation of p65/RelA NF-$\kappa$B is not impaired in caspase-8$^{-/-}$ or FLIP$^{-/-}$ MEFs. (**a**) WT MEFs, caspase-8$^{-/-}$, FLIP$^{-/-}$, and NEMO$^{-/-}$ MEFs were cultured with or without 70 ng/ml TNF for 20 min and stained with an anti-p65 antibody (green) and DAPI (blue), and visualized by immunofluorescence microscopy. Nuclear translocation of p65/RelA occurred within 20 min of TNF treatment, except in NEMO$^{-/-}$ MEFs, which were used as control to show defective p65/RelA translocation. (**b**) Absence of caspase-8 and FLIP in caspase-8$^{-/-}$ and FLIP$^{-/-}$ MEFs. Cell lysates from control and gene-deleted MEFs were probed for caspase-8 or FLIP. $\beta$-Actin was used as a loading control. (**c**) TNF-induced I$\kappa$B degradation is not impaired in caspase-8$^{-/-}$ and FLIP$^{-/-}$ MEFs. Caspase-8$^{lox/lox}$, caspase-8$^{-/-}$, FLIP$^{+/-}$, and FLIP$^{-/-}$ MEFs were treated with 100 ng/ml TNF for the times indicated. Cell lysates were probed for I$\kappa$B$\alpha$ and $\beta$-actin was used as a loading control

treatment with TNF the majority of p65/RelA staining was detected within the nucleus. To confirm that nuclear translocation of p65/RelA was dependent on an intact signaling pathway, we treated NEMO$^{-/-}$ MEFs with TNF, because NEMO, the regulatory subunit of the I$\kappa\kappa$ kinase complex, is required for activation of NF-$\kappa$B.[22] Consistent with published results,[23] in NEMO$^{-/-}$ MEFs p65/RelA failed to translocate to the nucleus after treatment with TNF (Figure 1a). By contrast, when treated with TNF, p65/RelA translocated to the nucleus normally in both caspase-8$^{-/-}$ and FLIP$^{-/-}$ MEFs, suggesting neither cell line has a defect in p65/RelA activation in response to TNF (Figure 1a).

Both FLIP and caspase-8 have been proposed to act upstream from I$\kappa$B degradation in the activation of NF-$\kappa$B.[14,17] To test this we examined degradation of I$\kappa$B in response to TNF. WT, caspase-8$^{-/-}$, and FLIP$^{-/-}$ MEFs were treated with TNF for various time periods and I$\kappa$B levels were determined by western blotting. In WT MEFs, I$\kappa$B was degraded after 5 min of TNF treatment, with maximal loss of I$\kappa$B occurring within 5–15 min. Subsequently, I$\kappa$B levels were restored to baseline by 60 min.[7,24] Significant loss of I$\kappa$B was also evident in caspase-8$^{-/-}$ and FLIP$^{-/-}$ MEFs in response to TNF (Figure 1c); however, unlike in WT MEFs, the baseline levels of I$\kappa$B had not been fully restored within 60 min in FLIP$^{-/-}$ MEFs. To determine whether restoration of I$\kappa$B levels was caspase-dependent, we tested whether I$\kappa$B levels were restored in FLIP$^{-/-}$ MEFs when caspases were blocked by the broad-spectrum inhibitor QVD-OPh (QVD).[25] As shown in Figure 1c, pre-treatment with QVD did not affect the rate at which I$\kappa$B levels changed.

These results suggest that the TNF-induced signaling pathways upstream from NF-$\kappa$B are not defective in caspase-8$^{-/-}$ or FLIP$^{-/-}$ MEFs. To confirm this, we infected WT, caspase-8$^{-/-}$, and FLIP$^{-/-}$ MEFs with a lentiviral vector encoding an NF-$\kappa$B-GFP reporter construct and assessed NF-$\kappa$B activation by GFP fluorescence using flow cytometry (Figure 2). TNF induced similar levels of fluorescence from the NF-$\kappa$B-GFP reporter in all MEF genotypes (Figure 2a, column-2). Furthermore, similar levels of GFP were produced from the NF-$\kappa$B reporter when MEFs were treated with smac mimetic (Figure 2a, column 3) or the TNF family cytokine TWEAK (Figure 2a, column 4). Once again, this suggests that neither caspase-8 nor FLIP is required for TNF to activate p65/RelA in this cell type.

Because FLIP was not required for TNF to activate p65/RelA in MEFs, we next tested whether overexpression of any of the FLIP isoforms was sufficient to activate NF-$\kappa$B. To do so we infected FLIP$^{-/-}$ MEFs with lentiviral constructs that express FLIP-L, FLIP-R, or FLIP-p43 when induced by 4-hydroxy tamoxifen (4HT) (Figure 2b). Contrary to what was observed in 293HEK cells,[17] overexpression of the FLIP isoforms at levels similar to those found in WT MEFs was not sufficient to activate NF-$\kappa$B (Figure 2c, column 2). Each of these MEF mutants was still able to activate NF-$\kappa$B normally in response to TNF, smac mimetic, or TWEAK (Figure 2c, columns 3–5).

Production and secretion of IL-6 in response to TNF has been shown to depend on NF-$\kappa$B signaling. Although nuclear translocation of p65/RelA, I$\kappa$B degradation, and NF-$\kappa$B activation were normal in caspase-8$^{-/-}$ and FLIP$^{-/-}$ MEFs, we wished to determine whether TNF could stimulate the production of IL-6 by caspase-8$^{lox/lox}$, caspase-8$^{-/-}$, FLIP$^{+/-}$, and FLIP$^{-/-}$ MEFs. To test this, we used ELISAs to measure IL-6 secreted by the MEFs after treatment with TNF for 24 h. Not only was IL-6 induced in caspase-8$^{-/-}$ MEFs, the levels secreted were at least as great as those produced by WT MEFs (Figure 2d). Similarly, TNF was able to stimulate IL-6 secretion by FLIP$^{-/-}$ MEFs, but they produced much less than FLIP$^{+/-}$ MEFs. However, pre-treatment with QVD, which prevented the cells from dying, restored IL-6 levels made by FLIP$^{-/-}$ MEFs so that they were similar to those made by the FLIP$^{+/-}$ MEFs (Figure 2d). We believe QVD increased IL-6 secretion by prolonging cell survival, because it had little effect on the amount of IL-6 produced by the WT (caspase-8$^{lox/lox}$) MEFs, which do not die in response to TNF (Figure 2d).

It has previously been proposed that for FLIP to activate NF-$\kappa$B, IKK$\gamma$/NEMO and TRAF2 are required.[17,18] We wanted to determine whether FLIP was bound to these proteins, either constitutively or in response to addition of TNF. To do this, we overexpressed FLAG-tagged FLIP-L and performed co-immunoprecipitation experiments using anti-FLAG antibody-conjugated beads. In the presence or absence of TNF, FLIP-L was able to co-immunoprecipitate full-length pro-caspase-8 (Figure 2e). However, FLIP-L was not able to bring down detectable levels of FADD, RIPK1, TRAF2, or cIAP1; adaptor proteins that have been shown to have roles in TNFR1 signaling.[26]

**Expression of FLIP-L, FLIP-R, or FLIP-p43 can block apoptosis of FLIP$^{-/-}$ MEFs induced by death receptor ligands.** FLIP is thought to inhibit caspase-8 activation either by preventing it from binding to FADD or by preventing pro-caspase-8 from auto-processing.[27] Given the extensive evidence showing that FLIP can modulate death-receptor signaling, we treated FLIP$^{+/-}$, FLIP$^{-/-}$, caspase-8$^{lox/lox}$, and caspase-8$^{-/-}$ MEFs with TNF alone, or TNF combined with a smac mimetic, to deplete cIAPs. Unlike FLIP$^{+/-}$ cells, FLIP$^{-/-}$ MEFs were killed by TNF alone (Figure 3a), whereas both FLIP$^{+/-}$ and FLIP$^{-/-}$ cells were killed by TNF plus smac mimetic (Figure 3b), indicating that, although the level of FLIP present in heterozygous MEFs could protect against TNF alone, it could not do so in the absence of cIAPs. Because TNF plus smac mimetic can kill WT MEFs, even the level of FLIP in FLIP$^{+/+}$ MEFs cannot stop TNF from inducing apoptosis in the absence of cIAPs.

To determine the mechanism by which MEFs died after treatment with TNF plus smac mimetic, we tested whether their death was inhibited by the broad-spectrum caspase inhibitor, QVD,[25] or the RIP1 kinase inhibitor, necrostatin-1[28] (Figures 3a and b). Because QVD strongly protected FLIP$^{-/-}$ MEFs from killing by TNF alone, as well as killing by TNF plus smac mimetic, and strongly protected FLIP$^{+/-}$ MEFs from killing by TNF plus smac mimetic, whereas necrostatin-1 had a minor effect, death of MEFs in these circumstances is due to a caspase-dependent mechanism, rather than by necroptosis, which depends on the kinase activity of RIPK1.[28]

The fact that caspase-8$^{-/-}$ but not caspase-8$^{lox/lox}$ MEFs were completely resistant to TNF plus smac mimetic-induced cell death (Figure 3c) is consistent with the results obtained

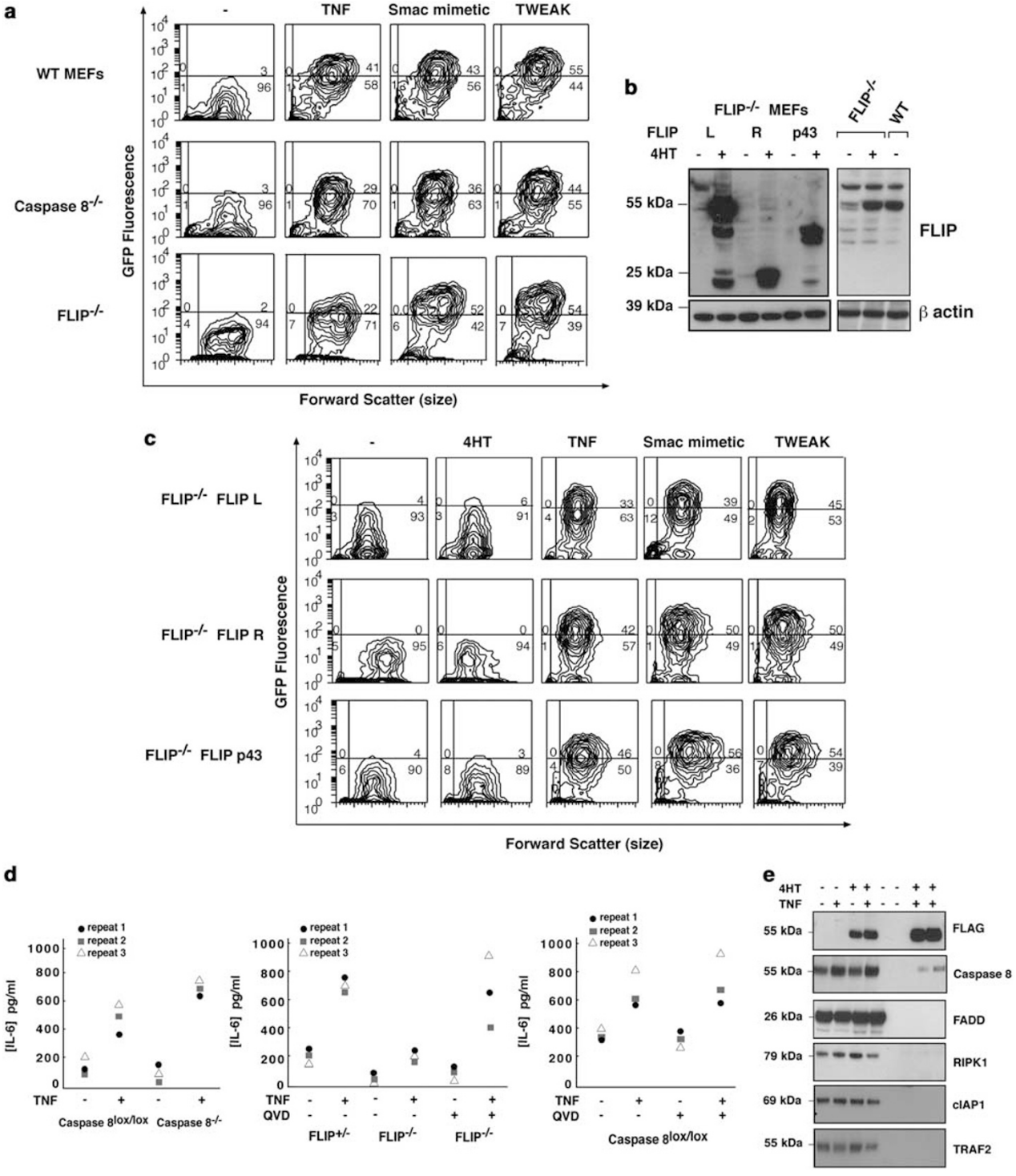


**Figure 2** TNF-induced NF-$\kappa$B activation is not impaired in caspase-8$^{-/-}$ or FLIP$^{-/-}$ MEFs. (**a**) WT, caspase-8$^{-/-}$, and FLIP$^{-/-}$ MEFs were infected with a lentiviral NF-$\kappa$B-GFP reporter. Cells were either treated with 100 ng/ml TNF, 500 nM smac mimetic, or 90 ng/ml TWEAK for 24 h. NF-$\kappa$B activation was determined by measuring the level of GFP fluorescence by flow cytometry. (**b**) Left panel: FLIP$^{-/-}$ MEFs bearing lentiviral vectors encoding 4HT-inducible FLIP isoforms were treated with 100 nM 4HT for 24 h. Right panel: FLIP$^{-/-}$ MEFs bearing 4HT inducible FLIP-L were treated with 100 nM 4HT for 24 h. WT MEFs were used as control. Cell lysates were immunoblotted for FLIP as indicated. $\beta$-Actin was used as a loading control. (**c**) FLIP$^{-/-}$ MEFs were either treated with 100 nM 4HT to induce FLIP expression or with 100 ng/ml TNF, 500 nM smac mimetic, or 90 ng/ml TWEAK for 24 h. NF-$\kappa$B activation was indicated by the level of GFP fluorescence determined by flow cytometry. (**d**) Induction of IL-6 is not impaired in caspase-8$^{-/-}$ MEFs. Caspase-8$^{lox/lox}$, caspase-8$^{-/-}$ FLIP$^{+/-}$, and FLIP$^{-/-}$ MEFs were treated with 100 ng/ml TNF for 24 h. FLIP$^{-/-}$ and caspase-8 $^{lox/lox}$ cells were pre-treated with 10 $\mu$M QVD and secreted IL-6 was quantified by ELISA. (**e**) FLIP-L bound to pro-caspase-8, but binding to adaptor proteins of the TNFR1-signaling pathway, was not detected. D645 cells were induced with 100 nM 4HT for 24 h to induce FLAG FLIP-L and then TNF was added for 2 h. FLIP-L was immunoprecipitated from cell lysates using anti-FLAG beads and immune complexes were eluted using 200 $\mu$g/ml FLAG peptide. Membranes were probed for FLAG-FLIP-L, caspase-8, RIPK1, TRAF2, cIAP1, and FADD as indicated. Tracks 1–4: Cell lysates. Tracks 5–8: Immunoprecipitates

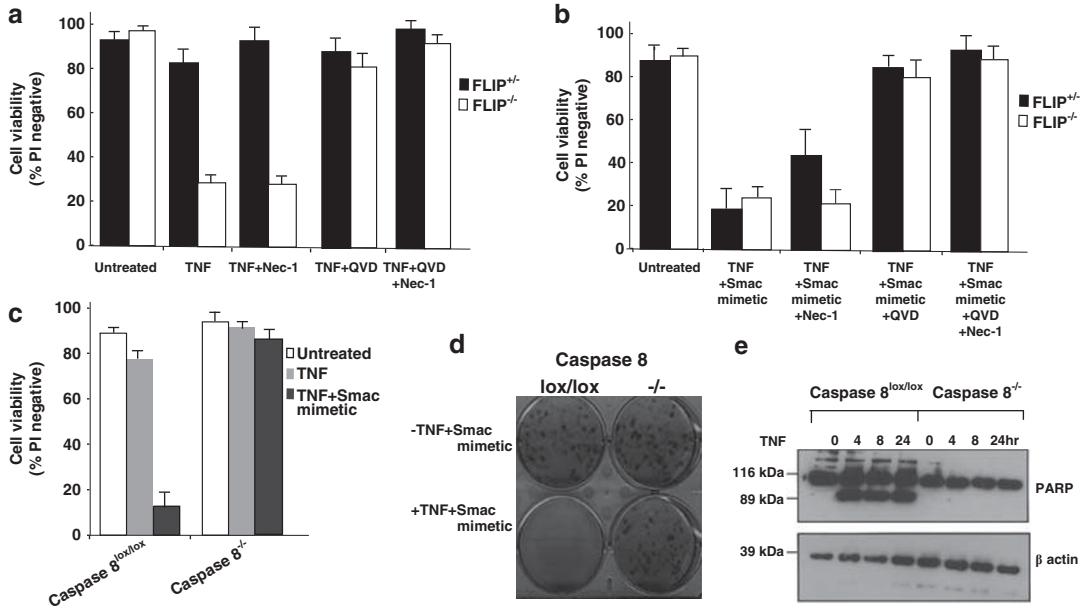


**Figure 3** Death of FLIP$^{-/-}$ MEFs caused by TNF alone or TNF plus smac mimetic is caspase-dependent. (**a**) FLIP$^{+/-}$ and FLIP$^{-/-}$ MEFs were pretreated with 50 $\mu$M necrostatin, 10 $\mu$M QVD, or both for 1 h and then 100 ng/ml TNF was added for 24 h. (**b**) FLIP$^{+/-}$ and FLIP$^{-/-}$ MEFs were pretreated with 50 $\mu$M necrostatin, 10 $\mu$M QVD, or both for 1 h and then 100 ng/ml TNF and 500 nM smac mimetic were added for 24 h. (**c**) Caspase-8$^{-/-}$ MEFs are resistant to TNF plus smac mimetic-induced cell death. Caspase-8$^{lox/lox}$ and caspase-8$^{-/-}$ MEFs were treated with TNF and smac mimetic for 24 h. (**a**–**c**) Cell viability was determined by PI exclusion and flow cytometry. The error bars show S.D. where $n = 3$ independently performed experiments. (**d**) Clonogenic survival of caspase-8$^{-/-}$ MEFs with TNF and IAP antagonist compound. Caspase-8$^{lox/lox}$ and caspase-8$^{-/-}$ MEFs were treated with TNF and smac mimetic for 24 h. Cells were re-plated and after 5 days fixed and stained with crystal violet. (**e**) Absence of PARP cleavage in TNF and smac mimetic-treated caspase-8$^{-/-}$ MEFs. Caspase-8$^{lox/lox}$ and caspase-8$^{-/-}$ MEFs were treated with TNF and smac mimetic for 4, 8 and 24 h. Cell lysates were immunoblotted for PARP as indicated. $\beta$-Actin was used as a loading control

with QVD, and implies but does not prove that QVD protects by inhibiting caspase-8. Furthermore, caspase-8 was not only necessary for rapid loss of plasma membrane integrity, but was also essential for TNF plus smac mimetic to kill the cells, because in clonogenic survival assays, caspase-8$^{-/-}$ MEFs treated with TNF plus smac mimetic survived and proliferated, and formed a similar number of colonies to untreated caspase-8$^{-/-}$ cells (Figure 3d). By contrast, treatment of caspase-8$^{lox/lox}$ MEFs with TNF plus smac mimetic dramatically reduced the survival of clonogenic cells (Figure 3d). In addition, cleavage of PARP was observed in WT MEFs treated with TNF plus smac mimetic, which was absent in caspase-8$^{-/-}$ MEFs (Figure 3e). Therefore, caspase-8 is essential for activation of the caspase cascade that causes cleavage of PARP.

Consistent with these observations, it has previously been reported that killing of Panc-1 cells by TNF plus smac mimetic is not inhibited by endogenous levels of FLIP, whereas some other types of cells that are not killed by TNF plus smac mimetic do die when FLIP levels are reduced by siRNA.[29] To assess whether absence of FLIP would sensitize MEFs to death ligands, we tested the sensitivity of FLIP$^{-/-}$ MEFs to TNF and FasL either alone or in combination with smac mimetic. As shown in Figure 4a, FLIP$^{-/-}$ MEFs were killed by TNF and FasL, whether or not smac mimetic was present. In comparison, FLIP$^{+/-}$ MEFs were less sensitive to TNF and FasL alone, but did die when they were added together with smac mimetic.

These observations raised the possibility that, although endogenous levels of FLIP could not protect MEFs against killing by TNF plus smac mimetic, elevated levels of FLIP might be able to do so. To test this, we infected FLIP$^{-/-}$ MEFs with inducible lentiviral vectors encoding FLIP-L, FLIP-R, or FLIP-p43. As shown in Figure 4b, induced expression of any of these isoforms of FLIP was able to protect the FLIP$^{-/-}$ MEFs from killing by TNF or FasL alone, as well as when these death ligands were combined with smac mimetic.

## Discussion

Various forms of NF-$\kappa$B can be activated by a wide range of signaling proteins, including the IKK kinase complex (IKK$\alpha$, IKK$\beta$, and IKK$\gamma$ (NEMO)), TRAFs, RIPK1 and NIK, and many others.[30] Because caspase-8 and FLIP were initially identified as an effector and an inhibitor of apoptosis, respectively, that act downstream from or independently of NF-$\kappa$B, it was surprising that they were both found to be required for activation of NF-$\kappa$B in certain circumstances. For example, it was reported that in human T-cell lines the FLIP-L cleavage product, FLIP-p22, bound to and activated NEMO,[18] and overexpression of FLIP-L activated NF-$\kappa$B in 293T cells.[17] Similarly, caspase-8 was reported to be required for activation of NF-$\kappa$B in 293T cells by cleaving FLIP-L to FLIP-p43, which bound to and activated TRAF2.[17] To resolve whether caspase-8 and FLIP acted upstream to activate NF-$\kappa$B; acted downstream after their induction by NF-$\kappa$B; or acted independently of NF-$\kappa$B, we took a genetic approach and used MEFs derived from gene-deleted mice.

Addition of TNF to caspase-8$^{-/-}$ and FLIP$^{-/-}$ MEFs showed that these gene products are not required for NF-$\kappa$B

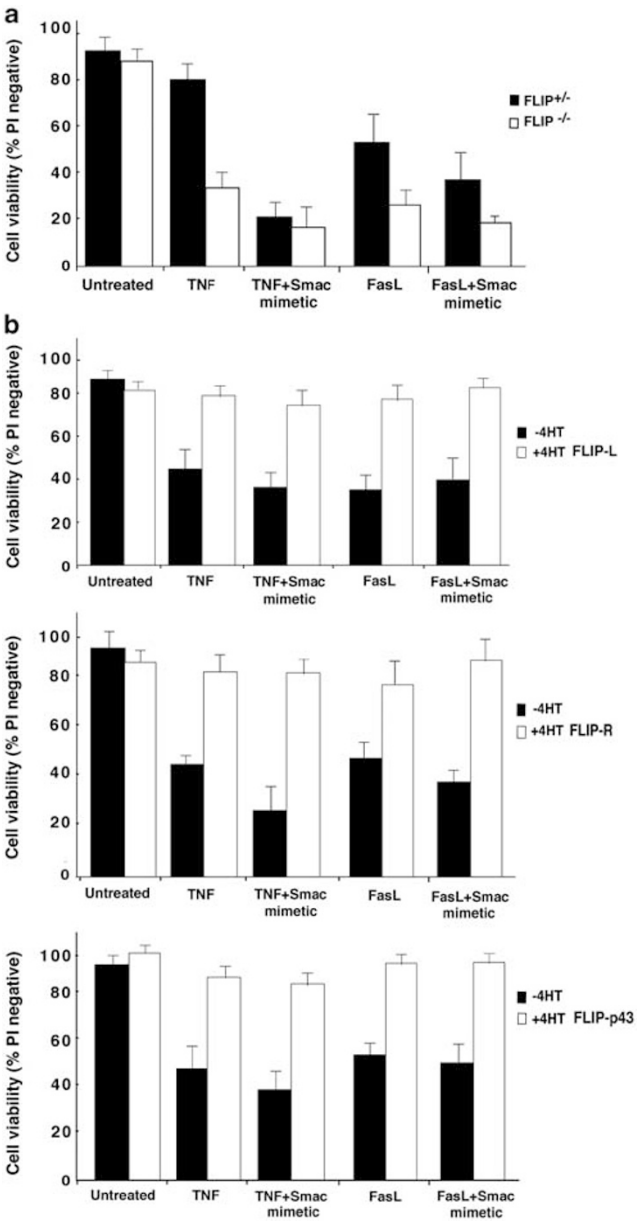


**Figure 4** FLIP-L, FLIP-R, and FLIP-43 can block death induced by death receptor ligands in the presence or absence of cIAPs. (**a**) FLIP$^{+/-}$ and FLIP$^{-/-}$ MEFs were treated with 100 ng/ml TNF or 10 ng/ml FasL in the presence or absence of 500 nM smac mimetic. Cell viability was determined by PI exclusion and flow cytometry. (**b**) FLIP$^{-/-}$ MEFs bearing the indicated 4HT-inducible FLIP isoform vectors were induced with 100 nM 4HT for 24 h, and treated with 100 ng/ml TNF or 10 ng/ml FasL in the presence or absence of smac mimetic for 24 h. Cell viability was determined by PI exclusion and flow cytometry. The error bars show S.D. where $n = 3$ independently performed experiments

activation, as degradation of I$\kappa$B, nuclear translocation of p65/RelA, and expression of NF-$\kappa$B-dependent genes occurred normally. This is consistent with a study by Varfolomeev *et al*.,[31] which demonstrated that TNF could trigger I$\kappa$B$\alpha$ phosphorylation and degradation in caspase-8-deficient fibroblasts. Whereas overexpression of FLIP has been reported to activate NF-$\kappa$B in transiently transfected 293T cells,[13,17] none of the FLIP isoforms were able to induce NF-$\kappa$B when overexpressed using an inducible lentiviral vector in MEFs (Figure 2).

Like most types of cells, WT MEFs are not killed by addition of TNF alone, but are sensitized to it by an inhibitor of translation, such as cycloheximide.[32,33] By contrast, p65/RelA$^{-/-}$, FLIP$^{-/-}$, and cIAP1$^{-/-}$ MEFs die in response to TNF alone,[7,34] as do cells in which cIAPs are depleted by a smac-mimetic compound.[7,24]

To determine the mechanism of TNF-induced death of cIAP-depleted cells, we added the broad-spectrum caspase inhibitor QVD,[25] and found that it prevented death of cells treated with TNF plus smac-mimetic compound. The key caspase inhibited by QVD is most likely caspase-8, because, unlike WT MEFs, caspase-8$^{-/-}$ MEFs were able to survive and produced colonies when treated with TNF and smac-mimetic compound. This demonstrates that in MEFs lacking cIAPs, caspase-8 is required for death induced by TNF, and other mechanisms, such as those involving RIPK1 or RIPK3, are not sufficient for cell death.

The role played by FLIP in regulation of cell death and transcription factor signaling in response to ligation of death receptors has been controversial. Whereas FLIP$^{-/-}$ MEFs have been reported previously to be highly sensitive to killing by TNF,[34] and FLIP has been found to inhibit death receptor-induced death of pancreatic cancer cells,[35] it has also been reported that physiological levels of FLIP activate caspases to induce apoptosis in a variety of cell types.[27]

We found that in FLIP$^{-/-}$ MEFs, overexpression of FLIP-L, FLIP-R, and FLIP-p43 inhibited apoptosis induced by TNF or FasL alone, or when they were added together with smac mimetic, presumably by preventing activation of caspase-8.

Shu *et al*.[36] have shown that in yeast, FLIP-L can bind to caspase-8, FADD, TRAF1, and TRAF2. We wished to examine interactions of FLIP-L with adaptor proteins of the TNFR1 pathway. We were able to detect caspase-8 co-immunoprecipitating with FLIP-L, but FLIP-L did not bring down detectable levels of FADD, TRAF2, RIPK1, or cIAP1. This is consistent with FLIP-L inhibiting caspase-8 by binding to it, rather than FLIP inhibiting cell death by interacting with FADD.

These experiments are consistent with a model in which FLIP is expressed in unstimulated MEFs at a level that confers protection against subsequent addition of TNF alone, and its levels are subsequently maintained by a TNF-activated NF-$\kappa$B-dependent pathway. Deletion of genes for FLIP itself, or genes for p65/RelA NF-$\kappa$B, or genes for proteins necessary for efficient activation of p65/RelA in response to TNF (such as TRAF2, cIAP1, NEMO, TAK1) thus render MEFs sensitive to killing by TNF alone. Consistent with this model, ectopic expression of FLIP can protect FLIP$^{-/-}$, TRAF2$^{-/-}$, TAK1$^{-/-}$, and smac mimetic-treated MEFs from killing by TNF.[37,38] Rather than FLIP acting as an upstream inducer of NF-$\kappa$B, as has been reported in HEK293 derivatives, in TNF-treated MEFs, NF-$\kappa$B acts as an upstream inducer of FLIP.

Rather than pro- and mature caspase-8 cleaving FLIP, which then activates NF-$\kappa$B, as has been reported in 293HEK and T-lineage cells, our results show that in MEFs caspase-8 is not required for activation of FLIP or NF-$\kappa$B. Caspase-8 is, however, necessary for TNF to induce apoptosis of MEFs. In unstimulated WT MEFs, enough FLIP is present to prevent

TNF from activating caspase-8 to cause cell death. TNF subsequently activates NF-$\kappa$B, driving the expression of enough FLIP to prevent subsequent activation of caspase-8 and apoptosis. Thus WT MEFs are not killed by TNF, but deletion of genes for FLIP, or genes for components of the signal transduction pathway required to maintain FLIP levels, renders MEFs sensitive to killing by TNF.

We have demonstrated that the death-inducing protease caspase-8, and the caspase-8 inhibitor, FLIP, were not required for activation of NF-$\kappa$B in MEFs. Rather, our results show that caspase-8 is necessary for death receptor- and IAP antagonist-induced death of MEFs, and FLIP is necessary to prevent death of MEFs induced by death receptor ligation.

## Materials and Methods

**Cell culture, transfections, constructs, and lentiviral infections.** All cell lines were maintained at 37 °C, 10% $CO_2$ in DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco, Melbourne, VIC, Australia), 50 $\mu$g/ml penicillin-G/50 U/ml streptomycin, 2 mM L-glutamine, and were passaged twice weekly. Sub-confluent cultures of 293T cells were transiently transfected with 1 $\mu$g of total DNA per 10-cm dish using Effectene (Qiagen, Clifton Hill, VIC, Australia) according to the manufacturer's specifications. The NF-$\kappa$B lentiviral reporter vector, pTRH1 mCMV NF-$\kappa$B dscGFP, was purchased from System Biosciences (Karrinyup, WA, Australia). Cre-recombinase and SV40 largeT antigen were cloned into the lentiviral vector pFU as described previously.[7] Mouse c-FLIP-L, FLIP-R, FLIP-p43, and human FLAG-FLIP-L were cloned into the 4HT-inducible lentiviral vector pF 5 $\times$ UAS.[7,39] After sequencing, all constructs were purified using Qiagen maxi/midi prep kits prior to transfection with Effectene. Lentiviruses were generated by transfecting sub-confluent 10-cm plates of 293T cells with lentiviral plasmids together with the packaging constructs, pCMV-$\Delta$R8 and pVSV-G, using Effectene as described previously.[40] After 48-h viral supernatants were collected, filtered, supplemented with 4 $\mu$g/ml polybrene, and added to target cells. Stably infected cells were selected in the presence of 5 $\mu$g/ml puromycin and 100–500 $\mu$g/ml hygromycin-B, or by screening for GFP fluorescence. Expression of pF 5 $\times$ UAS-inducible constructs was induced with 100 nM 4HT unless otherwise indicated.

**Antibodies and chemicals.** The primary antibodies used for western blot analysis were anti-FLAG (Sigma, Croydon, VIC, Australia; F-3165), anti-$\beta$-actin (Sigma, A-1978), anti-mouse RIPK1 (BD Transduction Laboratories, North Ryde, NSW, Australia; 610458), anti-p65 (Santa Cruz, Santa Cruz, CA, USA; SC-372), anti-human TRAF2 (Pharmingen, North Ryde, NSW, Australia; 558890), anti-cIAP1 (Alexis, San Diego, CA, USA), anti-mouse I$\kappa$B$\alpha$ (Santa Cruz, SC-371), anti-caspase 8 (Lorraine O'Reilly, WEHI), anti-mouse c-FLIP L (ProScience, Poway, CA, USA; clone Dave-2, XA1008), anti-PARP (Cell Signaling, Danvers, MA, USA; 9542), and anti-FADD (Assay designs, Ann Arbor, MI, USA; clone IF7, ADI-AAM-212-E). Anti-p65 (Santa Cruz, SC-372) was used for immunofluorescence microscopy and DAPI was obtained from Vector Laboratories (Homebush, NSW, Australia; H-1200). 4HT and QVD were purchased from Sigma. Necrostatin-1 and Compound-A were obtained from TetraLogic Pharmaceuticals (Malvern, PA, USA).

**Generation of MEFs.** Caspase-8 conditional knock-out MEFs were generated from E8.5 caspase-8 LoxP/LoxP embryos from mice provided by Dr Steve Hedrick (University of California, San Diego). Primary MEFs were immortalized by infection with SV40 largeT antigen-expressing lentivirus as described previously.[7] To delete caspase-8, the transformed MEFs were infected with a Cre recombinase-expressing lentivirus (pFU CreR SV40 Hygro) and deletion was confirmed by western blotting. FLIP$^{+/-}$ and FLIP$^{-/-}$ MEFs were a gift from Tak W Mak (Ontario Cancer Institute, Toronto) and were immortalized with SV40 largeT as described above.

**Cell death assays.** Cells were seeded at approximately 40% confluence onto 12-well tissue culture plates and were allowed to settle for 16–20 h. Smac-mimetic Compound-A (500 nM), 70 ng/ml human Fc-TNF$\alpha$, or 10 ng/ml human Fc-Fas were added to cells for 24 h, and cell death measured by uptake of propidium iodide (PI) using a FACSCalibur flow cytometer (BD Biosciences, North Ryde, NSW, Australia). A total of 10 000 events per sample were collected and cell death (% PI-positive cells) was quantified by using the WEASEL software (version 2.2.2; WEHI).

**Clonogenic survival assay.** WT and caspase-8$^{-/-}$ MEFs were plated at equal densities on six-well plates and cultured with or without TNF and smac mimetic for 24 h. After treatment, cells were treated with trypsin, re-suspended, washed, and re-plated. Cells were then grown for 5 days and fixed with glutaraldehyde, and colonies were stained with 0.1% crystal violet.

**NF-$\kappa$B-GFP reporter assays.** Cells were seeded onto 12-well tissue culture plates at approximately 40% confluence and were allowed to settle for 16–20 h. Smac mimetic (500 nM), 70 ng/ml human Fc-TNF, and 90 ng/ml TWEAK were added to cells for 24 h. GFP fluorescence of cells was measured by flow cytometry using FL1-channel fluorescence. A total of 10 000 events per sample were collected and quantified using WEASEL.

**Western blotting and immunoprecipitations.** Anti-FLAG immunoprecipitations were performed essentially as described previously[39] except 2 $\times$ 15-cm tissue culture plates were used per time point and immunoprecipitated complexes were eluted using 200 $\mu$g/ml FLAG peptide (AusPep, Tullamarine, VIC, Australia). Samples were separated on 4–12% polyacrylamide gels (Invitrogen, Mulgrave, VIC, Australia) and transferred to Hybond-C nitrocellulose membrane (GE, Rydalmere, NSW, Australia) for antibody detection. All membrane-blocking steps and antibody dilutions were performed using 5% skim milk in PBS containing 0.1% Tween-20 (PBST), and washing steps were performed with PBST. Western blots were visualized by enhanced chemiluminescence (GE).

**Immunofluorescence.** MEFs grown overnight on glass coverslips were treated with 70 ng/ml TNF for 20 min, immediately fixed with 4% paraformaldehyde for 20 min, washed in PBS, and permeabilized with 0.5% TritonX-100 for 5 min. Cells were blocked with 150 mM glycine in PBS, incubated with p65 antibody, washed four times with PBS, and then incubated with an anti-rabbit AlexaFluor488-conjugated secondary antibody (Invitrogen) and washed four times with PBS. All blocking steps and antibody incubations were performed using PBS containing 1% BSA for 30 min. Cells were viewed with an Olympus BX50 fluorescence microscope and the SPOT RT imaging software using a $\times$ 40 objective. Images were analyzed using the NIH ImageJ software (http://rsb.info.nih.gov/ij). All images were saved in JPEG format and imported into Freehand MX (Macromedia) for generation of collages.

**IL-6 ELISA assay.** Cells were seeded in 24-well plates at a density of 5 $\times$ 10$^4$ per well. Cells were cultured with or without 100 ng/ml Fc TNF and incubated for 24 h. Medium was collected and clarified by centrifugation, and assayed using the OptEIA Mouse IL-6 ELISA kit (BD Biosciences) as per the manufacturer's instructions. Assays were performed in triplicate in each of three independent experiments ($n = 3$).

## Conflict of Interest

DL Vaux is on the Scientific Advisory Board of TetraLogic Pharmaceuticals, a private biotechnology start-up. TetraLogic Pharmaceuticals provided the smac-mimetic compound. TetraLogic Pharmaceuticals did not fund the research, or have any role in directing or performing the experiments, or in interpreting the results.

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