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RIPK1 is not essential for TNFR1-induced activation of NF-κB

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On TNF binding, receptor-interacting protein kinase 1 (RIPK1) is recruited to the cytoplasmic domain of TNFR1, at which it becomes ubiquitylated and serves as a platform for recruitment and activation of NEMO/IKK1/IKK2 and TAK1/TAB2. RIPK1 is commonly thought to be required for the activation of canonical NF-κB and for inhibition TNFR1-induced apoptosis. RIPK1 has, however, also been reported to be essential for TNFR1-induced apoptosis when cIAPs are depleted. To determine the role of RIPK1 in TNF/IAP antagonist-induced death, we compared wild type (WT) and RIPK1$^{-/-}$ mouse embryonic fibroblasts (MEFs) treated with these compounds. On being treated with TNF plus IAP antagonist, RIPK1$^{-/-}$ MEFs survived, unlike WT MEFs, demonstrating a killing activity of RIPK1. Surprisingly, however, on being treated with TNF alone, RIPK1$^{-/-}$ MEFs activated canonical NF-κB and did not die. Furthermore, several cell types from E18 RIPK1$^{-/-}$ embryos seem to activate NF-κB in response to TNF. These data indicate that models proposing that RIPK1 is essential for TNFR1 to activate canonical NF-κB are incorrect.
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Signalling from TNFR1 in response to TNF results in the recruitment and assembly of a membrane-associated complex (complex I), which contains TRADD, RIPK1, TRAF2, cIAP1 and cIAP2. All of these components are thought to be required for the activation of NF-κB in response to TNF.[1] TNFR1 bears a cytoplasmic death domain (DD) and can also transmit apoptotic signals indirectly *via* TRADD.[2] However, most cells do not die when exposed to TNF, but only succumb when NF-κB signalling is blocked, for example, by deletion of p65 NF-κB (RelA), over-expression of a mutant IκBα, or treatment with the translation inhibitor cycloheximide (CHX).[3–5]

An essential role for RIPK1 in TNF-induced NF-κB activation has been inferred from overexpression, knockdown and knockout (KO) studies. For example, overexpression of RIPK1-induced NF-κB activation,[6,7] a RIPK1 mutant Jurkat T cell line was deficient in NF-κB activation,[7,8] and extracts from v-ABL-transformed RIPK1$^{-/-}$ B cells showed no binding to NF-κB probes in electrophoretic mobility shift assays.[4]

It is believed that on TNFR1 ligation, RIPK1 is K63 ubiquitylated by TRAF2,[9] which provides a platform for NEMO (IKKγ) to bind and allows TAB2/TAB3/TAK1 recruitment. TAK1 becomes ubiquitylated, and subsequently activates IKK2 in a phosphorylation-dependent manner. IKK2 in turn phosphorylates IκBα, which is K48 ubiquitylated and degraded. Degradation of IκBα allows cytoplasmic NF-κB dimers to translocate to the nucleus and transactivate pro-survival proteins such as $cFLIP_L$.[10,11] In this model, by activating NF-κB, RIPK1 shows a pro-survival function.

However, it has recently been shown that when cells are treated with an IAP antagonist in addition to TNF, reducing RIPK1 levels using shRNA prevents them from dying.[12] Therefore, in this case RIPK1 seems to have a pro-apoptotic function. To determine the effect of the complete absence of RIPK1 in TNFR1 cell death pathways, we generated mouse embryonic fibroblasts (MEFs) from wild-type (WT) and RIPK1$^{-/-}$ mice and analysed cell death and activation of NF-κB.

Results

To determine the effect of the complete absence of RIPK1 in TNFR1 cell death pathways, WT and RIPK1$^{-/-}$ MEFs were treated with TNF alone, or in combination with IAP antagonists and cell death was analysed by propidium iodide (PI) uptake using flow cytometry. As reported previously, SV40 large T (SV40T)-immortalized WT MEFs died when treated with the combination of TNF and the IAP antagonist compound A (comp A) (Figure 1a).[13] In marked contrast, RIPK1$^{-/-}$ cells survived in the presence of TNF and comp A, indicating that in the absence of cIAPs, RIPK1 is required to kill cells after TNF treatment, consistent with the observations of Wang *et al.*,[12] Gaither *et al.*[14] and Bertrand *et al.*[15] These results were confirmed by colony growth assays (Figure 1b). We noted,

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Abbreviations: RIPK1, Receptor Interacting Protein Kinase 1; CHX, Cycloheximide; TNF, Tumour Necrosis Factor; cIAP1, cellular inhibitor of apoptosis 1; DD, death domain; MEF, mouse embryonic fibroblast; PBS, phosphate buffered saline; PI, propidium iodide
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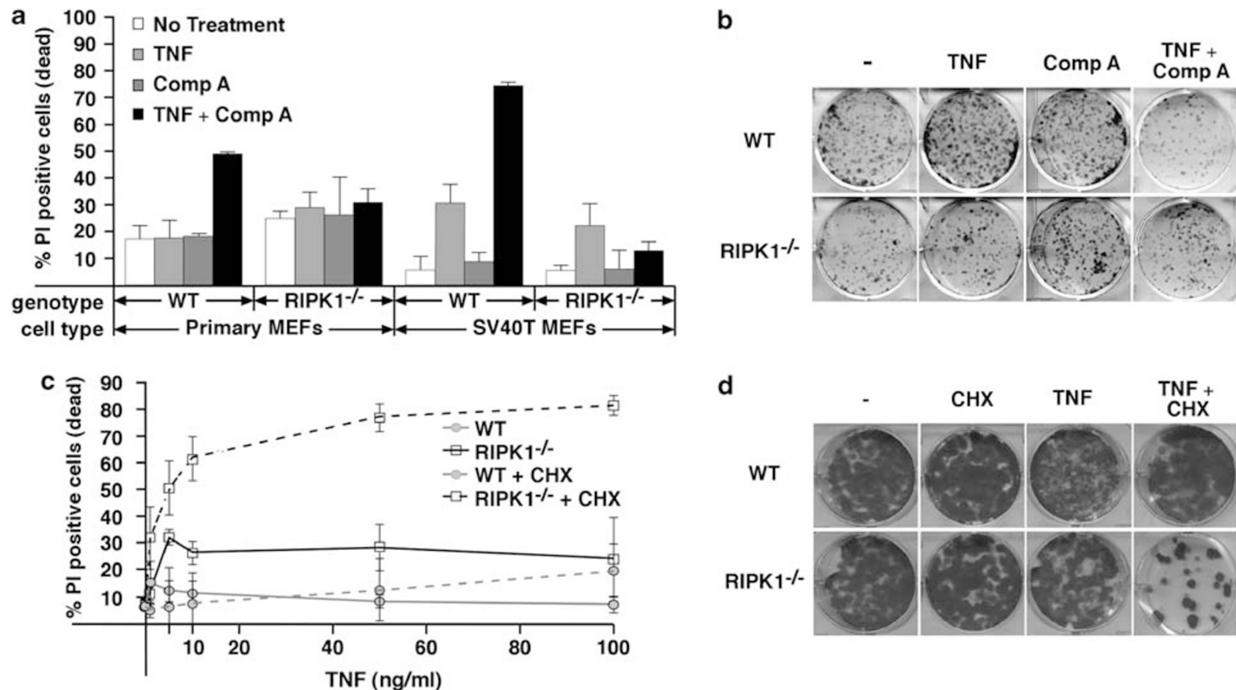

Figure 1 RIPK1 is required for death of MEFs treated with TNF + IAP antagonist, but is necessary for survival of MEFs treated with TNF + CHX. (**a**) TNF causes RIPK1-dependent death when IAPs are antagonised. WT and RIPK1$^{-/-}$ MEFs were treated with either 100 ng/ml TNF, 500 nM comp A or TNF + comp A for 24 h and cell death was assessed using PI staining and flow cytometry. Results are from independent experiments using MEFs generated from at least three embryos of each genotype. (**b**) WT and RIPK1$^{-/-}$ MEFs were treated with either 100 ng/ml TNF, 500 nM comp A or TNF + comp A for 24 h and plated in fresh medium for colony growth assays. (**c**) WT and RIPK1$^{-/-}$ SV40T MEFs were treated with various doses of TNF or TNF + 250 ng/ml CHX for 24 h. Cells were collected and assessed for cell death as in (**a**). Results are from three independent experiments conducted on a minimum of two independently derived WT and RIPK1$^{-/-}$ MEFs. Error bars represent S.E.M. throughout. (**d**) WT and RIPK1$^{-/-}$ SV40T MEFs were treated with 100 ng/ml TNF or TNF + 250 ng/ml CHX for 24 h and plated in fresh medium for colony growth assays

however, that RIPK1$^{-/-}$ cells were not more sensitive to TNF alone than WT MEFs (Figure 1a). We have previously noted differential sensitivity of SV40-transformed and primary MEFs to TNF treatment,[13,16] but neither primary nor transformed RIPK1$^{-/-}$ MEFs were more sensitive to TNF than their WT counterparts. These results question models in which RIPK1 protects cells from TNF killing. However, these models were to some extent based on experiments performed in the presence of a low dose of CHX.[4,12]

To test these findings, we treated cells with TNF in the presence of 250 ng/ml CHX,[4] and analysed cell death using PI uptake (Figure 1c) and confirmed it by colony growth assays (Figure 1d). The RIPK1$^{-/-}$ MEFs were much more sensitive to TNF + CHX than the WT MEFs, confirming a survival function for RIPK1 in the presence of CHX. However, neither cell type was sensitive to TNF alone. As NF-κB is required to protect cells from TNF-induced death,[5] these results suggested that NF-κB activation might be occurring in RIPK1$^{-/-}$ cells, contrary to the accepted model for induction of NF-κB by TNF. To test this possibility, we treated primary or SV40T-transformed MEFs with TNF, and assayed IκBα levels by western blotting. In WT cells, IκBα was degraded within 15 min and restored by 60 min as expected. Surprisingly, IκBα degradation and restoration also occurred in RIPK1$^{-/-}$ MEFs (Supplementary Figure S2a, S1a, S1b). In addition, TNF triggered translocation of the NF-κB subunit, RelA, to the nucleus, whether RIPK1 was present or not (Figure 2b). Furthermore, TNF induced similar increases in mRNA levels of NF-κB target genes in the presence or absence of RIPK1, in both primary and SV40T MEFs (Figure 2c). A *t*-test evaluation of the qPCR data shows that differences in induction of NF-κB target genes were significantly lower in RIPK1$^{-/-}$ MEFs compared with WT with the exception of A20 in primary cells and cIAP2 in SV40-transformed MEFs. Although there is a trend of reduced transcription in primary and SV40-transformed RIPK1$^{-/-}$ MEFs across these three TNF-induced genes, we were surprised to observe a TNF-induced transcription of these NF-κB-dependent genes in RIPK1$^{-/-}$ cells.

TNF induction of IL-6 has been shown to be dependent on RelA and in particular on phosphorylation of RelA in MEFs.[17] To further characterise the TNF-induced NF-κB response in RIPK1$^{-/-}$ MEFs, we performed an ELISA to measure IL-6 levels in both WT and RIPK1$^{-/-}$ MEFs. In two independent lines of MEFs generated from different matings, there was no loss of induction of this NF-κB-dependent gene by TNF in RIPK1$^{-/-}$ MEFs (Figure 2d). Our experiments show that, contrary to the widely held view, RIPK1 is not essential for the activation of canonical NF-κB pathways after ligation of TNFR1 in MEFs.

All our experiments were performed using recombinant Fc human TNF,[18] which is believed to mimic the activity of the membrane-bound form of the ligand.[18,19] Membrane-bound and soluble forms of TNFSF ligands often have different

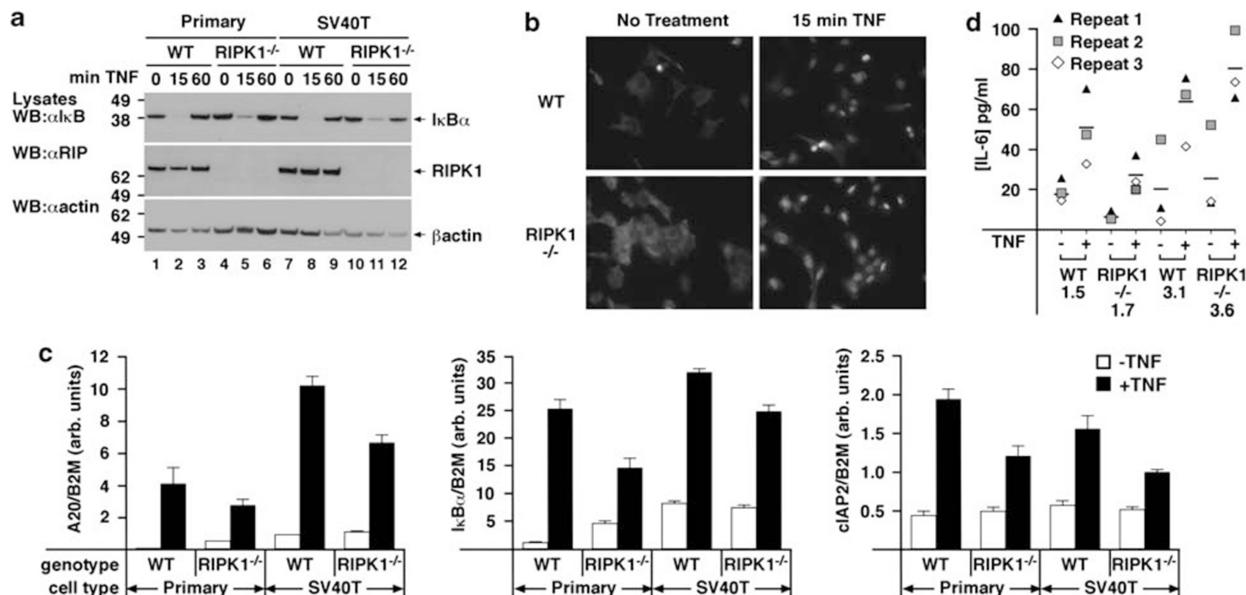

Figure 2 TNF-induced activation of NF-κB occurs in RIPK1$^{-/-}$ MEFs. (**a**) Degradation of IκBα was seen in both primary and SV40T MEFs from WT or RIPK1$^{-/-}$ genotypes when treated with 100 ng/ml TNF for 0, 15 or 60 min. Results are representative of three independent experiments. (**b**) Nuclear localisation of RelA by immunofluorescence occurs within 15 mins of TNF treatment in both WT and RIPK1$^{-/-}$ SV40T MEFs. (**c**) The expression of NF-κB target genes *A20*, *IκBα* and *cIAP2*, was determined by qPCR in primary and SV40T WT and RIPK1$^{-/-}$ MEFs after 1 h of exposure to 100 ng/ml TNF. Results are derived from MEFs generated from at least two embryos. Error bars represent S.E.M. (**d**) IL-6 production occurs in RIPK1$^{-/-}$ similar to WT SV40T MEFs after exposure to 10 ng/ml TNF for 24 h. Results are from two embryos of each genotype performed in three independent experiments

signalling potential[20] and sTNF is a poor activator of TNFR2.[21] WT and RIPK1$^{-/-}$ MEFs treated with either sTNF or Fc TNF responded identically (Supplementary Figure S1b), indicating that RIPK1 is not required in signalling induced by either soluble or Fc TNF.

To further exclude the possibility of a TNFR2-dependent degradation of IκBα in RIPK1$^{-/-}$ MEFs, we treated WT, TNFR1$^{-/-}$/RIPK1$^{+/+}$ and TNFR1$^{-/-}$/RIPK1$^{-/-}$ SV40T MEFs with TNF and measured IκBα levels by western blotting (Supplementary Figure S2a). Classical TNFR1-induced degradation of IκBα occurred within 15 min of TNF addition in WT MEFS and rebounded within 1 h. However, neither TNFR1$^{-/-}$RIPK1$^{+/+}$ nor TNFR1$^{-/-}$/RIPK1$^{-/-}$ SV40T MEFs demonstrated TNF-induced loss of IκBα (Supplementary Figure S2a). Similarly, WT and TNFR2$^{-/-}$ MEFs degraded IκBα normally in response to TNF whereas TNFR1$^{-/-}$ MEFs did not (Supplementary Figure S2b). Finally, we used a TNFR2-specific antibody to determine TNFR2 levels in WT, TNFR2$^{-/-}$ and RIPK1$^{-/-}$ MEFs by flow cytometry. Neither WT nor RIPK1$^{-/-}$ MEFs showed levels of staining above those observed in TNFR2$^{-/-}$ MEFs, showing that if TNFR2 is expressed by MEFs, it is at undetectable levels (Supplementary Figure S2c). Collectively, these results show that IκBα degradation in RIPK1$^{-/-}$ MEFs occurs through TNFR1, and not in a TNFR2-dependent manner.

RIPK1$^{-/-}$ mice die soon after birth so examining TNF responses in cell types other than fibroblasts is challenging. We therefore set up timed pregnancies from RIPK1 heterozygous crosses and monitored them until birth. Immediately after birth, pups were photographed and tail tissue was taken for typing (Figure 3a). There were no gross phenotypic differences among WT (data not shown), heterozygous (pups #2 and #3) and RIPK1$^{-/-}$ (pup #1) pups immediately after birth and no indication of liver pathology usually associated with excessive TNF sensitivity was observed. The pups were then killed and the thymus, liver, brain and lungcollected. All tissues looked phenotypically normal (data not shown). To minimise manipulation, the thymus was gently homogenised into single cell suspensions in PBS + 8% FCS by disrupting between the frosted ends of two sterile microscope slides. The liver and the lung were crudely sliced and incubated for 30 min in HEPES-buffered collagenase IV solution and single cells separated using a cell strainer. Cell suspensions were spun slowly and re-suspended immediately in a medium with or without 100 ng/ml TNF for 20 min. Cell recovery was, however, low and data available for western blotting consequently limited. Surprisingly, embryos collected at E18 and treated the same way gave improved cell recovery. As these analyses were performed immediately after killing the animals, and typing results were obtained subsequently, the original western blots were performed for all embryos in a blind manner with no knowledge of the genotype. Subsequently, a selection of genotypes was run on the same gel for easy comparison, however, same results were observed for all embryos regardless of the genotype (data not shown; see Supplementary Figure S3). Thymus cell suspensions from E18 embryos show clear degradation of IκBα within 20 min of TNF treatment and there was no discernible difference in the responses of WT or RIPK1$^{-/-}$ cells isolated from either organ (Figure 3b). RIPK1$^{-/-}$ cells collected from lung, potentially of an epithelial origin, however, did not respond to TNF as the WT cells did, indicating that RIPK1 might be important for TNF-induced degradation of IκBα in these cells (Figure 3c). RIPK1$^{-/-}$ hepatocytes isolated from E18 embryos also

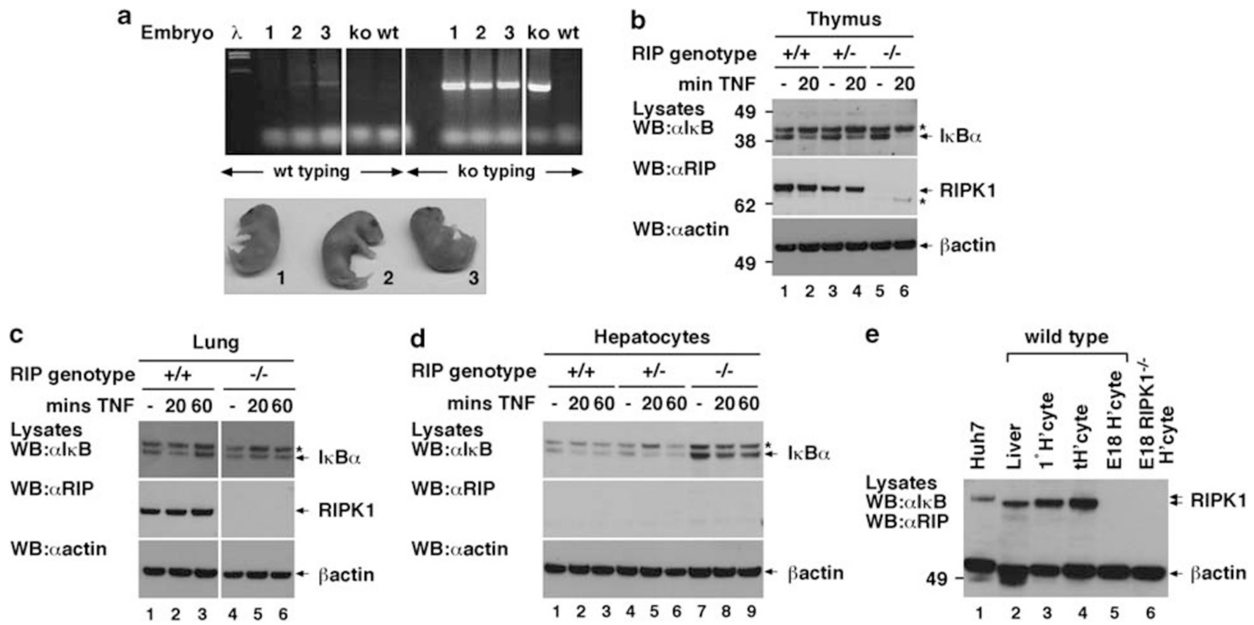

Figure 3 Lack of RIPK1 does not affect TNF-induced activation of NF-κB in multiple cell types. (**a**) Pictures of three live pups with indicated genotype. (**b**) Cells collected from thymus of the indicated genotype were incubated with TNF for the indicated time. Lysates were made, separated on SDS/PAGE gels transferred to nitrocellulose/PVDF membranes and western blotted with the indicated antibodies. (**c**) Cells collected from the lung were treated as in (**b**). (**d**) Hepatocytes isolated from livers of the indicated genotype were treated as in (**b**). (**e**) Lysates from untreated Huh7 (human), adult mouse WT liver, 1°H′cytes: primary adult WT mouse hepatocytes, tH′cytes: transformed adult WT mouse hepatocytes, primary E18 WT hepatocytes and primary E18 RIPK1$^{-/-}$ hepatocytes were treated as in (**b**)

responded like WT cells to TNF (Figure 3d). Surprisingly, we were unable to detect RIPK1 in WT E18 hepatocytes, suggesting that WT hepatocytes at this stage of development are RIPK1 null. This seems to be a stage-specific effect because RIPK1 was readily detectable in primary adult and transformed mouse hepatocytes (Figure 3e). This result further underlines the fact that RIPK1 is not required for TNF-induced NF-κB activation because E18 WT hepatocytes do not express RIPK1 and activate NF-κB in response to TNF. The lack of RIPK1 in E18 hepatocytes also suggests an explanation for the lack of any liver pathology in the RIPK1$^{-/-}$ pups.

Discussion

These results extend other findings and show that RIPK1 is required for cell death induced by IAP antagonist plus TNF in MEFs, but challenge the belief that RIPK1 has an essential role in the activation of NF-κB in response to TNF. First, IκBα degradation and NF-κB-dependent recovery of IκBα occur in both transformed and primary RIPK1$^{-/-}$ MEFs, as does translocation of RelA into the nucleus, and transcriptional activation of NF-κB-dependent genes. Second, although the loss of NF-κB activation observed in TRAF2/TRAF5, cIAP1/cIAP2 and RelA KO cells results in sensitisation to TNF in the absence of CHX,[5,13,22,23] loss of RIPK1 does not sensitise either primary or transformed MEFs to killing by TNF alone.

The activation of NF-κB in RIPK1$^{-/-}$ cells implies that ubiquitylation of RIPK1 is not essential for the activation of NF-κB by TNF. This result is consistent with a previous study that showed that RIPK1 was not required for the recruitment of IKK1/IKK2/NEMO to complex I in TNF-treated RIPK1$^{-/-}$ MEFs,[8] suggesting NF-κB activation is intact. In line with this, we observed that RIPK1$^{-/-}$ cells were able to secrete IL-6 in response to TNF similarly to WT. Lee *et al*[24,25] showed that RIPK1$^{-/-}$ cells did not induce IL6, but these differences could be due to the manner in which the MEFs were generated or the source of TNF. We and others have observed ubiquitylation of RIPK1 in response to TNF, which is lost in both cIAP1/cIAP2 double knockout (DKO) cells[23,26] and TRAF2/TRAF5 DKO cells,[22] or when cells are treated with IAP antagonist.[13,15,27] In addition, normal activation of canonical NF-κB is lost in cIAP1/cIAP2 DKO and TRAF2/TRAF5 DKO cells.[22,23] These results demonstrate that recruitment of TRAFs and IAPs is essential for NF-κB activation in response to TNFR1 activation but that, although RIPK1 ubiquitylation can serve as a marker for activation of NF-κB, it is not required for activation or recruitment of the IKK complex.

The role of K63-polyubiquitylated RIPK1 in TNF signalling has been questioned in several recent studies. First, although UBC13 was believed to be the E2 that functions together with TRAF2 to form K63-linked ubiquitin chains, TNF activated NF-κB normally in UBC13$^{-/-}$ cells.[28] Second, NEMO was recently shown to bind with higher affinity to linear (head-to-tail linked) polyubiquitin than to K63-linked polyubiquitin,[29] and NEMO itself can be conjugated to linear polyubiquitin chains through the action of HOIL-1 and HOIP.[30] Loss of HOIL-1 and HOIP greatly reduced NF-κB activation by TNF, suggesting that K63 ubiquitin-conjugated RIPK1 is not the only component of complex I that can recruit NEMO and activate IKK.[30,31] In addition, phosphorylation of TRAF2 by protein kinase C was shown to stimulate TRAF2 K63 ubiquitylation that was able to recruit IKK and the TAK1/TAB complex,[32] again suggesting complex I components other than RIPK1 can recruit IKK. Importantly, proteins that are

essential for NF-κB activation on TNF stimulation, such as RelA/p65 and NEMO, are early embryonic lethal.[33,34] In the case of RelA, this lethality is due to massive hepatocyte death and liver destruction at E14, which is rescued by the loss of TNF.[3] This is clearly distinct from the phenotype of RIPK1$^{-/-}$ mice that are born alive, but which fail to thrive and die within 3 days post-natally.[4] The fact that RIPK1$^{-/-}$ mice are normal until after birth strongly suggests that our findings can be extended to cell types other than MEFs. This is further emphasised by the fact that freshly isolated cells from the thymus and liver degraded IκBα in response to treatment with TNF with same kinetics as WT cells. Hepatocytes from E18 embryos were remarkable because WT E18 hepatocytes did not seem to express RIPK1. This would seem to be a developmental stage-specific lack of expression because RIPK1 was readily detectable in adult mouse liver and primary adult mouse hepatocytes. It is possible that RIPK1 is modified in E18 embryos and this precludes detection by the RIPK1 antibody we used, however, irrespective of this caveat both WT and RIPK1$^{-/-}$ hepatocytes degrade IκBα in response to TNF.

Cells collected from E18 lungs did not respond to TNF, therefore, it is possible that these cells require RIPK1 for full activation of NF-κB in response to TNF. It will be important, however, to perform experiments on multiple RIPK1$^{-/-}$ embryos to be confident of this interpretation. In experiments using a human T cell line, the IKK complex and TAK1 were not recruited to TNFR1 in the absence of RIPK1,[9] and extracts from TNF-treated v-ABL-transformed RIPK1$^{-/-}$ B cells failed to bind NF-κB element probes.[4] It is therefore possible that RIPK1 is important in NF-κB activation in a cell type- and/or species-specific manner. Indeed, in recent studies in which TRADD$^{-/-}$ MEFs failed to activate NF-κB in response to TNF, TRADD$^{-/-}$ bone marrow-derived macrophages (BMDM) did so to the same extent as WT BMDMs.[35,36] This implies that the signalling platform assembled on TNFR1 is more plastic than previously thought, and that the function of certain molecules in this complex is interchangeable.

Although our results challenge the current model for TNF-induced NF-κB, we confirmed that RIPK1$^{-/-}$ MEFs were sensitised to TNF to a greater extent than WT cells in the presence of low levels (250 ng/ml) CHX, as previously shown.[4] This suggests that in the presence of TNF plus CHX, RIPK1 promotes survival, but presumably not by activating NF-κB. Furthermore, RIPK1 is required for cell death triggered by TNF in the presence of an IAP antagonist. Exactly what switches RIPK1 from a pro-survival to a pro-death protein in the presence of CHX versus IAP antagonist remains to be determined, however, the finding that RIPK1 may have separable roles in the activation of complex II and its apparent redundancy in NF-κB activation will have significant implications in the interpretation of previous and future studies on TNFR1 signalling.

Materials and Methods

Generation of MEFs and other cell types. Wild-type and KO MEFs were generated from E15 embryos following standard procedures[13] and were either infected with SV40T-expressing lentivirus or maintained as primary MEFs. For other cell types, E18 embryos were collected, killed and the thymus, liver, lungs and neurons extracted. The tail was taken for typing. The thymus was gently homogenised into single cell suspensions in PBS + 8% FCS by disrupting between the frosted ends of two sterile microscope slides. The liver and the lung were crudely sliced and incubated for 30 min in HEPES-buffered collagenase IV solution (750 μg/ml, pH = 7.6). Single cells were separated using a cell strainer. Cell suspensions were spun at ~0.5 × *g* and re-suspended in a medium with or without 100 ng/ml TNF for 20 min. Cells were washed with PBS and lysed using DISC lysis buffer. The entire organ was used for each experiment. All cell lines were kept at 37°C, under 10% CO_2 in DMEM supplemented with 8% FBS, penicillin, streptomycin and L-glutamine and were passaged twice weekly.

Death assays. Cells were seeded on 12-well tissue culture plates at approximately 60% confluency and were allowed to adhere for 16–20 h. Comp A, described previously,[13] (500 nM), human Fc-TNF, as used in the study by Bossen *et al*,[18] (100 ng/ml), or CHX (250 ng/ml) were added to cells for 24 h and cell death was analysed by PI staining and flow cytometry. In each sample, 10 000 events were measured, and the cell death (% PI-positive cells) quantified.

Colony assays. After treatment as above, cells were trypsinised and replated in 6-well dishes. Cells were grown for 5 days, fixed and stained using 0.5% crystal violet in 20% (v/v) methanol/PBS.

Western blotting. Cells were treated with Fc TNF[18] or soluble TNF (Peprotech, Rocky Hill, NJ, USA) for the indicated times. Cells were then washed in ice-cold PBS, and lysed in DISC buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EDTA, Roche (Castle Hill, NSW, Australia) complete protease inhibitor cocktail) on ice. Cell lysate was spun at 14 000 × *g* for 5 min and the soluble material retained. Samples were separated on 4–12% polyacrylamide gels (Invitrogen, Sydney, NSW, Australia), and transferred to nitrocellulose or PVDF membranes for antibody detection. All membrane-blocking steps and antibody dilutions were performed with 5% skim milk in PBS containing 0.1% Tween 20 (PBS-T), and washing steps were performed with PBS-T. After incubating with HRP-coupled secondary antibodies, western blots were visualised by ECL (GE Healthcare, Rydalmere, NSW, Australia).

Antibodies. The primary antibodies used were anti-β-actin (Sigma Aldrich, Castle Hill, NSW, Australia), anti-RIPK1 (BD Sciences, North Ryde, NSW, Australia), anti-IκBα (Cell Signaling, Danvers, MA, USA), anti-TNFR2 (BD Transduction Laboratories) and anti-RelA (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

IL-6 ELISA. A total of 5×10^4 cells were plated in 24-well plates. Cells were either left untreated or treated with 10 ng/ml Fc TNF and incubated for 24 h. Medium was then taken and cleared of cells and debris by centrifugation and assayed using the OptEIA Mouse IL-6 ELISA kit (BD Biosciences) as per manufacturer's instructions. Assays were performed in triplicate for three independent times.

qPCR. Cells were collected using TRIzol (Invitrogen) and RNA extracted according to manufacturer's protocol. First strand cDNA synthesis was performed on 1 μg RNA using Superscript III and oligo dT using manufacturer's protocol (Invitrogen). cDNA samples were diluted 1:10 for qPCR and qPCR was performed using 2 × SYBR Green reaction mix (Roche) in a LightCycler 480 instrument (Roche). Complete primer sequences can be made available on request. Samples were analysed using a standard curve relative quantification. B2M was used to normalise each sample.

Immunofluorescence, image acquisition and processing. MEFs were grown overnight on glass coverslips and were treated with Fc TNF (100 ng/ml) for 15 min, fixed with 3.2% paraformaldehyde for 20 min, and permeabilized with 0.5% Triton X-100 for 5 min. Cells were blocked (PBS + 1% BSA), incubated with RelA antibody, washed, and incubated with anti-rabbit Alexafluor488-conjugated secondary antibody (Invitrogen) and were washed for four times. Cells were viewed with an Olympus BX50 fluorescence microscope and images taken using a ×40/0.85 Olympus objective lens, at room temperature, and SPOT RT imaging camera (Model 2.1.1) and software (Diagnostic Instruments, Perth, WA, Australia). All images were in TIF format and imported into Freehand MX (Adobe, Chatswood, NSW, Australia) for the compilation of figures.

Conflict of interest
J Silke is a consultant for, and DL Vaux is on the scientific advisory board of, TetraLogic Pharmaceuticals.

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