

The Prevalence of TNF α -Induced Necrosis over Apoptosis Is Determined by TAK1-RIP1 Interplay

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Abstract

Death receptor-induced programmed necrosis is regarded as a secondary death mechanism dominating only in cells that cannot properly induce caspase-dependent apoptosis. Here, we show that in cells lacking TGF β -activated Kinase-1 (TAK1) expression, catalytically active Receptor Interacting Protein 1 (RIP1)-dependent programmed necrosis overrides apoptotic processes following Tumor Necrosis Factor- α (TNF α) stimulation and results in rapid cell death. Importantly, the activation of the caspase cascade and caspase-8-mediated RIP1 cleavage in TNF α -stimulated TAK1 deficient cells is not sufficient to prevent RIP1-dependent necrosome formation and subsequent programmed necrosis. Our results demonstrate that TAK1 acts independently of its kinase activity to prevent the premature dissociation of ubiquitinated-RIP1 from TNF α -stimulated TNF-receptor I and also to inhibit the formation of TNF α -induced necrosome complex consisting of RIP1, RIP3, FADD, caspase-8 and cFLIP_L. The surprising prevalence of catalytically active RIP1-dependent programmed necrosis over apoptosis despite ongoing caspase activity implicates a complex regulatory mechanism governing the decision between both cell death pathways following death receptor stimulation.

Citation: Çöl Arslan S, Scheidereit C (2011) The Prevalence of TNF α -Induced Necrosis over Apoptosis Is Determined by TAK1-RIP1 Interplay. PLoS ONE 6(10): e26069. doi:10.1371/journal.pone.0026069

Editor: Wafik S. El-Deiry, Penn State Hershey Cancer Institute, United States of America

Received: July 12, 2011; **Accepted:** September 19, 2011; **Published:** October 10, 2011

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Funding: This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (TRR54) to C.S. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

TNF α is a pleiotropic cytokine regulating a variety of cellular responses such as proliferation, differentiation, and cell death. By binding to TNF receptor I (TNF-RI), a death receptor superfamily member, TNF α induces the formation of the plasma membrane-associated complex-I, including TRADD, TRAF2/5, cIAP1/2 and RIP1. Within complex-I, RIP1 is ubiquitinated depending on TRAF2/5 and cIAP1/2 and polyubiquitinated RIP1 (Ubi-RIP1) recruits TAK1 (TAK1/TAB2/TAB3) and IKK (IKK α /IKK β /NEMO) kinase complexes. Then, auto-activated TAK1 phosphorylates and activates IKK β , facilitating IKK β -mediated I κ B α degradation and downstream NF- κ B activation [1,2].

Ligation of TNF-RI by TNF α also leads to the assembly of a second, cytoplasmic multiprotein complex. Deubiquitination of RIP1 is prerequisite for the formation of complex-II comprising RIP1, RIP3, FADD and caspase-8, which is then activated by auto-cleavage and initiates apoptosis. Caspase-8 also cleaves RIP1 and inactivates it within complex-II [1,3]. TNF α -induced apoptosis is mainly studied in the absence of *de novo* protein synthesis or in cells lacking crucial activators of the NF- κ B pathway, since cells that can trigger TNF α -dependent NF- κ B activation express multiple anti-apoptotic genes and block complex-II-mediated apoptosis initiation [4,5,6,7].

TNF α -induced programmed necrosis, or necroptosis, is a recently defined alternative cell death pathway absolutely requiring RIP1 kinase activity and is described to dominate only when dying cells cannot activate caspase-8 [1,3]. Under these conditions, TNF α -induced complex-II acts as the pre-necrotic

'necrosome' complex, where catalytically active RIP1 and RIP3 trigger rapid reactive-oxygen-species (ROS) accumulation and subsequent cell death with morphological features reminiscent of necrosis [1,3,8,9,10]. The catalytic activity of RIP1 is required for the formation of the necrosome complex [8]. Since RIP1 is inactivated by caspase-8, prevention of RIP1 cleavage by caspase inhibition is thought to be required for efficient death receptor-mediated necrosis induction.

Recent studies indicated a role for cFLIP_L, the catalytically inactive homologue of caspase-8, in programmed necrosis inhibition [11,12]. Hetero-dimerisation of caspase-8 and cFLIP_L was shown to be necessary for caspase-8 mediated protection from necrosis [12]. However, depending on its expression level, cFLIP_L may inhibit or augment caspase-8 activity [13], hence its mechanistic contribution in necrosis is not completely clear.

As TAK1 is a critical component of TNF α -induced NF- κ B activation, cells lacking TAK1 expression undergo cell death following TNF α stimulation [14,15,16,17,18]. However, TAK1 knock-out (KO) cells are remarkably more sensitive to TNF α -induced cytotoxicity than other types of cells that cannot activate NF- κ B [18,19]. In addition, TAK1 KO mice die at an earlier embryonic stage than mice lacking IKK β or NEMO expression [4,5,19,20]. This shows that TAK1 has additional, NF- κ B-independent functions during embryonic development. It has been reported that TNF α stimulation of TAK1 KO keratinocytes led to a ROS-mediated, rapid cell death [18]. Moreover, it has recently been shown that in L929 cells, a murine fibrosarcoma cell line that undergoes caspase-independent programmed necrosis upon TNF α stimulation and is commonly used as a model system

for this type of cell death, down-regulation of TAK1 augmented the ongoing necrotic response [21]. Here, we dissect the molecular mechanism of TNF α -induced death of TAK1 deficient cells and show that ablation of TAK1 expression alone is sufficient for the induction of TNF α -mediated programmed necrosis in cells that are otherwise resistant to TNF α -induced cytotoxicity. We identified RIP1 as a critical mediator of TNF α -induced ROS accumulation and cell death in the absence of TAK1, indicating the induction of the necrotic pathway. TAK1 mediated the stabilization of polyubiquitinated RIP1 in complex-I and prohibited RIP1-dependent rapid necrosis, unravelling a novel functional connection between these two kinases in addition to their established cooperation in TNF α -induced NF- κ B signalling. Moreover, our results demonstrated that RIP1 kinase activity-dependent cell death proceeds even in the presence of ongoing caspase activity.

Results and Discussion

TAK1 hinders TNF α -induced, RIP1-mediated rapid cell death

To evaluate whether RIP1 plays a role in the death of TAK1 KO mouse embryonic fibroblasts (MEFs), cells were transfected with two different RIP1-targeting siRNA constructs along with a control siRNA. Loss of RIP1 completely prevented TNF α -induced cell death after 2.5 hours of stimulation and provided major protection after 6 hours (Fig. 1A). In addition, TNF α -induced ROS accumulation in TAK1 KO MEFs could be fully prevented by RIP1 down-regulation (Fig. 1B). A specific RIP1 kinase- and programmed necrosis-inhibitor, Necrostatin-1 (Nec-1) [22,23], and the ROS scavenger butylated hydroxyanisole (BHA) also completely blocked TNF α -mediated cytotoxicity (Fig. 1C) [18]. Moreover, Nec-1 was as effective as BHA in blocking TNF α -induced ROS accumulation in the absence of TAK1 (Fig. 1D). Taken together, these experiments show that catalytically active RIP1 is essential for TNF α -dependent, ROS-mediated rapid cell death in the absence of TAK1, indicating the triggering of the necrotic pathway.

Necrosis overrules apoptosis in TNF α -treated TAK1 deficient cells

Death receptor-mediated programmed necrosis has been defined to predominate in the absence of caspase activity [1,3]. However, TNF α stimulation induced the cleavage of caspase-8, caspase-3 and the caspase-8 target, RIP1 in TAK1 KO MEFs (Fig. 2A), similar to previous observations made in keratinocytes [18]. Treatment of TAK1 KO MEFs with the pan-caspase inhibitor zVAD-FMK completely abrogated caspase-8 and caspase-3 cleavage (Fig. 2B) as well as the cleavage of RIP1 and the caspase-3 target, PARP1 (Fig. S1A), nevertheless provided only minor protection from TNF α -dependent death (Fig. 2C). Moreover, zVAD failed to block TNF α -induced ROS accumulation in TAK1 KO cells (Fig. 2D). Similar observations were obtained by using another broad-spectrum caspase inhibitor, Q-VD-OPh (Fig. S1). These results indicate that TNF α -dependent caspase activation plays only a marginal role in the death of TAK1 KO cells. Of note, Nec-1 and BHA treatment attenuated caspase-8 and caspase-3 cleavage following stimulation, indicating that TNF α -induced ROS accumulation augmented caspase activation (Fig. 2E) [18]. Nec-1 and BHA provided full protection from TNF α -mediated rapid cell death without a complete block on caspase activation (Figure 1C and 2E), further supporting the conclusion that ROS-mediated necrosis is the primary cause of the rapid demise of TNF α -

stimulated TAK1 KO cells, giving no time to caspase-dependent apoptotic processes to mediate cell death.

TAK1 blocks TNF α -dependent necrosome formation

TNF α -mediated necrotic cell death requires the formation of the necrosome complex, including RIP1, RIP3, FADD and caspase-8, whose catalytic activity has to be blocked by either small-molecule inhibitors or virally expressed anti-apoptotic proteins [1,3]. Using FADD immunoprecipitation, we investigated whether TNF α could induce necrosome formation in the absence of TAK1. As expected, WT MEFs required co-administration of zVAD together with TNF α and CHX for efficient necrosome formation (Fig. S2). On the other hand, in TAK1 KO cells, all known components of the necrosome complex immunoprecipitated with FADD within 30-45 minutes of TNF α stimulation (Fig. 3A). Necrosome formation was fully inhibited by Nec-1 treatment, implicating an absolute dependence on catalytically active RIP1 (Fig. 3B). Thus, our study presents TAK1 as a key mediator of TNF α signalling that specifically blocks RIP1 kinase activity-dependent necrosome formation.

Surprisingly, in both WT and TAK1 deficient MEFs, the necrosome complex contained cFLIP_L (Fig. 3A, 3B and Fig. S2). Given its dual nature on the modulation of caspase-8 activation [13], further analysis of cFLIP_L within the necrosome complex may shed light on the dynamics of necrosome formation and function.

It is of note that at later time points, predominantly the cleaved forms of caspase-8 and cFLIP_L (43 kDa) were found in the necrosome complex, although the p43 fragment of caspase-8 was poorly identifiable in input samples (Fig. 3A and 3B). This indicated that caspase-8 in the necrosome was catalytically active and processed itself as well as cFLIP_L. Accordingly, treatment of TAK1 KO MEFs with TNF α and zVAD enhanced the incorporation of full length caspase-8, cFLIP_L and RIP1 (Fig. 3B). Whether this translates into an enhanced activation of the necrosome complex is an open question, nevertheless, these experiments show that RIP1 kinase activity-dependent necrosome formation can occur in the presence of ongoing caspase activity. Also, caspase-8-mediated cleavage and the assumed inactivation of RIP1 (Fig. 2A) [1,3] does not block programmed necrosis initiation following TNF α stimulation.

TAK1 stabilizes Ubi-RIP1 association with complex-I

In order to analyze whether TAK1 curbs the cytotoxic potential of RIP1 by regulating its incorporation into TNF α -induced complex-I, WT and TAK1 KO MEFs were stimulated with Flag-TNF α and complex-I was immunoprecipitated with an α -Flag antibody. Stimulus-dependent incorporation of Ubi-RIP1 [7,24] into complex-I was evident in both cell types, yet in TAK1 KO cells, the amount of Ubi-RIP1 decreased significantly at later time points compared to WT cells (Fig. 3C), indicating that TAK1 stabilizes Ubi-RIP1 in complex-I. In TNF α -treated TAK1 KO cells, the accelerated dissociation of RIP1 from complex-I was accompanied by RIP1-dependent necrosome formation within 30 minutes of stimulation. Hence, TAK1-mediated protection of cells from TNF α -induced rapid cytotoxicity might proceed via regulation of ubiquitinated-RIP1 incorporation into complex-I. In a similar manner, it has been shown that treatment of cells with Smac mimetic to diminish cIAP1/2 expression and Ubi-RIP1 association with complex-I also resulted in RIP1 kinase-activity-dependent caspase activation and subsequent cell death following TNF α stimulation [7].

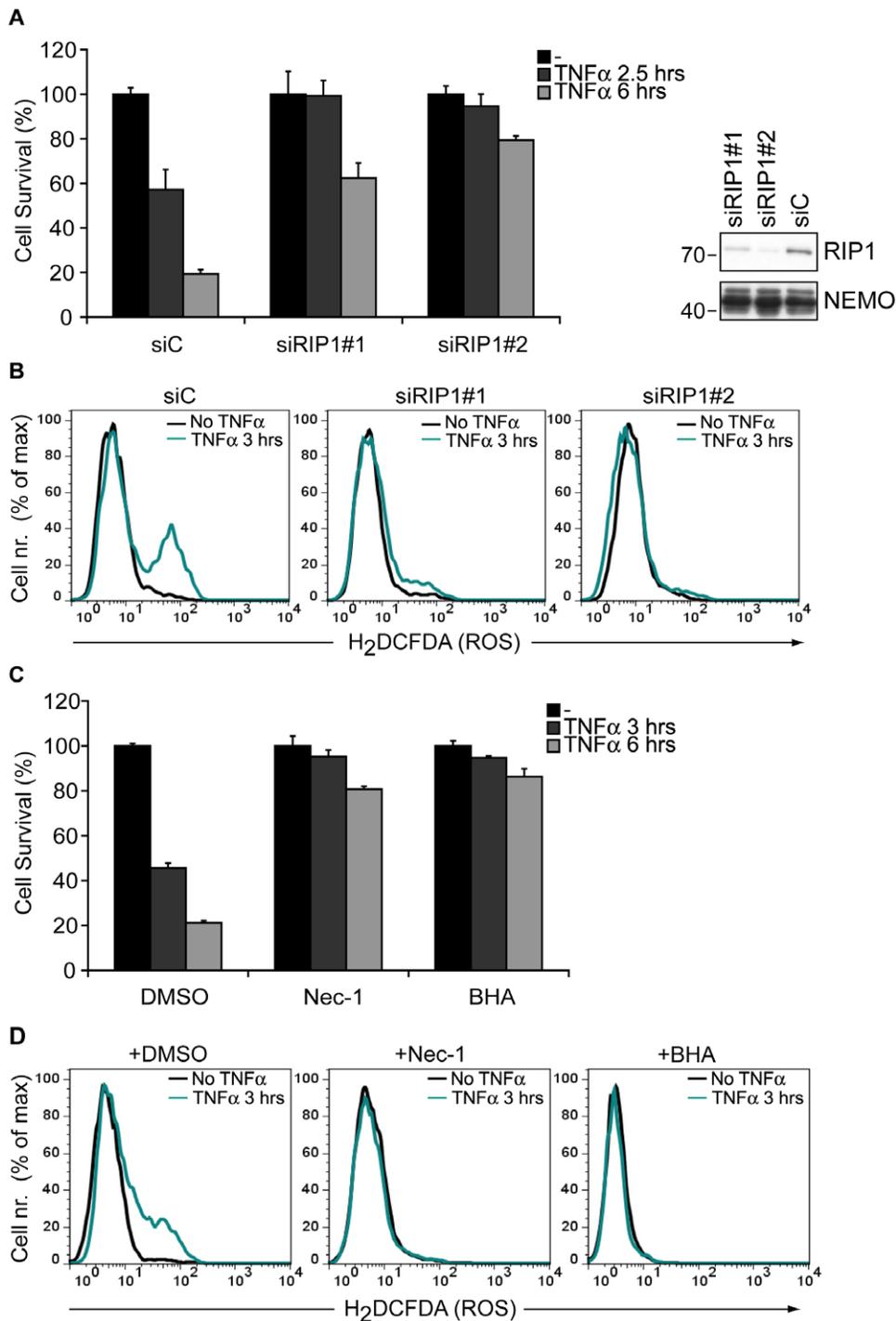


Figure 1. TAK1 impairs ROS-mediated cell death triggered by catalytically active RIP1. (A) *Left panel* TAK1 KO MEFs transfected with a control siRNA (siC) or with RIP1 targeting siRNAs were stimulated with TNF α and cell survival was analyzed by crystal violet staining (CV). The bar graphs depict mean values \pm SEM %. *Right panel* Cell lysates were analyzed by western blotting (WB) with α -RIP1 and α -NEMO antibodies to control the efficiency of RIP1 down-regulation. (B) TAK1 KO MEFs were transfected as in (A) and stimulated with TNF α . Intracellular ROS accumulation was analyzed by flow cytometry. (C) TAK1 KO MEFs were treated with DMSO or Necrostatin-1 (Nec-1) for 1 hour and then stimulated with TNF α . Butylated hydroxyanisole (BHA) was added together with TNF α . Cell survival was analyzed by CV. The bar graphs depict mean values \pm SEM %. (D) TAK1 KO MEFs were treated with DMSO, Nec-1 or BHA as in (C) and stimulated with TNF α for indicated time points. ROS accumulation was analyzed as in (B). doi:10.1371/journal.pone.0026069.g001

TAK1 has a kinase-independent cytoprotective function

In order to ensure that the sensitivity of TAK1 KO MEFs to TNF α -induced cytotoxicity was only due to the lack of TAK1

expression, cells were stably transfected with wild type TAK1. TAK1 KO MEFs were also reconstituted with a catalytically inactive mutant of TAK1 (TAK1-K63W [25]) to investigate

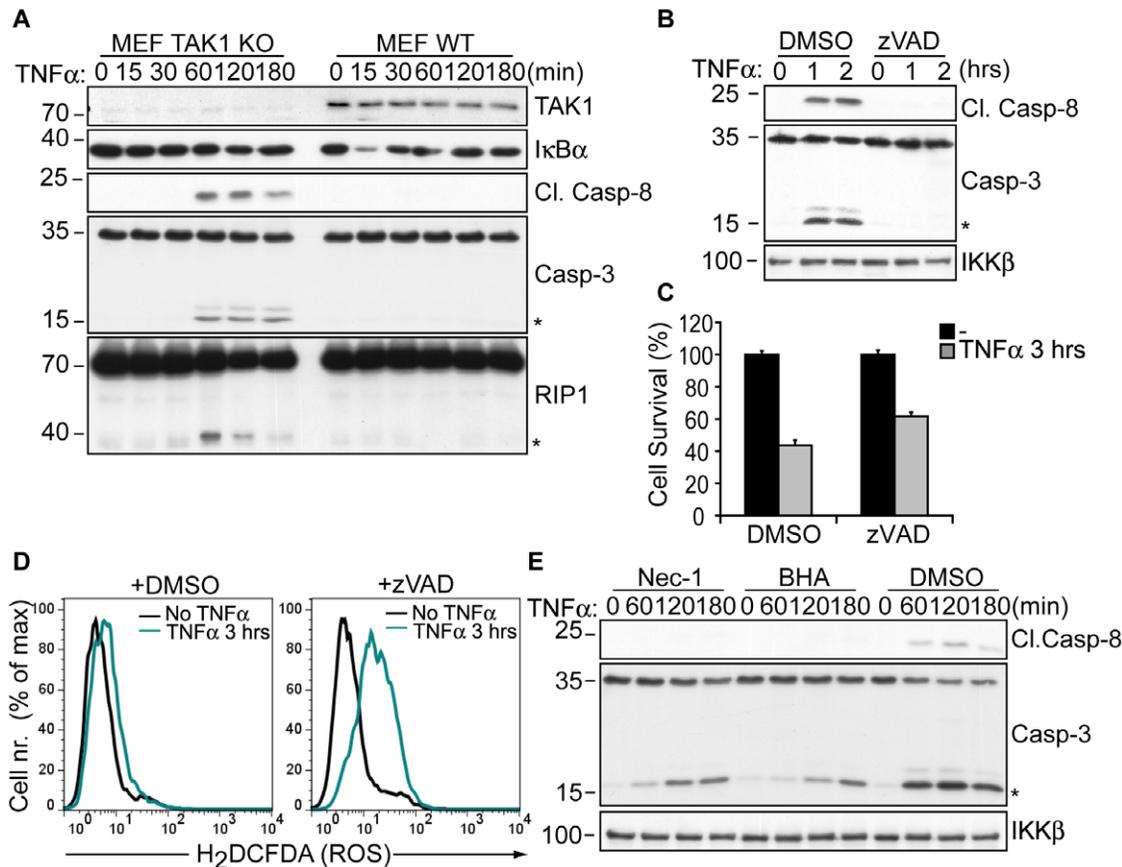


Figure 2. TNF α -induced caspase activity in TAK1 deficient cells is not essential for ROS-mediated cytotoxicity. (A) TAK1 KO and WT MEFs were stimulated with TNF α as indicated and analyzed by WB with the indicated antibodies to detect I κ B α degradation and the cleavage of RIP1, caspase-8 (p18) and caspase-3 (p15). (B) TAK1 KO MEFs were pre-treated with DMSO or zVAD for 1 hour, stimulated with TNF α and analyzed by WB for cleaved caspase-8, caspase-3 and IKK β . (C) TAK1 KO MEFs were pre-treated as in (B) and stimulated with TNF α . Cell survival was analyzed by CV. The bar graphs depict mean values \pm SEM %. (D) TAK1 KO MEFs were pre-treated as in (B) and stimulated with TNF α . ROS accumulation was evaluated by flow cytometry. (E) TAK1 KO MEFs were pre-treated with DMSO or Nec-1 for 1 hour, stimulated with TNF α and analyzed as in (B). BHA was added together with TNF α . (*) denotes the cleaved fragments of caspase-3 (p15) and RIP1. doi:10.1371/journal.pone.0026069.g002

whether TAK1 kinase activity was required for the protection from necrosis. By using a TAK1 kinase inhibitor (NP-009245) at 1 μ M, it has been suggested that inhibition of TAK1 catalytic activity further increased caspase-independent necrosis in L929 cells [21]. However, at this concentration, NP-009245 inhibited EGF and PMA-mediated JNK phosphorylation in WT and TAK1 KO MEFs equally well, indicating that NP-009245 did not enhance TNF α -mediated necrosis specifically via TAK1 inhibition (Fig. S3).

As expected, TAK1 KO cells reconstituted with TAK1-WT were capable of TNF α -induced I κ B α degradation, which was impaired in cells expressing TAK1-K63W (Fig. 4A). Nevertheless, reconstitution of TAK1 KO cells with either construct resulted in a prolonged association of Ubi-RIP1 with TNF α -induced complex-I compared to empty vector-transfected cells (Fig. 4B). This suggested a scaffold function for TAK1 to restrict the premature dissociation of RIP1 from complex-I. In parallel with this observation, TNF α -dependent necrosome formation was inhibited upon reconstitution with either TAK1-WT or TAK1-K63W (Fig. 4C). Moreover, expression of TAK1-WT or TAK1-K63W in TAK1 KO cells provided complete protection from TNF α -dependent cytotoxicity (Fig. 4D) and also blocked TNF α -induced caspase activation (Fig. 4E) as well as ROS accumulation

(Fig. 4F). These data demonstrate that TAK1 acts as an adaptor molecule in complex-I to prevent premature dissociation of Ubi-RIP1 and also functions independently of its kinase activity to block TNF α -induced, RIP1-mediated necrosis. Therefore, our data suggest that TAK1 may suppress the pro-necrotic activity of RIP1 by sequestering it within complex-I, highlighting a novel non-catalytic function of TAK1 in complex-I in addition to its role as a signal transducer for TNF α -mediated NF- κ B activation.

RIP1-dependent necrotic cell death is not only observed following death receptor stimulation, but also in acute pancreatitis [9,10], ischemia-induced brain injury [23] as well as myocardial infarction [26]. Hence, future studies may reveal whether TAK1 also modulates RIP1-dependent necrosis under these pathophysiological conditions and help further characterization of TAK1-RIP1 interplay in necrotic cell death.

Materials and Methods

Reagents and cytokines

Recombinant human TNF α and Flag-TNF α (Alexis) were used at 20 ng/ml and 200 ng/ml, respectively. Nec-1 (Biomol), BHA (Sigma), zVAD(oMe)-FMK (Imgenex) and Q-VD-OPH (Calbiochem) were utilized at 10 μ M, 100 μ M, 20 μ M and 20 μ M,

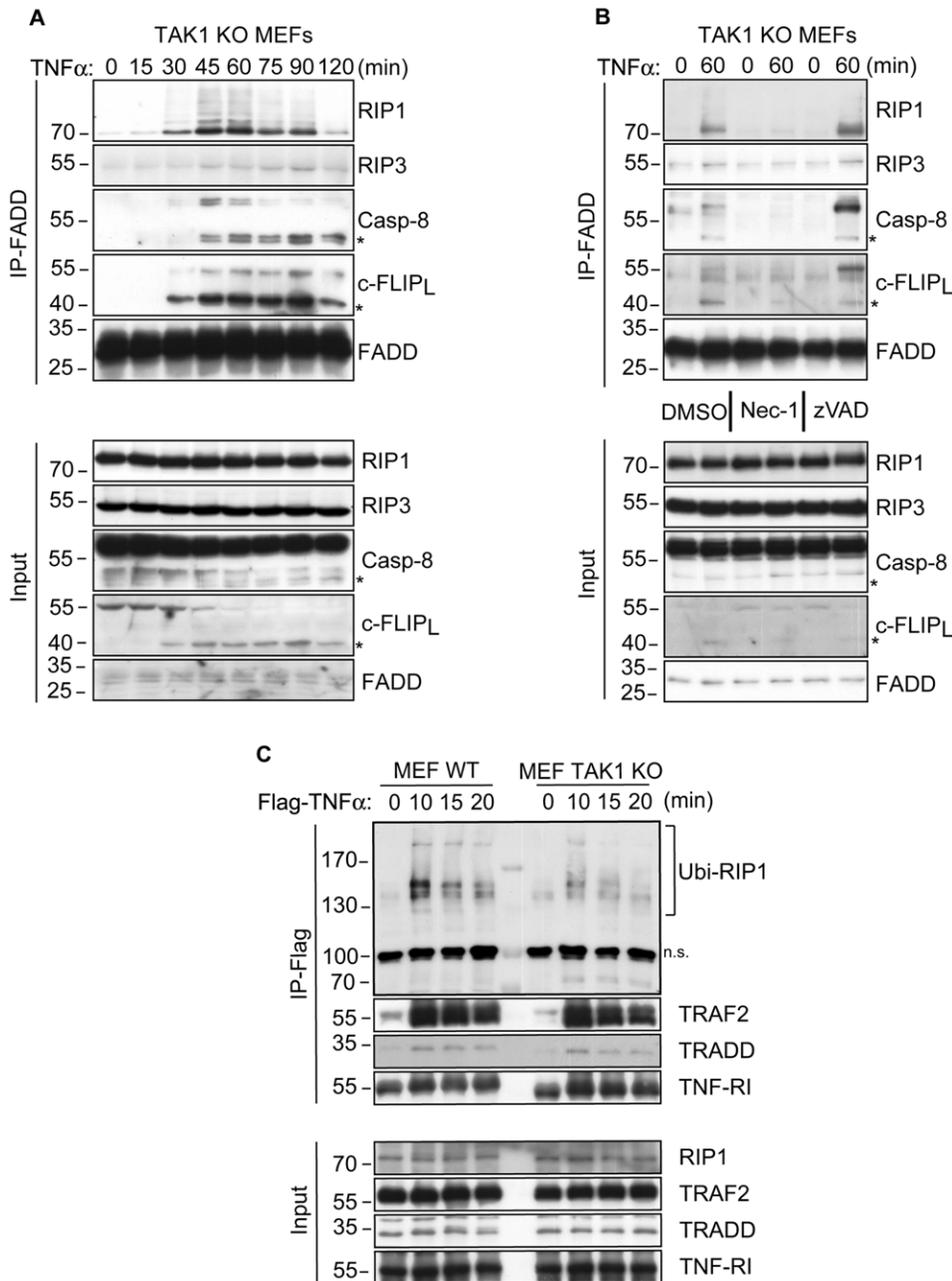


Figure 3. TAK1 prevents TNF α -induced necrosome formation and stabilizes polyubiquitinated RIP1 in complex-I. (A) TAK1 KO MEFs were stimulated with TNF α and lysed for immunoprecipitation with an α -FADD antibody to isolate the necrosome complex. Co-immunoprecipitated proteins were analyzed by WB with the indicated antibodies. (B) TAK1 KO MEFs were pre-treated with DMSO, Nec-1 or zVAD for 60 minutes and then stimulated with TNF α . Cell lysates were analyzed by WB as in (A). (C) WT and TAK1 KO MEFs were stimulated with Flag-TNF α for indicated time points. TNF α -bound TNF-RI and additional complex-I members were immunoprecipitated by α -Flag M2 affinity beads and analyzed by WB. Ubi-RIP1 depicts complex-I-associated polyubiquitinated RIP1. (*) denotes the p43 fragment of caspase-8 and cFLIPL. n.s., non-specific band.
doi:10.1371/journal.pone.0026069.g003

respectively. TAK1-inhibitor (NP-009245) was purchased from AnalytiCon Discovery. EGF (Invitrogen) and PMA (Calbiochem) was used at 25 ng/ml and 300 ng/ml, respectively.

Antibodies

The following antibodies were used for immunoprecipitation and western blot analyses: RIP1 (clone 38) and NEMO (clone 54) from BD Transduction, TAK1 (M-579), FADD (M-19), TRAF2 (C-20) and I κ B α (C-21) from Santa Cruz Biotechnology, IKK β (A10AG2) from Millipore, cleaved caspase-8 (#9429), caspase-3 (#9662) and

p-JNK (#9251) from Cell Signaling, JNK (51-1570GR) from BD Pharmingen, caspase-8 (1G12) and c-FLIPL (Dave2) from Enzo Life Sciences, RIP3 (2283) from Proscience, TNF-RI (ab 19139) and TRADD (ab 18914) from Abcam. Rabbit polyclonal antibody against PARP1 (288) was described previously [27].

DNA and siRNA constructs

Murine TAK1-WT and K63W were subcloned into pTRACER-CMV2 (Invitrogen) from pCMV-HA-TAK1-WT and K63W constructs (gift from Dr. Kunihiko Matsumoto, Nagoya

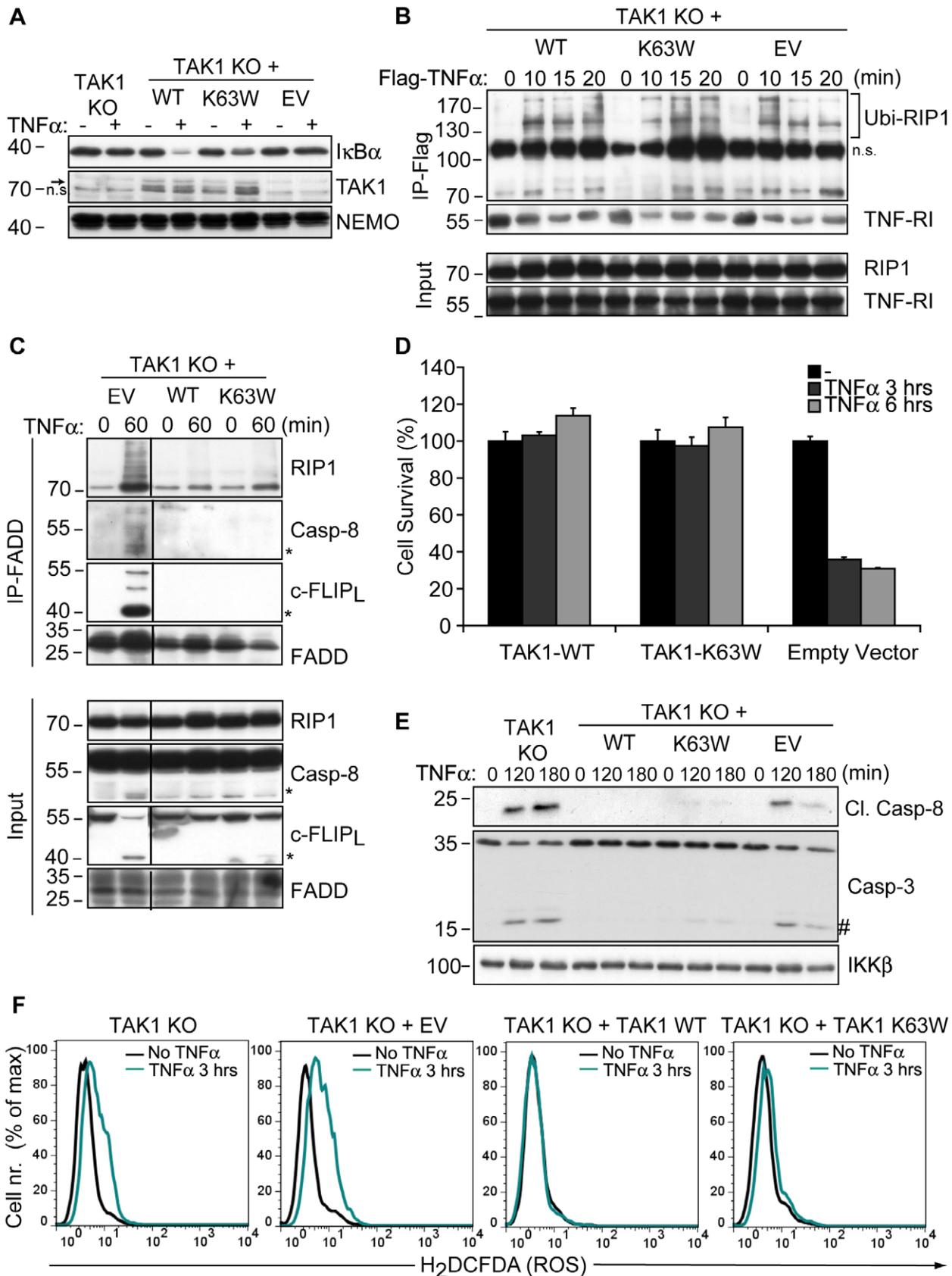


Figure 4. TAK1 blocks TNF α -induced necrosis independently of its kinase activity. (A) TAK1 KO MEFs transfected with a WT or a kinase-dead mutant (K63W) construct of TAK1 along with untransfected and empty-vector (EV) transfected cells were stimulated with TNF α and analyzed by WB for IκB α degradation as well as TAK1 and NEMO expression. (B) Cells were stimulated with Flag-TNF α and Ubi-RIP1 interaction with complex-I was

analyzed by immunoprecipitating Flag-TNF α -bound TNF-RI and associated factors by α -Flag M2 affinity beads as in Fig. 3C. (C) Indicated cells were stimulated with TNF α and necrosome formation was investigated by immunoprecipitating FADD as in Fig. 3A and 3B. (D) Cells were stimulated with TNF α and cell survival was analyzed by CV. The bar graphs depict mean values \pm SEM %. (E) Cells were stimulated with TNF α and caspase activation was assessed by WB analysis. (F) Cells were treated with TNF α and intracellular ROS accumulation was analyzed by flow cytometry. (*) denotes the p43 fragment of caspase-8 and cFLIP_L. (#) denotes cleaved caspase-3 (p15). n.s., non-specific band.
doi:10.1371/journal.pone.0026069.g004

University, Nagoya, Japan) between KpnI/XbaI restriction sites. RIP1 expression was downregulated by using two different siRNA constructs (#1 sense: 5' GCAGAGAGCUCGUGAGAAU 3' and #2 sense: 5' CCACUAGUCUGACUGAUGA 3'). Non-targeting control siRNA construct was 5' UCAAAUUGGUCCACGGAAU 3'. All siRNA duplexes were purchased from Eurogentec.

Cell culture and transfections

WT and TAK1 KO MEF cells [19] were a gift of Dr. Shankar Ghosh (Columbia University, New York, USA) and maintained in DMEM-GlutaMAX (Gibco) supplemented with 10% FCS (Gibco) and penicillin/streptomycin (100 U/ml and 100 μ g/ml). To reconstitute TAK1 deficient MEFs with pTRACER-TAK1-WT and pTRACER-TAK1-K63W along with empty pTRACER for empty vector transfection, cells were transfected by using Transfectin (Biorad) reagent according to manufacturer's instructions. Stably transfected monoclonal cells were selected with 80–100 μ g/ml Zeocin (Invitrogen) and expanded for experimentation. To downregulate protein expression, cells were transfected with different siRNA (10–20nM) constructs with the help of lipids from Silence Therapeutics according to manufacturer's instructions and harvested 3 days after transfection. All cells were starved overnight before analysis.

Immunoprecipitation and western blotting

The necrosome complex was isolated as described previously [8]. Briefly, cells ($\sim 1 \times 10^7$) were lysed in 1 ml necrosome buffer (20 mM Tris-HCl 7.5, 150 mM NaCl, 0.2% NP-40, 1mM EDTA, 10% glycerol, 3 mM NaF, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 50 mM Calyculin A (Cell Signaling), 500 μ M Pefablock (Roche) and protease inhibitor cocktail tablets (Roche)). Cleared lysates were incubated with α -FADD antibody for 2 hours and immunocomplexes were precipitated with protein G sepharose beads (GE Healthcare) for another hour. Co-precipitated proteins were analyzed by western blotting (WB). Standard procedures were followed for WB.

Flag-TNF α immunoprecipitation was done as described previously [24] with minor modifications. After stimulation with 200 ng/ml Flag-TNF α , cells ($\sim 1 \times 10^7$) were lysed in 500 μ l Flag-IP buffer (30 mM Tris-HCl pH 7.5, 120 mM NaCl, 1 mM EDTA pH 8.0, 1 mM KCl, 1% Triton X-100, 8 mM β -glycerophosphate, 20 mM NaF, 200 μ M Na₃VO₄, 500 μ M Pefablock and protease inhibitor cocktail tablets). Cleared lysates were incubated with α -Flag-M2 affinity gel (Sigma) for 3 hours. For uninduced samples, 200 ng of Flag-TNF α was added to the lysates just before immunoprecipitation. Immunocomplexes were analyzed by WB.

Intracellular ROS measurement with carboxy-H₂DCFDA and Propidium iodide staining

MEFs were stimulated for indicated time points and were supplemented with 10 μ M carboxy-H₂DCFDA (Invitrogen) in the last 30 minutes of incubation at 37°C. Cells were then trypsinized, washed and re-suspended in 1 ml PBS. Intracellular ROS accumulation was analyzed by using the FL1 channel of a BD FACSCalibur flow cytometer. Propidium iodide (PI, Sigma) was added (2 μ g/ml) to each sample just before measurement. PI staining was visualized by using the FL3 channel. Data was

analyzed by using FlowJo software. In all experiments, cells were FSC/SSC gated before analysis. ROS accumulation was visualized in the living population (PI negative cells).

Crystal violet staining (CV)

Following medium removal, MEFs (in 12-well plates) were incubated for 5 minutes with 1 ml PFA/PBS (4%, RT). PFA/PBS was replaced by 1 ml of 0.01% CV solution (Sigma, in ddH₂O) and incubated for 20–30 minutes. Plates were washed 3–4x with tap water and let dry briefly. CV was solubilised with methanol, and its absorbance was measured at 540 nm. All treatments were done in at least triplicates. The graphs depict mean values \pm SEM %.

Supporting Information

Figure S1 Caspase inhibition by Q-VD-OPh does not block TNF α -induced ROS accumulation and death in TAK1 KO MEFs. (A) TAK1 KO MEFs were pre-treated with DMSO, Q-VD-OPh (20 μ M) or zVAD-FMK (20 μ M) for one hour and then incubated with or without TNF α for another hour. The efficiency of caspase inhibition was assessed by WB analysis of cleaved PARP1 and RIP1. (*) denotes the cleaved products of PARP1 and RIP1 (B) TAK1 KO MEFs were pre-treated with DMSO or Q-VD-OPh (20 μ M) for one hour and then stimulated with TNF α as indicated. ROS accumulation was analyzed by flow cytometry. (C) TAK1 KO cells were pre-treated as in (A) and (B), and stimulated with TNF α for 3 hours. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. The percentage of dead (PI positive) cells is indicated in each histogram.
(TIF)

Figure S2 TNF α -induced necrosome formation in WT MEFs requires inhibition of protein synthesis and caspase activity. WT MEFs were pre-treated for 1 hour with cycloheximide (CHX (Sigma), 1 μ g/ml) and/or zVAD (20 μ M) as indicated and stimulated with TNF α for 4 hours. Necrosome formation was evaluated by WB analysis with the indicated antibodies following α -FADD immunoprecipitation.
(TIF)

Figure S3 Limited specificity of the TAK1 inhibitor NP-009245. (A) WT and TAK1 KO MEFs were pre-treated with DMSO or NP-009245 (denoted as NP, 1 μ M) for 1 hour and stimulated with EGF (25 ng/ml) or PMA (300 ng/ml) for 15 minutes. The inhibitory effect of NP-009245 was investigated by WB analysis of stimulus-dependent JNK phosphorylation, which was efficiently blocked in both TAK1 KO and WT MEFs. (B) WT MEFs were pre-treated with DMSO or increasing concentrations of NP-009245 for 1 hour and subsequently incubated with TNF α for 15 minutes. The inhibitory effect of NP-009245 on TAK1 catalytic activity was evaluated by WB analyses of I κ B α degradation and JNK phosphorylation. 50 nM of NP-009245 was sufficient to block TNF α -induced JNK phosphorylation; while I κ B α degradation was only blocked at 1 μ M (C) WT MEFs were pre-treated as in (B) and then stimulated with TNF α for 3 hours. Cell survival was analyzed by crystal violet staining. NP-009245 induced cell death already at 250 nM. (D) WT MEFs

were pre-treated as in (B) and stimulated with TNF α for 1 hour. Cells were lysed and immunoprecipitated with an α -FADD antibody to subsequently analyze RIP1 co-immunoprecipitation. Weak RIP1 co-immunoprecipitation with FADD was only observed at 1 μ M of NP-009245.

(TIF)

Acknowledgments

We are indebted to Dr. Sankar Ghosh and Dr. Kunihiro Matsumoto for providing us with the indicated cells and constructs. We are deeply grateful

to Dr. Michael Hinz and Dr. Michael Stilmann for helpful discussions during the preparation of this manuscript. We also thank Maria Mühlbauer, Mareen Kamarys and Sonja Kumsteller for their excellent technical help.

Author Contributions

Conceived and designed the experiments: SCA CS. Performed the experiments: SCA. Analyzed the data: SCA CS. Wrote the paper: SCA CS.

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