The cytoplasmic nuclear receptor RARγ controls RIP1 initiated cell death when cIAP activity is inhibited

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Tumor necrosis factor (TNF) has a critical role in diverse cellular events including inflammation, apoptosis and necroptosis through different signaling complexes. However, little is known about how the transition from inflammatory signaling to the engagement of death pathways is modulated. Here we report that the cytoplasmic retinoic acid receptor gamma (RARγ) controls receptor-interacting protein kinase 1 (RIP1)-initiated cell death when cellular inhibitor of apoptosis (cIAP) activity is blocked. Through screening a short hairpin RNA library, we found that RARγ was essential for TNF-induced RIP1-initiated apoptosis and necroptosis. Our data suggests that RARγ initiates the formation of death signaling complexes by mediating RIP1 dissociation from TNF receptor 1. We demonstrate that RARγ is released from the nucleus to orchestrate the formation of the cytosolic death complexes. In addition, we demonstrate that RARγ has a similar role in TNF-induced necroptosis in vivo. Thus, our study suggests that nuclear receptor RARγ provides a key checkpoint for the transition from life to death.
The inflammatory cytokine tumor necrosis factor (TNF) induces diverse cellular responses including apoptosis and necroptosis, The molecular mechanism of TNF signaling has been intensively investigated. It is known that TNF triggers the formation of a TNF receptor 1 (TNFRI) signaling complex by recruiting several effectors such as TNFR1-associated death domain protein (TRADD), receptor-interacting protein kinase 1 (RIP1) and TNFR-associated factor 2 (TRAF2) to mediate the activation of the transcription factor nuclear factor-κB (NF-κB) and mitogen-activated protein (MAP) kinase pathways. Importantly, under certain conditions, this TNFRI signaling complex (complex I) dissociates from the receptor and recruits other proteins to form different secondary complexes for apoptosis and necroptosis. It is known now that necroptosis needs RIP3 and mixed lineage kinase-domain-like (MLKL) in the necrosome, Apoptosis is initiated through the recruitment of the death domain protein Fas-associated death domain protein (FADD) to form complex II. FADD then recruits the initiator caspase protease Caspases-8, -10. The physiological roles of these death proteins and the cross-talk between necroptosis and apoptosis have been elegantly demonstrated recently in animal models.

Both TRADD and RIP1 proteins have a death domain and interact with TNFR1 directly. TNF can induce cell death through either TRADD- or RIP1-initiated pathways. It has been shown that TNF triggers TRADD-mediated apoptosis when de novo protein synthesis is inhibited, but engages RIP1-initiated apoptosis when RIP1 ubiquitination by E3 ligases baculoviral IAP repeat-containing protein (IAP)1/2 is blocked. However, both TRADD- and RIP1-initiated cell death becomes necroptotic when caspase activity is suppressed. In the case of de novo protein synthesis inhibition, TRADD needs to recruit RIP1 to mediate TNF-induced necroptosis. RIP1-initiated cell death also occurs in cells in response to other death factors such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), although some proteins such as cyldonastas (CYYLD) and cellular FLICE-like inhibitory protein (cFLIP) have been suggested to have a role in regulating the formation of complex II/necrosome. Little is known about how the transition from the TNFR1 complex to the cell death complexes is modulated.

Retinoic acid receptors (RARs), RARα, RARβ and RARγ belong to the super family of nuclear hormone receptor and act as transcription factors after activation by RA. RARs regulate the expression of a large number of genes that are critical for cell growth, differentiation and cell death. Although the localization of these RARs is predominantly nuclear, however, cytoplasmic localization of RARs have been reported in some types of cells, but the function of the cytosolic RARs is unknown.

Here we report that RARγ has a critical role in RIP1-, but not TRADD-, initiated cell death in response to TNF and other death factors treatment. We found that RARγ is released from the nucleus to orchestrate the formation of the cytosolic cell death complexes. Our findings suggest that the nuclear receptor RARγ functions as a critical checkpoint of RIP1-initiated cell death.

**Results**

**RARγ is required for cell death initiated by RIP1.** To identify additional components of TNF-induced necroptosis, we used a retroviral short hairpin RNA (shRNA)-mediated genetic screen to identify genes whose knockdown resulting in resistance to necroptosis. The pseudo-kinase protein MLKL was identified as a key mediator of necroptosis through screening a kinase/phosphatase shRNA library. Another shRNA library used in our screening is one targeting cancer-implicated genes and this library of 1,841 shRNAs targets 1,272 human genes. HT-29 cells were infected with the retroviral shRNA library and were treated to undergo necroptosis by the combination of TNF-α, Smac mimetic and the caspase inhibitor z-VAD-fmk (TSZ) (Supplementary Fig. 1). Surviving cell clones were selected for confirmation of necrotic resistance and for identification of the corresponding shRNAs by PCR and DNA sequencing. Among the 60 selected clones, 7 clones had the shRNA targeting the *rarγ* gene.

RARγ has two major splicing isoforms and the full-length RARY is a 454-amino acid protein. The seven isolated RARY-shRNA clones had the same shRNA that targets the 3′-untranslated region 2,680–2,700 of *rarγ*. Three of the seven clones (RARY-shRNA-α, -B and -C) were used for further testing. Compared with the control firefly shRNA cells (cont-shRNA), all three RARY-shRNA clones were resistant to necroptosis (Fig. 1a). The necroptosis resistance of these three RARY-shRNA clones is comparable to that of RIP1-shRNA cells. The expression levels of RARY, RARα, RIP1, RIP3, TRADD, MLKL, CYLD and TRAF2 in the three RARY-shRNA clones and cont-shRNA cells were examined by western blotting (Fig. 1a) and the result indicated that the RARY-shRNA specifically knocked down the level of RARY protein and did not affect the expression of other related proteins.

RARγ-shRNA-A clone was then used as a representative in the rest of the experiments. To rule out off-target effect of the RARY-shRNA on necroptosis, a RARY-shRNA resistant RARY protein (rRARγ) was introduced back into the RARY-shRNA-A HT-29 cells. The ectopic expression of the resistant RARY restored the sensitivity of the cells to TNF-induced necroptosis, indicating that the resistance of RARY-shRNA-A cells to necroptosis is specifically due to the knockdown of RARY (Fig. 1b). The specific contribution of RARY in necroptosis was further confirmed by using two different RARY-shRNAs (RARγ-shRNA-1 and RARY-shRNA-2) in a variety of cell lines (Supplementary Fig. 2). Importantly, knockdown of RARY had no effect on TNF-induced activation of NF-κB and MAP kinase, Janus kinase and TSZ-induced RIP1 autophosphorylation (Supplementary Fig. 3).

To examine whether RARY is specifically required for necroptosis, we treated these cells with TNF-α and Smac mimetic (TS) to induce RIP1-initiated apoptosis. As previously shown, RIP1-shRNA cells were resistant to TS-induced apoptosis. Surprisingly, RARY-shRNA cells behaved similarly and were resistant to TS-induced apoptosis compared with cont-shRNA cells (Fig. 1c). We then examined whether RARY is involved in TNF-induced TRADD-initiated apoptosis and necroptosis by treating these cells with TNF-α plus the protein synthesis inhibitor cycloheximide with/out zVAD (TC or TCZ). Knockdown of RARY had little effect on TC-induced apoptosis or TCZ-induced necroptosis while RIP1 knockdown sensitized the cells more resistant to TCZ-induced necroptosis (Fig. 1d). Therefore, these results suggest that RARY is likely involved in TNF-induced RIP1-initiated apoptosis and necroptosis. Similar observations were made in several other cell lines (Supplementary Fig. 2). To further verify this, we then generated TRADD knockdown cells in cont-shRNA and RARY-shRNA HT-29 cells (Fig. 1e). The cells with TRADD knockdown were still sensitive to cell death by TS or TSZ although their sensitivity was slightly decreased (Fig. 1f). Importantly, RARY knockdown protects cells from cell death in RARY-shRNA-A + TRADD-shRNA cells. Therefore, RARY is specifically required for TNF-induced RIP1-initiated apoptosis and necroptosis.

In addition, although RARα is previously reported to be involved in apoptosis, we found that RARα knockdown had no effect on the sensitivity of HT-29 cells to TSZ-induced RIP1-initiated apoptosis or necroptosis (Supplementary Fig. 4a).
Fig. 1 RARγ is required for TNF-induced RIP1-dependent apoptosis and necroptosis. a Cell death analysis of HT-29 clones cont-shRNA, RARγ-shRNA-A, RARγ-shRNA-B, RARγ-shRNA-C or RIP1-shRNA when treated with TSZ for 24 h was determined by PI staining using flow cytometry (left panel) (*P < 0.05 versus cont-shRNA; ANOVA). The bars represent the mean ± s.e.m. of three experiments. Western blot analysis of cells as mentioned in left panel; cell lysates were probed with antibodies as indicated (right panel). b Cell death determination by PI staining using flow cytometry of HT-29 cont-shRNA, RARγ-shRNA-A or RARγ-shRNA-A cells reconstituted with RARγ-shRNA resistant RARγ protein (RARγ-shRNA-A-rRARγ) when treated with DMSO or TSZ for 24 h (left panel) (*P < 0.05 versus cont-shRNA. #P < 0.05 versus RARγ-shRNA-A; ANOVA). The bars represent the mean ± s.e.m. of three experiments. Western blot analysis of cells as mentioned in left panel; cell lysates were probed with antibodies as indicated (right panel). c, d HT-29, RARγ-shRNA-A, or RIP1-shRNA cells were treated with necrotic (DMSO, TS, or TSZ) or apoptotic (CHX, TC or TCZ) conditions for 24 h. PI positive population was determined by flow cytometry. (*P < 0.05 versus cont-shRNA; ANOVA). The bars represent the mean ± s.e.m. of three experiments. Western blot analysis of HT-29 cont-shRNA cells infected with TRADD-shRNA, or RARγ-shRNA-A cells infected with TRADD-shRNA lentivirus (RARγ-shRNA-A + TRADD-shRNA). Cell lysates were probed with antibodies as indicated. e Cells from f were treated with DMSO, TS, or TSZ for 24 h and the PI-positive population was determined by flow cytometry. (*P < 0.05 versus cont-shRNA; ANOVA). The bars represent the mean ± s.e.m. of three experiments. All blots above are representative of one of three experiments.
Also, RARα does not have any redundant role with RARγ in the process since knockdown of RARα in RARγ-shRNA-A cells did not further increase the resistance of the cells to TNF-induced RIP1-initiated apoptosis and necroptosis (Supplementary Fig. 4b).

**Cytosolic RARγ mediates cell death initiated by RIP1.** RARγ localizes in the nucleus as a ligand-dependent transcription factor, but may have a cytosolic localization. We next examined the cellular localization of RARγ during the process of cell death. We first transfected HeLa cells with a green fluorescent protein (GFP)-tagged RARγ plasmid and found that the GFP-RARγ protein almost exclusively localized in the nucleus (Fig. 2a and Supplementary Fig. 5). Although TNF-α alone did not have any effect on the localization of RARγ, TS treatment caused a dramatic increase of GFP-RARγ presence in the cytoplasm (Fig. 2a and Supplementary Figs 5 and 6). Importantly, GFP-RARα remained predominantly in the nucleus under TS treatment (Supplementary Fig. 7). The increased cytosolic localization of RARγ during RIP1-initiated apoptosis was further verified by examining the endogenous RARγ protein by nuclear and cytosolic fractionation experiments. The cytosolic level of the endogenous RARγ protein was significantly higher, while the nuclear RARγ amount was reduced, in the TS-treated HeLa cells compared with the non-treated control or TNF-treated cells (Fig. 2b). Nuclear protein PARP1 and cytosolic protein GAPDH were used as controls in this experiment. The cytosolic localization of RARγ was also observed during RIP1-initiated necroptosis by immunostaining the endogenous RARγ in TSZ-treated HT-29 cells. Although the endogenous RARγ protein mainly localized in the nucleus in non-treated cells, the level of cytosolic RARγ was dramatically increased in TSZ-treated cells (Fig. 2c). Also, inhibition of TSZ-induced necroptosis by the specific RIP1 inhibitor, Necrostatin-1, had no effect on the dramatic increase of RARγ localization in the cytoplasm (Supplementary Fig. 8). These results indicated that RARγ was released to the cytoplasm from the nucleus during RIP1-initiated apoptosis and necroptosis.

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**Fig. 2** Cytosolic RARγ mediates apoptosis and necroptosis. a Confocal microscopy of HeLa cells transfected with RARγ-GFP plasmid and treated with DMSO, TNF-α or TS for 2 h. (blue DAPI; green: RARγ) (scale bar: 10 μm). b The total (T), cytosolic (C) and nuclear (N) fractions analysis of HeLa cells treated with DMSO, TNF-α or TS for 4 h. Fractions were analyzed by immunoblotting with the indicated antibodies. c Confocal microscopy of HT-29 cells treated with DMSO or TSZ for 2 h. Cells were stained with nuclear DAPI (blue), anti-F-actin (green) and anti-RARγ (red) antibodies (scale bar: 10 μm). d Confocal microscopy of HT-29 cells treated with Smac-minic for 2 h. Cells were stained with nuclear DAPI (blue) and anti-RARγ (green) antibodies (scale bar: 10 μm). e Scheme for the mutation of RARγ in NLS (upper left panel). The total (T), cytosolic (C) and nuclear (N) fractions of HT-29 RARγ-shRNA-A cells infected with resistant RARγ-NLS mutation lentiviral plasmid (RARγ-shRNA-A-RARγ-NLSmut). The cell lysates were by immunoblotted with the indicated antibodies (lower left panel). HT-29 cont-shRNA, RARγ-shRNA-A or RARγ-shRNA-A-rRARγ-NLSmut cells were treated with TSZ for 24 h and PI-positive population was determined by flow cytometry (right panel). (*P < 0.05 versus cont-shRNA; #P < 0.05 versus RARγ-shRNA-A; ANOVA). The bars represent the mean ± s.e.m. of three experiments. All images and blots above are representative of one of three experiments.
Interestingly, Smac-mimetic treatment alone was sufficient to trigger the release of RARγ from the nucleus (Fig. 2d and Supplementary Fig. 9a, b). As TNF treatment alone without Smac mimetic induces necroptosis in mouse L929 cells (Supplementary Fig. 2c), we then examined RARγ localization in L929 cells and found that unlike in HT-29 and HeLa cells, RARγ, but not RARα, localizes in both the cytoplasm and the nucleus in the non-treated cells (Supplementary Fig. 10). Also, it is known that TZ treatment without Smac mimetic induces necroptosis in bone marrow-derived macrophages (BMDMs) (Supplementary Fig. 9a, b). We next examined whether cellular IAPs (cIAPs) are required for RARγ nuclear localization. Although loss of cIAP1 has limited effect on RARγ localization, the dominant negative cIAP2 mutant, cIAP2 H570A, which blocks both cIAP1 and cIAP2 function (Supplementary Fig. 11a), enables RARγ to localize in both the cytoplasm and the nucleus, indicating that cIAPs may be required for RARγ nuclear localization (Supplementary Fig. 12a). Consistently, TZ treatment without Smac mimetic triggers necroptosis in the cIAP2 H570A MEFs (Supplementary Fig. 12b). Taken together, these results suggest that the release of RARγ from the nucleus by Smac mimetic is most likely through inhibiting cIAPs function.

To address whether the cytosolic or the nuclear RARγ has a role in TNF-induced cell death, we mutated the RARγ nuclear localization signal (NLS) in the RARγ-shRNA-resistant RARγ M

### Fig. 3 RARγ has a role in the formation of death complexes.

#### a
Western blot analysis of HT-29 cont-shRNA, and RARγ-shRNA-A treated with DMSO, TS or TSZ for 24 h and cell lysates were immunoblotted with the indicated antibodies.

#### b
Immunoprecipitation of HT-29 cont-shRNA or RARγ-shRNA-A cells treated with TSZ for the indicated time. Cell lysates were collected and immunoprecipitated with anti-Caspase-8 antibody or anti-TNFR1 antibody.

The immunoprecipitated complexes were analyzed by with the indicated antibodies. Sequential immunoprecipitation of HT-29 cont-shRNA or RARγ-shRNA-A cells treated TSZ for 2 h. First IP: TNFR1 complex I was immunoprecipitated using anti-TNFR1 antibody. Second IP: the remaining lysates were immunoprecipitated again with anti-TNFR1 antibody. Third IP: the remaining lysates were then immunoprecipitated with anti-TRADD antibody. The immunoprecipitated complexes were analyzed by with the indicated antibodies. (M: marker). All blots above are representative of one of three experiments.
Fig. 4 RARγ C fragment are important for protecting cell from TNF-induced necroptosis via interaction with RIP1. a Immunoprecipitation of HT-29 cells treated with TSZ for the indicated times. Cell lysates were immunoprecipitated with anti-RARγ antibody and analyzed by immunoblotting with the indicated antibodies. b HEK293T cells were co-transfected with wild-type V5-RARγ (WT) or the V5-RARγ-NLSmut (NLS) and with or without FLAG-RIP1 plasmids. Cell lysates were immunoprecipitated using anti-FLAG (RIP1) antibody and analyzed with the indicated antibodies. c Scheme of RARγ and RIP1 genes (upper panel). HEK293T cells were co-transfected with different fragment of RARγ-V5 (F: full; N: 1–201aa; C: 188–454aa) and with or without FLAG-RIP1 plasmids; or with different fragment of His-Xpress-RIP1 (F: full; N: 1–324aa; C: 324–671aa; DD: 560–669aa) and with or without RARγ-V5. Cell lysates were immunoprecipitated using anti-FLAG (RIP1) or anti-V5 (RARγ) antibody and analyzed with the indicated antibodies. d HT-29 RARγ-shRNA-A cells infected with rRARγ-C. Cell death analysis of HT-29 cont-shRNA, RARγ-shRNA-A, and RARγ-shRNA-A + rRARγ-C when treated with TSZ for 24 h was determined by PI staining using flow cytometry (upper left panel). (*P < 0.05 versus cont-shRNA; #P < 0.05 versus RARγ-shRNA-A; ANOVA). The bars represent the mean ± s.e.m. of three experiments. Western blot analysis of cells as mentioned in left panel (upper right panel). Confocal microscopy of HT-29 cells infected with GFP-RARγ-C plasmid (lower left panel) (blue: DAPI; green: RARγ). Western blot analysis of HEK293T cells were transfected with p-EGFP, p-EGFP-RARγ and p-EGFP-RARγ-C by using anti-GFP and anti-Actin antibodies (lower right panel). All blots and images above are representative of one of three experiments.
plasmid (rRARγ-NLSmut). When this rRARγ-NLSmut protein was introduced back into the RARγ-shRNA-A HT-29 cells, the mutant RARγ localized in the cytoplasm and was able to fully rescue TSZ-induced necroptosis (Fig. 2e). These data suggested that the cytosolic RARγ is critical for TSZ-induced necroptosis.

**RARγ is essential for formation of cytosolic death complexes.** RIP3 autophosphorylation is an early event in TNF-induced necroptosis. In RARγ knockdown cells, RIP3 phosphorylation was abolished (Fig. 3a). This result indicated that RARγ functions upstream of RIP3 phosphorylation during necroptosis. We then examined whether RARγ is required for the formation of the
necroptosome. It is known that the necroptosome consists of multiple proteins including RIP1, RIP3, FADD and Caspase-8, and can be detected by immunoprecipitating Caspase-8\(^7\)–\(^9\). HT-29 cells expressing RAR\(\gamma\)-shRNA or cont-shRNA were treated with TSZ and collected for immunoprecipitation with a Caspase-8 specific antibody. The necroptosome components, RIP1, RIP3 and FADD were efficiently pulled down by immunoprecipitating Caspase-8 in TSZ-treated cont-shRNA cells, but the amounts of RIP1, RIP3 and FADD proteins pulled down by Caspase-8 immunoprecipitation were almost unnoticeable in RAR\(\gamma\)-shRNA cells (Fig. 3b). Consistent with previous reports\(^9\), some TRADD protein was also detected in the necrototic complex in the control cells. However, no RAR\(\gamma\) protein was found in the same complex. These results suggested that RAR\(\gamma\) knockdown prevented the formation of the necroptosome. We found that RAR\(\gamma\) is also required for the formation of apoptotic complex Iia (Supplementary Fig. 13).

It is believed that TRADD and RIP1 dissociate from the receptor and recruit other proteins to form the secondary death complexes\(^1\)–\(^8\), \(^8\)–\(^38\). As RAR\(\gamma\) is required for the formation of the necroptosome, but is not a part of the complex, we next examined whether RAR\(\gamma\) has any role in the formation of the TNFR1 complex by immunoprecipitating TNFR1. RAR\(\gamma\) knockdown has no effect on the formation of TNFR1 complex, because TNFR1 immunoprecipitation efficiently pulled down RIP1 and TRADD proteins in both con-shRNA and RAR\(\gamma\)-shRNA cells (Fig. 3c). Consistent with previous findings\(^6\), \(^8\), no FADD and RIP3 proteins were found in the TNFR1 complex. Again, no RAR\(\gamma\) protein was detected in the TNFR1 complex. These results indicated that RAR\(\gamma\) is not required for the assembling of the TNFR1 complex. As it has been suggested that TNFR1 endocytosis may be required for downstream signaling\(^39\), we checked whether RAR\(\gamma\) knockdown affects TNFR1 internalization\(^40\) and found that RAR\(\gamma\) knockdown has no effect on TNFR1 internalization (Supplementary Fig. 14). We then investigated whether RAR\(\gamma\) knockdown affects the dissociation of TRADD and RIP1 proteins from TNFR1 by examining the presence of the non-receptor-bound TRADD/RIP1 complex. After two sequential immunoprecipitation of TNFR1, all TNFR1-bound TRADD and RIP1 were removed from the cell lysates. Next, the TRADD immunoprecipitation with the remaining lysates pulled down RIP1 only from con-shRNA cells, but not from RAR\(\gamma\)-shRNA cells, indicating that the TRADD and RIP1 complex did not dissociate from TNFR1 in the absence of RAR\(\gamma\) (Fig. 3d). Therefore, it is possible that RAR\(\gamma\) may regulate the dissociation of complex I from the TNFR1 receptor. As RAR\(\gamma\) is not in either the TNFR1 complex or the necroptosome, we then examined what TNF effector proteins it may interact with by immune-precipitating RAR\(\gamma\) protein. Interestingly, both RIP1 and FADD were co-precipitated with RAR\(\gamma\) as early as 1 h after TSZ treatment (Fig. 4a). The amount of RIP1 and FADD peaked at 2 h and decreased by 3 h after treatment. Only a marginal amount of TRADD and no RIP3 were detected in the RAR\(\gamma\) precipitants. These results suggested that RAR\(\gamma\) forms a complex with RIP1 and FADD that is different from the necroptosome. This observation raised the possibility that RAR\(\gamma\) may interact with RIP1 and FADD. We then evaluated the interaction between RAR\(\gamma\) with each of these proteins by cotransfection/immunoprecipitation experiments. Immunoprecipitating FLAG-RIP1 clearly pulled down RAR\(\gamma\) and more efficiently the cytoplasmic RAR\(\gamma\)-NLSmut, but none of the FADD, TRADD or RIP3 immunoprecipitation did so (Fig. 4b and Supplementary Fig. 15a–c). In addition, we found that RAR\(\alpha\) did not interact with RIP1 (Supplementary Fig. 15d). The interaction of RIP1 and RAR\(\gamma\) was also confirmed by immunoprecipitating the endogenous RIP1 following TSZ treatment (Supplementary Fig. 16). These results suggested that RAR\(\gamma\) specifically interacts with RIP1.

To better understand how RAR\(\gamma\) interacts with RIP1, we generated several truncated RAR\(\gamma\) and RIP1 proteins and examined which regions of these two proteins interact (Fig. 4c). We found that the C-terminal region of RAR\(\gamma\), which does not include the transcription activation domain, DNA binding domain and the NLS site, is fully capable to interact with RIP1 protein, most likely through RIP1’s RIP homotypic interaction motif domain (Fig. 4c). The C-terminal region of RAR\(\gamma\) still contains the activation function 2 region that does in fact interact with coactivator proteins to confer ligand inducible transcriptional activation to RAR\(\gamma\). Importantly, when a RAR\(\gamma\)-shRNA resistant c-terminal region of RAR\(\gamma\) was expressed in RAR\(\gamma\)-shRNA-A cells, it localized in the cytoplasm and rescued the sensitivity of the cells to TSZ-induced necroptosis (Fig. 4d). These results further supported our conclusion that the cytosolic RAR\(\gamma\) is required for RIP1-initiated cell death.

Both TRADD and RIP1 contribute to the formation of the TNFR1 complex\(^4\). To test whether TRADD is important for TNFR1 and RIP1 interaction in necroptosis, we next examined TNFR1 and RIP1 interaction in TRADD knockdown cells. Immunoprecipitating TNFR1 pulled down RIP1 similarly in the control and TRADD-shRNA cells, indicating that TRADD knockdown does not have any effect on RIP1 recruitment to TNFR1 in necroptosis (Fig. 5a). Therefore, TNFR1 and RIP1 interaction is independent of TRADD in necroptosis. Next, we tested whether RAR\(\gamma\) affects TNFR1 and RIP1 interaction. HEK293T cells were co-transfected with FLAG-TNFR1, DsRed-RIP1 and increasing amounts of V5-RAR\(\gamma\)-NLSmut plasmids. Immunoprecipitation of TNFR1 clearly co-precipitated RIP1 protein. However, in the presence of increasing amounts of RAR\(\gamma\), the TNFR1 and RIP1 interaction gradually diminished (Fig. 5b, top panel). When the cell lysates after TNFR1 pull-down were used to immunoprecipitate V5-RAR\(\gamma\), increasing levels of RIP1 were detected (Fig. 5b, bottom panel). These results indicated that RAR\(\gamma\) disrupts the TNFR1 and RIP1 interaction by pulling away RIP1 protein from TNFR1.

As the RAR\(\gamma\), RIP1 and FADD complex seems to be transient as seen in Fig 4a, and the necroptosome does not have the
RARγ protein, we wondered whether RARγ protein was removed from the complex when other proteins such as RIP3 was recruited. We then co-transfected HEK293T cells with V5-RARγ-NLSmut, RIP1-Myc and increasing amounts of DsRed-RIP3 plasmids and immunoprecipitated V5-RARγ. The interaction between RARγ and RIP1 was significantly decreased in the presence of increasing amounts of RIP3 (Fig. 5c, top panel). When the cell lysates after V5-RARγ pull-down were immunoprecipitated with an anti-DsRed antibody, increasing amounts of RIP1 were pulled down (Fig. 5c, bottom panel). These results indicated that the RARγ and RIP1 interaction was disrupted by RIP3. To confirm that the absence of RARγ in the necroosome is due to the recruitment of RIP3, we performed RIP3 immunoprecipitation experiments (Fig. 3b) were done at 2 and 4 h time points after TSZ treatment when RIP3 is already recruited, we also checked whether RARγ could be pulled down by Caspase-8 at 1 h before RIP3 is recruited in wild-type (wt) MEFs. As shown in Fig. 5d, RARγ is indeed present in the complex at 1 h after TSZ treatment in wt cells. Importantly, while the presence of RARγ in this complex disappears at 2 and 4 h time points in wt cells, RARγ presence in the complex is sustained in RIP3−/− MEFs (Fig. 5d). These results suggest that RARγ may be a component of the TNFR1 complex IIa and is competed off when RIP3 is recruited to form necroosome/complex IIb. The presence of RARγ in the TNFR1 complex IIa was confirmed when Caspase-8 was immunoprecipitated in TSZ-treated HeLa cells (Supplementary Fig. 17), which does not have RIP3 expression. Taken together, the above data suggests that RARγ initiates the formation of the necroosome by recruiting RIP1 away from TNFR1 to form the TNFR1 complex IIa.

RARγ is required for cell death initiated by RIP1 in vivo. RARγ has two major splicing isoforms, RARγ1 and RARγ2, and RARγ1 is the major functional isoform and early studies reported that RARγ complete knockout mice have growth defect and may die prematurely. To study the physiological role of RARγ in cell death, we generated RARγ knockout (RARγ1−/−) mice using the CRISPR/Cas9 system. The Cas9 mRNA was co-microinjected with two single guide RNAs (sgRNAs), which target two different regions of the first coding exon of the mouse rary1 gene (Fig. 7a), into the cytoplasm of mouse zygotes. The founder mice were genotyped by PCR and DNA sequencing. We selected two different founder mice that carried frame shift mutations for line expansion and subsequent studies. The line used in the below animal experiments has a ~200 bp deletion (Fig. 7b). The RARγ1-KO mice appear normal phenotypically.

From the RARγ1−/− mice we generated MEFs and checked for loss of RARγ expression and made sure that the expression levels of RIP1, RIP3, MLKL and CYLD in RARγ1−/− MEFs are not altered (Fig. 7c). The primary RARγ1−/− MEFs were resistant to TSZ-induced RIP1-initiated necroptosis while they are still sensitive to TC- or TCZ-induced TRADD-initiated cell death.

**Fig. 6** RARγ is involved in death receptor and genotoxic stresses-induced cell death. a Cell death analysis of HT-29 cont-shRNA and RARγ-shRNA-A treated with DMSO, TSZ, TRAIL + S, CH11 + S or FasL + S for 24 h (left panel), or treated with DMSO, TRAIL + S, CH11 + S or FasL + S for 24 h (right panel). Cell death was determined by PI staining using flow cytometry. (*p < 0.05 versus cont-shRNA; ANOVA). The bars represent the mean ± s.e.m. of three experiments. b HT-29 cont-shRNA, and RARγ-shRNA-A treated with FasL + S for the indicated times. Cell lysates were immunoprecipitated with anti-Caspase-8 antibody and analyzed with the indicated antibodies. Blots are representative of one of the three experiments.
Histologic H&E staining of liver shows focal necrotic cells and red blood cells (arrow). (survival curve generated and analyzed by immunoblotting using indicated antibody. Genomic DNA from wild-type (RARγ+/+) and homozygous CRISPR-knock-out (RARγ−/−) MEFs were treated with DMSO, TS or TSZ for 24 h (left panel) or with CHX, TC or TCZ for 24 h (right panel). Cell death was determined by PI staining using flow cytometry. Body temperature curve; log rank test). Mouse number used in each experiment was indicated. All blots and gel above are representative of one of three experiments. (Fig. 7d) and the immortalized RARγ−/− MEFs were resistant to both TS- and TSZ-induced cell death (Supplementary Fig. 18a) confirming the results obtained with RARγ-shRNAs. In addition, RARγ deletion also protects the primary BMDMs cells from TSZ-induced necroptosis (Supplementary Fig. 18b). Next we used a mouse model of systemic inflammation that resembles clinical sepsis to examine the role of RARγ in cell death in vivo. It has been demonstrated that inhibition of caspases by z-VAD-fmk strongly sensitizes mice to TNF-induced shock involving RIP1/RIP3-dependent necrosis. The wt and RARγ−/− littersmates were injected with a combination of TNFα and z-VAD-fmk (TZ). We monitored the body temperature of the mice for 72 h and although both wt and RARγ−/− littersmates had similar hypothermia initially, the wt mice had continued drop in body temperature whereas most RARγ−/− mice recovered (Fig. 7e). The wt mice succumb by 24–36 h, whereas 60% of the RARγ−/− mice survive up to 4 days (Fig. 7f). When these mice were examined 6 and 12 h after treatment, we find that hematoxylin and eosin staining of the liver showed focal necrotic cells in wt mice, evidenced by pyknotic nuclei and eosinophilic cytoplasm whereas the RARγ−/− livers had significantly less necrosis (Fig. 7g and Supplementary Fig. 19). Interestingly, both wt and RARγ−/− mice have similar mortality in the TNF-induced, TRADD-mediated cell death model when mice were treated with TNF and D-galactosamine (G), which is a metabolic inhibitor of transcription (Fig. 7h)46, 47. In addition, TRADD−/− mice were not resistant to TZ-induced lethality (Supplementary Fig. 20). These results suggested that RARγ has a critical role in RIP1/RIP3-mediated necroptosis, but not TRADD-mediated cell death in vivo.
RARγ is involved in necroptosis by TCZ in TRADD null MEFs.

Our results indicated that RARγ is not required for TC- or TCZ-induced cell death, which is initiated by TRADD (Figs. 1d and 7d). However, a recent study reported that TRADD−/− MEFs are still sensitive to TCZ-induced necroptosis.

To investigate whether RARγ has a role in TCZ-induced necroptosis in TRADD−/− MEF cells, we first examined the formation of the TNFR1 complex IIa and necrosome in response to TCZ treatment by immunoprecipitating Caspase-8 in wt and TRADD−/− MEFs. As seen in Fig. 8a, the complex IIa components RIP1 and TRADD were co-precipitated with Caspase-8 at 2 h after treatment while the necrosome component RIP3 was detected in the complex at 4 h in wt cells. However, RIP1 and RIP3 were not detected in the Caspase-8 immunoprecipitants until 6 and 8 h after TCZ treatment in TRADD−/− MEFs (Fig. 8a). Importantly, although RARγ was not detected in either complex IIa or necrosome in wt cells, the protein was present in the complex IIa at 6 h after TCZ treatment, but not in the necrosome when RIP3 was recruited at 8 h, in TRADD−/− MEFs (Fig. 8a). These results suggest that TRADD has a major role in the formation of the TNFR1 complex IIa and necrosome in response to TCZ in wt cells and that RARγ may have a role in the process in TRADD−/− MEFs. The major role of TRADD in TCZ-induced necroptosis was supported by the fact that TRADD−/− MEFs are much less sensitive to

Fig. 8 RARγ is involved in TCZ-induced necroptosis in TRADD−/− MEFs. a WT and TRADD−/− MEFs treated with TCZ for the indicated times. Cell lysates were immunoprecipitated using anti-Caspase-8 antibody and immunoblotted with the indicated antibodies. b WT or TRADD−/− MEFs transfected with sicontrol or siRARγ were treated with TSZ for 24 h. Cell death was determined by PI staining using flow cytometry (left panel). Western blot analysis of cells as mentioned in left panel; cell lysates were probed with antibodies as indicated (right panel) (*P < 0.05 versus WT + sicontrol. #P < 0.05 versus TRADD−/− + sicontrol; ANOVA). The bars represent the mean ± s.e.m. of three experiments. c WT and TRADD−/− MEFs treated with TCZ in indicated time points. The localization of endogenous RARγ assessed by immunofluorescent staining (blue: DAPI; green: RARγ) (scale bar: 10 μm). All blots and images above are representative of one of three experiments.
TCZ-induced necroptosis in comparison with wt cells.\(^4\)\(^8\) (Fig. 8b). Notably, knockdown of RARγ in TRADD\(^−/−\) MEFs further reduced the sensitive of the cells to TCZ-induced necroptosis, indicating that RARγ is needed for RIP1 to mediate TCZ-induced cell death in TRADD\(^−/−\) cells (Fig. 8b). Although we found that RARγ is specifically released from the nucleus by Smac mimetic (Fig. 2 and Supplementary Fig. 5–9), TCZ, but not TC, treatment resulted in its release from the nucleus nonspecifically since the treatment had the same effect on RARα (Fig. 8c and Supplementary Fig. 21).

**Discussion**

Apoptosis and necroptosis are two types of programmed death that are physiologically and pathologically relevant. Although the molecular mechanism of apoptosis is well studied, the regulation of necroptosis is starting to be understood. TNF-induced apoptosis and necroptosis could be initiated by TRADD or RIP1 respectively when de novo protein synthesis is blocked or IAP E3 ligases are inhibited.\(^1,\)\(^3\) To engage these death pathways, respectively when de novo protein synthesis is blocked or IAP E3 ligases are inhibited, apoptosis and necroptosis could be initiated by TRADD or RIP1. Further, we showed that it is the cytoplasmic RARγ, not the nuclear RARγ, which mediates the formation of cytosolic death complexes. We demonstrated that RARγ interacts with RIP1 and is critical for RIP1 dissociation from the receptor. The critical role of RARγ in RIP1-initiated cell death is further supported through studying the RARγ-null cells and mice. Our data also indicated that RARγ has a broad role in cell death, because it is involved in RIP1-initiated cell death in response to other death factors, FasL and TRAIL.

To identify additional components of TNF-induced necroptosis, we used a retroviral shRNA-mediated genetic screen to identify genes whose knockdown resulting in resistance to necroptosis. In our first screening with a kinase/phosphatase shRNA library, MLKL was identified as a key mediator of necroptosis. We identified one clone of targeting RIP1 and six clones of targeting MLKL, but none of clones targeting RIP3 in this screening. Clearly, the shRNAs of each gene have different penetration/efficiency since RIP3 was not identified in our screening. We did not hit other known proteins of this pathway such as TNFR1, as they are not included in this library. In our current study, RARγ was identified by screening the shRNA library targeting cancer-related genes and this library of 1841 shRNAs targets 1,272 human genes, in which none of the...
known necroptosis-related genes is included. We identified seven clones targeting RARγ in this screening.

TNF triggers the formation of TNFR1 signaling complex. Under apoptotic or necrotic conditions, TNFR1:complex dissociates from the receptor and recruits other proteins to form complex II or necrosome11,12. When protein synthesis is blocked, cFLIP protein is degraded and TRADD-initiated cell death is carried out although TRADD needs to recruit RIP1 for mediating necroptosis6 (Fig. 9a). In the case of IAP E3 ligases inhibition, RIP1-mediated cell death happens. It is thought that the blockage of RIP1 ubiquitination by cIAP1/2 is sufficient for RIP1 to recruit FADD and RIP3 to engage apoptosis and necroptosis respectively6,13. However, we found that when RARγ was knocked down, the non-ubiquitylated RIP1 was stuck in the TNFR1 complex and no complex IIa or necrosome was formed (Fig. 3 and Supplementary Fig. 13). As knocking down of RARγ does not affect TNFR1 internalization (Supplementary Fig. 14), our data suggests that RARγ is necessary for RIP1 to dissociate from the TNFR1 receptor and to initiate the formation of complex IIa and the subsequent necrosome (Fig 4 and Supplementary Figs 13 and 17). Our in vivo study with RARγ−/− mice further supported that RARγ is involved in RIP1-initiated necroptosis, but not in TRADD-initiated cell death, as RARγ−/− mice are resistant to TZ, but not TG treatment (Figs. 7e−h), whereas TRADD−/− mice are resistant to TG, but not TZ treatment47 (Supplementary Fig. 20). The residual lethality of RARγ−/− mice in response to TZ treatment may be due to the presence of RARβ2. Interestingly, we found that RARγ has a role in TCZ-induced necroptosis in TRADD−/− MEFs because it is released from the nucleus non-specifically by the TCZ treatment (Fig 8 and Supplementary Fig. 21). These findings further supported our conclusion that RARγ is required for RIP1-initiated cell death.

The functions of RARs have been extensively studied as nuclear transcription factors and the RARs are predominantly nuclear even in the absence of their ligands. In addition to their functions in regulating cell proliferation and differentiation, RARs are implicated indirectly in apoptosis through their nuclear transcriptional activity29,31. However, our data indicated that the involvement of RARγ in RIP1-initiated cell death does not rely on its transcriptional activity (Figs 2 and 4c, d). Instead, RARγ has a role in RIP1-initiated cell death as a cytosolic protein. Though RARs are nuclear proteins, RARγ could be found in the cytoplasm when overexpressed and the subcellular localization of RARγ is highly regulated32. Our data indicated that this TS or TSZ-induced cytoplasmic localization is specific to RARγ because the localization of RARα is not altered (Fig. 2 and Supplementary Figs 5–7). Interesting, Smac mimicetic alone is sufficient to induced the release of RARγ from the nucleus (Supplementary Fig. 9). Overexpression of RXRα also did not have any effect on RARγ release from the nucleus in response to TS treatment in HeLa cells (Supplementary Fig. 22). Currently, it seems that the RARγ cytoplasmic localization is induced by Smac mimicetic through inhibiting cIAP1/2 function because RARγ localizes in both the cytoplasm and the nucleus in the cIAP2(H570A) MEFs (Supplementary Fig. 12). The finding that RARγ localizes in both the cytoplasm and the nucleus in L929 cells (Supplementary Fig. 9) provides new insights about why TNF induces necroptosis in these cells without the presence of Smac mimicetic. In addition, while RA promotes the nuclear localization of RARα, RA pre-treatment has little effect on cell death and the cytoplasmic localization of RARγ in response to TSZ in HT-29 cells, but however, RA pre-treatment did indeed partially block TNF-induced necroptosis in L929 cells (Supplementary Fig. 23).

Our study reveals a function for the nuclear receptor RARγ as a key regulator of RIP1-initiated cell death when cIAP activity is inhibited. As RIP1-initiated cell death is a vital cellular response triggered by death factors, the engagement of this pathway is finely regulated. As shown in Fig. 8b, the requirement of RARγ, which is released from the nucleus, provides a critical checkpoint for the transition from survival signaling to death machinery of RIP1-initiated cell death. This safety control by RARγ allows cells to engage death pathways only when they are fully committed to die.

Methods

**Reagents and antibodies.** TNF-α and z-VAD-fmk were purchased from R&D. Cyclohexamide (CHX) from Sigma. Smac mimicetic was a gift from S. Wang (University of Michigan, Ann Arbor, Michigan, USA). TRAIL from Novoprotein. Fasl from Upstate Biotech. CH11 from MBL Medical and Bio Lab.

All antibodies are at concentration of 1 μg ml−1 and used at 1:1,000 dilution unless otherwise stated. Anti-RARγ (C-15) (sc-550) for human, anti-RARα (C-20) (sc-551), anti-caspase-8 (C-20) (sc-6136), anti-cIAP2 (sc-7944) and anti-Fas (C-20) (sc-715) from Santa Cruz; anti-RARγ (610459) and anti-FADD (610400) from BD Biosciences; anti-RARγ1 (ab9505) for mouse, anti-RIP3 (ab27106), anti-MLKL (ab184718) for human, anti-MLKL (ab172868) for mouse and anti-cIAP1 (ab2399) from Abcam; anti-RIP3 (2283) for mouse from ProSci, anti-TRADD (55-473) from Upstate; anti-TRAF2 (MA3277) and anti-TNFR1 (AF-425-PB) from R&D; anti-Acetin (A3853) (dilution 1:10,000), anti-FLAG (F2921) (dilution 1:5,000) and anti-GFP (G6539) (dilution 1:5,000) from Sigma; anti-V5 (R960-25) (dilution 1:5,000) from Invitrogen; anti-PARP1 (BML-SA250-0050) from Enzo Life Science; anti-GAPDH (NB500-22) (dilution 1:5,000) from Novus Biologicals; anti-cleaved-caspase-3 (9664) and anti-cleaved-caspase-8 (9657) from Cell Signaling Technology; anti-DsRed (63292) (dilution 1:5,000) from Clontech.

**shRNA library screening.** shRNA screening was performed using a retroviral shRNA library targeting cancer-related genes33. HT-29 cells were cultured in 15 cm dishes and infected using 8 μg ml−1 polybrene (Millipore). Duplicated infections were carried out. At 24 h after the infection, media were replaced with 2 μg ml−1 puromycin. After a 4-day puromycin selection, cells were treated with TSZ (TNF-α 30 ng ml−1, Smac 10 nM and z-VAD-fmk 20 μM) for 48 h. The media were replaced with fresh completed medium containing 2 μg ml−1 puromycin. Two days later, the cells were treated with TS for an additional 48 h. The colonies were picked and the isolated genomic DNA was sequenced for PCR and DNA sequencing analysis. The shRNA (5′-ATAATAAAGGGCTTCTCCTG-3′) targeting human RARγ gene was identified. The firefly luciferase shRNA (cont-shRNA) was used as a negative control shRNA.

**Generation of RARγ knockout mice by CRISPR.** All animal experimental protocols were performed in accordance to the NIH Animal Care & Use Committee guidelines. The RARγ knockout mice were generated using the CRISPR/Cas9 mediated genome editing technology. Two sgRNAs were designed to target the 1st coding exon (Exon 1) of the mouse gene, one (5′-gggagggacttcccctgcggc-3′) targeted shortly after the translation initiation codon (ATG) and the other one (5′-gggagggacttcccccaaggc-3′) targeted near the end of Exon 3. The two sgRNA DNA constructs were made using OriGene's gRNA Cloning Services (Rockville, Maryland) and were then used as templates to synthesize sgRNAs using MEGAshortscript T7 Kit (Invitrogen). The two sgRNAs were co-microinjected with Cas 9 mRNA into the cytoplasm of fertilized mouse eggs at the concentration of 100 μg ml−1 of Cas9 and 20 μg ml−1 of each sgRNA. The injected zygotes were cultured overnight in M16 medium. Those embryos reached 2-cell stage of development were implanted into the oviduct of pseudopregnant surrogate mothers. Mice born to these surrogate mothers were genotyped by PCR amplification of the region surrounding the CRISPR cutting site followed by DNA sequencing. Founder mice with X and Y base pair deletions were bred with wt C57BL/6 mice to establish two independent RARγ knockout mouse lines, which were used in subsequent studies.

**Cell culture and treatment.** HT-29, MCF-7, HeLa, HEK293T, L929 and MEFs cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g ml−1 glucose, 10 % fetal bovine serum and 2 mM l-glutamine with 100 units penicillin per ml and 100 units streptomycin per ml.

**Cell death treatment:** Necroptosis was induced by TNF-α (30 ng ml−1), Smac (10 nM) and z-VAD-fmk (20 μM) or TNF-α (30 ng ml−1), CHX (10 μg ml−1) and z-VAD-fmk (20 μM). Apoptosis was induced by TNF-α (30 ng ml−1) and Smac (10 nM) or TNF-α (30 ng ml−1), CHX (10 μg ml−1). Necroptosis was induced by TRAIL (250 ng ml−1), Fasl. (2 ng ml−1) or CH11 (100 ng ml−1) with Smac (10 nm) and z-VAD-fmk (20 μM) treatment for 24 h, respectively. Apoptosis was made by TRAIL (250 ng ml−1), Fasl. (2 ng ml−1) or CH11 (100 ng ml−1) with Smac (10 nm) for 24 h, respectively.

**Preparation of primary MEFs.** Primary MEFs were prepared as described by Pobezinskaya et al.37. Briefly, embryos at day 13.5 of gestation were isolated from mice.
pregnant mice. Each embryo was incubated for 30 min at 37 °C in a separate Eppendorf tube with Cellstripper solution (Cellgro). Cells were gently ‘mashed’ by being pipetting, then filtered through cell strainers and cultured in DMEM with 10% (vol/vol) fetal bovine serum (FBS).

**Macrophage differentiation in vitro.** Bone marrow-derived monocytes were isolated from mouse femurs and tibias and cultured for 2 h. The attached monocyte enriched cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) FBS and 2 mM glutamine, with penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹) and 20 mM −glycerol phosphate, 1 mM sodium vanadate and 1 mM β-glycerol phosphate, 1 mM sodium vanadate and 1 mM β-glycerophosphate (PMSF). The lysates were separated by 4–20% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. The proteins were visualized by enhanced chemiluminescence according to the manufacturer’s (Amersham) instructions.

For immunoprecipitation, the lysates were precipitated with antibodies (1 µg) and protein-G agarose bead by incubation at 4 °C overnight. The beads were washed four to six times with 1 ml M2 buffer and the bound proteins were removed by boiling in SDS buffer and resolved in 4–20% SDS-polyacrylamide gels for western blot analysis.

For endogeneous RAR immunoprecipitation, crosslinking of cellular proteins was performed before the cell lysis. The cells were washed four to six times with 1 ml M2 buffer and the bound proteins were removed by boiling in SDS buffer and resolved in 4–20% SDS-polyacrylamide gels for western blot analysis.

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