Quantitative analysis of phosphoproteome in necroptosis reveals a role of TRIM28 phosphorylation in promoting necroptosis-induced cytokine production

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INTRODUCTION

Necroptosis is a form of regulated necrotic cell death that promotes inflammation. In cells undergoing necroptosis, activated RIPK1 kinase mediates the formation of RIPK1/RIPK3/MLKL complex to promote MLKL oligomerization and execution of necroptosis. RIPK1 kinase activity also promotes cell-autonomous activation of proinflammatory cytokine production in necroptosis. However, the signaling pathways downstream of RIPK1 kinase in necroptosis and how RIPK1 kinase activation controls inflammatory response induced by necroptosis are still largely unknown. Here, we quantitatively measured the temporal dynamics of over 7000 confident phosphorylation-sites during necroptosis using mass spectrometry. Our study defined a RIPK1-dependent phosphorylation pattern in late necroptosis that is associated with a proinflammatory component marked by p-S473 TRIM28. We show that the activation of p38 MAPK mediated by oligomerized MLKL promotes the phosphorylation of S473 TRIM28, which in turn mediates inflammation during late necroptosis. Taken together, our study illustrates a mechanism by which p38 MAPK may be activated by oligomerized MLKL to promote inflammation in necroptosis.

Cell Death and Disease (2021)12:994; https://doi.org/10.1038/s41419-021-04290-7

TNFα, an important proinflammatory cytokine implicated in a multitude of human diseases, is one of the most extensively studied triggers of necroptosis [13]. In TNFα-stimulating cells, RIPK1 is rapidly recruited into complex I that is transiently formed in association with activated TNFR1 [14]. Under certain specific cellular conditions, RIPK1 kinase may be activated in complex I [15]. Activated RIPK1 in turn mediates the formation of RIPK1/RIPK3/MLKL complex (complex IIb or necrosome), which promotes the oligomerization of MLKL and execution of necroptosis [16–18]. The activation of RIPK1 and formation of necrosome robustly promote the sustained activation of NF-κB and MAPK to mediate cell-autonomous proinflammatory cytokine production [1]. However, the downstream phosphorylation signaling events regulated by RIPK1 kinase that promote proinflammatory cytokine production remain poorly understood.

TRIM28, a large multi-domain protein (110 kDa), is a member of human Tripartite motif-containing (TRIM) protein family and also known as KAP1 (Krüppel-Associated Box [KRAB]-Associated Protein 1) or TIF1-β (Transcriptional Intermediary Factor 1β) [19]. TRIM28 is known to be phosphorylated in response to a variety of extracellular stresses such as DNA damage to attenuate its binding with heterochromatin protein 1 (HP1), which results in transcriptional activation of target genes to promote DNA repair and cell survival [20]. TRIM28 is involved in regulating endothelial...

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Received: 27 April 2021 Revised: 13 September 2021 Accepted: 23 September 2021 Published online: 23 October 2021

Official journal of CDDpress
inflammatory responses by maintaining expression of TNF receptors \[21\]. p38 MAP kinases, including p38-α (MAPK14), -β (MAPK11), -γ (MAPK12/ERK6), and -δ (MAPK13), are members of mitogen-activated protein (MAP) kinase family \[22\] and essential regulators of MAP kinase transduction cascades mediated by proinflammatory cytokines and environmental stress \[23\].

Inhibition of p38 has been shown to inhibit the transcription of proinflammatory cytokines in necroptosis \[1\]. However, it is unclear how p38 is activated in necroptosis, nor do we know the involvement of TRIM28 or its phosphorylation in necroptosis.

In the present study, we conducted a systemic quantitative analysis of phosphoproteome regulated by RIPK1 kinase across a
time course from complex I to complex IIb formation during necroptosis of FADD-deficient Jurkat cells induced by TNFα. Our study identified a prominent RIPK1-dependent phosphorylation component in the global phosphoproteome in late-stage of necroptosis, which was highly enriched in the pathways of gene expression and inflammatory response. We characterized an example of such late-stage phosphorylation events, the phosphorylation of Ser473 TRIM28, and showed the activation of p38 during necroptosis induced by its interaction with oligomerized MLKL mediates this phosphorylation event. Finally, we found that the phosphorylation of Ser473 TRIM28 promoted the production of proinflammatory cytokines in necroptosis. Since the activation and oligomerization of MLKL is mediated by the interacting RIPK1 and RIPK3 in amyloid conformation during necroptosis, our study may provide an example as how functional amyloids promote inflammation.

RESULTS

Quantitative analysis of RIPK1 kinase-dependent phosphoproteome in necroptosis

Necroptosis of FADD-deficient Jurkat cells are defective upon TNFα [3] began ~4 h after addition of TNFα, which was effectively inhibited by Nec-1s (Fig. S1A). Activation of RIPK1 kinase, as indicated by its activation biomarker p-S166 RIPK1 [24], was detectable within 15 min of TNFα addition and peaked at 2–4 h and effectively blocked by Nec-1s (Fig. S1B). Quantitative mass spectrometry analysis was performed at indicated time points (Fig. S1C). We identified 8722 phosphorylation sites with localization probability over 75% confidence including 153 novel sites previously unidentified, and quantitatively measured 7045 phosphosites (Table S1). Most of peptides have coefficient of variations (CVs) among 6 replicates below 25% (Fig. S1D). Total proteome analysis identified 4448 proteins and 41974 peptides with FDR lower than 1%. Among those, 3315 proteins were quantified in at least two replicates across all the time points (Table S2). The log2Ratio distribution of observed peptides used for quantification of phosphosites and proteome is normal, and more changes were found at the phosphosites levels than at protein levels (Fig. S1E), suggesting that the changes of phosphosites are most likely independent from the changes in protein abundance.

A principal component analysis (PCA) shows that the phosphoproteomes of replicates at each time point after treatments of TNFα or TNFα + Nec-1s are clustered tightly, demonstrating the good reproducibility of the measurements (Fig. 1A). Interestingly, the phosphoproteomes stimulated by TNFα are clearly distinct from that treated with TNFα plus Nec-1s at all time points analyzed. In addition, the phosphoproteomes at early time points after the addition of TNFα or TNFα + Nec-1s at 15 min and 0.5 and 2 h show distinctions from that of late time point at 4 h (Fig. 1A), demonstrating the temporal dynamics in TNFα-induced and RIPK1-dependent phosphoproteomic changes during necroptosis. From these results, we conclude that the overall phosphoproteomes stimulated by TNFα vs. that of TNFα + Nec-1s are substantially different, indicating a significant RIPK1-dependent phosphorylation component in the global phosphoproteome of FADD-dependent Jurkat cells stimulated by TNFα.

As shown in the volcano plots (Fig. S2A), we defined the phosphosites with log2Ratios ≥ 0.485 or ≤ −0.485 and p-value < 0.05 as significantly upregulated or downregulated by TNFα stimulation, respectively. Several known phosphosites on proteins in response to TNFα stimulation at 15 min were found to be significantly increased, including Ser418-CYLD, Thr53-ATF7, Thr69/T71-ATF2 and Ser320-RIPK1 [25], which were not affected by the treatment with Nec-1s, providing a validation for this study. We defined the phosphosites that were inhibited or rescued over 50% by Nec-1s as regulated by RIPK1 kinase (Figs. 1B and S2B). Notably, the pattern of phosphoproteome during necroptosis shows temporal specific features (Fig. 1B), and consistent with the dynamic changes of RIPK1 kinase activation during necroptosis shown in Fig. S1B. Nec-1s treatment exerts increasingly prominent effects on the phosphoproteome at late stage of necroptosis (Fig. 1B and Fig. S2B, C). For example, the increased levels of p-S473 TRIM28, p-S612 NUP98 and p-S515 TOMM22 found only at the late necroptosis were suppressed in Nec-1s-treated samples. Some phosphosites, such as p-S510 ACIN1, p-S813 MADD and p-S497 ATG2B, were upregulated by TNFα stimulation at 15 min, 2 h and 4 h, but were only inhibited by Nec-1s at 2 h and 4 h, indicating that those phosphosites might be regulated distinctly in the early and late stage of necroptosis (Fig. 1B). The analysis of sequence consensus motif for phosphosites upregulated by TNFα that were inhibited by Nec-1s showed that RXXpSP motif was significantly enriched at all four time points (Fig. 1C).

The regulated pathways in the early time points (15 min, 0.5 and 2 h) of TNFα-treated FADD-deficient Jurkat cells are distinguishable from that with TNFα + Nec-1s, and while at late 4 h time point, there were considerable convergence in the regulated pathways, suggesting the dominance of RIPK1-dependent regulation in necroptosis with prolonged TNFα treatment (Fig. 1D). Significant enrichments of the pathways relevant to gene expression and MAPK signaling at late time points were affected by Nec-1s (Fig. 1D). These results highlight the role of RIPK1 kinase in mediating signal transduction with prolonged TNFα stimulation during necroptosis. Interestingly, using MCODE algorithm we identified substantial enrichments of seven protein complexes that were regulated in RIPK1-dependent manner during late necroptosis (Fig. 1E and
In addition to the genes involved in the apoptotic execution, the RIPK1-dependent phosphoproteome is enriched with the complexes involved in the nuclear import and export, mRNA splicing, B-cell activation, cytoskeleton organization, and snRNA transcription (Fig. 1E and Table S3).

RIPK1-dependent phosphorylation of S473-TRIM28 during late necroptosis

We next focused on Ser473 TRIM28, one of the most robustly phosphorylated sites in response to TNFα and was inhibited by Nec-1s (Fig. 2A). Validated by western blotting, the phosphorylation of S473 TRIM28 in FADD-deficient Jurkat cells was elevated at 2 h and further boosted at 4 h after TNFα addition, which was obviously inhibited by Nec-1s (Fig. 2B). We found that the phosphorylation of S473 TRIM28 was also detectable in necroptotic HT-29 cells induced by TNFα + SM164 + zVAD (TSZ). The phosphorylation of S473 TRIM28 was slightly upregulated at early time points between 30 min and 2 h with TSZ treatment, which was not inhibited by Nec-1s. In contrast, the phosphorylation of S473 TRIM28 induced at late time points from 4 h to 10 h with TSZ stimulation was inhibited by Nec-1s (Fig. 2C). Thus, the phosphorylation of S473 TRIM28 was regulated in RIPK1-independent manner in early time points and RIPK1-dependent manner in late time points during necroptosis.

The phosphorylation of S473 TRIM28 was detected in HT-29 cells treated with TNFα alone in early time points from 30 min to 2 h, which was not inhibited by Nec-1s (Fig. 2A). In HT-29 cells treated with TNFα + SM164 (TS), the phosphorylation of S473 TRIM28 was detected from 30 min until 4 h but was not inhibited by Nec-1s (Fig. 2C). These results suggest that RIPK1-dependent phosphorylation of S473 TRIM28 in necroptosis is mechanistically different from that induced by TNFα only and TS.

In addition, we found that the phosphorylation of S473 TRIM28 was also detected in necroptosis induced by treatment with TNFα/CH2/zVAD (TCZ) but cannot be inhibited by Nec-1s (Fig. S3C). However, treatment with cycloheximide alone could induce the phosphorylation of S473 TRIM28 (Fig. S3D). Thus, the increased phosphorylation of S473 TRIM28 induced by cycloheximide is likely to mask the RIPK1 kinase regulated phosphorylation of S473 TRIM28 in necroptosis induced by TCZ.

S473 and S824 TIRM28 are phosphorylated by ATM/ATR kinases and Chk1 in response to DNA damage [20, 26]. As shown in Fig. S4A, C and D, treatment with etoposide, a DNA damage inducer, efficiently induced phosphorylation of S473 TRIM28 rather than S824 that was not inhibited by Nec-1s, RIPK3 inhibitor GSK’872, MLKL inhibitor NSA or Chk1 inhibitor, nor was RIPK1 kinase activation induced by etoposide. The phosphorylation of H2A.X S139, a marker for DNA damage, was not detected across the time course of TSZ stimulation in these cells (Fig. S4B). These results indicate that the regulation of S473 TRIM28 phosphorylation in necroptosis is distinct from that induced by DNA damage.

S473 TRIM28 is a residue highly conserved in its orthologues among mammalian species, including human, mouse, rat, cattle, and dog (Fig. 2D). Consistently, phosphorylation of S473 TRIM28 was induced at 5 h after TSZ stimulation, which was inhibited by treatment with Nec-1s in mouse embryonic fibroblasts (MEFs) (Fig. 2E).

Taken together, we demonstrate that the phosphorylation of S473 TRIM28 is induced in late stage of necroptosis in RIPK1-dependent manner in both human and mouse cells, but is mediated by a mechanism distinct from that of apoptosis and DNA damage.
**TRIM28 interacts with MLKL and S473 TRIM28 is phosphorylated in complex Iib**

We next investigated how TRIM28 may be phosphorylated in late necroptosis in HT-29 cells and MEFs. The phosphorylation of S473 TRIM28 induced by TSZ was inhibited by RIPK3 inhibitor GSK872 and MLKL inhibitor NSA without affecting RIPK1 kinase activation, and totally abolished in RIPK1−/− and MLKL−/− MEFs (Fig. 3A–D). These results suggest that the phosphorylation of S473 TRIM28 is likely mediated by the activation of MLKL, which is dependent upon RIPK1 activation and the formation of complex Iib.

We found that TRIM28 was constitutively bound with MLKL in control and necroptotic cells and p-S473 TRIM28 was also in
complex with MLKL (Fig. 3E). Treatment with Nec-1s inhibited the phosphorylation of S473 TRIM28 but did not affect its binding with MLKL. We also detected enhanced interaction of p-S473 TRIM28 with activated RIPK1 (Fig. 3F). We further confirmed the interaction of TRIM28 with MLKL by co-overexpression in 293T cells (Fig. 3G) and the pseudo-kinase domain of MLKL mediates its binding with TRIM28 (Fig. S4E–G). Thus, TRIM28 interacts with MLKL in unstimulated cells and p-S473 TRIM28 is a part of complex IIIb during necroptosis.

MLKL Q356A mutant can spontaneously aggregate and induce cell death [27]. Interestingly, we found that TRIM28 was recruited to oligomerized MLKL Q356A upon the induction of expression, and the phosphorylation levels of S473 TRIM28 associated with MLKL Q356 was inhibited by NSA (Fig. 3H, I). Taken together, we demonstrate that TRIM28 interacts with MLKL and is phosphorylated at S473 in complex IIIb downstream of MLKL oligomerization during necroptosis.

**p38 activation induced by MLKL oligomerization mediates S473 TRIM28 phosphorylation in necroptosis**

Next, we explored the mechanism that mediates the phosphorylation of S473 TRIM28 in complex IIIb. Using mass spectrometry, we identified several kinases, including STK4, WNK1, PRKCD, PRKCI and ADK, bound to oligomerized MLKL after Dox induction of expression of MLKL Q356A and the addition of NSA inhibited the binding (Table S4). However, knocking down any of those candidate kinases did not affect the phosphorylation of S473 TRIM28 in necroptosis (Fig. S5A). Strikingly, treatment with p38 inhibitor abolished the phosphorylation of S473 TRIM28 in HT-29 cells induced by TSZ but IKK and MK2 inhibitors had no effect, and TAK1 inhibitor S2-Z partially inhibited p38 phosphorylation (Fig. 4A). p38 knockout abolished the phosphorylation of S473 TRIM28 compared to that of WT HT-29 cells during necroptosis induced by TSZ (Fig. 4B, C).

Furthermore, we transfected an inducible MLKL dimerization construct with fused FKBP at C terminus of MLKL into HT-29 cells. The addition of AP20187 effectively induced MLKL oligomerization and cell death, which can be inhibited by NSA; p38 inhibitors had no effect on cell death induced by MLKL oligomerization (Fig. 4D). Consistently, interaction of TRIM28 with MLKL and phosphorylation of S473 TRIM28 was induced by MLKL oligomerization, which was inhibited by the treatment with NSA and p38 inhibitors (Fig. 4E). Finally, we expressed flag-FKBP-MLKL in WT and RIPK3 knockdown HT29 cells. Induction of MLKL dimerization by AP20187 is sufficient to activate p-S473 TRIM28 in both WT and RIPK3-kd HT29 cells. Knockdown of RIPK3 even slightly increased the p-S473 TRIM28 (Fig. S5B). Taken together, these results suggest that phosphorylation of S473 TRIM28 in necroptosis is mediated by p38, which is activated upon binding with oligomerized MLKL.

TAK1-MKK3 axis can activate p38 and subsequently result in MK2 phosphorylation downstream of TNFR1 and TRs pathways [22]. Treatment with S2-Z robustly inhibited p38 activation and phosphorylation of S473 TRIM28 during early time points (0.5–2 h) after induction of necroptosis when phosphorylation of MLKL was not yet detected; treatment with Nec-1s had no effect on either p38 activation or phosphorylation of S473 TRIM28 during the early time points (Fig. S5C). In contrast, p38 activation and phosphorylation of S473 TRIM28 at late time points (4–6 h) during necroptosis were inhibited by Nec-1s but not S2-Z (Fig. S5C). In addition, knockout of MK3 or MK2 had no effect on p38 activation or phosphorylation of S473 TRIM28 during necroptosis (Fig. S5D–E). We also found that knockdown of both MKK3/MMK6 had no effect on p-p38 or p-S473-TRIM28 (Fig. S5F). Taken together, these results suggest that p38 activation and phosphorylation of S473 TRIM28 during necroptosis is mediated by two mechanisms, the first mechanism is activated before MLKL activation and is TAK1-dependent and RIPK1-independent, while the second mechanism is TAK1 independent and is dependent upon RIPK1 and MLKL activation.

**Phosphorylation of S473 TRIM28 regulates cytokine production in necroptosis**

We next examined the effect of S473 TRIM28 phosphorylation on necroptosis. The cell death and cell viability assays showed that TRIM28 KO had no effects on cell death induced by TSZ in HT-29 cells (Fig. 5A–D). The cell death assays showed that TRIM28 KO promoted cell death 10 h after stimulation of TNFα alone but had no effects on cell death induced by TS or TC (TNFα/CHX) in FADD-deficient Jurkat cells (Fig. S6E–G). However, there was no obvious effect on cell death in both HT-29 and FADD-deficient Jurkat cells reconstituted with TRIM28 S473 mutations by indicated stimulations of TS, TNFα only or TS compared to that of WT cells (Fig. S6C–F). Moreover, TRIM28 knockout and TRIM28 S473 mutations did not affect the oligomerization and phosphorylation of S473 TRIM28 in necroptosis (Fig. S6H–I). These results suggest that phosphorylation of S473 TRIM28 is an event downstream of MLKL.

TRIM28 is localized in nucleus and acts as a transcription repressor, and phosphorylation of TRIM28 S473 is known to promote gene transcription [28]. We demonstrated that phosphorylated TRIM28 was present in nucleus and co-localized with DAPI in necrotic cells by immunofluorescence (Fig. 5A). RNAseq analysis showed that the expression of 536 genes was upregulated and that of 380 genes downregulated with p-value < 0.05 and log2Ratio > 2 or < -2 by TSZ stimulation in TRIM28 WT reconstituted TRIM28 KO HT29 cells (Fig. 5B, Fig. S7A, and Table S5). Among those 536 TSZ-upregulated genes, the expression levels of 193 genes were further upregulated in TRIM28 KO cells and among them, the expression of 133 genes was upregulated in both TRIM28 KO and TRIM28 S473D reconstituted TRIM28 KO cells compared to that of WT TRIM28 reconstituted cells (log2Ratios > 0). Among these 133 genes, the expression of 88 genes was upregulated by TRIM28 S473D compared to TRIM28 S473A (log2Ratio > 0), and the expression levels of 46 genes have increased log2Ratios > 0.26 as shown in the heat map (Fig. 5C). The expression of several genes in inflammatory signaling pathways (highlighted in Fig. 5C), such as...
as CXCL8, IL17C, RELB, MAPK6, TNFAIP3, IFN\(\gamma\)R2, SQSTM1, was upregulated at the transcriptional levels in necroptosis and enhanced by TRIM28 KO and the expression of TRIM28 S473D while inhibited by the expression of TRIM28 S473A (Fig. 5D).

Moreover, the mRNA expression levels of TNF\(\alpha\) and CXCL8 examined by qPCR were dramatically increased in HT-29 and TRIM28 WT reconstituted HT-29 cells by induction of necroptosis, further enhanced by TRIM28 KO and TRIM28 S473D and suppressed by TRIM28 S473A (Fig. 5E). Taken together, we demonstrate that phosphorylation of S473 TRIM28 during necroptosis is involved in regulating transcription of genes associated with inflammation.
MLKL oligomerization induces p38 activation to mediate the phosphorylation of Ser473 TRIM28. A HT-29 cells were treated with the indicated inhibitors followed by TSZ treatment for 4 h. The cell lysates were analyzed by western blotting using indicated antibodies. B p38 KO cells were engineered by CRISPR–Cas9 technology and p38 expressing vector was reconstituted in HT-29 cells. WT and p38 KO HT-29 cells were treated with TSZ in the presence or absence of Nec-1s at indicated time points. The cell lysates were analyzed by western blotting using indicated antibodies. C WT and p38 reconstituted p38 KO HT-29 cells were treated with TSZ in the presence or absence of Nec-1s as indicated. Western blots were analyzed using indicated antibodies. D E HT-29 cells were transfected with an expression vector of flag-tagged MLKL fused with two AP20187-binding (FKBP/V) domains. The cells were pretreated with indicated compounds for 4 h followed by treatment with AP20187 to induce MLKL oligomerization. Cell viability was determined by CellTitre-Glo after treatment with AP20187 for 2 h (D Left). Cell lysates were separated by non-reducing SDS/PAGE and analyzed by western blotting using MLKL antibody (D Right). The cell lysates were immunoprecipitated by anti-Flag resin after treatment with AP20187 for 2 h and analyzed by western blotting using MLKL antibody (E). acMLKL, oligomerizable MLKL, endo, endogenous. TNFα, 20 ng/mL; zVAD, 25 μM; SM164, 50 nM; Nec-1s, 10 μM; NSA, 4 μM; PTPCA-1, 10 μM; p38i PH-228828, 4 μM; S7–2, 500 nM; MK2i MK2-IN-1, 10 μM; AP20187, 20 μM. Data were presented as mean ± SEM. **p-test p < 0.01, n = 3. n.s., not significant.

**DISCUSSION**

We present a quantitative phosphoproteomic study that reveals the dynamic time-dependent changes of RIPK1 kinase-dependent phosphoproteome during necrosis induced by TNFα in FADD-deficient Jurkat cells. Our study reveals a set of RIPK1-dependent phosphorylation events in late necrosis. The binding of p38 with oligomerized MLKL mediates a set of late phosphorylation events that includes the phosphorylation of TRIM28. Our results suggest that the RIPK1-dependent phosphorylation events in late necrosis may be mediated by the downstream events of RIPK1-dependent signaling. Consistently, the sequence consensus motif for Nec-1s inhibited phosphosites found in this study was RXXpSP, which is similar to that of p53 sequence motif previously reported for RIPK3-dependent phosphoproteome [29]. Furthermore, since we found that the formation of necrosome and oligomerization of MLKL could promote the activation of p38 MAPK, which mediates the phosphorylation of substrates with the sequence consensus motif of p53/p53 [30], the RXXpSP motif found by this study may also reflect the activation of MAPK during necrosis. Since the interacting RIPK1 and RIPK3 in necrosome (complex Iib) may be present in the conformation of amyloid fibers and phosphorylation of MLKL by RIPK3 promotes the oligomerization of MLKL [31], our results suggest the possibility that p38 activation in binding with MLKL is sensitive to its conformation and is activated by the oligomerized MLKL, which in turn mediates the phosphorylation of Ser473 TRIM28 to promote the transcription of proinflammatory cytokines (Fig. 6). Thus, the activation of p38 and phosphorylation of Ser473 TRIM28 may provide a mechanism by which functional amyloid fibers promote inflammatory response. In addition, our analysis also demonstrated a significant enrichment of the proteins involved in gene transcription, splicing and nuclear imports whose phosphorylation were regulated in RIPK1-dependent manner during late-stage necrosis. Thus, it is possible that there is a coordinated activation of proinflammatory responses in necrosis at multiple levels of gene expression.

p38 MAPKs have established roles as key signaling molecules that regulate the production of proinflammatory cytokines in response to environmental stresses and are implicated in the pathogenesis of many inflammatory-driven conditions [32]. Therapeutic inhibition of p38 MAPKs for attenuating inflammation has been investigated in the last 3 decades. The therapeutic potential of p38 MAPK inhibitors was initially explored in clinical trials for the treatment of human inflammatory conditions such as rheumatoid arthritis and Crohn’s disease, but the progression of such studies was prevented due to poor clinical efficacy and unacceptable side effects. Our study demonstrates the role of RIPK1 activation in promoting the activation of p38 in mediating inflammation activated by necrosis. RIPK1 inhibitors have been advanced into multiple of human clinical studies beyond Phase I including for the treatment of rheumatoid arthritis and Crohn’s disease [5]. Thus, targeting RIPK1 might provide an alternative means to inhibit p38-mediated inflammatory signaling.

Here, we describe a dual role of TAK1 and RIPK1 in regulating the phosphorylation of Ser473 TRIM28 during necrosis. TAK1 (TGFB-β activated kinase 1) is a MAPK kinase kinase family serine threonine kinase that is known to be involved in the signaling cascades involving p38 [33]. TAK1 activation has also been shown to mediate the phosphorylation of Ser321 mRIPK1/S320 hRIPK1 during the early stage of necrosis [34]. Since the early TAK1-dependent phosphorylation of Ser473 TRIM28 during necrosis is also inhibited by p38 KO and p38 inhibitors, TAK1 may cooperate with RIPK1-RIP3-MLKL axis to promote inflammatory response activated by necrosis. Since TAK1 can exert an inhibitory activity on RIPK1 [34], it is conceivable that the activation of TAK1 may inhibit the subsequent RIPK1 activation under certain condition to reduce inflammation. Moreover, we delineate a landscape of dynamic phosphoproteomes downstream of RIPK1 kinase activation during necrosis and reveal the mechanism by which p-Ser473 TRIM28, one of the most upregulated phosphosites in necrosis, is controlled and its function in controlling inflammation under chronic inflammatory conditions.

**MATERIALS AND METHODS**

**Reagents**

The following commercial reagents were used in this study: TNFα (Novoprotein, C0008), S7–2-Oxoozeanol (S7–2) (Sigma-Aldrich, O9890), PTPCA-1 (MCE, HY−10074), PH−797804 (MCE, HY−10403), LY2288280 (MCE, HY−13241), MK2i (MCE, HY−12834A), GSK872 (Calbiochem, 530389), NSA (Necrosulfonamide) (Calbiochem, 480073), MK−8776 (Selleck, SCH900776), PD−407824 (Sigma-Aldrich, PZ0111), BHT (Sigma-Aldrich, 34750), BHA (Necrosulfonamide) (Calbiochem, 480073), MK−8776 (Selleck, SCH900776), PD−407824 (Sigma-Aldrich, PZ0111), BHT (Sigma−Aldrich, 34750), BHA (Sigma−Aldrich, 20021), AP20187 (MCE, HY−13992). R−7−C1−O−Nec−1(Nec−1s), zVAD.fmk and SM164 were made by custom synthesis. Final concentrations of the compounds used in all experiments: TNFa, 20 ng/mL; zVAD, 25 μM; SM164, 50 nM; Nec−1s, 10 μM.

**Cell culture**

HEK293T cells, MEFs, FADD-deficient Jurkat cells, and HT-29 cells were originally obtained from the American Type Culture Collection (ATCC). HEK293T cells and MEFS were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, 11965) with 10% (vol/vol) fetal bovine serum (FBS; Thermo Fisher Scientific, 10082-147). FADD-deficient Jurkat cells were cultured in RPMI 1640 medium deficient in l-lysine and l-arginine with 10% (vol/vol) fetal bovine serum deficient in l-lysine and l-arginine, using stable isotope labeling by amino acids in cell culture (SILAC). Recipes of SILAC were obtained from Pandey Lab. HT-29 cells were cultured in McCoy’s 5A medium (Thermo Fisher Scientific, 16600082) with 10% (vol/vol) FBS. All media were supplemented with 1% (vol/vol) penicillin (100 U/mL) and streptomycin (100 U/mL). All of the mammalian cell lines were maintained at 37°C with 5% CO2. The cells were tested routinely using a TransDetect PCR Mycoplasma Detection Kit (Transgen Biotech, FM311-01) to ensure that they were mycoplasma free.
Generation of knockdown and knockout cell lines

Cells were stably infected with shRNA against p38 (CCGGTACCTCTGTG-TACTCTTAC TCGAGTAAAGAGTACACAGGAAGTACTTTTTG), TRIM28-1 (CCGGGTACTGTCTATTTGCAACGTCTCGAGACGTTGCAATAGACAGTACTTTTTG), TRIM28-2 (CCGGCTGAGGACTACAACCTTATCTCGAGATAAGGTTGTAGTCCTCGTTTTTG) or scramble control in the pLKO.1 lentiviral background. For CRISPR-Cas9 system-mediated gene knockout, we used guide RNAs against p38 (CACCAGGCAAAAACCGGGATATTG (F), AAACCCACACGCACACCATCTTTCATTTTGTG (R), MK2 (CACCAGAAGTCCCTCGTTTGCCTG (F), AAACCCACACGCACACCATCTTTCATTTTGTG (R)), TRIM28 (CACCAGCAGACACACCTGTGAGG (F), AAACCCACACGCACACCATCTTTCATTTTGTG (R)) and MK3 (CACCAGGCAAAAACCGGGATATTG (F), AAACCCACACGCACACCATCTTTCATTTTGTG (R)) in

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were transfected with different vectors using Polyethylenimine (PEI, generated by infecting MEFs with lentiviruses carrying Lenti-CRISPR v2-obtained by selection with 5-fluoroouracil) four hours after infection, cells stably expressing shRNA or sgRNA were infected with viral supernatant fractions at a final concentration of 8 μg/mL puromycin.

Fig. 6 A schematic model for phosphorylation of Ser473 TRIM28 mediated by p38 MAPK in regulating cytokine production in necroptosis. The activation of RIPK1 kinase promotes its interaction with RIPK3 to form complex Ibb (necrosome), which leads to the activation of RIPK3. The activated RIPK3 in turn mediates the phosphorylation of MLKL to promote its oligomerization. Oligomerized MLKL interacts with p38, which promotes the activation of p38. The activated p38 in turn mediates the phosphorylation of Ser473 TRIM28. The Ser473 phosphorylated TRIM28 promotes proinflammatory cytokine production.

the Lenti-CRISPR v2 lentiviral background. TRIM28 KO HT-29 cells were generated by CRISPR technology and TRIM28 knock down FADD-deficient Jurkat cells were generated by shRNA. RIPK1 and MLKL MEFs were generated by infecting MEFs with lentiviruses carrying Lenti-CRISPR v2-sgRIPK1 and Lenti-CRISPR v2-sgMLKL. For viral packaging, HEK293T cells were transfected with different vectors using Polyethylenimine (PEI, Polysciences, 23966-2). Viral supernatant fractions were collected at 48 h post-transfection. Cleared supernatant fraction with virus particles was filtered through a 0.45 μm filter. Polybren (8 mg/mL) was supplemented to viral supernatant fractions at a final concentration of 8 μg/mL. Twenty-four hours after infection, cells stably expressing shRNA or sgRNA were obtained by selection with 5 μg/mL puromycin.

Construction, transfection of plasmids, and reconstitution cell lines Transient transfections of HEK293T cells were performed using PEI according to the manufacturer’s instructions. In brief, HEK293T cells were seeded in 6-well plates. When cells were 95% confluent, plasmids were transfected using PEI reagent at a ratio of 1:3, and each well was transfected with a total of 2 μg DNA per well for 24 h. Full-length cDNAs for human MLKL and TRIM28 were PCRamplified from HT-29 cells and cloned into pcDNA3.1 vector using Q5 High-Fidelity DNA Polymerase (NEB, M0491) with appropriate tags. MLKL T357E/S358D and FKBP F36V were PCR-amplified from corresponding wild-type sequences with point mutation primers using PfuUltra II Fusion HS DNA polymerase (Agilent, 600674), then cloned into pMSCV plasmid with Flag tag using ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd, C112) and transfected into HT-29 cells, and the reconstitution product was named as acMLKL. Tet-On vector carrying Flag-MLKL-Q536A was transfected individually into HEK293T cells, in which system MLKL oligomerization could be induced by doxycycline. For add-backs, TRIM28 WT, S473A, S473D and S473E sequences were cloned into pMSCV vector also using ClonExpress II One Step Cloning Kit. TRIM28 WT, S473A, S473D and S473E were reconstituted into TRIM28 KO HT-29 and TRIM28 knock down FADD-deficient Jurkat cells. Flag-p38 was reconstituted into p38 KO HT-29 cells. All plasmids were verified by DNA sequencing.

Immunoblotting and co-immunoprecipitation (Co-IP) Antibodies against the following proteins were used for western blot analysis: RIPK1 (Homemade), phospho-RIPK1 (S166) (CST, 65746), MLKL (Homemade; Abcam, ab183770; GeneTex, GTX107538), phospho-MLKL (T357/S358) (Abcam, ab187091), TRIM28 (Abcam, ab22533; CST, 4123), phospho-TRIM28 (S473) (Biolgend, 644602; Biolegend, 654102), phospho-TRIM28 (S473) (Abcam, ab70369), H2AX (CST, 2595), phospho-H2AX (S139) (Millipore, 05-636), Chk1(Santa Cruz, sc-8408), phospho-Chk1 (S296) (CST, 90178), phospho-Chk1 (S345) (CST, 2341), MK2 (CST, 3042), phospho-MK2 (T344) (CST,3007), p38 (CST, 9212), phospho-p38 (CST, 4511; CST, 9216), MK3 (CST, 8535), MK66 (CST, 8550), phospho-MK3/MK66 (CST, 12280), I KK (CST, 89435), phospho-IKK (CST, 20785), RIPK3 (Homemade), phospho-RIPK3 (T544), GP (Santa Cruz, sc-9996), HA (SAB, TS01), Akt (Santa Cruz, 81178), a-Tubulin (MBL, M175-3), GAPDH (Sigma-Aldrich, G5262), Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, A11001), Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (Thermo Fisher Scientific, A11012), Goat anti-Rabbit IgG (H + L) Secondary Antibody, HRP (Thermo Fisher Scientific, 31460), Goat anti-Mouse IgG (H + L) Secondary Antibody, HRP (Thermo Fisher Scientific, 31430). For Co-immunoprecipitation, cell lysates were prepared in following lysis buffer: 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 10% glycerol supplemented with 0.5 mM PMSF, 20 mM N-ethylnemaldeide (N-EM), 1× protease inhibitor cocktail (Bimake, B14001), 10 mM β-glycerol phosphate, 5 mM NaF, and 200 μM Na3VO4. Cells were lysed on ice for 1 h and centrifuged at 15,000 rpm for 15 min at 4 °C. The cell lysates were incubated with indicated antibody overnight at 4 °C and immunocomplex was captured by protein G agarose (Thermo Fisher Scientific, 20399). After extensive washes, beads were boiled in loading buffer and eluted products were separated by SDS-PAGE, which were transferred to nitrocellulose membrane and analyzed with indicated antibodies.

Immunofluorescence For immunofluorescence analysis, HT-29 cells were grown on coverslips. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% TritonX-100 (Sigma-Aldrich, T8787) and blocked with 5% bovine serum albumin (BSA). Blocked cells were incubated with indicated primary antibody overnight at 4 °C. Cells were then incubated with secondary antibody for 1 h at room temperature. Fluorescence imaging was done on Leica SP8 Confocal System.

Quantitative reverse-transcription PCR Total RNA was extracted with Trizol Reagent (Thermo Fisher Scientific, 15596026) and reverse transcription was performed with Reverse Transcriptase M-MLV (RNase H-) (Takara, 600674), then both cloned into pMSCV plasmid with Flag tag using ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd, C112) and transfected into HT-29 cells, and the reconstitution product was named as acMLKL. Tet-On vector carrying Flag-MLKL-Q536A was transfected individually into HEK293T cells, in which system MLKL oligomerization could be induced by doxycycline. For add-backs, TRIM28 WT, S473A, S473D and S473E sequences were cloned into pMSCV vector also using ClonExpress II One Step Cloning Kit. TRIM28 WT, S473A, S473D and S473E were reconstituted into TRIM28 KO HT-29 and TRIM28 knock down FADD-deficient Jurkat cells. Flag-p38 was reconstituted into p38 KO HT-29 cells. All plasmids were verified by DNA sequencing.

Analysis of cytotoxicity and viability The rates of cell death were measured in triplicate in a 384-well plate using ToxLight Non-destructive Cytotoxicity BioAssay Kit (Lonza, LT07-217). General cell survival was measured by the ATP luminescence assay CellTiterGlue (Promega, G7573) according to the manufacturer’s protocol and the results were expressed as percentages of luminescence intensity per well relative to that of the viability in the untreated wells. The intensity of luminescence was determined in an EnSpire Multimode Plate Reader (PerkinElmer). Data were collected using PerkinElmer EnVision Manager Version 1.13 software.

RNA-Seq One microgram of total RNA was used for stranded mRNA library preparation according to the manufacturer’s protocol (http://www.interchim.fr/en/B/BOEJM0.pdf). Libraries were quantified using both Qubit and Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) to detect the total RNA samples concentration. Libraries were run on NextSeq at 150 nucleotide and pair-end sequence read length. Reads were mapped to the Human Genome GRCh38.p12 with STAR using the default parameters. Reads were assigned to all gene in the GENCODE version 29 GTF file using
the featureCounts. Reads per gene were then analyzed using the DESeq2 R package from Bioconductor. Genes upregulated and downregulated with TSZ treated were selected by comparing DMSO-treated T28 cells cultivated at 37 °C. Expression differences were determined using drug as the independent categorical variable modeled with DESeq2 using the DESeq analysis for statistical significance. Differentially expressed genes were selected using the absolute log2 fold change ≥ 1.5 and p-value ≤ 0.05.

Mass spectrometry and data analysis

The SILAC strategy was used for quantification of RIPK1 kinase-dependent phosphoproteome. The “light” (K0, R0), “medium” (K4, R6) and “heavy” (K8, R10) labeled cells were treated with DMSO, TNFa and TNFa + Nec-1s, respectively. Cells were harvested in lysis buffer after treatments for 15 min, 0.5, 2, and 4 h. The protein concentrations were measured. Equal amounts of proteins in three differentially labeled cells were mixed for each time point and trypsin digested. The resulting peptides were separated by high-pH reversed-phase fractionation. A part of peptides of each fraction were subjected to phosphopeptide enrichment by TiO2 and analyzed by mass spectrometry. The cysteine carbamidomethylation was set as a static modification, and the N-acetylation, S/T/Y phosphorylation and methionine oxidation were set as variable modifications. The false discovery rate (FDR) at peptide level was based on target-decoy searching with a peptide false discovery rate (FDR) of ≤ 1%.

 statistical analysis

The cell data are presented as mean ± SEM (standard error of mean) of nine oxidation were set as variable modifications. The false discovery rate (FDR) at peptide level was based on target-decoy searching with a peptide false discovery rate (FDR) of ≤ 1%.

Statistical analysis

The data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Statistical significance was determined using unpaired two-tailed Student's t-test for comparison between two groups, one-way ANOVA followed by Tukey's multiple comparisons test for comparison among multiple groups, and Pearson's correlation test. A p-value < 0.05 was considered statistically significant.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomexchange.proteomeexchange.org) via the iProX partner repository [34] with the dataset identifier PXD005313. The RNAseq data have been deposited to Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) with the dataset identifier GSE171214.

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ACKNOWLEDGEMENTS

We thank Dr. Liming Sun of Shanghai Institute of Biochemistry and Cell Biology for providing expression constructs of truncated MLKL. This work was supported in part by the China National Natural Science Foundation (21837004, 91849204, 92049303 and 91849109), the Shanghai Municipal Science and Technology Major Project (2019SHZDZX02), the Chinese Academy of Sciences (XDB39030200), China National Natural Science Youth Foundation (31701210), and the Science and Technology Commission of Shanghai Municipality (18JC1420500).

AUTHOR CONTRIBUTIONS

JY and BS designed and directed the experiments; RZ and ZY conducted majority of the experiments; CFS and LS analyzed parts of the mass spec data; JZ conducted RNAseq experiments and analyzed the RNAseq data; XJL, WL, KZZ, TZ, GL, and MMZ, conducted specific experiments; YYZ directed part of the mass spec data analysis and NL directed RNAseq experiments and data analysis.

COMPETING INTERESTS

JY is a consultant for Denali Therapeutics and Sano. The other authors declare no competing interests.

ETHICS STATEMENT

This study does not involve human participants, human data or human tissue, thus there is no requirement for ethical approval.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41419-021-04290-7.

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