Thioredoxin-1 actively maintains the pseudokinase MLKL in a reduced state to suppress disulfide bond-dependent MLKL polymer formation and necroptosis

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Necroptosis is an immunogenic cell death program that is associated with a host of human diseases, including inflammation, infections, and cancer. Receptor-interacting protein kinase 3 (RIPK3) and its substrate mixed lineage kinase domain-like protein (MLKL) are required for necroptosis activation. Specifically, RIPK3-dependent MLKL phosphorylation promotes the assembly of disulfide bond-dependent MLKL polymers that drive the execution of necroptosis. However, how MLKL disulfide bond formation is regulated is not clear. In this study we discovered that the MLKL-modifying compound necrosulfonamide cross-links cysteine 86 of human MLKL to cysteine 32 of the thiol oxidoreductase thioredoxin-1 (Trx1). Recombinant Trx1 preferentially binds to monomeric MLKL and blocks MLKL disulfide bond formation and polymerization in vitro. Inhibition of MLKL polymer formation requires the reducing activity of Trx1. Importantly, shRNA-mediated knockdown of Trx1 promotes MLKL polymerization and sensitizes cells to necroptosis. Furthermore, pharmacological inhibition of Trx1 with compound PX-12 induces necroptosis in multiple cancer cell lines. Altogether, these findings demonstrate that Trx1 is a critical regulator of necroptosis that suppresses cell death by maintaining MLKL in a reduced inactive state. Our results further suggest new directions for targeted cancer therapy in which thioredoxin inhibitors like PX-12 could potentially be used to specifically target cancers expressing high levels of MLKL or MLKL short isoforms.

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‡ The abbreviations used are: RIP, receptor-interacting protein; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; MLKL protein, mixed lineage kinase domain-like protein; Z-VAD-FMK, benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone; ROS, reactive oxygen species; NSA, necrosulfonamide; NTD, N-terminal domain; Trx1, thioredoxin-1; IP, immunoprecipitation; ASK1, apoptosis signal-regulating kinase 1; SDD-AGE, semi-denaturing detergent agarose gel electrophoresis; Dox, doxycycline; aa, amino acids; TetR, Tet repressor; DmrB, dimerization domain; LDH, lactate dehydrogenase.

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and Ser-358 located within the C-terminal kinase-like domain of human MLKL (27, 28). Active MLKL molecules co-localize with various cellular compartments and interact with a specific subset of phosphatidylinositol phospholipids (29–34). MLKL then forms oligomers that somehow disrupt the integrity of the plasma membrane through a process that is not fully understood. Previous reports show that MLKL oligomers are stabilized by intermolecular disulfide bonds (29, 30, 35). Recently we demonstrated that MLKL forms disulfide bond-dependent amyloid-like fibers to promote necroptosis. MLKL mutant with multiple cysteine residues changed to serine residues could not form intermolecular disulfide bonds and is greatly compromised in its ability to induce necroptosis (36). These results suggest that MLKL activity is tightly regulated by its redox state. Interestingly, mitochondrial dysfunction and ROS production are observed during necroptosis (37). In murine cells, TNF-α-induced necroptosis can be prevented by co-treating cells with ROS-scavenging molecules such as butylated hydroxyanisole (BHA) or N-acetylcysteine (NAC) (38). More recently, it was shown that mitochondrial ROS induced RIPK1 autoprophosphorylation, bolstering the idea that oxidation plays an important role in the mammalian necrotic pathway (39). However, whether similar oxidative events drive MLKL activation in human cells remains a topic of much debate.

Here, we provide evidence that thioredoxin-1 (Trx1), a 12-kDa thiol oxidoreductase, suppresses necroptosis by blocking MLKL disulfide bond formation and polymerization. Trx1 functions as an anti-oxidizing enzyme that is central for regulating cellular redox balance. Trx1 protects cells from oxidative stress by catalyzing thiol disulfide exchange reactions that results in the reduction of disulfide bonds on specific protein targets, thereby modulating their activities (41, 42). Proteins regulated by Trx1 include cell surface receptors, kinases, transcription factors, and other signaling molecules that are involved in a multitude of biological processes including cell death (42–44). In this study we found that necrosulfonamide (NSA), a small molecule inhibitor of MLKL (23), cross-linked Cys-32 of Trx1 to Cys-86 of human MLKL. In vitro interaction studies revealed that Trx1 interacted with MLKL and maintained MLKL in a reduced state. Knockdown of Trx1 by shRNA enhanced MLKL tetramer and polymer formation. Moreover, Trx1 knockdown cells were more sensitive to necroptosis upon induction of RIPK3 and MLKL protein expression. These results were further corroborated with the use of a commercially available Trx1 inhibitor, PX-12, which disables the recycling of Trx1 by thioredoxin reductase. PX-12 treatment promoted RIPK1–RIPK3–MLKL necosome formation, RIPK3-dependent MLKL phosphorylation, MLKL polymerization, and ultimately caspase-independent necrotic cell death. Overall, these findings point to Trx1 as a suppressor of necroptosis that functions at the step of MLKL polymer formation.

Results

**NSA cross-linked Cys-32 of thioredoxin-1 to Cys-86 of human MLKL**

NSA is a synthetic compound that inhibits necroptosis in human cells (23). NSA contains two potential Michael acceptors that covalently conjugate cysteine residues on target proteins. Mutation of either Michael acceptor renders NSA non-functional (23). By irreversibly conjugating Cys-86 of human MLKL protein, NSA prevents necroptosis without affecting RIPK1–RIPK3–MLKL necosome complex formation or RIPK3-dependent MLKL phosphorylation (23, 27, 36). We observed that NSA cross-linked MLKL to an endogenous protein in NTD-DmrB-FLAG cells, which stably express a truncated MLKL transgene containing the N-terminal domain (NTD) fused to an interaction-inducible DmrB domain under the control of a doxycycline (Dox)-inducible promoter (Fig. 1A). Western blot analysis revealed that NSA, but not NSA-D1, produced a prominent extra protein band that migrated near 55 kDa (lanes 2 and 3, Fig. 1B), which is ~12–15 kDa larger than NTD-DmrB itself. This indicated that NSA requires two cysteine-reactive Michael acceptor moieties, one of which is lacking in NSA-D1 to successfully cross-link MLKL to a 12–15-kDa endogenous protein. As expected, NSA cross-linking required Cys-86 of MLKL, which is a known targeting site for NSA (lane 4, Fig. 1C).

Next, we set out to determine the identity of the protein cross-linked to NTD-DmrB by NSA. NTD-DmrB–NSA–protein complexes were isolated from whole cell extracts by FLAG-tag affinity purification and subjected to SDS-PAGE for silver-staining (Fig. 1D). The 55-kDa gel band of interest was excised and subjected to LC/MS analysis to determine the protein composition. The list of candidate proteins was narrowed down to those with a molecular weight between 12 and 15 kDa. One of the proteins that fit this criterion was thioredoxin-1 (Trx1). Trx1 is a small cytoplasmic oxidoreductase that catalyzes the reduction of disulfide bonds on specific protein substrates. To verify that Trx1 was cross-linked to MLKL by NSA, we ectopically expressed either wild-type or a C86S mutant HA-FLAG-MLKL full-length protein in HEK293T cells and treated these cells with NSA for 16 h. Anti-Trx1 Western blotting of the FLAG-IP product confirmed that Trx1 was cross-linked by NSA to wild-type MLKL (lane 2) but not to C86S mutant (lane 4) (Fig. 1E). MLKL-NSA-Trx1 cross-linking was also confirmed through ectopic expression of HA-tagged Trx1 in HeLa:GFP-RIPK3:MLKL cells, which stably express GFP-RIPK3 and MLKL-HA-FLAG under the control of a Dox-inducible promoter (Fig. 1F). In HA-Trx1-expressing cells, NSA cross-linked MLKL to both endogenous Trx1 and HA-Trx1, producing two cross-linking products (lane 4, Fig. 1F).

The ability of Trx1 to reduce disulfide bonds is attributed to two reactive cysteine residues, Cys-32 and Cys-35, located within its active site (40, 41). To determine which cysteine of Trx1 reacts with NSA, we mutated Cys-32 and Cys-35 to serine individually or in combination and tested MLKL cross-linking. FLAG-IP revealed that C32S, but not C35S, failed to conjugate MLKL, indicating that Cys-32 is necessary for NSA cross-linking (lane 4, Fig. 1G). This suggests that the catalytic domain of Trx1 mediates the interaction with MLKL.

**Trx1 interacted with MLKL in a signal-dependent manner**

Given that NSA cross-links Trx1 to MLKL under normal conditions, we tested whether Trx1 could directly bind to MLKL in cells. However, attempts to verify their interaction by standard MLKL or Trx1 immunoprecipitation assays were not
To overcome this challenge, we first immobilized recombinant HA-Trx1-His6 purified from BL21 bacteria cells to nickel-agarose beads and incubated them with cell extracts prepared from HeLa:GFP-RIPK3:MLKL cells. Nickel beads containing wild-type Trx1 were able to pull down MLKL (lane 2, Fig. 2A), which was not the case with the beads containing the CS2 (C32S/C35S) mutant (lane 3, Fig. 2A). Next, immobilized Trx1 nickel beads were incubated with either control or T/S/Z-induced necroptotic cell extracts prepared from HT-29 cells (Fig. 2, B and C). Both wild-type Trx1 and the C35S mutant bound MLKL (lanes 3–6, Fig. 2C). Interestingly, C35S mutant showed even higher affinity with MLKL than wild-type (compare lanes 5 and 3), similar to a previous report that C35S mutant constitutively interacted with its known substrate apoptosis signal-regulating kinase 1 (ASK1) with higher affinity (44). Importantly, Trx1-binding affinity with MLKL was significantly higher in control extracts than extracts prepared from T/S/Z-treated cells (lanes 3 and 4 and lane 5 and 6, Fig. 2C), suggesting that Trx1 preferentially associates with monomeric MLKL molecules under normal condition.

Trx1 reduces MLKL disulfide bonds to inhibit MLKL polymer formation

Trx1 reduces disulfide bonds on specific target proteins, thereby maintaining the reducing environment of the cytoplasm, preventing undesired protein aggregation and regulating redox signaling pathways (40, 41). Previous reports indicate that MLKL tetramers are stabilized by intermolecular disulfide bonds (29, 30, 35), which is also the case for larger MLKL polymers (36). As shown in Fig. 3A, T/S/Z treatment induced MLKL phosphorylation in HeLa:GFP-RIPK3:MLKL cells. Phosphorylated MLKL complexes were purified by FLAG-IP and analyzed by Western blotting. The arrowhead at 72 kDa points to the NSA-cross-linked MLKL-Trx1 complex. Each band corresponds to the expected MLKL molecular weight of 55 kDa. The NSA adduct was confirmed in the experiment using NSA-D1 variant (Fig. 3B). In addition, endogenous Trx1 was conjugated to MLKL by NSA (lane 4, G). The NSA-D1 variant of NSA produces a lower molecular weight adduct due to the smaller conjugate. Both endogenous Trx1 and HA-Trx1 conjugated to MLKL by NSA (lane 4, G). A panel of HA-tagged mutant Trx1 proteins were ectopically expressed in HeLa:GFP-RIPK3:MLKL cells followed by NSA treatment. FLAG-IP products were analyzed by Western blotting. In HA-Trx1-C52 mutant, both Cys-32 and Cys-35 were mutated to serine.
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Reducing SDS-PAGE (lanes 2, Fig. 3B). The MLKL tetramers were stabilized by disulfide bonds because incubation with 5 mM β-mercaptoethanol for 30 min at 30 °C reduced tetramer back to the monomeric state (lane 4, Fig. 3B). Next, semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) was used to resolve megadalton-size MLKL polymers that are resistant to SDS exposure. T/S/Z treatment resulted in MLKL polymer formation (lane 2, Fig. 3C), which were dissociated after incubation with 5 mM DTT for 30 min at 30 °C (lane 4, Fig. 3C). Altogether, these results confirm that active MLKL tetramers and polymers are stabilized by disulfide bonds.

To address the mechanism by which Trx1 regulates MLKL function, we employed a cell-free system that was recently reported (36) to assess MLKL polymerization in vitro. Recombinant GST-NTD-FLAG protein was purified from BL21 bacteria cells. Overnight incubation of GST-NTD-FLAG at 37 °C resulted in MLKL polymer formation (lane 2, Fig. 3D). Importantly, GST-NTD incubated at 4 °C did not polymerize suggesting that the process is temperature-sensitive (lane 1). Moreover, incubating polymers with DTT resulted in total dissociation of the polymers (lane 3, Fig. 3D), confirming the importance of disulfide bond formation for in vitro MLKL polymerization. To test the effect of Trx1 in this system, 5 μM GST-NTD-FLAG protein was incubated with increasing amounts of recombinant Trx1 (3 μM, 10 μM, and 30 μM) overnight at 37 °C. Wild-type Trx1 inhibited MLKL tetramer formation in a dose-dependent manner (lanes 2–4), whereas the CS2 mutant did not (lanes 5–7, upper panel, Fig. 3E). Similarly, wild-type Trx1 but not CS2 mutant blocked MLKL polymer formation (upper panel, Fig. 3F), suggesting that the Trx1 reductase activity is required to keep MLKL in a monomeric state. Protein levels of recombinant GST-NTD-FLAG and HA-Trx1-His6 in the reaction were visualized by Coomassie Blue staining (lower panel, Fig. 3F). These results confirm that MLKL is a substrate of Trx1, and Trx1 maintains MLKL in a reduced monomeric state in vitro to prevent MLKL polymerization. shRNA-mediated Trx1 knockdown promoted MLKL polymerization and sensitized cells to necroptosis

Trx1 is an essential gene and, therefore, cannot be successfully knocked out in cells (45). To overcome this challenge and test whether Trx1 suppresses MLKL activation, we stably introduced a Dox-inducible Trx1 shRNA cassette into the genome of HeLa:GFP-RIPK3:MLKL cells by lentiviral transduction (Fig. 4A). shTrx1 cells grew at similar rates as parental cells and exhibited normal morphology. After 72 h of Dox treatment, cell extracts from parental and shTrx1 cells were collected and probed for Trx1, RIPK3, and MLKL protein levels by Western blotting. Dox treatment induced GFP-RIPK3 and MLKL expression (lanes 2 and 4, Fig. 4A) and greatly reduced Trx1 expression by shTrx1 (lane 1, Fig. 4A). Notably, even without Dox, Trx1 expression was already reduced (lane 3), suggesting the occurrence of leaky shRNA expression. Inhibition of Trx1 is known to activate ASK1 to induce apoptosis (43, 44). Therefore, to eliminate the Trx1 reduction-induced apoptosis effect, Z-VAD-FMK was included in all the analysis for Trx1 knockdown or inhibition experiments. Next, cell extracts were subjected to non-reducing SDS-PAGE analysis to test whether knockdown of Trx1 promoted MLKL tetramer formation. Indeed cells with diminished levels of Trx1 presented higher amounts of MLKL tetramers (lane 4, Fig. 4B). Moreover, MLKL polymers were also detected by SDD-AGE in Trx1 knockdown samples (lane 4, Fig. 4C). Lastly, cell death measurement indicated that reduction of Trx1 protein resulted in significantly higher sensitivity to necroptosis (columns 2 and 4, Fig. 4D). These results suggest that Trx1 functions to keep MLKL in a reduced state to suppress MLKL polymerization and activation (Fig. 4E).

To address the possibility that the cross-linking product MLKL-NSA-Trx1 might contribute to NSA’s ability to block cell death, we tested the effect of NSA in shTrx1 cells. In cells that had reduced levels of Trx1, MLKL-NSA-Trx1 was not detectable (lane 4, Fig. 4F). However, NSA was fully capable of blocking T/S/Z-induced cell death (columns 5 and 6, Fig. 4G), suggesting that the cross-linking product MLKL-NSA-Trx1 is not required for NSA to block necroptosis. This confirms our previous result that NSA could directly block MLKL polymerization in vitro (36).

**Trx1 inhibitor PX-12 induced necroptosis in HeLa:GFP-RIPK3:MLKL cells**

Because Trx1 knockdown sensitized cells to necroptosis, we tested if chemical inhibition of Trx1 activity exhibited the same
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This could have significant implications in cancer biology, as induction of necroptosis in tumors could potentially enhance immune response to cancer cells, resulting in heightened anti-tumor immunity (46, 47). Therefore, we employed a commercially available Trx1 inhibitor PX-12, which irreversibly binds to Cys-73 of Trx1, and prevents its two active site cysteines from being reduced by thioredoxin reductase (48). We first tested the PX-12 effect in HeLa:GFP-RIPK3:MLKL cells, which express RIPK3 and MLKL transgenes under the control of a Dox-inducible promoter (lane 2, Fig. 4A). Cells were treated with or without Dox for 24 h followed by treatment with Z-VAD-FMK and increasing amounts of PX-12 ranging from 3 μM to 30 μM for an additional 16 h. Cells with Dox treatment exhibited significantly higher sensitivity to PX-12 than those that were not given Dox (Fig. 5A). At 10 μM, PX-12 induced ~60% cell death (column 8), similar to the effect of T/S/Z (column 10). Importantly, co-treatment with NSA rescued PX-12-induced cell death, confirming the involvement of MLKL (column 13, Fig. 5A). We also stained the cells with the cell-impermeable DNA dye SYTOX Green. This confirmed that the observed cell death in PX-12/Z treatment is likely necroptosis and not apoptosis (Fig. 5B).

After determining that RIPK3-MLKL expression sensitized cells to PX-12-induced cell death, we evaluated the status of canonical biomarkers of necroptosis. PX-12 treatment induced MLKL phosphorylation at a putative RIPK3-specific phosphorylation site, Ser-358, indicating that RIPK3 kinase activity was positively stimulated during PX-12-induced cell death (lane 2 of Input, Fig. 5C). Next, we performed a FLAG-IP and detected RIPK1, RIPK3, and MLKL recruitment to the necrosome complex.
plex after PX-12/Z treatment (lane 2, Fig. 5C). Moreover, PX-12 induced MLKL tetramer (lane 2 and 5, Fig. 5D) and polymer formation (lane 2, Fig. 5E), demonstrated by non-reducing SDS-PAGE and SDD-AGE, respectively. Taken together, Trx1 inhibition by PX-12 led to necrosome formation, MLKL polymerization, and necroptosis (Fig. 5F).

**PX-12 induced RIPK3-independent necroptosis in NTD-DmrB cells**

Many cancer cells do not express RIPK3, including HeLa cells (21). To expand the potential usage of PX-12 in anti-cancer therapy, we tested PX-12 in NTD-DmrB-FLAG cells, which are generated in a HeLa cell background. We have shown previously that the addition of Dimerizer is sufficient to induce the homo-interaction of the DmrB domain, which leads to MLKL polymerization and ultimately necroptosis (36) (left panel, Fig. 6A). To test whether the expression of NTD-DmrB sensitized cells to necroptosis after PX-12 treatment, we treated cells with or without Dox for 24 h followed by increasing concentrations of PX-12 (1 μM–10 μM) in combination with Z-VAD-FMK for an additional 16 h. Cell death was measured by CellTiter-Glo assay. Cells expressing NTD-DmrB were significantly more sensitized to PX-12-induced cell death, which could be blocked by co-treatment with NSA (compare columns 4, 9, and 14, Fig. 6A). Cell death was confirmed to be necrotic in nature by SYTOX Green staining (Fig. 6B). PX-12 also appeared to stabilize NTD-DmrB and promoted the formation of tetramers and high molecular weight polymers (lanes 2, Fig. 6, C and D, respectively). These results suggest that inhibition of Trx1 can induce necroptosis in a RIPK3-independent manner by directly promoting MLKL polymerization (Fig. 6F).

**Discussion**

Necroptosis has garnered increasing attention over the past several years for its implications in host immune defense and disease. At the heart of the necroptotic pathway is RIPK1, RIPK3, and MLKL, the major components of the necrosome complex. In mammalian cells, RIPK3-dependent MLKL activation leads to the formation of lethal MLKL polymers (36). Current models of necroptosis indicate that MLKL polymers are responsible for disrupting the integrity of the plasma membrane. MLKL polymers were previously shown to be stabilized...
by intermolecular disulfide bonds as reducing agents such as DTT and β-mercaptoethanol readily dissociate oligomers and polymers in vitro (29, 30, 36). Yet, the mechanistic details as to how these polymers are formed remains unresolved. Herein, we identified Trx1, a thiol oxidoreductase, as a MLKL-binding partner. As depicted in our model in Fig. 4E, Trx1 interacts with MLKL under normal conditions and actively maintains MLKL in a reduced inactive state. When the necroptotic signal comes, MLKL is recruited to the necrosome and dissociates from Trx1. This is demonstrated by the observation that Trx1 interaction with MLKL decreased significantly in T/S/Z-treated cell extracts (Fig. 2C). Within the necrosome, RIPK1 and RIPK3 form large polymers and recruit many copies of MLKL. Phosphorylation of MLKL by RIPK3 leads to MLKL conformational changes, which allow locally concentrated MLKL to form intermolecular disulfide bonds. Without the reducing power of Trx1, disulfide bond-linked MLKL tetramers further polymerize to form amyloid-like polymers to induce necroptosis.

Interestingly, only a fraction of total MLKL was cross-linked to Trx1 (Fig. 1, B and C), suggesting that the majority of MLKL is not associated with Trx1 at any given time. One explanation is that the interaction between Trx1 and MLKL may be transient, which is common among an enzyme and its substrates. Previous reports indicate that the associations between Trx1 and its substrates are relatively weak and short-lived, particularly during oxidative stress. For example, Trx1 maintains ASK1 in a reduced, inactive state under normal conditions; however, increased oxidative-stress dissociates Trx1-ASK1 and induces apoptosis (43, 44). Therefore, NSA may primarily trap Trx1 molecules that come into contact with MLKL intermittently, which may explain why we were not able to co-immunoprecipitate Trx1 with MLKL using standard IP assays. As shown in Fig. 2, this problem was overcome by increasing the amount of available Trx1 using recombinant protein. Trx1 immobilized nickel beads were able to pull down endogenous MLKL from HT29 cell extracts. Notably, Trx1 preferentially bound monomeric MLKL, suggesting that active MLKL polymers have a lower affinity for Trx1 binding (Fig. 2C). Thus, recruitment into necrosome and subsequent alterations in MLKL conformation, potentially via RIPK3-dependent phosphorylation, may lead to Trx1-MLKL dissociation. To better understand this phenomenon, further analysis is required to determine the precise conformational changes in MLKL that affect Trx1 binding.
Mitochondrial dysfunction and ROS production have been observed during necroptosis, leading researchers to hypothesize that oxidation plays an important role during necroptosis (37, 38). For instance, RIPK1 was recently shown to be activated by ROS generated during TNF-α/H9251-induced necroptosis in L929 murine cells (39). Here, we demonstrate that Trx1 directly exerts its oxidoreductase activity on MLKL to suppress necroptosis. This is supported by our findings that genetic and pharmacological inhibition of Trx1 enhanced MLKL activation as seen by polymer formation, resulting in higher sensitivity to necroptosis. The extent of necroptosis induced by Trx1 inhibition highly correlates with MLKL expression level. As shown in Figs. 5 and 6, PX-12 led to much higher levels of necroptosis when MLKL expression was induced with Dox treatment. Interestingly, NTD-DmrB-FLAG cells are as sensitive to PX-12 treatment as the HeLa:GFP-RIPK3:MLKL cells even though NTD-DmrB-FLAG cells do not express RIPK3 and the expression level of NTD-DmrB transgene is ∼5 times lower than MLKL-HA-FLAG (data not shown). We believe this is because the NTD-DmrB protein does not contain the autoinhibitory C-terminal kinase-like domain, which renders it more sensitive to necrotic signals than the full-length protein. Although NTD-DmrB is an artificially constructed transgene, there are short isoforms of MLKL that exist in nature, such as NP_001135969.1 (263 aa) and XP_011521238 (293 aa), which have a truncated C-terminal kinase-like domain. It is conceivable that inhibition of Trx1 with PX-12 could potentially induce these short isoforms to polymerize and activate necroptosis (right panel, Fig. 6E).

Overall, these findings provide a deeper understanding of the regulatory networks that control MLKL activation. This is the first line of evidence to implicate Trx1 as a binding partner and suppressor of MLKL in cells. Reports linking Trx1 inhibition to apoptosis in cells is well-established (42–44); however, our results expand cell death outcomes induced by Trx1 inhibition to necroptosis. Future studies will be directed toward whether Trx1 inhibition induces necroptosis in tissues.

A new concept in cancer immune therapy is to induce necroptosis in tumors to enhance immune response against cancer cells leading to heightened anti-tumor effect (46, 47).

Figure 6. Inhibition of Trx1 activity with PX-12 induced RIPK3-independent MLKL polymerization and necroptosis in NTD-DmrB cells. A, left panel, illustration of Dimerizer-induced NTD-DmrB polymerization. Right panel, NTD-DmrB-FLAG cells were treated with DMSO, Dox, or Dox plus NSA for 24 h followed by PX-12 (1–10 μM) or Dimerizer treatment for another 16 h. Cell survival was quantified by CellTiter-Glo assay. Z-VAD-FMK (20 μM) was included in all treatments to prevent apoptosis. Triplicate samples were used for each treatment. Data are presented as the mean ± S.D. (p < 0.005, one-way analysis of variance analysis). B, NTD-DmrB-FLAG cells were treated as in A followed by SYTOX Green and Hoechst staining. The scale bar represents 20 μm. C and D, NTD-DmrB-FLAG cells were induced with Dox for 24 h followed by 10 μM PX-12/Z or Dimerizer/Z treatment for 6 h. Cell extracts were analyzed by non-reducing SDS-PAGE (C) and SDD-AGE (D), respectively. E, immunoblot. F, working model. In some cells, expressed MLKL isoforms do not contain the full-length auto-inhibitory C-terminal kinase-like domain. Inhibition of Trx1 in these cells can directly induce disulfide bond-dependent MLKL polymer formation independent of RIPK3 to promote necroptosis.
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The notion that inhibition of Trx1 with PX-12 sensitizes some cells to necroptosis could have significant implication in that regard. So far PX-12 has been mainly used to trigger apoptosis in cancer cells (49). However, it did not show significant efficacy in multiple clinical trials (50, 51). Our data point to a new direction for cancer treatment with PX-12, which is to induce necroptosis in tumors with elevated MLKL levels or with MLKL short isoform expressions. Specifically targeting these cancer patients will potentially result in better outcome with PX-12 treatment.

Materials and methods

General reagents

Recombinant TNF-α, Smac-mimetic, and anti-human RIPK3 were prepared as described before (21). The following reagents and antibodies were used: Z-VAD-FMK (ApexBio), Necrostatin-1 (Calbiochem), necrosulfonamide (Millipore), Dimerizer (Clontech, 635058), anti-FLAG M2 antibody and affinity gel (Sigma), anti-human MLKL (GeneTex, GTX107538), anti-phospho-Ser-358 of human MLKL (Abcam, ab187901), anti-RIPK1 (BD Biosciences, 551042), and anti-lactate dehydrogenase (Abcam, ab53292). The amounts of reagents used were 20 ng/ml TNF, 100 nM Smac-mimetic, 20 μM Z-VAD-FMK, 20 μM Dimerizer, and 5 μM NSA. Generally, cells were treated for 16 h for cell death analysis. For cell lysates used for Western blotting, SDD-AGE, or immunoprecipitation, cells were treated for 6 h before harvesting.

Cell culture and stable cell lines

HT-29 and HeLa cells were cultured in DMEM (high glucose) supplemented with 10% fetal bovine serum. All the HeLa stable lines were generated in the background of previously reported HeLa-TetR cells, which expressed the Tet repressor (TetR) (26).

MLKL knock-out HeLa line—MLKL knock-out in the HeLa-TetR background was generated according to the protocol described in Cong et al. (52). Briefly, oligo targeting human MLKL with the sequence GCTGCCCTGGAGGAGGCTAGG was cloned into the gRNA vector. It was cotransfected with Cas9-expressing vector into HeLa-TetR cells. MLKL knock-out was confirmed by Western blotting and sequencing.

NTD-DmrB-FLAG line—Amino acids 1–190 of human MLKL fused to the DmrB domain with a C-terminal 3×FLAG was driven by a Dox-inducible promoter. This construct was stably integrated in the MLKL knock-out HeLa cells.

HeLa:GFP-RIPK3:MLKL line—Dox-inducible GFP-RIPK3 and Dox-inducible MLKL-3×FLAG were stably expressed in the MLKL knock-out HeLa cells. For Dox-inducible expression, 50 ng/ml doxycycline was added for 24 h.

Mass spectrometric analysis

Mass spectrometric analysis was done as described before (23). Briefly, protein band was excised and de-stained and reduced followed by in gel trypsin digestion. The peptides were extracted and analyzed by a QSTAR XL mass spectrometer (AB ScieX).

Transfection

Polyjet (SignaGen Laboratories) was used for transfection according to the manufacturer’s protocol. 1 × 10⁵ cells were seeded in 10-cm dishes 24 h before transfection. Cells were harvested 48 h later.

Protein extraction and Western blotting

Cells were scraped and washed in ice-cold PBS before lysing in protein extraction buffer (PEB) composed of 20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, and freshly added protease and phosphatase inhibitors. Cells were rotated at 4 °C for 15 min before centrifugation at 20,000 × g for 20 min to clear out insoluble debris. Protein concentrations were determined by Coomassie Plus Protein Assay Reagent (Thermo Scientific). Samples were boiled in 5× SDS sample buffer (250 mM Tris, pH 6.8, 5% β-mercaptoethanol, 0.02% bromphenol blue, 30% glycerol, 10% SDS) for 5 min, resolved by SDS-PAGE, and transferred onto PVDF membranes (Bio-Rad). Membranes were incubated in 5% Blotting-Grade Blocker (Bio-Rad) reconstituted in phosphate-buffered saline (PBS) solution with 0.1% Tween 20 (PBS-T). Primary and secondary antibodies were also diluted in 5% PBS-T milk solution.

Immunoprecipitation assay

Protein samples were diluted to a final concentration of 1 mg/ml in 1 ml of PEB and incubated with 10 μl of FLAG (M2)-agarose beads (Sigma) and rotated overnight at 4 °C. M2 beads were washed with 1 ml of ice-cold extraction buffer 3 times and directly boiled in 1× SDS sample buffer. Silver-staining was performed using ProteoSilver Plus Silver Stain kit (Sigma).

Cell survival assay

Cell survival was measured using CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer’s protocol (Promega). Cells were seeded at 2 × 10³ cells/well in white flat-bottom 96-well plates (Corning) 24 h before treatment. Luminescence was measured using a BioTek Synergy 2 plate reader.

Semi-denaturing detergent agarose gel electrophoresis

SDD-AGE gels were made using TAE buffer (40 mM Tris, pH 8.6, 20 mM acetate, 1 mM EDTA) containing 1% agarose and 0.1% SDS. Protein samples were mixed with 4× sample buffer (2× TAE buffer, 20% glycerol, 8% SDS) at room temperature and run at a constant 60 V for 5 h in TAE buffer containing 0.1% SDS. Proteins were transferred onto PVDF membranes by capillary action using TBS buffer (20 mM Tris, pH 7.4, 150 mM NaCl). Membranes were treated in accordance with the Western blotting procedure.

Protein purification

Recombinant proteins were purified from Rosetta DE3 bacterial cells grown in LB media. Cultures were grown at 37 °C to an A600 of 0.6, then shifted to 18 °C and allowed to induce expression with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 16 h. The bacteria were pelleted by centrifugation at 3000 × g for 10 min, washed once in ice-cold PBS, and re-pelleted before being resuspended in 40 ml of binding buffer. For HA-Trx1-His6, binding buffer contained 20 mM Tris, pH 8.6, 20 mM acetate, 1 mM EDTA) containing 1% agarose and 0.1% SDS. Protein samples were mixed with 4× sample buffer (2× TAE buffer, 20% glycerol, 8% SDS) at room temperature and run at a constant 60 V for 5 h in TAE buffer containing 0.1% SDS. Proteins were transferred onto PVDF membranes by capillary action using TBS buffer (20 mM Tris, pH 7.4, 150 mM NaCl). Membranes were treated in accordance with the Western blotting procedure.
Thioredoxin-1 suppresses MLKL polymerization and necroptosis

8.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 20 mM imidazole, 10 mM β-mercaptoethanol, 1 mM PMSF, and an EDTA-free protease inhibitor mixture. Cells were lysed by sonication pulsing 15 times for 30 s each time. Lysates were centrifuged at 10,000 × g for 20 min and filtered before incubation with 100 μl of nickel-nitrilotriacetic acid-agarose beads (Qiagen). Binding was performed at 4 °C for 30 min. Nickel beads were washed 3 times in binding buffer lacking β-mercaptoethanol and eluted with binding buffer containing 250 mM imidazole. 100-μl fractions were collected and dialyzed against PBS.

For recombinant GST-NTD-FLAG, 100 μl of glutathione-agarose beads (Pierce) were used with binding buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 20 mM DTT, 1 mM PMSF, and EDTA-free protease inhibitor mixture. GST-NTD-FLAG was eluted in 100-μl fractions with binding buffer containing 10 mM reduced L-glutathione and dialyzed against PBS.

Cell staining
Cells were seeded at 1 × 10⁶ cells/well in a 12-well plate (Cellstar). SYTOX Green and Hoechst dyes were added directly to culture media at 1 μM and incubated for 10 min before imaging with a BioTek Cytation 3 plate reader.

In vitro GST-NTD-FLAG polymerization assay
Recombinant GST-NTD-FLAG was used at a final concentration of 5 μM in 20 μl of PBS. HA-Trx1-His₆ was used at a final concentration of 3 μM, 10 μM, and 30 μM. To induce polymerization, samples were incubated at 37 °C for 16 h, and 50 ng of GST-NTD-FLAG was used for SDD-AGE.

Trx1 shRNA knockdown
Trx1 shRNA was cloned into a modified pSuperior-puro (Oligoengene) vector. The Dox-inducible shTrx1 cassette was then subcloned into a lentiviral transfer vector containing hygromycin-resistant gene (Addgene). Lentivirus was produced by transfecting HEK293T cells with Trx1-shRNA viral vector, pMD2.G, and psPAX2 vectors in a 10-cm plate. Lentiviral media was collected at 48 h and 72 h post-transfection, filtered through a 0.45-μm sterile filter, and stored at 80 °C.

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