Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death

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Necroptosis is considered to be complementary to the classical caspase-dependent programmed cell death pathway, apoptosis. The pseudokinase Mixed Lineage Kinase Domain-Like (MLKL) is an essential effector protein in the necroptotic cell death pathway downstream of the protein kinase Receptor Interacting Protein Kinase-3 (RIPK3). How MLKL causes cell death is unclear, however RIPK3-mediated phosphorylation of the activation loop in MLKL trips a molecular switch to induce necroptotic cell death. Here, we show that the MLKL pseudokinase domain acts as a latch to restrain the N-terminal four-helix bundle (4HB) domain and that unleashing this domain results in formation of a high-molecular-weight, membrane-localized complex and cell death. Using alanine-scanning mutagenesis, we identified two clusters of residues on opposing faces of the 4HB domain that were required for the 4HB domain to kill cells. The integrity of one cluster was essential for membrane localization, whereas MLKL mutations in the other cluster did not prevent membrane translocation but prevented killing; this demonstrates that membrane localization is necessary, but insufficient, to induce cell death. Finally, we identified a small molecule that binds the nucleotide binding site within the MLKL pseudokinase domain and retards MLKL translocation to membranes, thereby preventing necroptosis. This inhibitor provides a novel tool to investigate necroptosis and demonstrates the feasibility of using small molecules to target the nucleotide binding site of pseudokinases to modulate signal transduction.

Significance

The four-helix bundle (4HB) domain of Mixed Lineage Kinase Domain-Like (MLKL) bears two clusters of residues that are required for cell death by necroptosis. Mutations within a cluster centered on the \textit{c} helix of the 4HB domain of MLKL prevented its membrane translocation, oligomerization, and ability to induce necroptosis. This cluster is composed principally of acidic residues and therefore challenges the idea that the 4HB domain engages negatively charged phospholipid membranes via a conventional positively charged interaction surface. The importance of membrane translocation to MLKL-mediated death is supported by our identification of a small molecule that binds the MLKL pseudokinase domain and retards membrane translocation to inhibit necroptotic signaling.

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cardiolipin, which facilitates its disruption of membrane integrity (17), a model supported by a subsequent paper (18).

Here, we show that the MLKL 4HB domain is sufficient to induce necroptosis and identify several charged residues clustered on two faces that are required for this function. Surprisingly the polarity of several of these charged residues is not conserved between mouse and human MLKL, and alanine substitution of negatively charged residues on the α4 helix of the 4HB domain disrupted function. This finding challenges the importance of phospholipid binding to the killing activity of the 4HB domain and illustrates that membrane association cannot solely be attributed to the interaction of poorly conserved basic residues within the MLKL 4HB domain. Intriguingly, mutation of a second cluster of residues on the 4HB domain did not preclude membrane localization or oligomerization but did prevent cell death, illustrating that additional function(s) beyond membrane translocation are required for the 4HB domain to induce cell death. MLKL oligomerization and membrane translocation were also inhibited by a small molecule, compound 1, which we identified on the basis of its affinity for the nucleotide binding site of the MLKL pseudokinase domain. These data support a model for MLKL function whereby the pseudokinase domain of MLKL holds the 4HB domain in check until phosphorylated by RIPK3, which causes a conformational change in the pseudokinase domain to allow the 4HB domain to oligomerize and associate with membranes. Activation of MLKL can be thwarted by a small MLKL binding molecule, indicating the feasibility of targeting the nucleotide binding or “pseudokinase” sites of pseudokinases, a hitherto unexplored class of therapeutic targets.

Results

The N-Terminal 4HB Domain of MLKL Is the Necroptotic Effector Domain.

We sought to define the contributions of MLKL’s component domains to necroptotic signaling in light of our recent X-ray crystal structure and functional analysis of full-length mouse MLKL (1). We inducibly expressed a suite of mouse MLKL truncation constructs (Fig. L4) in Mlkl−/− and wild-type mouse dermal fibroblasts (MDFs). Their capacity to induce cell death was determined by propidium iodide (PI) uptake using flow cytometry, in the absence or presence of the necroptotic stimulus, TNF (T), Smac mimetic (S), and the pan-caspase inhibitor Q-VD-OPh (Q). TNF initiates signaling upon ligation of TNF Receptor 1 on the cell surface, Smac mimetic inhibits the E3 ubiquitin ligase activity of the cellular Inhibitor of Apoptosis proteins known to ubiquitinate and prevent the participation of RIPK1 in apoptotic and necroptotic signaling, and Q-VD-OPh inhibits the activity of caspase-8, thereby preventing the cleavage and inactivation of RIPK1 (19, 20). As we previously showed (1), Mlkl−/− MDFs are sensitive to TS-induced apoptosis but are resistant to TSQ-induced necroptosis, and constructs encoding untagged full-length mouse MLKL reconstitute TSQ-induced necroptosis in Mlkl−/− MDFs (Fig. 1B). Surprisingly, tagging the N terminus of MLKL with the eight-amino-acid FLAG-tag prevented full-length MLKL from reconstituting necroptosis in Mlkl−/− MDFs (Fig. 1C), despite being expressed (Fig. S1A). Expression of different C-terminal pseudokinase domain constructs (Fig. 1D and E) also failed to reconstitute the necrotic pathway when overexpressed in Mlkl−/− MDFs. On the contrary, inducible expression of MLKL(124–464), encompassing the brace and pseudokinase domain, inhibited TSQ-stimulated cell death by ∼50% in wild-type MDFs compared with the uninduced controls (Fig. 1F).

These data point to an essential role for the N-terminal domain of MLKL in necroptotic signaling, while implicating the C-terminal pseudokinase domain as a suppressor of MLKL-mediated cell death. Indeed, inducible expression of untagged MLKL constructs that lacked the pseudokinase domain in either Mlkl−/− or wild-type MDFs led to constitutive cell death in the absence of TSQ stimulation (Fig. 1G and H). The capacity of MLKL(1–180) to induce cell death was independent of caspase and RIPK3 kinase activities (Fig. 1G) or the presence of RIPK3 (Fig. 1H). We principally characterized MLKL fragments encompassing residues 1–180 because our monoclonal MLKL antibody (3H1) recognizes the brace region (Fig. 1A), and like the full-length MLKL, addition of an N-terminal FLAG- or HA-tag prevented its killing activity in wild-type and Mlkl−/− cells (Fig. 1C and J and Fig. S1 B and C). However, the 4HB domain alone was sufficient to induce cell death in the absence of TSQ stimulation in both Mlkl−/− and wild-type MDFs (Fig. 1K and L). These results demonstrate that the 4HB domain alone mediates the killing function of MLKL, and this function is suppressed in the context of full-length MLKL by its pseudokinase domain until activation by RIPK3-mediated phosphorylation.

Two Charged Clusters on the 4HB Surface Are Essential for MLKL-Induced Cell Death.

Having established that the 4HB domain of MLKL mediates necroptotic cell death, we performed alanine-scanning mutational analysis to define key residues(s) required for this function. We selected residues on the surface of the 4HB domain, based on our recent mouse MLKL structure (Fig. 1A) (1), and mutated constructs of typically two to three residues to alanine (Fig. 2A). We did not mutate residues that participate in the helical core of the 4HB domain because such mutations easily disrupt domain folding and/or stability. Mutations were introduced

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Fig. 2. Residues required for 4HB killing cluster into two motifs. (A) Alignment of MLKL orthologs. Groups of mutated residues are indicated by lowercase letters. Residues in red when mutated to Alanine prevented 4HB killing in both Mikt−/− and wild-type cells; mutation of residues highlighted in orange prevented 4HB killing in Mikt−/− but not wild-type cells. (B) Three biologically independent MDF cell lines derived from wild-type and mutant constructs and each assayed in two independent experiments. Cell lines were induced for 20 h (white bars) or not (black bars) before viability was quantitated. All data are plotted as mean ± SEM. (C) Depiction of the 4HB domain (Protein Data Bank ID code 4BTF) (1) drawn using PyMOL software with residues colored according to the scheme in panel A.

into the MLKL(1-180) construct, as expression of these untagged constructs could be monitored using the 3H1 antibody. Wild-type and mutant MLKL(1-180) constructs were inducibly expressed in Mikt−/− (Fig. 2A and 2B) and wild-type (Fig. 2A and 2B) MDFs, and were deemed to have retained necroptotic killing function when the percentage of PI-positive cells was ≥threefold higher than in uninduced controls. As before, induction of wild-type MLKL(1-180) killed both Mikt−/− and wild-type MDFs (Figs. 1 G and H and 2A and 2B), but a subset of the mutant MLKL(1-180) constructs were unable to kill, despite expressing similar levels as wild-type MLKL(1-180) (Fig. S2B).

One group of MLKL(1-180) mutants were unable to induce cell death in either Mikt−/− or wild-type MDFs: C18S/C24S/C28S, K22A/R30K, R63A/D65A, K80A/K81A, H98A/E99A, E102A/K103A, R105A/D106A, E109A/E110A, and LLLL112–115AAA (highlighted in red in Fig. 2A and C). On the other hand, the Y15A/E16A, E70A/N72A, and E76A/K77A mutations prevented MLKL(1-180)-induced cell death when expressed in Mikt−/− MDFs, but not wild-type MDFs. These residues (highlighted in orange in Fig. 2A and C) are therefore essential for 4HB domain killing but can induce cell death via a mechanism dependent on endogenous MLKL.

The mutations that compromise 4HB domain function in cell death were centered on two clusters positioned on opposing sides of the domain (Fig. 2C). Cluster 1 centered around the α1 helix (E102/K103, R105/D106, E109/E110, and L112–L115) and included residues from the adjacent α3 helix (R63/D65), and cluster 2 centered around the C-terminal part of the α1 helix, the N-terminal part of the α2 helix, and the connecting α1–α2 loop (Y15/E16, K22/R30, and C18/C24/C28) and includes residues from the α3–α4 loop that was disordered in the MLKL structure (K80A/K81A).

Membrane Localization of MLKL Is Not Sufficient to Induce Cell Death. We sought to establish the localization of MLKL by performing subcellular fractionation and Blue-Native PAGE of wild-type MDFs ± TSO-induced necroptosis. These studies revealed that MLKL resides in the cytoplasm (C) of a healthy cell in a complex that migrates similarly to the 146-kDa molecular weight marker (complex I; Fig. 3A) until necroptosis is induced, when it translocated to the 0.025% digitonin-insoluble, 1% digitonin-soluble cell fraction (containing most biological membranes; M) and formed part of a complex that migrated above the 480-kDa molecular weight marker (complex II; Fig. 3A) with concomitant depletion from the cytoplasm. These complexes, formed from endogenous (rather than tagged or overexpressed) MLKL, were extracted from cells and resolved on Blue-Native PAGE under nondenaturing conditions in the presence of the N-ethyl maleimide, a compound that reacts with free thiols. Previous demonstrations of MLKL oligomerization in situ have used nonreducing SDS/PAGE (15–18). Although the physiological relevance of the observed disulfide bonding and the reported oligomer stoichiometry to MLKL function remains to be established, all reports to date (15–18) support the idea that MLKL association with the plasma membrane is an essential step for the induction of necroptotic cell death. We tested this hypothesis by monitoring membrane translocation of wild-type MLKL(1-180) when expressed in Mikt−/− MDFs (Fig. 3B). MLKL(1-180) translocated from the cytoplasm to the membrane following induction of protein expression, and like full-length MLKL, MLKL(1-180) was incorporated into a higher molecular weight complex (complex II) by Blue-Native PAGE. Based on migration relative to molecular weight markers in Blue-Native PAGE, we speculated that cytoplasmic full-length MLKL (Fig. 3A) and MLKL(1-180) (Fig. 3B) formed homotrimeric, Allosteric ultracentrifugation studies of recombinant MLKL(1-169) confirmed that the MLKL 4HB and brace exists as a stable homotrimer in solution with a Kd of 7.2 ± 0.7 × 10⁻⁵ μM² (Fig. S3 A–C), and like the cytosolic fraction of cells expressing MLKL(1-180) (Fig. 3B), recombinant MLKL(1-169) comigrated with the 66-kDa marker in Blue-Native PAGE (Fig. 3C).

Accumulation of MLKL(1-180) at the membrane correlated with the time course of cell death postinduction in Mikt−/− MDFs in the absence of TSO stimulation (Fig. 3D). This suggested that lack of necroptotic potency among the loss-of-function mutants might arise from defective translocation to the membrane fraction. We therefore selected eight MLKL(1-180) constructs for closer examination. The cluster 1 mutants—R63A/D65A, E102A/K103A, R105A/D106A, E109A/E110A, and LLLL112–115AAA—showed negligible or no capacity to incorporate into high–molecular-weight complexes in the membrane fraction, despite high levels of expression (Fig. 3D). Small amounts of R105A/D106A and E109A/E110A mutants were detected in membrane fractions, but notably did not assemble into high molecular complexes (complex II; Fig. 3D). Interestingly, in contrast to the cluster 1 mutants, the cluster 2 mutant, Y15A/E16A, and to a lesser extent C18S/C24S/C28S and N-terminally FLAG-tagged wild-type MLKL(1-180) were capable of membrane translocation and formed the high–molecular-weight complexes typical of cell death–inducing complexes, but did not induce cell death in Mikt−/− MDFs. Despite observing less translocation of
MLKL and the 4HB domain of MLKL form high-molecular-weight complexes in biological membranes. (A) Blue-Native PAGE showed that endogenous MLKL translocated from cytoplasm to membrane fraction in wild-type MDFs following TSQ treatment. (B) MLKL(1–180) similarly translocated to the membranes of MLKL(1–180) MDVs following induction. (C) Recombinant MLKL(1–169) resolved by Blue-Native PAGE. (D) Membrane complex (complex II) formation monitored by Blue-Native PAGE after a 6-h induction of wild-type MLKL(1–180), N-FLAG-tagged MLKL(1–180), cluster 2 mutants (Y15A/E16A and C185/C245/C285), and cluster 1 mutants (R63A/D65A, E102A/K103A, R105A/D106A, E109A/E110A, and LLLL112–115AAAA). Data presented were obtained from three independent experiments performed on two biological replicate cell lines.

The C185/C245/C285 than Y15A/E16A mutant to the membrane fraction, both mutations are spatially adjacent (forming “cluster 2”) and exhibit deficits in cell death signaling. These data demonstrate that the formation of high-molecular-weight, MLKL-containing complexes in biological membranes alone is insufficient to initiate cell death. We then examined the cluster 1 mutations, R105A/D106A and E109A/E110A, and the cluster 2 mutation, Y15A/E16A, in the context of full-length MLKL. Cluster 1 mutations completely compromised the ability of full-length MLKL to reconstitute necrotic signaling in MLKL(1–180) MDVs and partially antagonized endogenous MLKL to reduce TSQ-induced death in wild-type MDFs (Fig. S4 A and D). However, in the context of full-length MLKL, the cluster 2 mutant, Y15A/E16A, restored TSQ sensitivity to MLKL(1–180) MDVs (Fig. S4 A and D). These data support the idea that cluster 2 is dispensable for MLKL to assemble into a high-molecular-weight, membrane-associated complex, but is crucial for orienting protomers within the complex or binding to a downstream effector to induce death, a function that can be complemented by the pseudokinase domain in the context of full-length MLKL.

A Small-Molecule Compound That Binds the MLKL Nucleotide Binding Site Retards Membrane Association to Inhibit Necroptosis. Based on these observations and our recent studies (1, 14), we hypothesized that the pseudokinase domain functions as a switch that, until activated, restrains the necrotic activity of the 4HB domain. Our data suggest a model whereby release of the 4HB domain occurs following a conformational change in the pseudokinase domain induced by RIPK3 phosphorylation. Previously, using thermal shift assays, we established that the pseudokinase domain of MLKL could engage nucleotides in a conformation-independent, noncatalytic manner (1, 14, 21), although the biological significance of nucleotide binding is unclear. To probe the role of nucleotide binding in MLKL function, we screened a library of 367 small molecules (22) against the recombinant mouse MLKL pseudokinase domain using a thermal stability shift assay (21, 23) and identified compound 1 as an MLKL inhibitor (Fig. 4 A and B). Subsequent analyses by Surface Plasmon Resonance (SPR) provided further validation that compound 1 bound the MLKL pseudokinase domain (Fig. 4 C and Fig. S5 A), yielding a value of 9.3 μM. Saturating difference NMR (STD–NMR) studies inferred that compound 1 bound the nucleotide binding site in the MLKL pseudokinase domain, because compound 1 competed with either ATP or ADP for binding to MLKL (Fig. S5 B). This idea was further supported by thermal shift assays where compound 1 exhibited diminished binding to K219M MLKL, a mutant known to possess defective ATP binding compared with wild-type MLKL (Fig. S5 C) (1). We next examined the capacity of compound 1 to inhibit TSQ-induced necroptosis in cells. Compound 1 rescued 50% of wild-type MDFs from TSQ-induced necroptosis, with an IC50 < 50 nM, with >50-fold greater potency than Nec-1, when 1 ng/mL TNF was used (Fig. 4D). However, supraphysiological TNF concentrations (100 ng/mL) overwhelmed the ability of compound 1 to inhibit necroptosis, leading to an IC50 value of 100–500 nM with maximally ~50% of cells protected from TSQ-induced death (Fig. S5D). Although compound 1 bound the MLKL pseudokinase domain with a relatively high Kd in vitro, the efficiency of compound 1 as an inhibitor of necroptosis in cells may be attributed to the slow off-rates observed in SPR studies. This indicates that despite a significant energy penalty during the association phase, the resulting complex, between compound and protein is relatively stable (Fig. 4C). We observed that compound 1 affected cell viability at high concentrations (above 5 μM; Fig. 4D), presumably due to off-target effects. Consequently, we used 1 μM compound 1 in subsequent experiments. Compound 1 has previously been described as a nanomolar inhibitor of the protein kinase, VEGFR2 (24), raising the possibility that inhibition of VEGFR2 might block necroptosis. However, sorafenib, a potent VEGFR2, Ret, and c-Kit inhibitor, was unable to inhibit necroptosis in wild-type MDFs (Fig. S5E). Although we cannot exclude the possibility that compound 1 inhibits other targets in addition to MLKL, the experiments with sorafenib suggest that inhibition of VEGFR2, Ret, and c-Kit cannot prevent necroptosis.

To establish the mechanism by which compound 1 binding to the MLKL pseudokinase domain inhibited necroptosis, we examined whether compound 1 might prevent phosphorylation of MLKL by its upstream activator, RIPK3, using in vitro kinase assays, but neither the catalytic activity of recombinant RIPK3 nor RIPK3-mediated phosphorylation of MLKL were inhibited by compound 1 (Fig. S5 F). On the contrary, in the presence of 10 μM of compound 1, RIPK3-mediated phosphorylation of MLKL was enhanced. Mass spectrometry analyses confirmed that phosphorylation of established activation loop substrate residues S345 and S347 (1) was enhanced (Fig. S6), consistent with compound 1 increasing solvent exposure of the MLKL activation loop. We therefore tested whether compound 1 inhibited the membrane localization of endogenous MLKL in wild-type MDFs following TSQ-induced necroptosis. TSQ stimulation of MDFs led to the accumulation of MLKL in the membrane fraction over a 6-h time course, and this was reproducibly retarded in three independent experiments by preincubation of MDFs with 1 μM of compound 1 (Fig. 4E). Consistent with a mode of action targeting the MLKL pseudokinase domain, compound 1 (i) did not protect MDFs from MLKL(1–180)-mediated death (Fig. S5G) and (ii) conferred no significant protection on the predominantly apoptotic death arising from TS stimulation (Fig. S5D). Collectively, these
Compound 1, a small molecule targeting the nucleotide binding site of the MLKL pseudokinase domain, retards MLKL membrane translocation and inhibits necroptosis. (A and B) Compound 1 was identified as an MLKL interactor using a thermal stability shift assay. (C) Compound 1 binding to the MLKL pseudokinase domain was validated by SPR. Sensorgrams show the kinetics of compound 1 binding (at a given concentration from 6.25 to 200 μM) to MLKL (colored curves) with fit to model overlaid (black curves). y axis, time (s); x axis, response unit (RU) levels. (D) Compound 1 and Nec-1 inhibited necrotic death of wild-type MEFs stimulated with TSQ (1 ng/mL TNF, 500 nM compound A, 10 μM Q-VD-OPh) in a dose-dependent manner. Data shown are the mean ± SEM for three independent experiments. (E) Compound 1 (1 μM) retarded MLKL translocation to the membrane. Cytoplasmic and membrane fraction purity and protein abundance are illustrated by control blots for GAPDH and VDAC1. Data are representative of three independent repeats. (F) A model for MLKL activation and the mechanism of action of compound 1.

Findings support a model in which binding of an ATP mimetic to the pseudokinase domain of MLKL can jam the switch mechanism, thereby preventing RIPK3-mediated phosphorylation of MLKL from inducing a conformational change within the pseudokinase domain to unleash the necrotic death effector, the 4HB domain (Fig. 4F).

Discussion

Although the pseudokinase MLKL has been confirmed as a necrotic effector downstream of the protein kinase RIPK3, it is unclear how it induces cell death. We show that the 4HB domain of MLKL is sufficient to induce necroptosis and is able to oligomerize and translocate to membranes without an ectopic oligomerization domain or any other stimulus. Two clusters of amino acids on opposing faces of the 4HB domain are required for killing by this domain, and mutations in these clusters affect the ability of the 4HB domain to either form high-molecular-weight complexes and/or localize to membranes or to induce death once incorporated into high-molecular-weight, membrane-associated complexes. Notably, solvent exposure of both 4HB domain clusters in our recent structure of full-length MLKL (1) is highly suggestive that the crystallized conformation represents an activated form of MLKL. Endogenous MLKL also translocates to the membrane fraction following a necrotic stimulus, and this translocation and ensuing cell death can be inhibited by a small molecule that binds the ATP binding site of MLKL.

Several groups have shown that MLKL can oligomerize, but there is no consensus regarding the stoichiometry of the oligomer—Cai et al. reported it contains three protomers (15), whereas others claim fourteen (16) and possibly six (17) units. Chen et al. showed that the N-terminal domain of MLKL(1–130) was sufficient to trigger necroptosis, formed tetramers, and translocated to lipid rafts in the plasma membrane (16). However, to induce death in their hands, forced oligomerization of the 4HB domain with an inducible dimerization domain was required. In contrast, we show that the MLKL 4HB domain is sufficient to induce death by necroptosis and does not require fusion to an exogenous oligomerization domain. Additionally, our Blue-Native PAGE and analytical ultracentrifugation studies support the idea that both endogenous MLKL and the N-terminal domain of MLKL exist as stable homotrimers in the cytoplasm, before their incorporation into membrane-associated, high-molecular-weight complexes and subsequent necrotic cell death.

We were intrigued by the poor conservation of charged residues in the 4HB domain between orthologs from different species, often with oppositely charged amino acids (Fig. 2A). We therefore chose to replace charged residues, as well as a partially conserved, solvent-exposed hydrophobic stretch (L141–L145), with alanine, and potential metal coordinating cysteines C126, C128, which were also partially conserved, with serine. None of these mutations are expected to disrupt the structure of the 4HB domain, and accordingly, the expression levels of all mutants were comparable to wild-type MLKL (1–180). When we tested these mutants in wild-type and MLKL+/−/− cells, we identified three classes of mutants: The first class did not affect MLKL function, and these mutants were dispersed over the α1, α2, and α3 helices and the α3–α4 loop; the second completely prevented 4HB domain killing whether expressed in MLKL+/−/− or wild-type cells; and the third class of mutants, located on the α1 and α3 helices, presumably retained the ability to interact with other MLKL because they killed wild-type but not MLKL+/−/− cells. Loss-of-function mutations clustered into two groups on opposite faces of the 4HB domain, suggesting that the 4HB domain performs two independent activities required to kill cells. Although the cluster 2 mutants retained the ability to form high-molecular-weight complexes (complex II) in membranes, mutations on the opposite face of the 4HB domain (cluster 1; on the α4 helix) prevented formation of complex II in the membrane fraction. While membrane localization is required for MLKL complex II formation or complex II formation is required for membrane localization currently remains unclear.

It is notable that the polarity of the charged residues that we mutated is not well conserved among MLKL proteins from different species, although the presence of charged residues in these positions is. For example, mouse MLKL has a glutamate or aspartate in positions 16, 56, and 65, whereas human MLKL has a lysine in these positions. Human MLKL has glutamates in positions 20 and 70, whereas mouse MLKL has lysines in these positions. This suggests, in the possibility of charged pairs interacting between MLKL protomers, although the charged inversion is not conserved in all species. Recent studies suggested that MLKL can bind negatively charged phospholipids (17, 18), and this function was attributed to nine basic residues in the 4HB domain because simultaneous charge reversal of all nine residues compromised phospholipid binding (18). In contrast, our observation that the requirement for a charged residue, rather than strictly a basic residue, argues against charged residues being important for interaction with the phospholipids in the plasma membrane and does not support the idea that phospholipid, and thus membrane, binding is mediated by these poorly conserved basic residues via a simple positive–negative charge pairing. In contrast to previous observations (15–17), we observed by Blue-Native PAGE that endogenous MLKL exists in a cytoplasmic complex of ~150 kDa (complex I) in the absence of exogenous necrototic stimuli, but is incorporated into a much...
larger complex (complex II) in membranes following treatment with necroptotic stimuli. A similar phenomenon was observed upon induction of MLKL(1–180) expression in MLKL−/− dermal fibroblasts, indicating a correspondence between assembly of an MLKL-containing, high-molecular-weight complex in biological membranes and necroptotic signaling. The composition of the high-molecular-weight, membrane-associated complex (complex II), however, is currently unclear and remains a subject of ongoing investigation.

Having established the importance of pseudokinase site integrity for the MLKL pseudokinase domain to function as a negative regulator of necroptosis (Fig. 1F and ref. 1), we hypothesized that small molecules that bind the nucleotide binding site of MLKL might antagonize necroptosis. Initially, we identified an ATP mimetic (termed compound 1) that bound recombinant mouse MLKL pseudokinase domain, which we subsequently showed inhibited TSQ-induced death of MDFs by delaying MLKL translocation to the membrane. Compound 1 therefore not only represents a valuable reagent to inhibit necroptosis to aid discovery in this field but, more broadly, provides an important proof-of-principle that targeting catalytically dead pseudoenzymes represents a feasible, emerging therapeutic avenue.

Materials and Methods

Expression Constructs. Mouse MLKL cDNA (encoding residues 1–464), PCR-derived mutants, or a library of MLKL(1–180) mutants (DNA2.0, CA) were cloned into the downstream-inducible, purorogenic selectable vector pTRE3G Puro (28), as previously (1, 11, 25). Lentiviruses were generated in HEK293T cells (26) before injection of target cells and selection/maintenance in 5 μg/mL puromycin.

Reagents and Antibodies. Recombinant hTNF-Fc (27), rat anti-mouse MLKL monoclonal antibody (clone 3H1; available from Millipore, cat. no. MABC604) (1) and compound 1 (available from Synkinase, Australia) were produced in-house. Smac mimetic, compound A, was described previously (26). GO-OPH was from R&D Systems. Anti-β-actin and anti-FLAG (M2) antibodies were purchased from Sigma Aldrich; anti-VDAC1 (AB10527) was purchased from Millipore; anti-GAPDH was purchased from Cell Signaling Technologies; and HRP-conjugated secondary antibodies were from GE Healthcare or Jackson Immunoresearch, with the ECL reagent from Millipore.

Cell Lines and Cell Death Assays. Three biologically independent MDF cell lines were generated from wild-type, MLKL−/−, and Ripk3−/− mice and cell death assays performed as described previously (1). Cells were attached over 4 h in the presence of 10 ng/mL doxycycline before addition of death stimuli (Fig. 1), or incubated with 10 ng/mL doxycycline for 20 h (Fig. 2 and Fig. S2), to induce protein expression from stably transfected constructs and PI-positive cells quantified by flow cytometry.

Fractionation and Blue-Native PAGE. MDFs were stimulated with TSQ (WT MDFs) or doxycycline [MLKL(1–180)] and permeabilized in buffer containing 0.025% digitonin. Cytosolic and crude membrane fractions were further solubilized in 1% digitonin, resolved by Bis·PAGE or BioRad, and analyzed by Western blotting with a variety of cytokine/chemokine antibodies.

SPR Binding Experiments. The kinetics of compound 1 (6.25–200 μM) binding to MLKL(1–179) was determined by SPR (Biacore T200, GE Healthcare) following protein capture via Ni2+/NTA chelation. The Kd was determined from a global fit of data to a two-state kinetic interaction model.

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