Identification of MLKL Binding Proteins as Novel Regulators of Necrosis

College of Veterinary Medicine, University of Missouri

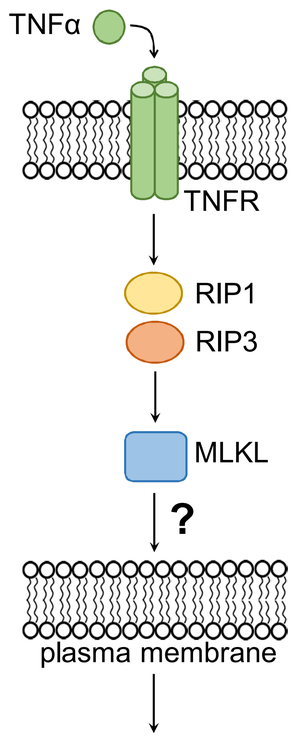
KNW Deininger, KD Marshall, M Gutiérrez-Aguilar and CP Baines

Department of Biomedical Sciences, Dalton Cardiovascular Research Center
University of Missouri, Columbia, MO 65211

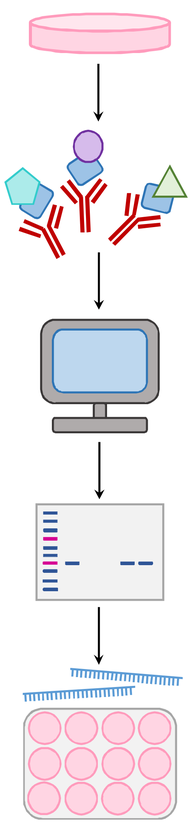
BACKGROUND

- Programmed cell death plays a vital role in both physiological and pathological processes from limb development during embryogenesis to disease pathogenesis in multiple organs.
- Until recently, apoptosis was thought to be the only form of programmed cell death and necrosis was assumed to occur “accidentally”, yet new findings suggest that necrosis may be just as programmed.
- Activation of TNFα receptors has been shown to trigger an intracellular kinase cascade that leads to necrosis; one kinase essential for TNFα-induced necrosis is mixed lineage kinase domain-like (MLKL).
- However, despite MLKL’s importance in mediating necrosis, the downstream targets of MLKL remain unknown.

MLKL Mediates TNFα-induced Necrosis



STUDY OVERVIEW & OBJECTIVES



Transfect HEK-293 cells with GFP (control) and MLKL-Myc-FLAG plasmids

Immunoprecipitate the MLKL-protein complexes using an anti-FLAG antibody

Identify the proteins in the MLKL complexes using LC-MS/MS

Verify the MLKL-protein interactions by co-immunoprecipitation assays

Knockdown the candidate proteins in MEFs using siRNA and test the effects on TNFα-induced necrosis

RESULTS

Overexpression of MLKL in 293 Cells

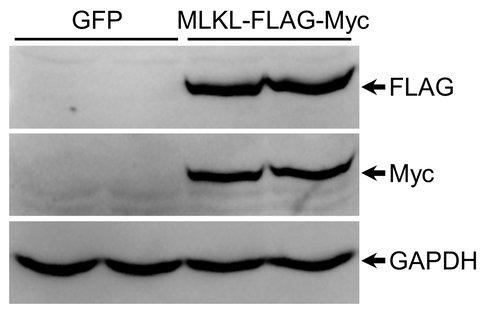


Figure 1: Overexpression of MLKL in 293 cells. *HEK-293 cells were transfected for 48 hours with a plasmid encoding GFP (control) or a plasmid encoding MLKL with c-terminal Myc and FLAG tags. Cells were lysed and subjected to Western blotting for FLAG and Myc. GAPDH was used as a control.*

Immunoprecipitation of MLKL

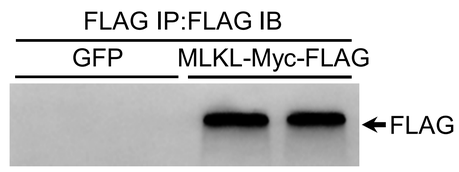


Figure 2: Immunoprecipitation of MLKL from 293 cells. *HEK-293 cells were transfected for 48 hours with a plasmid encoding GFP (control) or a plasmid encoding MLKL with c-terminal Myc and FLAG tags. Cells were lysed and MLKL immunoprecipitated using an anti-FLAG antibody. The complexes were then subjected to Western blotting for FLAG.*

Proteomic Identification of MLKL-Binding Proteins

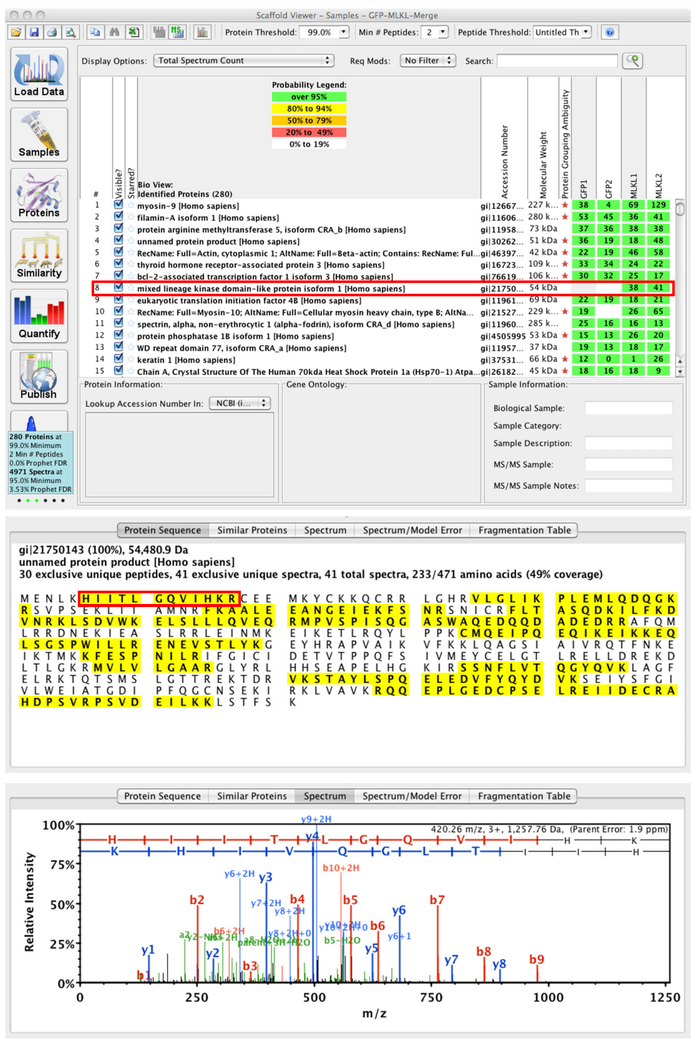


Figure 3: Proteomic identification of MLKL binding proteins. *HEK-293 cells were transfected for 48 hours with a plasmid encoding GFP (control) or a plasmid encoding MLKL with c-terminal Myc and FLAG tags. Cells were lysed MLKL immunoprecipitated using an anti-FLAG antibody. The complexes were eluted from the agarose beads using a FLAG peptide. The eluates were then digested with trypsin and the peptides subjected to LC-MS/MS using a Proxeon LC system coupled to an LTQ Orbitrap mass spectrometer. The data were then searched against NCBI database using Sorcerer, and the results examined using Scaffold.*

Candidate MLKL Binding Proteins

Protein	GFP Counts	MLKL Counts	Subcellular localization
EFhd2	0,0	2,2	Plasma Membrane (Lipid rafts)
Annexin-A2	2,2	7,8	Plasma Membrane (Lipid rafts)
CD59	0,2	5,3	Plasma Membrane
RGS14	0,0	2,2	Plasma Membrane
Nucleophosmin	0,0	2,4	Nucleus, Cytoplasm
Peroxiredoxin-1	0,0	4,4	Cytoplasm
Tropomyosin-α3	0,0	6,11	Contractile Fibers, Cytoskeleton
Tropomyosin-α4	2,1	9,18	Contractile Fibers, Cytoskeleton
Drebrin-1	4,5	17,18	Cytoskeleton
α-actinin-4	1,0	21,10	Cytoskeleton
Gelsolin	0,0	3,3	Cytoskeleton

Interaction of EFhd2 and Annexin-A2 With MLKL

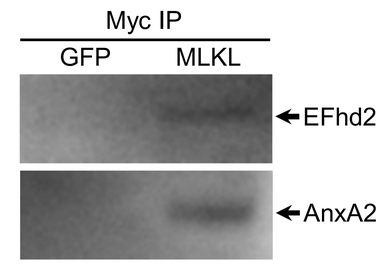


Figure 4: Immunoprecipitation of MLKL from 293 cells. *HEK-293 cells were transfected for 48 hours with a plasmid encoding GFP (control) or a plasmid encoding MLKL with c-terminal Myc and FLAG tags. Cells were lysed and MLKL immunoprecipitated using an anti-Myc antibody. The complexes were then subjected to Western blotting for EFhd2 and Annexin-A2.*

Effects of EFhd2 and Annexin-A2 siRNA on TNFα-Induced Necrosis

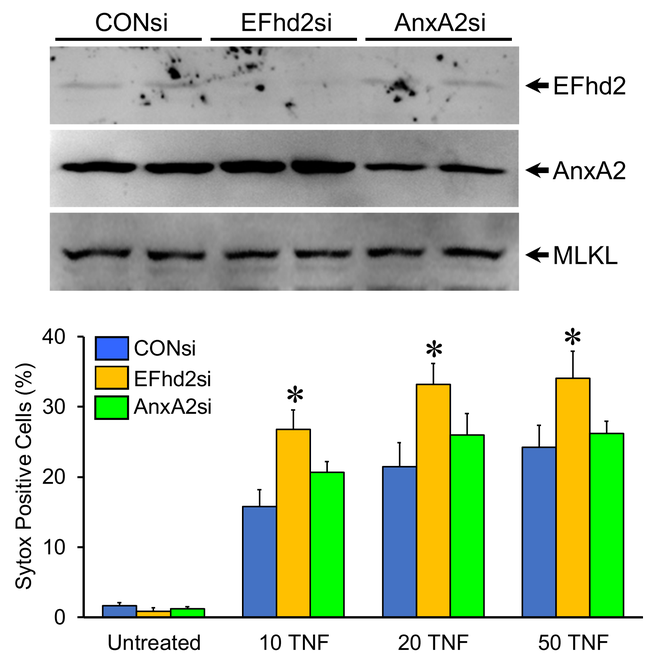


Figure 5: Effects of EFhd2 and Annexin-A2 siRNA on TNFα-induced necrosis. *Mouse embryonic fibroblasts (MEFs) were transfected for 48 hours with control, EFhd2, or Annexin-A2 siRNAs. The cells were lysed and Western blotted for EFhd2, Annexin-A2, and MLKL. Having confirmed knockdown of each protein, transfected MEFs were then exposed to TNFα (10, 20, or 50ng/mL) in the presence of a caspase inhibitor (zVAD-FMK, 20μM) for 4 hrs. Necrosis was then measured using the Sytox Green fluorescent vital dye. Error bars = SEM. *P<0.05 vs. CONsi (n=3).*

CONCLUSIONS

- We successfully identified several novel MLKL binding proteins.
- The lipid raft proteins EFhd2 and Annexin-2 interacted with MLKL.
- EFhd2, but not Annexin-A2, appears to be a negative regulator of TNFα-induced necrosis.
- Future studies will evaluate the other MLKL-binding proteins and their potential role in TNFα-induced necrosis.

ACKNOWLEDGEMENTS

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