RESEARCH HIGHLIGHT

RHIMoving fibrils of death

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Necroptosis relies on interactions between RIP Homotypic Interaction Motif (RHIM)-containing proteins to form an RHIM-amyloid structure called the necrosome. Recent findings by Wu et al. in *Cell Research* now demonstrate that HSPA8, a member of the 70-kDa heat shock protein (HSP70) family, negatively regulates necroptosis by inhibiting and reversing RHIM-amyloid fibril formation, thereby safeguarding tissues from damage.

Necroptosis, a regulated form of lytic cell death, plays a significant role in various human diseases. Advances over the past decade have enhanced our understanding of the signaling cascades underlying necroptosis and have led to the development of therapeutic strategies aimed at halting this inflammatory type of cellular demise. However, recent insights emphasize that the mechanisms governing necroptosis are far more intricate than currently appreciated, with significant implications for health and disease.

Necroptosis can be activated upon ligation of death receptors (DRs) of the tumor necrosis factor receptor (TNFR) family, the T cell receptor, Toll-like receptors (TLRs) 3 and 4, and the intracellular receptor Z-DNA binding protein 1 (ZBP1, also known as DAI or DLM-1).[1] Necroptosis is mediated by Receptor Interacting serine/threonine-Protein Kinase (RIPK) 3 and executed by the pseudokinase mixed lineage kinase domain-like (MLKL). RIPK3 is activated by RIPK1 following DR ligation, the adapter protein TRIF upon TLR3/4 ligation or by ZBP1 directly. These proteins all hold a RIP Homotypic Interaction Motif (RHIM) that mediates the recruitment of RIPK3 via RHIM:RHIM interactions, initiating the RHIM-mediated assembly of additional RIPK3 molecules and growth of the RHIM fibril. The resulting macromolecular amyloid-like structure, termed the "necrosome", serves as a platform for the recruitment, phosphorylation and oligomerization of MLKL. Consequently, MLKL translocates to disrupt the integrity of membranes, including the plasma membrane, resulting in cell death.

Central to necroptosis are RHIM interactions. The RHIM is a conserved ~18 amino acid motif that comprises a tetrad sequence (I/V)-Q-(I/V/L)-G flanked by hydrophobic sequences.[2] Interacting RHIMs adopt a cross-β-sheet configuration that creates the hydrophobic core of the amyloid fibril. Since RHIM-amyloid fibrils have a specific cellular purpose, they are recognized as "functional amyloids", which contrasts with disease-related amyloids implicated in disorders like Alzheimer's disease (β-amyloid), Parkinson's disease (α-synuclein) and TDP43-proteinopathies.

Necroptosis is strictly regulated, and perturbations of known necroptosis-regulatory proteins often lead to exacerbated cell death, culminating in tissue damage and inflammation.[3] However, it is expected that many necroptosis-regulatory proteins remain undiscovered. In a recent study published in *Cell Research*, the Sun group[4] endeavored to discover novel necroptosis-inhibitory proteins. Besides confirming the well-known necroptosis suppressor caspase-8,[5] they uncovered the protein HSPA8 as a negative regulator of necroptosis. HSPA8 is a member of the 70-kDa heat shock protein (HSP70) family, which are co-chaperones that regulate various cellular processes, including the folding of newly synthesized proteins, protein transport and protein degradation.[6] TNFR1-, TLR3-, TLR4- and ZBP1-induced necroptosis were all inhibited by HSPA8, which suggested that HSPA8 functions downstream in the necroptotic pathway. The authors further showed that other HSP70 family members did not suppress necroptosis. However, previous studies identified that HSP90 promotes necroptosis by regulating the stabilization, activation and functioning of RIPK1, RIPK3 and MLKL,[7] indicating that the HSP70 family exhibits both necroptosis-promoting and -suppressing roles.

HSPA8 is comprised of an N-terminal nucleotide binding domain that holds ATPase activity, a linker domain, and a C-terminal substrate binding domain (SBD). Mechanistically, the HSPA8 SBD interacted with the RHIM of RIPK3 independently of the VQVG tetra-peptide core. Rather, the interaction between HSPA8 and all human RHIM-containing proteins hinged on a central hydrophobic amino acid (L, M or I) within a hexapeptide motif at the RHIM's C-terminus, which was predicted by AlphaFold to fit into a hydrophobic pocket within the SBD of HSPA8. Employing electron microscopy and Thioflavin T fluorescence assays, commonly used to determine growth and integrity of amyloid fibrils, the authors demonstrated that HSPA8 inhibits RHIM fibril growth, likely due to steric hindrance of RHIM interactions. Notably, the study showed that HSPA8 could bind to pre-formed RIPK3 fibrils in vitro. This disintegrated the fibrils, which required HSPA8's ATP hydrolyzing ability but was independent of co-chaperones.

Importantly, the Sun group evaluated the necroptosis-suppressing implications of HSPA8 under homeostatic and pathological conditions in vivo. Wild-type (WT) animals treated with the HSPA8 inhibitors Pifithrin-μ or Apoptazole developed hypothermia and exhibited disruption of the small intestine and colon architecture, which showed heightened MLKL phosphorylation. Systemic inflammatory response syndrome, modeled by TNF administration, is known to involve necroptosis and was exacerbated by HSPA8 inhibition in WT mice. Both effects were not observed in *ripk3*$^{-/-}$ or *mlkl*$^{-/-}$ mice. Thus, HSPA8 suppresses

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necroptosis under homeostatic and inflammatory conditions in vivo.

This study identified HSPA8 as a novel negative regulator of necroptosis. Since HSPA8 catalytically disassembles functional RHIM amyloids, the authors dubbed HSPA8 an "amyloidase", which further hinders RHIM interactions to inhibit RHIM fibril growth with the purpose of blocking necroptosis. Since RIPK3 has other, non-necroptosis-related functions that require its activation, it would be of interest to investigate whether these functions are also impacted by HSPA8. The present study underscores the need for careful assessment of potential side effects when considering therapeutic strategies targeting HSPA8.

The discovery of an "amyloidase" hints at the existence of additional proteins that may regulate functional amyloid signaling within living cells. It is tantalizing to speculate that defects in such regulation may contribute to the formation of disease-related amyloids.

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ADDITIONAL INFORMATION

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