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**Meeting Report**

# An appointment with death, 生死有时: 2013 Cold Spring Harbor Asia meeting 'Mechanisms and Functions of Non-Apoptotic Cell Death'

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The significance of trade with China for the economies of the west has, it seems, only recently been fully appreciated. Following this metaphor, the existence of non-apoptotic forms of cell death has been simultaneously noted and neglected. However, a series of key discoveries highlighting that these other forms of cell death are genetically programmed and have physiological significance has seen an appreciation of their importance emerge. Organized by Junying Yuan, Jiahuai Han, Masayuki Miura and Peter Vandenabeele this was the first international meeting devoted solely to non-apoptotic cell death.

The most-studied form of programmed necrosis is induced by tumor necrosis factor (TNF), now called 'necroptosis' to distinguish it from unprogrammed, trauma induced, necrosis. The kinases, RIPK1 and RIPK3 and the pseudokinase MLKL form the 'necrosome' or 'ripoptosome' downstream of TNF:TNFR1 signaling. Caspase-8 plays an inhibitory role in the formation of this complex by cleaving RIPK1 and RIPK3. Jiahuai Han started the meeting by demonstrating that the kinase, p90RSK, phosphorylates and inactivates caspase 8. Phosphorylation of caspase-8 by p90RSK depends on p90RSK recruitment to the necrosome via its interactions with MLKL and RIPK3. While p90RSK does not influence TNF-induced necroptosis, p90RSK knockdown increases the number of TUNEL-positive cells, and may help explain why apoptosis is enhanced in the absence of RIPK3.

Liming Sun expanded on her recent discovery of MLKL with the observation that its oligomerization is important for its necroptotic function. Many in the field believe that necroptosis can exacerbate inflammatory diseases but the lack of markers for necroptotic cells slows progress in the area. Liming showed that phospho-specific MLKL antibodies specifically recognize necroptotic cells and these may overcome this bottleneck. Pharmacological intervention in such diseases is therefore an exciting frontier and Liming reported on two lead compounds that block necroptosis, one of which targets RIPK1 with very high potency. Yigong Shi reported on the co-crystal structure of RIPK1 kinase domain and three chemically distinct necrostatins revealing that these RIPK1 inhibitors all bed into the same hydrophobic pocket in RIPK1 and providing an explanation for their novel allosteric inhibition mechanism.

Alexei Degterev, presented a novel fluorescence based, thermo shift assay to study inhibitors using recombinant RIPK1 and Bill Kaiser explored novel RIPK3 inhibitor compounds. Remarkably, at high concentrations, these RIPK3 inhibitors induce apoptosis! Bill confirmed this was an on-target effect by showing that cells without RIPK3 no longer died. These data generate a pretty puzzle; loss of the protein does not induce apoptosis but inhibition of RIPK3 kinase activity can. This suggests that inhibiting the kinase activity of RIPK3 results in a 'gain of function' activity presumably resulting in activation of caspase 8, but the precise mechanism for this activation is unknown. In support of these findings, Yves Dondelinger from the group of Peter Vandenabeele showed that in a model of TNF-induced apoptosis (TNF + IAP and TAK1 inhibitors) RIPK3 was required, but in a kinase- and MLKL-independent manner.

The therapeutic potential of inhibiting necroptosis was highlighted by Andreas Linkermann. He tested several knockout mice for their ability to resist kidney damage; *Ripk3*$^{-/-}$ mice exhibited significantly decreased sensitivity to both acute and sustained renal ischemia/reperfusion injury (IRI) and cisplatin-induced damage and combined absence of RIPK3 and cyclophilin D provided strong protection against kidney IRI.

While these results suggest that inhibiting necroptotic cell death will be therapeutic in pathological conditions, it is comforting for drug developers that mouse development can proceed almost normally provided both necroptotic and

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apoptotic cell death pathways are blocked. This was nicely demonstrated in an epic series of mouse crosses from Doug Green and Bill Kaiser's labs, which showed that combined loss of caspase-8 and RIPK3 was sufficient to prevent the post-natal lethality observed in RIPK1 knockouts. Similarly, the unrestrained caspase-dependent cell death, which results in embryonic lethality at E10.5 observed in cFLIP knockout mice, could be rescued by combined loss of FADD (to prevent activation of the apoptotic pathway) and RIPK3 (to prevent activation of the necroptotic pathway). These results lead to the surprising conclusion that dysregulation of either the apoptotic or necroptotic pathway is the key factor that drives embryonic lethality in the single knockouts. Hiroyasu Nakano presented work showing the destructive power of apoptosis and necroptosis when unleashed by the conditional loss of c-FLIP in intestinal epithelial cells (IECs) or hepatocytes. While equally catastrophic and leading to death within 2 days of birth, there are interesting differences: IEC death occurred in a predominantly TNFR1-dependent manner, while the three death ligands TNF, FasL and TRAIL were implicated in hepatocyte death.

These findings show that inhibiting necroptosis has therapeutic potential, but how necroptotic death occurs is still unknown. Hao Wu shed some light with crystallographic evidence for the formation of high order, amyloid fibril like, supra-oligomeric RIPK1 RIPK3 structures that may play a role in the spatio/temporal control of the necroptotic cell death pathway.

John Silke reported on two developments; the structure of MLKL and a newly minted MLKL knockout mouse. Cells from the knockout mouse confirm work from Xiaodong Wang's group identifying MLKL as a key player in the necroptosis pathway. Furthermore *Mlkl*$^{-/-}$ mice are viable and healthy, exactly like *Ripk3*$^{-/-}$ mice. The crystal structure allowed the design of constitutively active MLKL mutants that indicate that phosphorylation of MLKL by RIPK3 is the molecular on switch-promoting cell death downstream of RIPK3.

Andrew Oberst used a chemical approach to activate RIPK3 by fusing an FV, inducible dimerization domain. Chemical dimerization rapidly induces necroptosis with high selectivity. One unexpected finding with this system was that RIPK1 knockdown increased RIPK3-induced necroptotic cell death, suggesting that RIPK1 might not only activate RIPK3 by phosphorylation, but also inhibit spontaneous autoactivation of RIPK3.

Ubiquitin-mediated control of cell death signaling pathways is particularly important and highlighted by the fact that depletion of cIAP E3 ligases predisposes cells to necroptotic cell death. Avi Ashkenazi provided further evidence showing how TRAF2 tags caspase-8 with K48-ubiquitin chains within a Death Receptor signaling complex, thereby limiting its half-life post activation. By setting a timer on activated caspase-8, TRAF2 may be involved in determining whether caspase-independent cell death occurs. Several papers show that loss of FADD can predispose cells to necroptotic cell death and Jaewhan Song discussed recent findings that the E3 ligase MKRN1 mediates ubiquitylation and degradation of FADD. Consistently, knockdown of MKRN1 sensitized cells to apoptosis by facilitating caspase-8 activation.

Although necroptosis was a star of the meeting, there are other types of non-apoptotic cell death. Zheng-Hong Qin showed how the lysosomal transmembrane protein Dram1 works beyond its established role in autophagy, linking autophagic cell death with apoptosis. He showed that DRAM1-mediated recruitment of Bax to the lysosome leads to cathepsinB release, tBid cleavage and apoptosis via the mitochondrial pathway of caspase activation. Shigeoni Shimizu described how MEFs from *Bax*$^{-/-}$*Bak*$^{-/-}$ mice die from both conventional (ATG5 dependent) and alternative (ATG5 independent) autophagy following etoposide treatment. As depletion of Bax and Bak inhibited ATG5-independent autophagic cell death, *Bax*$^{-/-}$*Bak*$^{-/-}$*Atg5*$^{-/-}$ triple knockout MEFs showed superior viability to *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. Additional data suggested that this 'alternative macroautophagy' pathway is evolutionarily conserved in *S. cerevisiae*.

Sharad Kumar and Eric Baehrecke talked about the role autophagy plays in intestine deletion during *Drosophila* development, and how caspases have no clear impact on this cell death. This *Drosophila* intestine model is a fruitful realm to study the spatial and temporal regulation of autophagy and the crosstalk between autophagic regulation of cell size and nutrition-mediated growth signaling pathways.

In *C. elegans*, where the fate of every cell has been mapped, it is possible to get personal about cells. Shai Shaham described how the death of the male-specific Linker cell is required to open up a passage for sperm into the cloaca. This death occurs independently of caspase activity and an RNAi screen revealed that a polyQ repeat protein, PQN-41, was required. The nucleus of the dying Linker cell is highly crenellated, and reminiscent of the crenellated nuclei of dying neurons in Huntington's disease patients that can also be driven by PolyQ expansion, raising the interesting hypothesis that the two forms of death may be related.

Nektarios Tavernarakis's work in *C. elegans* demonstrates that heat-shock-induced necrosis in *C. elegans* is caused by dysregulated calcium release from the Golgi complex and can be rescued by the upregulation of HSP-16, which modulates the Golgi calcium pump, PMR-1. HSP-16 protection is evolutionary conserved, and pre-conditioning of mammalian cells with mild heat stress also leads to the induction of cytoprotective factors.

Space restrictions preclude us from discussing many other interesting presentations and work. To give some local flavor some of the delegates visited Soochow University in Suzhou, hosted by Sudan He and were told of the university's plans to expand the Cyrus Tang Haematology center with 15 PIs to 150 PIs! A similar breathtaking feeling of new vistas being opened up in the field of non-apoptotic cell death was the main take home message from this exciting conference.