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From zero to sixty: cell death signaling in the city that never sleeps

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INTRODUCTION

In a blistery winter of New York City, over 120 researchers congregated at the Icahn School of Medicine at Mount Sinai for the first Cell Death Signaling conference (Figure 1). Manhattan's Upper East Side was the setting for 2 days of presentations to discuss a topic relevant to all life: cell death. The mechanisms by which cells generate and respond to death signals remain of tremendous interest, and this is particularly true with regard to diseases in which cell death regulation is disrupted. As such, this conference served to bring together cutting edge research from the fields of cell death, metabolism, immunology, autophagy and cancer. In addition, the conference coincided with the sixtieth birthday of Douglas R Green PhD, *Oncogene* Editor-in-Chief. In celebration of his many contributions and accomplishments, speakers highlighted the influence Doug has on the cell death community, both scientifically and personally. As a result, state of the art cell death research was juxtaposed with a celebration of scientific accomplishments, and here we provide a summary of the 25 presentations and describe general themes emerging in the field.

IF IT AIN'T BROKE, DON'T KILL IT: MECHANISMS OF CELL DEATH AND MAINTENANCE

A meeting about cell death signaling would be incomplete without appropriate coverage of the different forms of caspase-mediated cell death. Guy Salvesen (Sanford-Burnham Institute for Medical Research, La Jolla, CA, USA) discussed the discovery and history of caspases, the evolutionarily conserved proteases of cell death, as well as the pathways for which they are required. Apoptosis, the cell death pathway defined by caspase activation, occurs when effector caspases-3/-6/-7 cleave a variety of cellular substrates resulting in the

phenotypic hallmarks of apoptosis. The effector caspases can be activated downstream of two distinct pathways: extracellular signals can interact with cell-surface receptors (such as those in the TNF receptor family) and lead to accumulation and activation of initiator caspase-8. Alternatively, intrinsic cell stress (such as damage or nutrient deprivation) signals through the BCL-2 family of proteins, initiating permeabilization of the outer mitochondrial membrane (OMM), release of proteins from the inner-membrane space (cytochrome *c*), formation of the apoptosome, and finally, activation of initiator caspase-9 and downstream effector caspases.

Other forms of cell death include necroptosis, which occurs in the absence or restriction of caspase action, and pyroptosis, a recently defined form of cell death that utilizes alternative caspases-1/-11. Seamus Martin (Trinity College Dublin, Dublin, Ireland) discussed the consequences of cells dying through these mechanisms and the resulting uncontrolled release of internal components into the surrounding environment. Cells succumbing to unregulated methods of death create an inflammatory response when released chemokines and cytokines signal resident neutrophils and macrophages. In addition, released cellular components (ATP, DNA fragments) function as damage associated molecular patterns (DAMPs) and initiate pro-inflammatory signaling cascades through pattern recognition receptors (PRRs). However, Martin remarked that the list of cellular proteins or molecules that may act as DAMPs remains largely unexplored.

In contrast, regulated secretion of cytokines occurs during pyroptosis when pathogen-associated molecular patterns bind cytosolic PRRs and activate the inflammasome. Thirumala-Devi Kanneganti (St Jude Children's Research Hospital, Memphis, TN, USA) introduced work demonstrating that in response to *Aspergillus* infection, AIM2 and NLRP3 orchestrate the formation of the inflammasome proteins ASC and caspase-1, resulting in the proteolytic cleavage and activation of IL-1 β and IL-18. These cytokines create an inflammatory response through recruitment of leukocytes and lymphocytes. Interestingly, work by William Evans (St Jude Children's Research Hospital) demonstrated that patients with prednisolone-resistant acute lymphoblastic leukemia (ALL) had increased expression of NLRP3 and caspase-1. This over-expression results in inappropriate caspase activation, increased secretion of IL-1 β and IL-18, and cleavage of the glucocorticoid receptor, making patients insensitive to the corticosteroid drug. Patient response to prednisolone remains prognostic of ALL outcomes and the observed increased expression of inflammasome NLRP3 may serve as a potential new therapeutic target.

PRECARIOUSLY PLACED ON A PRECIPICE: FACTORS DETERMINING LIFE OR DEATH

Proteins of the extrinsic or intrinsic apoptotic pathway can initiate both pro-survival and pro-death responses. Although pro-survival proteins engage repair machinery (for example, autophagy) and inhibitors of apoptosis (for example, c-FLIP and IAPs), the accumulation of pro-apoptotic proteins results in commitment to cell death. Ruslan Medzhitov (Yale University, New Haven, CT, USA) postulated that the outcome of the antagonistic relationship between pro-survival or pro-apoptotic proteins could be stochastic. He described a model where stochastic cell fate decisions evolved as populations of cells were

limited by a finite pool of growth factors or differentiation signals. A subset of the population would die, thus ensuring that surviving cells would receive adequate signal transduction from exogenous ligands. Commonly, this stochastic result could be the consequence of asynchronous cell cycling or intrinsic gene expression ‘noise,’ and then would be propagated through feed-forward loops.

In addition to pathways responding to growth factors or differentiation signals, cells evolved pathways to respond to pathogens or damaged organelles. Eric H. Baehrecke (University of Massachusetts, Worcester, MA, USA) presented modern mechanisms of autophagy signaling highlighting conserved proteins (for example, ULK1, Beclin-1 and ATG5/7) and their role in organelle quality control and removal of pathogenic bodies. Douglas Green (St Jude Children’s Research Hospital) presented his work on a form of phagocytosis that non-canonically utilizes the autophagic machinery. Termed LAP (LC3-associated phagocytosis), this process forms a single-membrane vesicle not requiring ULK1 pre-initiation, which is normally required in nutrient deprivation-driven autophagy. Interestingly, Beclin-1, ATG5 and ATG7 are all required for proper endosome maturation, fusion with lysosomes and suppression of an inflammatory response similar to canonical autophagy in response to infection. Therefore, autophagy represents a vital process for mitigating cellular damage or infection without the engagement of cell death machinery.

Aside from stochastic regulation, downstream responses to cell death signals are modulated by both anti- and pro-apoptotic proteins. In the intrinsic pathway of apoptosis, the BCL-2 family serves both as executioner and survival factors. Mitochondrial outer membrane permeabilization (MOMP) is the hallmark of this pathway and represents a commitment step for cell termination. The effector pro-apoptotic proteins BAK and BAX are the key mediators of MOMP, forming homo-oligomeric complexes in the OMM, and eventually, pores to release cytochrome *c*. However, the nature of how these proteins form high molecular-weight complexes (and proteolipid pores in the OMM) remains largely unclear. Ruth Kluck (The Walter and Eliza Hall Institute, Parkville, Australia) used cysteine-crosslinking and loss-of-function screens in BAK to propose a model where dimerization precedes higher order oligomerization. According to her model, following activation by BID or BIM, BAK alpha helices associate with the membrane and form a ‘latch’ domain for dimerization, whereas the $\alpha 9$ domain forms a transmembrane anchor. Activation by BID or BIM is antagonized by the role of the anti-apoptotic BCL-2 proteins, which can be upregulated in response to cellular stress. One particular anti-apoptotic member of the BCL-2 family, MCL-1, is commonly over-expressed in a variety of cancer types. MCL-1 is also crucial for healthy tissue development and therefore pharmacological inhibition needs to be tumor specific to reduce severe side effects. This dilemma was highlighted by work from Joseph Opferman (St Jude Children’s Research Hospital) using BCR-ABL⁺ ALL cells as a model for tumor response to BCL-2 inhibitors. He demonstrated that there currently exists no specific and potent MCL-1 inhibitor, but that this void in targeted cancer therapeutics is driving design of new drugs.

In the receptor-mediated machinery of the extrinsic pathway of apoptosis, a critical modulator of life and death is RIP1 kinase. As presented by Peter Vandenabeele (University of Ghent, Ghent, Belgium), RIP1 serves as a scaffold in the TNF-family receptor complex

and recruits adapter proteins for the activation of NF κ B resulting in pro-survival/-inflammatory signaling. In contrast, the kinase activity of RIP1 is required for activation of RIP3, MLKL, and other proteins characteristic of necroptosis. This molecular switchboard is governed by specific ubiquitinylation chains on RIP1. The linear M1-linked ubiquitinylation of RIP1 provides an interaction site for NEMO of the IKK complex, favoring activation of NF κ B. Henning Walzack (University College London, London, UK) focused on the antagonistic relationship between the E3 ubiquitin ligase LUBAC and the deubiquitinating enzymes OTULIN and CYLD, the latter having a role in promoting cellular necroptosis. However, downstream gene induction of the TNF-receptor family also contributes to cellular outcome. For example, targets of NF κ B signaling include the IAPs, which create a positive feedback mechanism for cell survival. It was recently discovered by Thomi Brunner (University of Konstanz, Konstanz, Germany) that the expression of IAPs in intestinal epithelia is dramatically reduced, resulting in an extreme sensitivity of the tissue to TNF signaling. Although it has been known that many inflammatory diseases of the gastric-intestinal track are mediated by TNF signaling, it was unknown how these cells succumbed to death. This work now supports a model where it is not TNF-mediated inflammation of the tissue but rather necroptosis that kills the intestinal epithelia and subsequently engages an inflammatory response.

A WATCHED POT NEVER DEREGULATES: IMMUNOLOGIC SURVEILLANCE AND DISEASE

Tumor immunity is an increasingly relevant topic in cancer biology and many presentations focused on the relevance of the immune system in disease and cancer. The immune system must be tightly regulated, switching from silent removal of apoptotic bodies to active termination of foreign pathogenic bodies or neoplastic tissue. The former is commonly found in the intestinal epithelium where apoptosis occurs regularly as new cells are differentiated in the crypt and migrate into villi. Using a model of low-dose diphtheria toxin, Julie Blander (The Icahn School of Medicine at Mount Sinai, New York, NY, USA) characterized the professional phagocytes that detect and remove these apoptotic cells. She found that the responding macrophages upregulate genes responsible for homeostasis, catabolism and lipid processing; in contrast, dendritic cells (DCs) that engulf these bodies downregulate genes for cytokines, activators of Complement and MAPK—but upregulate suppressive factors. Interestingly, the genes that are down regulated in DCs represent genes containing known single-nucleotide polymorphisms associated with Inflammatory Bowel Disease. Vijay Kuchroo (Harvard University, Boston, MA, USA) added to this theme by describing the different subtypes of T helper cells and their role in immunologic surveillance. Of particular note, he discussed the newly defined Fc γ 2-secreting TIGIT⁺ T_{reg} subpopulation, which selectively inhibits T_H1 and T_H17 cells from releasing IFN γ and IL-17, respectively, and initiating an immune response. Collectively, these works highlighted the necessity of immunological regulation in normal lymphocyte activity.

One of the hallmarks of tumor biology is the ability of neoplastic cells to evade surveillance by utilizing mechanisms of immune inhibition. Gerard Evan (University of Cambridge, Cambridge, UK) described that RAS-driven Myc deregulation in a variety of cancers causes

defects in immuno-surveillance by inhibiting T cells through PD-1 signaling. In this way, he emphasized that tumor immunology is a fundamental attribute of tumor survival. Inhibitory cytokines within the tumor microenvironment also inhibit activation of local leukocytes in response to tumorigenic tissue. In his work, Dario Vignali (University of Pittsburgh, Pittsburgh, PA, USA) demonstrated that tumors use IL-35 to promote their growth, which upon neutralization results in immune-mediated shrinkage of tumor mass. Vignali went on to show that the receptor complex of Sema4a:Nrp1 on T cells/DCs and T_{regs}, respectively, inhibits regulation of the T_{reg} master transcription factor FoxP3. By selectively targeting Nrp1 on T_{regs}, tumors demonstrate limited immune evasion without consequential autoimmune manifestations, collectively suggesting that the Sema4a:Nrp1 complex may serve as a useful therapeutic target. Huseyin Mehmet (Proteostasis Therapeutics, Cambridge, MA, USA) presented work on a novel X-linked immunodeficiency that predisposes patients to chronic viral infections and lymphomas. In this disease, the MAGT1 magnesium transporter gene is mutated preventing rapid and transient Mg²⁺ influx following antigen-receptor stimulation. This abrogation of Mg²⁺ signaling impairs PLC-mediated Ca²⁺ influx in T, but not B, cells and impairs natural killer (NK)-activating receptor NKG2D in NK and CD8⁺ cells. This deregulation of the immune system fundamentally disturbs surveillance mechanisms and permits both pathogens and tumorigenic cells to evade detection and removal.

The regulatory function of p53 represents another highly disrupted surveillance mechanism in malignant cells. Although most research focuses on deletion of p53 as a critical event in tumorigenesis, Scott Lowe (Memorial Sloan Kettering Cancer Center, New York, NY, USA) presented his work on the 4-megabase deletion of the 17p11B3 locus commonly accompanying *trp53* loss in tumors. When mice harboring this deletion were compared against mice with loss of *trp53* alone, they demonstrated dramatically higher tumor development due to loss of *trp53*-neighboring genes. Interestingly, a similar phenomenon is observed by the amplification of the *Mdm2* locus versus over-expression of the gene alone. MDM2, known mostly for its regulation of p53, has also been implicated in regulating hypoxia-induced factor-1 α (HIF-1 α). Gerry Melino (MRC Toxicology Unit, Leicester, UK) observed that mice deficient in *TAp73* displayed enhanced vascularization. In normal cells, TAp73 binds the regulatory subunit HIF-1 α marking it for MDM2-mediated ubiquitinylation and subsequent degradation. In human lung cancer data sets, TAp73 levels strongly correlated with patient prognosis, indicating that it may become a useful biomarker in the clinic. Vishva Dixit (Genentech, Inc., South San Francisco, CA, USA) connected several common themes with his work on understanding the importance of DUBs in the maturation of T_H17 cells. Drawing analogy with the USP7-MDM2-p53 axis of regulation, the E3 ligase UBR5 regulates activity of the T_H17 transcription factor ROR γ T, but also autoubiquitinates itself leading to its degradation. DUBA/OTUD5 operates to remove the ubiquitin from UBR5 thereby increasing its half-life and providing prolonged ROR γ T inhibition. This work supports the notion that focus on upstream DUBs is necessary to fully comprehend the regulation of transcription factors linked to disease.

FEEDING THE BEAST: NUTRIENT DEPENDENCE AND UTILIZATION IN DISEASE

Cells within malignant tumors require increased levels of glucose and oxygen to sustain their high demands for energy. The differential between the nutrient requirements in tumor and normal tissue provides opportunities for therapeutic intervention. Christina Munoz-Pinedo (The Bellvitge Institute for Biomedical Research, Barcelona, Spain) described a new form of cell stress in response to glucose deprivation in rhabdomyosarcoma. Characterized by ATF4 activation but independent of CHOP, this response mechanism promotes autophagy and endoplasmic reticulum collapse causing co-localization of death receptor DR5/TRAIL-R2 with downstream adapter molecules. This congregation achieves threshold levels of pro-death signaling. Karen Vousden (The Beatson Institute, Glasgow, UK) discovered that serine utilization is drastically altered in tumor cells. Healthy cells use the non-essential amino-acid primarily for glutathione synthesis but in cancer cells, particularly those with loss of *trp53* or *p21*, serine is utilized for nucleotide synthesis and to bolster key enzymes of glycolysis. Similar to increased glucose uptake found in the Warburg effect, serine uptake is preferentially increased in tumors compared with normal tissue. Mice that were fed a serine/glycine-deficient diet demonstrated reduced tumor progression for a time before adapting and engaging the serine synthesis pathway. This pathway utilizes glycolytic intermediates and therefore cells adapting to serine deprivation exhibit greater dependence on oxidative phosphorylation machinery to provide energy. Thus, using inhibitors of oxidative phosphorylation with the serine-deprived diet displayed a synergistic effect on tumor growth inhibition without affecting normal tissue.

The discovery of key metabolic enzymes playing a role in tumorigenesis is a topic of interest in cancer biology. A new connection was described by Jean-Ehrland Ricci (INSERM, Paris, France), who identified glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an indicator of cancer aggressiveness and vascularization in non-Hodgkin's lymphomas (NHL). This occurs due to interactions between GAPDH and TRAF2, which promotes NF κ B-mediated transcription of HIF-1 α , which subsequently transcribes both *Vegf* and *Gapdh*. Of particular interest, GAPDH-dependent activation of this pathway also increases CD20. Current standard of care for NHL is combination anti-CD20 immunotherapy with conventional chemotherapeutics, but 30–40% of patients experience therapeutic failure. Therefore, weak GAPDH expression may serve as a useful indicator of which patients will not respond to the immunotherapy.

Tak Mak (Princess Margaret Cancer Centre, Toronto, Canada) reviewed the role of tumor microenvironments and genetic alterations modulating production and accumulation of reactive oxygen species (ROS). In response to ROS accumulation, cancer cells upregulate antioxidant gene expression and activity during tumor progression. He went on to demonstrate that expression of antioxidants proceeds in a step-wise manner correlating with tumor maturation. Combined inhibition of glutathione and thioredoxin disrupts management of increased ROS levels resulting in synergistic death of tumor cells. Anna Colell (IIBB-CSIC, Barcelona, Spain) supported the notion of detrimental ROS accumulation in a different context, Alzheimer's disease. Her work suggested that increased cholesterol in the

cellular and mitochondrial membrane might have an important role in the disease. High levels of cholesterol form lipid rafts and promote the self-aggregation of cerebral amyloid- β . This leads to mitochondrial dysfunction and ROS production resulting in PI3K-mediated activation of autophagy/mitophagy. Moreover, cholesterol blocks mitochondrial glutathione, further promoting ROS accumulation and engagement of mitophagy machinery. Therefore, it appears that these two different pathologies hinge on the sustained action of autophagy in order to maintain homeostasis.

THE DEATHBED: CLOSING THOUGHTS

The conference brought together work from multiple fields focused on the cellular machinery determining the ultimate fates of a cell: to live or to die. The speakers emphasized that dysfunction of numerous fundamental mechanisms including autophagy, metabolism, immune surveillance and propagation of death signals is highly relevant in disease etiology and treatment. There were several underlying themes during the conference. One was the increased relevance of the tumor microenvironment in disease progression. Several talks demonstrated this concept by highlighting suppression of the immune system, altered nutrient delivery and utilization or inter-cellular signaling in survival of malignant tissue. Another common theme of the conference was the development of prognostic indicators for use in the clinic. The presented research demonstrated that understanding the fundamental mechanisms of cell death signaling is the key to the development of novel therapeutics. In addition, continued investigation into the cell death signaling pathways has provided putative biomarkers, which may predict patient outcome to particular standards of care. Finally, the general tone of the conference centered on integrating distinct disciplines and approaches in order to identify avenues of pathway dependence. In addition to providing possibilities for targeted therapy development, identifying these interlinked disease-specific pathways elucidates more targets for synthetic lethality upon combination therapy. Furthermore, research is finding more potent and effective combinations of targeted therapy that may serve to efficiently eliminate disease tissue at lower and more tolerated doses. With all of the exciting cell death signaling discoveries contributing to the fields of cancer biology, immunology and metabolism, there is undoubtedly much anticipation for the next Cell Death Signaling conference.

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ABBREVIATIONS

ATF4	activating transcription factor 4
ATG5	autophagy protein 5
ATG7	autophagy protein 7
BAK	BCL-2 homologous antagonist killer
BAX	BCL-2 associated X protein
BCL-2	B-cell lymphoma 2
BH	BCL homology
BID	BH3-interacting domain death agonist
BIM	BH3-interacting mediator of cell death
c-FLIP	Cellular FLICE (FADD-like IL-1-converting enzyme)-inhibitory protein
CHOP	C/EBP homologous protein
CYLD	cylindromatosis
DAMP	damage-associated molecular pattern
DR5/TRAIL-R2	death receptor 5/TNF-related apoptosis-inducing ligand receptor 2
DUB	deubiquitinase
DUBA/OTUD5	deubiquitinating enzyme A/OTU (ovarian tumor) deubiquitinase 5
ER	endoplasmic reticulum
Fgl2	fibrinogen growth factor-like 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HIF-1α	hypoxia-induced factor-1 α
IKK	I κ B kinase
IL	interleukin
LAP	LC3-associated phagocytosis
MAGT1	magnesium transporter 1
MCL-1	myeloid cell leukemia sequence 1
MDM2	mouse double minute 2
MLKL	mixed lineage kinase domain-like protein
NEMO	NF κ B essential modulator
NFκB	nuclear factor κ -light chain enhancer of activated B cells
NK	natural killer
NKG2D	natural killer group 2, member D

Nrp1	neuropilin 1
OTULIN	OTU DUB with linear linkage specificity
PD-1	programmed cell death protein 1
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
PRR	pattern recognition receptor
RIP1	receptor interacting protein kinase 1
RIP3	receptor interacting protein kinase 3
RORγT	RAR-related orphan receptor gamma
Sema4a	semaphorin 4A
TRAF2	TNF receptor-associated factor 2
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	tumor necrosis factor
UBR5	ubiquitin protein ligase E3 component n-recognin 5
ULK1	unc-51 like autophagy activating kinase 1
USP7	Ubiquitin-specific-processing protease 7.

Figure 1.

Cell Death Signaling 2015 guests and speakers. Speakers and invited guests of the meeting congregated at the Temple of Dendur in the Metropolitan Museum of Art in New York City's Upper East Side. The guest of honor, Douglas R Green PhD, was greeted by his colleagues and friends who traveled great distances to celebrate him and his many achievements. This evening served as the kick-off event for a 2-day conference at the Icahn School of Medicine at Mount Sinai that would encompass the most recent scientific findings in the fields of cell death, metabolism, immunology, autophagy and cancer, while honoring Dr Green's contributions to these fields.