Cell Death and Differentiation (2011) 18, 1944–1947

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Meeting Report

International EMBO Molecular Medicine Workshop 2011 on 'Cell Death and Disease'

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Cell Death and Differentiation (2011) **18,** 1944–1947; doi:10.1038/cdd.2011.108; published online 26 August 2011

Obergurgl, Austria, 10–14 March 2011

Superbly organised by Andreas Villunger, this friendly and lively meeting took place by the ski slopes of Obergurgl. The third in a series of cell death conferences held in this Tyrolean village, the speakers focused on their recent findings in the classic and alternative cell death signalling pathways and provided insights into how these pathways modulate cancer and immune signalling.

miRNAs, Synthetic Lethality and Cancer

Carlo Croce (Columbus, OH, USA) took us from early chromosome breakpoint analysis of tumours, which paved the way for the cloning of human oncogenes such as *Bcl-2* and *c-Myc*, and illustrated that microRNA sequences can be similarly identified. Global miRNA signatures of indolent and aggressive subsets of CLL and solid tumours revealed some commonality between multiple cancer types. miRNAs are attractive tumour therapies and provide useful blood biomarkers for early tumour detection. As miRNAs have multiple targets, the challenge will be to identify these and establish their contribution to malignant transformation. The importance of miRNAs in cancer was further demonstrated by Jean Christophe Marine (Leuven, Belgium) who showed that in a mouse model of retinoblastoma, monoallelic loss of the miRNA-processing enzyme Dicer1 accelerated tumour progression. Further studies indicated that Dicer1 loss is synthetically lethal with p53 inactivation, in cells where the retinoblastoma protein is inactivated. Identification of microRNAs, downstream of Dicer1, that are responsible for synthetic lethality in p53-deficient cells may highlight key therapeutic targets to selectively treat retinoblastoma.

Daniel Murphy (Würzburg, Germany) presented data from a synthetic lethal siRNA screen, which identified two kinases from the AMP-activated protein kinase (AMPK) family as potential targets in Myc-overexpressing tumours. Depletion of either kinase resulted in apoptosis *in vitro* and therapeutic efficacy in a Myc-driven mouse model of hepatocellular carcinoma. Thus, small molecule inhibition of AMPK-related kinases may prove effective against tumours overexpressing Myc. However, care should be taken when using rapamycin-based therapies, as rapamycin rescued cells depleted of either kinase from Myc-induced lethality. Jochen Prehn (Dublin, Ireland) provided further caution, as he implicated AMPK as a key switch in the decision between cell survival and death. Transient AMPK activation protects against apoptosis through the activation of GLUT transporters and stimulation of glucose metabolism. However, prolonged AMPK activation can lead to apoptosis via Bim and Bmf. Therefore, identifying what determines whether AMPK has a protective or destructive effect will have important implications for a range of human diseases.

The p53 Family

Following DNA damage, cells either undergo cell cycle arrest or apoptosis. As p53 is stabilised in both situations, additional factors must determine which response prevails. Ulrich Maurer (Freiburg, Germany) showed that in response to DNA damage, glycogen synthase kinase-3, through phosphorylation of Tip60, regulates p53 acetylation and p53-dependent Puma expression. This mechanism may provide a link between phosphatidylinositol 3-kinase signalling and the p53-mediated decision for life or death.

Gerry Melino (Leicester, UK) provided new data on p63 in ear development. *p63* null mice have supernumary auditory hair cells, and patients with *p63* mutations and the syndrome ectrodactyly, ectodermal dysplasia and cleft lip/palate have a reduced number of cochlea coils. Gerry also described how the p53 family of proteins might protect the female germline; p63 is involved in follicle maturation, p73 in blastocyst development, and p53 controls implantation. Andreas Strasser (Melbourne, Australia) explained that mouse oocytes respond to DNA damage by apoptotic cell death, requiring Puma and Noxa. *Puma*/*Noxa* double knockout (DKO) oocytes are protected against this type of death, but progeny are developmentally normal and fertile. Although much emphasis is placed on p53 as a tumour suppressor, *Puma*/*Noxa* DKO mice do not get tumours, nor do *Puma*/*p21* DKO mice.

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Consistent with this, Alexander Egle (Salzburg, Austria) showed that *Puma*/*p21* DKO mice were resistant to developing thymic lymphomas after repeated doses of non-lethal irradiation. This tumour-resistant phenotype appeared to be related to differential regulation of cell cycle and DNA-damage repair in irradiated haemopoietic stem cells *versus* wild-type counterparts.

Tumour Evasion of the Immune Response and Chemotherapy

Thomas Brunner (Konstanz, Germany) reported that glucocorticoid synthesis by colon cancer cells might assist them in escaping immune surveillance. Intestinal cells produce cortisol through stimulation of the nuclear receptor, liver receptor homolog-1 (LRH-1), and steroidogenic enzymes. LRH-1 was restricted to the crypt base in normal colon, but exhibited uniform expression in colonic tumours. Secretion of cortisol by human colon cancer cells likely suppresses activation of infiltrating T cells as well as inducing their death, as these functions were inhibited with 11-β hydroxylase inhibitor.

Jan Paul Medema (Amsterdam, The Netherlands) expanded on his work showing that high Wnt activity functionally designates a chemotherapy-resistant, CD133-positive 'cancer stem cell' (CSC) population among colon tumours. Class I histone deacetylase (HDAC) inhibitors can induce differentiation of CSCs and sensitise them to chemotherapy. Therefore, even apoptosis-resistant CSCs might be targeted by combination treatments involving HDAC inhibitors.

Autophagy, Metabolism and Disease

Xin Lu (Oxford, UK) considered the role of *ASPP2* in epithelial tumours, where it exhibits reduced expression (e.g., in breast, lung and hepatocellular carcinoma) and is a haploinsufficient tumour suppressor. She showed a p53-independent function of ASPP2 in maintaining tight junction integrity and cell polarity through regulating localisation of the tight junction protein, PAR3. She also proposed a link between cell polarity and autophagy.

Jennifer Martinez (Memphis, TN, USA) showed that phagocytosis of apoptotic, necrotic or necroptotic cells caused Atg7 and Beclin-1-dependent LC3 translocation to the phagosome. Thus, LC3-associated phagocytosis is utilised to efficiently remove dying cells and limit autoimmune responses.

Inês Castro (Leicester, UK) showed that autophagy degrades defective mitochondria, with an accumulation of unfolded proteins, via the PINK1/Parkin pathway. Both *Pink1* and *Parkin* mutant flies exhibit an atypical wing posture due to abnormal mitochondria in the indirect flight muscles. A similar phenotype was observed upon mitochondrial accumulation of unfolded ornithine transcarbamylase (OTC). Furthermore, these mutant flies had locomotor defects. Parkin suppressed mitochondrial dysfunction and locomotor defects caused by OTC accumulation by promoting autophagy, a process involving the *Drosophila* orthologue of mammalian *p62*, *Ref(2)P*. Inês suggested that defects in the regulation of mitochondrial quality by the PINK1/Parkin/Ref(2)P pathway may be relevant to the aetiology of Parkinson's disease.

Seamus Martin (Dublin, Ireland) explained how mutant Ras^{V12} induced Noxa in normal human ovarian epithelial cells, leading to caspase-independent cell death with features of autophagy. shNoxa or shPuma restored colony forming efficiency upon switching on Ras^{V12}; moreover, A1 and Mcl-1, which are efficient binders of Noxa, blocked cell death more efficiently than Bcl-2 or Bcl-xL. Furthermore, shBeclin-1 and siAtg5 both conferred protection. Beclin-1 binds Bcl-xL, Mcl-1 and Bcl-2, the first two of which dissociate from Beclin-1 when Ras^{V12} is switched on; Puma displaces Bcl-xL from Beclin-1, and Noxa displaces Mcl-1 from Beclin-1. This mechanism may serve to limit the oncogenic potential of deregulated Ras signals.

Cristina Muñoz-Pinedo (Barcelona, Spain) analysed cell death induced by inhibition of glycolysis by glucose deprivation or treatment with 2-deoxyglucose (2-DG), a non-metabolisable glucose analogue. As tumours have increased glycolytic flux, such approaches may target tumour cells for cell death. In rhabdomyosarcoma cells, interesting differences were noted between the two methods of inhibiting glycolysis; glucose withdrawal resulted in necrosis, whereas 2-DG induced caspase-dependent apoptosis associated with decreased Mcl-1 and increased Noxa. Clearly, understanding the consequences of anti-glycolytic treatments will be crucial in predicting tumour response.

The Bcl-2 Family in Immunity

Eric Eldering (Amsterdam, The Netherlands) described how TCR or BCR clonal diversity is regulated by the Mcl-1/Noxa rheostat. Antigen stimulation induces Noxa, the ablation of which leads to lower antigen affinity and greater clonal diversity in the T-cell response. *Noxa* null mice take longer to recover from infection, indicating that Noxa has a role in ensuring the most effective clones of the adaptive immune system survive. Eleonora Ottina (Innsbruck, Austria) showed evidence that A1 may be important in positive selection of thymocytes, B-cell maturation and granulopoiesis.

Cytotoxic lymphocytes cosecrete perforin and granzyme B to induce death of target cells. Jamie Lopez (Melbourne, Australia) showed that perforin likely forms a pore to permit granzyme B entry. However, detailed kinetic analysis showed only a small window of opportunity for granzymes to gain entry into the target cell before plasma membrane repair.

The Bcl-2 Family in Cancer

Suzanne Cory (Melbourne, Australia) discussed how increased Mcl-1 is a mechanism for resistance to the BH3 mimetic, ABT-737. Mcl-1 siRNA or Noxa overexpression can enhance its effectiveness. ABT-737 was also discussed by Martin Schuler (Essen, Germany) as a co-treatment to overcome resistance to EGFR antibody-based therapy. A novel use of ABT-737, outside of cancer treatment, was introduced by Douglas Fairlie (Melbourne, Australia). Douglas suggested that BH3 mimetics could be employed to treat schistosomiasis by targeting pro-survival Bcl-2 orthologues in the schistosome, resulting in parasite death.

The concept of direct activator BH3 proteins acting by 'Mode 1' and sensitiser BH3 proteins acting via 'Mode 2' was discussed by Doug Green (Memphis, TN, USA). Doug proposed that Mode 2 is more efficient and is engaged at higher stresses, whereas Mode 1 is more easily derepressed. Peter Czabotar (Melbourne, Australia) used an alternative approach to investigate the interactions of the Bcl-2 family. Met74E Bax cannot bind anti-apoptotic Bcl-2 family members, but can oligomerise; it reduces the colony forming ability of *Bax*/*Bak* DKO mouse embryonic fibroblasts and sensitises cells to ABT-737 more effectively than wild-type Bax. This suggests that anti-apoptotic proteins provide some restraint on Bax, suppressing a 'Mode 2'-type of death.

Cell Signalling from Soluble Intracellular Complexes

Peter Vandenabeele (Ghent, Belgium) described TNF signalling and necroptosis, a RIP kinase-mediated form of programmed necrosis. RIP kinases are crucial in determining the TNF response: knockdown of RIPK1 prevented necrosis, but sensitised to apoptosis, whereas knockdown of RIPK3 led to protection against both forms of cell death. The cytoprotective importance of RIPK3 was illustrated by comparing RIPK3 knockout and wild type mice injected with TNF; in wild type mice, TNF induces systemic inflammatory response syndrome, but RIPK3 knockout mice were completely protected. Andrew Oberst (Memphis, TN, USA) discussed how caspase-8 and c-FLIP$_L$ protect against TNF-induced necroptosis. This was achieved by caspase-8 and c-FLIP$_L$ forming a catalytically active complex that inhibited the stable formation of a RIPK1/RIPK3/FADD complex. Therefore, c-FLIP$_L$ can protect against both apoptosis and necroptosis.

Martin Leverkus (Heidelberg, Germany) and Pascal Meier (London, UK) reported on an intracellular complex containing caspase-8, c-FLIP, FADD and RIPK1 termed the 'Ripoptosome'. This is formed in the absence of cIAPs and can be recruited to different platforms such as those formed by death receptors or Toll-like receptor-3 (TLR3). Martin showed that upon ligation of TLR-3, the complex was recruited to the adaptor protein TRIF and both apoptosis and necroptosis were induced. Pascal reported that treating cells with SMAC mimetics or etoposide results in reduced levels of IAPs, facilitating ripoptosome formation and c-FLIP recruitment. John Silke (Melbourne, Australia) showed that the IAP family modulates RIPK1 recruitment to the TNFR1 platform and, by analysing cIAP1/cIAP2 or cIAP1/XIAP DKO mice, illustrated that this critically regulates RIPK1- and TNFR1-mediated death *in vivo*.

David Wallach (Rehovot, Israel) discussed the mitochondrial-associated RIG-1 complex, a ribonucleic acid sensor, to which caspase-8 and RIPK1 are recruited in response to viral infection. The RIG-1 complex activates IRF3, which drives transcription of type I interferons. The complex is stabilised by RIPK1 ubiquitination and dissociated by caspase-8-mediated cleavage of RIPK1. Therefore, caspase-8 can restrict RIG-1 signalling without compromising its initial activation. Caspase-8 knockdown in basal epidermal keratinocytes leads to fatal inflammation associated with enhanced phosphorylation of IRF3. Hence, this work revealed a further function for caspase-8 in restricting inflammation.

Maria Andree (Cologne, Germany) discussed XIAP as a key regulator of immune responses to intracellular infection, for example by *Shigella flexneri*. XIAP serves to regulate the NOD1 signalling cascade, induce NF-κB activation and upregulate IL-8 secretion. *Shigella* dampens the pro-apoptotic response to infection by selective release of SMAC from mitochondria, thus inhibiting XIAP. Philipp Jost (Munich, Germany) described how cytokine secretion in macrophages following activation of NOD2 depends (in part) on XIAP, whereas Mads Gyrd-Hansen (Copenhagen, Denmark) proposed that the E3 ligase activity of XIAP was required for its potentiation of NF-κB activation. Interestingly, X-linked lymphoproliferative disease is associated with XIAP mutations and defects in NF-κB activation.

Cell Signalling from Membrane-Associated Complexes

Arton Philip Kater (Amsterdam, The Netherlands) studied the lymph node microenvironment that is postulated to favour chemoresistant CLL. He reported that BAFF and APRIL, two TNF receptor ligands, are abundantly expressed and accelerate tumorigenesis in a mouse model of CLL, suggesting a rationale for targeting the TNF-receptor pathway.

Henning Walczak (London, UK) described the identification of the linear ubiquitin chain assembly complex (LUBAC) as a novel component of the TNF-receptor signalling complex (TNF-RSC). Recruitment of LUBAC to the TNF-RSC is apparent within seconds and is dependent on TRADD, TRAF2/5 and cIAP1/2. LUBAC consists of HOIL-1, HOIP and Sharpin, which together form an E3 ligase that creates linear ubiquitin chains stabilising TNF-RSC and enhancing recruitment of the NEMO-IKK and TAB/TAK complexes. Sharpin is mutated in mice with chronic proliferative dermatitis and the skin lesions in these mice can be prevented by TNF knockout. Therefore, Henning proposed that Sharpin prevents TNF-induced inflammation by balancing the output of TNF signal transduction. This may open new therapeutic avenues for the treatment of autoimmunity.

Laura Dickens (Leicester, UK) described a novel structural model of the TRAIL DISC, derived from mass spectrometry analysis of the proteins present in the affinity-purified complex. This analysis suggested that the relative abundance of proteins within the DISC does not fit with current hypotheses. Laura challenged existing views of the DISC structure and left delegates considering the challenges of redrawing the TRAIL DISC.

Inna Lavrik (Heidelberg, Germany) addressed the contribution of DED-proteins in the CD95 DISC. In each HeLa cell, there are around 250 000 molecules of caspase-8, but only 530 molecules of c-FLIP$_S$ and 320 molecules of c-FLIP$_L$. Therefore, c-FLIP affinity for the DISC is much higher than that of caspase-8. c-FLIP$_L$ can act in a pro-apoptotic or anti-apoptotic manner depending on DISC stoichiometry, the amount of c-FLIP$_{S/R}$ and the extent of CD95 stimulation. Also cleaved c-FLIP$_L$ (p43) can disable, promote or inhibit NF-κB activation depending on whether its levels are low, medium or high, respectively.

Closing Remarks

As the meeting concluded, the weather closed in, resulting in cancellation of the 3rd Apoptosis Ski Slalom competition. Prizes were awarded to PhD students who gave exceptional short talks: Inês Castro, Eleonora Ottina and Maria Andree. Congratulations to them, and a big thank you to the organisers, sponsors and participants who made the meeting outstanding.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This meeting was an EMBO Molecular Medicine Workshop and was also sponsored by BD, Biozym, eBioscience, Eppendorf, Genentech, Invitrogen, Microsynth, VWR, Innsbruck Biocenter and *Cell Death and Differentiation*. We would like to thank the speakers who gave us permission to include unpublished work and apologise to those whose work we were unable to include due to space limitations. We would also like to thank Professor Marion MacFarlane for her helpful comments.