Beyond BRAF: Mechanisms of Resistance and Therapeutic Development

Summary of the 26th Nikolas Symposium, Athens, Greece May 5-8, 2016

The 26th Nikolas Symposium returned to the theme of targeted therapies in histiocytosis. The Symposium Mission of a 'search for a rational cure' has reached a pivotal phase. The somatic mutations in MAPK pathway genes that cause histiocytosis have largely been identified. Owing to the occurrence of these mutations in other cancers, notably melanoma, pharmaceutical companies have invested heavily in developing targeted therapies. Dramatic successes have been reported in the treatment of patients with advanced disease using these drugs. The symposium considered two important themes in oncology relevant to histiocytosis: how to conduct trials that allow patients with histiocytosis to gain access to drugs, and how to pre-empt resistance to drugs. Honours were given to the family of Dr Bob Arceci and to Dr Maarten Egeler for their contributions to the field of histiocytosis.

Sesssion I. Introduction. The meeting was opened by **Dr Barrett Rollins** (Boston) and **Dr Maarten Egeler** (Toronto). Dr Egeler introduced participants to the clinical features and treatment of LCH, outlining the current international treatment trial in children and young adults (LCH IV). One of the challenges facing investigators and physicians is that LCH IV was conceived prior to the discovery of somatic mutations such as BRAF V600E and the advent of targeted drugs. Careful planning is now required to preserve the integrity of the trial for the majority of low risk patients who will be cured with standard therapy while also ensuring that high-risk patients can access the new treatments [1].

Session II. Cell of Origin and Clonal Heterogeneity. Dr William Mifsud (London) and **Dr Jennifer Picarsic** (Pittsburgh) presented the pathology of LCH. They highlighted the heterogeneity of lesions and the difficulty of making a firm diagnosis in some tissue sites, notably inflamed skin and skin-draining lymph node where Langerhans cell hyperplasia can be a reactive finding. S100, CD1a and Langerin continue to be the mainstay of pathological diagnosis of LCH. Non-LCH lesions such as Erdheim Chester Disease present a much greater challenge as they remain a pathological diagnosis of exclusion and the presence of foamy macrophages and giant cells can mimic a wide range of other states. Unlike other haematopoietic neoplasms, it is not possible to grade histiocytic lesions with any prognostic impact. Genetic testing will help to resolve some of these problems but also has limitations when there are very few LCH cells present, their distribution is heterogeneous or DNA has been destroyed during the processing. Although it is possible to take sections of every biopsy for DNA sequencing to identify the mutation causing LCH, in many centres this does not occur because molecular diagnosis is not yet a standard of care that will be funded/reimbursed and there are no specific consent procedures in place to conduct sequencing on a research basis. New antibody reagents including VE1, specific for BRAFV600E, work in melanoma but high background staining has been observed in macrophages, compromising its use in histiocytosis [2]. Phospho-MEK and –ERK specific antibodies hold some promise but validation of their sensitivity and specificity is a protracted process.

Dr Carl Allen (Houston) gave a presentation on the evolving biology of LCH. He emphasized that clinical staging remains the basis of risk stratification. Low risk single system LCH has 100% survival. High risk multisystem disease can be cured with conventional cytoreductive therapy although up to 50% suffer at least one relapse or reactivation within 3 years of diagnosis that can increase the risk of long-term sequelae including potentially late neurodegenerative disease. Resistance to initial therapy is rare but identifies all patients at risk of death and requires swift salvage therapy. In molecular terms, Dr Allen reminded the audience that LCH cells have many transcriptomic differences compared with epidermal LCs and that p-ERK is a universal

finding regardless of BRAF mutational status. In the search for the remaining disease genotypes, it was noted that p-MEK is higher in lesions carrying BRAF or MAP2K1 mutation that those with unknown mutations. The genomic landscape is relatively unperturbed in histiocytosis with mutually exclusive MAPK pathway activating mutations, similar to the pattern observed in hairy cell leukaemia and pilocytic astrocytoma. Dr Allen discussed the potential prognostic associations with genotype such as the suggestion that BRAFV600E conferred a higher rate of progression than other mutations. He also described a powerful proteomic classifier that could identify CNS LCH compared with glioma. Mutated BRAF is undetectable in CSF and not useful diagnostically, however, circulating alleles are detectable in myeloid cells and T cells at the time of active disease in patients who go on to develop neuro-degeneration. This critical information suggests that neuro-degeneration may be a delayed but direct manifestation of infiltration by mutated precursor cells into the brain, rather than a para-neoplastic or other indirect phenomenon. In keeping with this, Dr Allen showed brain pathology from one patient with clear peri-vascular infiltration of LCH cells and described several cases in which BRAF inhibitor therapy had induced radiological and clinical responses. These findings extend the previous experience of the Houston team that chemotherapy can halt or even reverse neurodegeneration if given early enough.

Dr Julien Haroche (Paris) described Erdheim Chester Disease (ECD), a histiocytic disorder still described by exclusion of LCH pathologically but related to LCH by molecular genetics [3]. He described the original 1930 case series containing every known major feature of ECD. Symmetrical long bone involvement, present in 96% of patients, was described as 'iconic'. Peri-nephric, fat, tibia and xanthelasmata are the best sites for observing characteristic pathology and obtaining nucleic acid for sequencing. The Paris case series now includes 119 male and 44 female patients with a 16% overlap with LCH, Rosai Dorfman or both conditions. Although vastly better recognised in the last decade, diagnostic delays are still common with a median interval of 4 years between onset of symptoms and diagnosis, ranging up to 25 years in one case. Major risk sites include the brain and heart. Interferon alpha remains the most tested therapy although responses have been seen with anakinra, infliximab and toculizumab. Vemurafenib has induced major responses in difficult patients but has a significant side effect profile due to activation of wild-type BRAF. Dabrafenib (BRAFi), Trametinib (MEKi) and Cobimetinib (MEKi) are also promising in early phase studies. The LOVE study of treatment interruption demonstrated that treatment is probably required at some level for life, to prevent reactivation. No resistance has yet been reported.

In the final presentation, **Dr Ben Ebert** (Boston) described his work on Clonal Haematopoiesis of Indeterminate Potential, (CHIP) [4]. This striking observation was made by simply re-analysing exome sequencing data without setting a threshold for the variant allele frequency but adding a filter for known somatic mutations in myeloid malignancy. In practice, clones can be detected from about 1% frequency and are found in 10% of 60 year olds and 20% of 70 year olds. Most cases have a single mutation, DNMT3A, TET2 and ASXL1 being the most common, with a mean allele frequency of 0.12 (24% of cells carrying heterozygous mutation). BRAFV600E was observed at a low rate of 2/17,000 (tenfold higher than the incidence of histiocytosis or hairy cell leukaemia in adults). Clonal haematopoiesis converts to myeloid malignancy at 0.5-1.0% rate per year and confers a 10-fold risk of haematological malignancy over 5 years. All cause mortality was increased, notably due to the increased risk of vascular death that surpassed the risk due to hypertension or hypercholesterolaemia. Dr Ebert also described a second series of studies relating the effect of imids in targeting transcription factors IKZF1 (ikaros), IKZF3 (aiolos) and casein kinase 1A1 (CK1A) to the E3 ubiquitin ligase for degradation [5, 6]. These observations explain the therapeutic effect of lenalidomide in myeloma (dependent upon IKZF1) and 5q-MDS (dependent upon CK1A). His work has also elucidated the amino acid residue within the E3 ligase complex that confers imid sensitivity on humans but not rodents. Dr Ebert concluded by observing that LCH has been successfully treated by imids suggesting a role for IKZF1/3 in

LCH cell development or dependence of LCH upon TNF□□□ □□ □□□□□□□□ □□□□□□□ □□ □□□□□□□□□□□□ □□□□□□□□□□

Session III. Single cell analysis of lineage. The question of how gene expression shapes the function of cells was revisited by **Dr Joachim Schultze** (Bonn), using combined time-dependent ChIP-seq and RNA-seq, to examine the process by which macrophages respond to different signals to take up specific roles in the tissues. The previous model of several decades described polarisation of monocyte-derived macrophages into two phenotypes either M1 or M2 that were designated pro or anti-inflammatory. Dr Schultze showed that up to 15 different stimuli induce distinct macrophage states, controlled by modules of regulatory genes. Although tissue macrophage populations have tissue specific enhancer landscapes that correlate closely with gene expression, inflammatory monocyte-derived cells all have a relatively uniform and open chromatin profile allowing functional groups of transcription factors to effect a second tier of rapid stimulus-specific transcriptional regulation [7].

High dimensional analysis of cell phenotype was also discussed by **Dr Evan Newell** (Singapore) who presented 45-parameter CyTOF data to interrogate diversity in immune cell populations relevant to understanding antigen specificity, function, proliferation status, trafficking and senescence [8]. Traditional 2D visualisation methods are overwhelmed by n>10 parameters owing to the exponential increase in possible pairwise combinations (2^n) and require the development of dimension reduction techniques such as PCA, tSNE or clustering. These approaches have redefined tissue lymphocyte populations by revealing unprecedented diversity and exposing simple associations previously documented between chemokine receptor expression and function to be mostly spurious. CyTOF can be powered up to screen for up to 1,000 TCR clonotypes using combinatorial 'bar-codes' of 4/14 different heavy metals. This allows mapping of the immuno-dominant epitopes of tumour infiltrating T cells and how lymphocyte pouplations are modulated by co-stimulatory blockade in vivo.

Session III was closed by the two Pritchard Scholars, **Dr Rikhia Chakraborty** (Houston) and **Dr Ben Durham** (New York) who both described recent molecular genetic studies in LCH and other histiocytic disorders [9, 10]. Dr Chakraborty outlined two new mutations: FAM37-BRAF fusion in LCH discovered by whole genome sequencing and ERK mutation in JXG. In the absence of BRAF mutation, MEK is frequently inactive suggesting an alternative ERK activation pathway or feedback inhibition. Phospho-flow of ERK in primary LCH cells can even be exploited to predict sensitivity to targeted therapy when the mutation is unknown. At a genome level, Dr Chakraborty also reported hypermethylation of protein tyrosine phosphatase genes that might contribute to MAPK pathway activation in LCH. She also updated the audience on the Houston triad-based GWAS study that shows promising signals in SMAD6, part of the TGFb signal transduction pathway, known to be active in LCH cells. Dr Durham described a plethora of new mutations in LCH and non-LCH histiocytosis including copy number variations in MAPK genes MAP2K2 (MEK2) and MAPK7 (ERK5), mutation of MLL3, CDKN1B deletion or LOH of RB1, TP53, NF1, and NF2. Although drug resistance has not yet been reported in histiocytosis, vemurafinib-resistance in hairy cell leukaemia has been attributed to secondary N/KRAS mutation. He also showed proof of the hematopoietic origin of ECD by transplantable NRAS mutated ECD in immunodeficient NSG-SGM3.

Session IV. Defining success in targeting single pathway cancers and drug development. **Dr George Demetri** (Harvard) gave a talk entitled 'Combination strategies and orthogonal approaches to overcome resistance in kinase-driven cancers.' He discussed the impact of lessons learned in the last 2 decades of targeted therapy for GIST, a tyrosine kinase receptor mutated tumour sensitive to imatinib. He observed the paradigm shift in approach to treating cancer from empiric clinical trials to 'knowledge-based' intervention and recalled that the molecular clue to GIST originated with the description of CD117 (CKIT) expression by pathologists. This was

primarily important in classification and then in molecular genetics since 65% of GIST is C-KIT mutated and the majority of the remainder, PDGFRA-mutated. Similar to LCH, single mutations are mutually exclusive and as anticipated but unproven in LCH, genotype correlates with risk (PDGFRA mutated GIST rarely metastasizes). Molecular therapy requires new tools for response evaluation. GIST treated with TKI therapy rapidly becomes metabolically inactive but is very slow to regress and contains residual tumour cells. Thus trial endpoints based on radiological or pathological evaluation can be too stringent. In this context, BRAF-mutated cells are known to persist in the circulation of LCH patients in spite of dramatic clinical responses. Resistance to therapy is seen in about 10% of patients with GIST, and involves the slow expansion of resistant clones present at low frequency at diagnosis. New generation drugs are able to overcome some resistance mutations and early tumour eradication has become a potentially achievable goal through more active therapy. Alternative approaches maybe effective in GIST and histiocytosis including antibody therapy (CD117 is the target in GIST), co-stimulatory blockade with PD1/PDL1 inhibitors and IDO inhibitors. Dr Demetri concluded by advocating 'expansion trials' in which successful phase I agents can be accelerated into phase III and recommended that 'age anonymous' trial designs are developed to capture high risk disease in adults. **Dr Hubert Caron** (Roche) described the regulatory challenges that face pharmaceutical companies in testing new drugs and explained recent legislation that compels industry to offer treatment to children in early phase clinical trials. This is seen as an important advance in making new medicines more rapidly available to children with rare diseases, such as LCH. Previously, paediatricians have often had no alternative but to use adult formulations of new drugs without a license. Roche has anticipated these changes by creating a paediatric development team to integrate age appropriate study designs. Successful modern approaches include the use of Phase I/II 'Matrix' protocols in which phase I compounds are tested on molecular targets that may span cancers of multiple different tissues. He mentioned a number of potential targets in LCH that are over-expressed including c-myc, BCL2, MDM2, and CSFR1.

Session V. Mechanisms of Resistance to RAF inhibitors.

Unfortunately Dr Neal Rosen was unable to attend to discuss the characterization of oncogenic RAF and MEK alleles. The programme was closed by **Dr Levi Garraway** (Boston) who has pioneered the analysis of why cancer becomes resistant to drug therapy, at the molecular level. He began with the example of melanoma which often shows a dramatic response to therapy followed by a rapid relapse. Resistance can be modelled in the laboratory by exposing cell lines to drug and looking for escape mutants. Random events can now be amplified by ENU mutagenesis or interrogated by high throughput screening of cDNA, shRNA or CRISPR libraries [11]. Both clinical and laboratory observations indicate that hitting the pathway hard improves durability of responses but the resistance can still emerge. Dr Garraway characterised 3 mechanisms of resistance: pathway reactivation; pathway bypass; and pathway indifference. These were all first characterised in the laboratory and the findings matched to clinical observations. For example BRAF inhibition can lead to pathway reactivation by secondary RAS or ERK mutation. In vitro cDNA library screens identify cAMP, G-protein coupled receptors and Protein Kinase A as bypass routes that can become activated. Transcriptional profiling identified signatures that correlate with pathway indifference although this was often a transcription factor network modulation rather than a specific tractable target [12]. It was predicted that resistance to targeted therapy will emerge in histiocytosis by the same three mechanisms. The importance of clinical resampling was emphasized, combined with the power of single cell genomics to reveal pre-defined heterogeneity from which resistant clones could be predicted to emerge [13]. The concept of a 'resistome' was elaborated; the set of about 160 loci that coalesce into 10 or so patterns to confer resistance to targeted therapy. Dr Garraway concluded with a discussion of resistance to immunotherapy. High resolution molecular analyses permit the mapping of neoantigen generation and associated lymphocyte responses associated with

prognosis. Tumours are clearly under selective immune pressure as they frequently accumulate mutation in antigen presentation pathways including the peptide binding site of HLA [14]. In summary it was proposed that effective therapy of cancer will require high-order combination therapy incorporating alternating bespoke drugs with scheduled interruption and frequent tumour re-sampling. The use of cytotoxics will continue; although empirically selected, they target the apoptosis pathway with high efficacy.

In summary, the 26th Nikolas symposium placed LCH and related disorders firmly on the map of cancer medicine. Many critical advances, from DNA-based diagnosis and understanding of biochemical abnormalities, to the testing of appropriate drugs and surmounting of drug resistance, are being drawn from mainstream discoveries in the field of oncology. Reciprocally, a number of insights derived from histiocytosis, have redefined the envelope of 'cancer'.

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