Genomics and Metabolomics in Dendritic Cells: Are there clues for LCH causes and cures?

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The Nikolas Symposia

The mission of the annually held Nikolas Symposium is to find a rational cure for Langerhans Cell Histiocytosis (LCH) (Beverley et al., 2005). This meeting is sponsored by Paul and Elizabeth Kontoyannis whose son Nikolas developed LCH in infancy but has survived his battle with this disease. The symposium is an interactive forum of basic scientists and clinicians who discuss different aspects of biology and clinical features of the disease, and attempt to apply this information towards an improved understanding and treatment of LCH. A focus of the symposium is the biology of dendritic cells (DC) to which the Langerhans cells belong. Although LCH is a rare disease, the organizers believe that the research stimulated by this symposium will not only improve our understanding of LCH, but will also increase our insight into normal DC and other disorders in which dendritic cells are involved.

Introduction

The Nikolas Symposium 2011 has been a very exciting and memorable one for several reasons. Looking back at the meeting, it leaves extreme emotions of great satisfaction and hope, as well as feelings of sadness and loss. First, recent advances in LCH research have provided a major breakthrough in our understanding of the underlying cause of the disease. Finally, LCH researchers have found a needle in the haystack, which holds the promise of a true rational cure. But simultaneously we feel hurt by the recent decease of Dr. Ralph Steinman, the 2011 Nobel laureate, who was an inspiring member of the steering committee of the symposium for several years and the founding father of dendritic cell research. We are proud that he was in our midst and that he could contribute his great insights to this Nikolas Symposium.

Langerhans cell histiocytosis (LCH) is a rare disease, presenting in various clinical forms, that is characterized by the accrual of cells with features of immature Langerhans cells (LC), a subtype of dendritic cells (DC) (reviewed in (Beverley et al., 2005; Egeler et al., 2010). LCH primarily presents with single or multifocal granuloma-like lesions in different organs with skin and bone being most frequently affected. More severe forms of LCH, in which multiple organ systems are involved, may resemble acute leukemia and occur especially in younger children. In LCH lesions of both single and multisystem disease, the presence of LCH cells is a constant factor but other infiltrating cell types are often abundant and are thought to have a profound influence on the biological behavior of the LCH cells. The disease is sporadic, occurring in a broad age range from the newborn to the elderly, but peaks between 1-4 years of age. The incidence in the pediatric age range has been estimated at 2-5 per million per year.

For several years, a central question in LCH research has been whether LCH cells develop due to an intrinsic defect, and thus are (pre-)malignant, or represent activated DC that accumulate as a consequence of a dysregulated immune response to environmental triggers. Interestingly, supportive evidence for both scenarios exists (Egeler et al., 2010). Considering LCH a reactive condition is suggested by the clinically variable behavior with frequent remissions, the cytologically benign appearance of the cells and their non-clonal relationship in cases of pulmonary LCH. The granulomatous, inflammatory nature of the LCH lesion has been linked to abnormal production of IL-17A , in part by LCH cells themselves (Coury et al., 2008), but these findings could not be confirmed by others (Peters et al., 2011). On the other hand, the clonal relationship of LCH cells in non-pulmonary cases, their maturation arrest, cell cycle dysregulation, and sometimes aggressive clinical behavior as well as the high disease concordance rates observed in twins argue in favor of a genetic origin. This notion was strengthened by a recent breakthrough in LCH research, showing a mutation of the BRAF protein, which is part of the RAS - MAPK signaling cascade, in almost 60% LCH cases (Badalian-Very et al., 2010; Badalian-Very et al., 2012). To discuss these exciting findings in more depth, and to contemplate the physiological consequences for LCH cell behavior, this year's symposium was dedicated to the genomic and metabolic dysregulation of cancer cells, and dendritic cells in particular.

The pathology of the various forms of histiocytosis was introduced by **Dr. Malone**, who used involvement of either antigen-presenting (i.e. DC) or antigen-processing cells (i.e. macrophages) as leading distinguishing principle. Besides anatomical location and morphology of the cells, characteristics related to their respective functions, such as phagocytosis, concentration of lysosomes and expression of surface molecules determine their identification. High level CD68 and CD163 identify macrophages, while different types of DC- and LC-related cells express CD1a, S100, Langerin (CD207), Fascin and/or Factor XIIIa. Histiocytic disorders are further divided into diseases that are composed of pathologically benign cells and malignant forms. This presentation focused in particular on the benign forms. LCH is the most frequent DC-related histiocytosis, while macrophage-type disorders mostly present as hemophagocytic lymphohistiocytosis (HLH). Phenotypically, LCH cells are characterized by a round morphology and large cytoplasm, expressing CD1a and Langerin as distinctive markers, although these are not always co-expressed. The racket-shaped cytoplasmic Birbeck granules are characteristic of LC, but may be absent in LCH cells. Together with LCH cells, a variety of other cell types is found in LCH lesions, such as eosinophils, T-cells, macrophages and multinucleated giant cells.

Different factors complicate the pathological diagnosis of histiocytic disorders, such as their rare occurrence, the developmental and phenotypic heterogeneity of the histiocytic cells and variable lesional composition. The plasticity of lesional histiocytes is illustrated by the occasional loss of a characteristic marker profile over time or the (infrequent) finding of co-existence or sequential development of LCH and juvenile xanthogranuloma (JXG), which is characterized by accumulation of dermal dendritic cells with a distinctive FXIIIa+ CD1a– S100– phenotype (Hoeger et al., 2001). LCH diagnosis may be complicated further by coexisting macrophage activation syndrome (Favara et al., 2002) and accumulation of activated histiocytes in lymph node sinuses, also known as Rosai Dorfman disease (O'Malley et al., 2010). Moreover, in several cases LCH developed from a histiocytic disorder that initially did not match the diagnostic LCH criteria. Thus, the natural history of LCH lesions are highly variable, with the "LCH cell" as the hallmark.

Current notions in DC biology relevant for LCH

In his keynote contribution to this symposium, **Dr. Steinman** provided an overview of DC development and heterogeneity, and further focused on the application of DC in vaccination (Steinman, 2012). Ground-breaking research of recent years, has revealed the origins of DC to a large degree. A common precursor for macrophages and DC (MDP) in the bone marrow is characterized by expression of receptors for both M-CSF and Flt-3L (Liu et al., 2009; Geissmann et al., 2010). Stimulation with Flt-3L then gives rise to common DC precursors, which generate plasmacytoid DC (pDC) and classical (conventional) DC. Conversely, M-CSF stimulation of MDP leads to development of monocytes, which may differentiate into macrophages, but, upon stimulation with Gram-negative triggers, also into CD209a$^+$/DC-SIGN$^+$ lymph node DC (Cheong et al., 2010). Thus, different subsets of DC are generated *in vivo*, so far best characterized in mice. These subsets can be separated into CD8$^+$ and CD8$^-$ conventional DC, plasmacytoid DC and monocyte-derived DC, respectively. The DC subsets are characterized by differential expression of surface markers, often lectins that may function as antigen-uptake receptors, and different signaling sensors, such as Toll-like receptors.

Dr. Steinman's work has been instrumental in the appreciation that, in the steady state, DC induce immune tolerance (Hawiger et al., 2001; Steinman and Nussenzweig, 2002). This is essential to avoid subsequent autoimmunity and chronic inflammation during infection. One of the tolerance mechanisms is via the peripheral induction of Foxp3$^+$ regulatory T cells (iTreg). In an *in vitro* approach, only DC appeared to be capable of stimulating this conversion of Foxp3$^-$ cells, and TGF-beta and all-trans retinoic acid were important tolerogenic factors (Sela et al., 2011). When tested *in vivo*, these iTreg appeared to be functionally stable and provided long term protection in an allogeneic graft versus host disease model.

Although the past decades have generated great insights in immune function and manipulation, Dr. Steinman stressed that further challenges are to produce new vaccines that are safe and effective and have a rational basis. These should be applicable in fighting cancer and infections such as HIV and *M.tuberculosum*, but others should generate durable tolerance against allergies and asthma, against autoimmunity, and against transplanted organs. The critical role of DC in skewing the balance between tolerance and immunity places this cell type central in these new developments. Targeting antigen specifically to DC can be done very efficiently via fusion antibodies that are aimed at DC surface lectins, such as DEC-205,

and carry antigen sequence in their Fc part (Idoyaga et al., 2011). For an effective immune response, simultaneous stimulation of activating receptors is essential. Pilot studies in non-human primates and, subsequently, human volunteers showed that TLR3- and MDA5-targeting synthetic RNA (poly-ICLC) was a superior adjuvant for the induction of Th1 and humoral immune responses (Stahl-Hennig et al., 2009).

Dr. Collin discussed the current insights in human DC heterogeneity, elaborating on the putative origin of the cells that become dysregulated in LCH. He distinguished three different possibilities: epidermal LC, monocytic precursors, or local Langerin$^+$ DC. LC are autonomous, self-renewing cells that are maintained under steady state conditions independently from circulating precursor cells. They persist even in the rare condition in which DC, monocytes and B-/NK-lymphocytic cells are absent, known as DCML deficiency (Bigley et al., 2011; Collin et al., 2011). Since LCH lesions also accumulate at sites that normally contain no LC, a direct LC origin seems to be less likely. The dermis is the preferred site for skin LCH lesions and appears to contain different subsets of DC and macrophages (Haniffa et al., 2009; Teunissen et al., 2012). Two populations of migratory DC can be distinguished: CD1c$^+$ CD1a$^+$ DC and CD14$^+$ FXIIIa$^+$ DC. The latter are probably the normal counterparts of JXG cells, while the former bear phenotypic similarity to LCH cells. Both DC populations appear to be dependent on circulating precursors. In contrast, non-migratory, tissue-fixed macrophages are autonomous and characterized by high level autofluorescence, melanin content and expression of FXIIIa and CD209. A small fraction of CD1a/1c$^+$ dermal DC express Langerin, although the expression levels are not as high as in LC. As observed before in mouse studies, these dermal Langerin$^+$ CD1a/1c$^+$ DC are maintained independently of epidermal LC, and appear to be absent in DCML deficiency patients.

In a series of *in vitro* studies, Dr. Collin showed that Langerin expression by myeloid cells appears to be a rather flexible trait. For instance, it was found to be inducible in culture in a fraction of precursors stimulated with either Flt-3L/GM-CSF/TNF-α or GM-CSF/IL-4/TGF-β. While these mobilized stem cell cultures take 14 days to develop, even in 18h cultures Langerin expression was induced in peripheral blood lineage marker-negative, HLA-DR$^+$ cells stimulated with the latter combination of cytokines. Within this subset, Langerin appeared to be expressed in particular by the CD14$^-$ CD1c$^+$ population, which may thus represent circulating pre-DC. Taken together, these findings suggest that cells from different origins may be induced to express Langerin, and assume an LCH-like phenotype, depending on the local environmental conditions.

The role of bone marrow niches, and particularly mesenchymal stromal cells (MSC), in the pathogenesis of human hematopoietic dysregulation was presented by this year's Pritchard fellow, **Dr. Vaiselbuh**. She described a model in which bioengineered 3D scaffolds coated with human MSC were implanted subcutaneously in NOD/SCID mice to study the effects on pediatric acute myeloid leukemogenesis (Vaiselbuh et al., 2010). The niche scaffolds creates a human tumor microenvironment that forms a sanctuary for AML cells. The stromal compartment mimics a normal BM niche, including presence of blood vessels, adipocytes and osteoclasts. After four months in the mouse, the human leukemic cells exit the niche and spread via the bloodstream, thus mimicking leukemia relapse. To study the niche influence on evolutionary changes in leukemic cells, Dr. Vaiselbuh studied the microRNA profiles of AML cells before implantation (d.0), after 1 month, representing niche retention, and after 4 months upon niche exit. MicroRNAs are ~22 nt. small non-coding RNAs, which are important regulators of mRNA stability and known to be involved in regulation of normal hematopoiesis and leukemogenesis (Marcucci et al., 2011). Interestingly, distinct microRNAs were found to correlate with the stage of leukemia development related to niche interaction. It is hypothesized that different microenvironmental factors, derived from the niche stromal cells, stimulate the pathogenic leukemia development. This notion is reinforced by the recent finding that abnormal niche stromal cells can induce myelodysplasia and secondary leukemia in a mouse model (Raaijmakers et al., 2010). Thus, it would be very interesting to study the role of stromal cell abnormalities in initiation and maintenance of LCH lesions by activating by-standing Langerhans cells (or precursors) and attracting other types of inflammatory cells. The stromal cell line DOR-1, derived from an LCH bone lesion (Gogusev et al., 2005), would provide an excellent tool to study this in the available *in vivo* model.

Dr. Soumelis elaborated on the variety of signals that elicit distinct responses in human plasmacytoid DC (pDC). These signals can have different origins, and in infection the most important ones are either microbial, generating especially TLR-signals, or cytokine-mediated. Given the multiplicity of environmental signals, the cellular outcome is highly dependent on the integration of the different signals. To shed light on this complex matter, Dr. Soumelis studied the interaction between pDC-activating signals,

i.e. IL-3 or GM-CSF (Ghirelli et al., 2010)and heat-killed influenza virus (Flu) and/or inhibitory signals, such as mediated by glucocorticoid exposure. PDC appeared to be highly sensitive to GC-induced apoptosis (Lepelletier et al., 2010). However, they were protected from apoptosis induction by viral TLR stimulation, but not by microbe-independent stimuli, such as IL-3 or GM-CSF. This protection was dependent on TLR-induced autocrine TNF-alpha and IFN-alpha, which collectively increased the expression ratio between anti-apoptotic genes, such as Bcl-2 and Bcl-xL, versus pro-apoptotic genes such as caspase-8.

In a second model system, gene expression profiling was used to study the interaction between activating cytokine- and Flu-mediated pathways in pDC. To this end, pDC were treated *in vitro* with either IL-3 or with Flu virus, or with both triggers simultaneously. Theoretically, different outcomes are plausible. Independent processing of both signals will cause only additive effects. In contrast, interaction of signal pathways may lead to either (i) inhibition, where one stimulus without significant effect annuls the positive or negative effect of the other stimulus, (ii) negative or positive synergy, where both stimuli reinforce each others effects beyond mere addition, or (iii) restoration, where the combined stimuli show a lesser effect than each of the individual stimuli. Upon stimulation of pDC in this approach with IL-3 and/or Flu, of all classifiable genes, 8344 were found to follow an additive signal integration, while 1394 genes showed an interactive profile. Of the latter, most (852) showed a restorative profile. Negative synergy (339) was observed more frequently than positive synergy (33). Interestingly, in 85% of all cases where genes were found to be regulated in an interactive, so non-additive manner, the Flu-induced changes appeared to be dominant. Together, these findings lead to the conclusion that integration of cytokine and TLR signals by human pDC is multimodal as regulation of different genes may follow different profiles of interaction. In this, the TLR signal is dominant over IL-3-induced signals in human pDC.

State of the art in cancer genomics and metabolomics

As various contributions at this symposium demonstrated, technical developments in molecular research have transformed the biomedical field immensely over the last years. This concerns especially the application of genomic tools in cancer research. **Dr. Meltzer** provided an overview of current developments in this field, addressing questions about how genomic data and derived biological information could be obtained best, and how this could be translated optimally for patients' benefit. Current methodology in cancer genomics enables the assessment of numerous aspects, including transcription factor activity, gene expression, chromatin modification and DNA methylation as well as gene copy number and sequence variation. Especially sequencing technologies are developing extremely rapidly, and these may soon replace current microarray-based assessments. Alterations in all genomic aspects can be involved in carcinogenesis, and thus should be considered in an integrated approach to obtain a full picture of the pathogenesis of a proliferative deviation such as LCH.

To illustrate current molecular developments, Dr. Meltzer elaborated examples from his research on different malignancies. In alveloar rhabdomyosarcoma, the PAX3 –FKHR fusion gene encodes a pathogenic fusion transcription factor. Using chromatin immunoprecipitation, a genome-wide map of binding sites of this unique transcription factor could be identified, which facilitates the further understanding of disease pathogenesis (Cao et al., 2010). In a practical sense, research progression is often hampered by the scarcity of clinical samples. To study the role of DNA methylation patterns, methods were established that allowed the use of paraffin-embedded archival tumor tissue instead of fresh, frozen material (Killian et al., 2012). Using this, a positive correlation was found between cancer aggressiveness and DNA methylation profile in estrogen receptor-positive breast cancers (Killian et al., 2011). With the possibility to obtain sequence data relatively easily, management of the complexity of information becomes a challenge. In this respect, it may be more realistic to aim for sequencing of exomes, i.e. all exons, or of the approximately 100 most important cancer genes in samples rather than obtaining full genome sequence data. The availability of fast third generation sequencing technology strongly reduces both time and cost, thus making wide implementation a feasible option.

Dr. Ventura discussed the contribution of microRNAs to malignant transformation and development in general. He focused on the role of the microRNA cluster 17~92, also known as oncomir-1, since it is expressed at high levels in various malignancies. This cluster consists of 6 individual microRNAs (miR-17, 18a, 19a, 20a, 19b-1 and 92-1), which are transcribed in a single primary microRNA. MiR17~92 expression is induced by the transcription factor c-Myc or N-Myc, and these microRNAs play an important role in oncogenesis by inhibiting cell death. In a model of Myc-induced B-cell lymphoma, Dr. Ventura showed that the cluster is required to suppress apoptosis (Mu et al., 2009). Among the six microRNAs that are encoded by the cluster, miR-19a and miR-19b are absolutely required and largely sufficient to recapitulate the oncogenic properties of the entire cluster. Accordingly, mice lacking only miR-19 show increased resistance to Myc-driven tumorigenesis. A putative molecular target of these microRNAs is the tumor suppressor PTEN.

So far, the involvement of microRNAs in human hereditary syndromes has remained largely undiscovered. Dr. Ventura elaborated findings in the human Feingold syndrome, which is characterized by microcephaly, short stature and digital abnormalities. In the majority of cases this syndrome is caused by a loss of function mutation in the *MYCN* gene. However, those cases with normal *MYCN* appear to have a hemizygous deletion of the miR17~92 cluster (de Pontual et al., 2011). A mouse model, lacking a single allele of these microRNAs, phenocopies several key features of affected humans, indicating the functional involvement of the miR17~92 cluster with N-Myc-related effects in skeletal development. This is confirmed by the original findings in the full knockout model, where complete absence leads to severe skeletal defects, as well abnormal lung and heart development, causing neonatal death (Ventura et al., 2008). In a series of mutant mice, in which distinct members of the cluster were deleted, the contribution of individual microRNAs was determined to skeletal as well as lung and B-lymphocyte development. Deletion of miR-17 and -20 caused defects in skeletal and B-cell development, while deletion of miR-18a or miR-19a and -19b did not give rise to apparent developmental deviations. Thus, different members of the cluster miR17~92 have both unique and overlapping functions in various aspects of development.

In his contribution, **Dr. Dang** illustrated the intricate relationship between oncogenic pathways in cells and their metabolic reprogramming. Seminal findings early in the last century by Warburg already indicated that cancer cells have an altered cellular metabolism, characterized by high level glucose uptake and lactate production (aerobic glycolysis), even when enough oxygen is available (reviewed in (Koppenol et al., 2011)). The possibility to image LCH lesions with high sensitivity using FDG-PET scanning (Phillips et al., 2009) suggests that also LCH cells follow this paradigm. For tumor cells this metabolic switch is functional as essential elements for cellular biomass are generated. The *MYC* oncogene, which encodes the transcription factor c-Myc, contributes to tumorigenesis in many human cancers by integrating metabolism, cell growth and proliferation (reviewed in (Dang, 2012)). C-Myc has numerous target sites in the genome, especially related to regulation of metabolic processes. Identification of a cell type-independent Myc core signature indicates the primordial function of Myc in RNA processing, ribosome and mitochondrial biogenesis and biomass accumulation (Ji et al., 2011). Of note, ribosomes constitute approximately 2/3 of the cell mass, and therefore control of ribosomal biosynthesis closely relates to biomass regulation. Myc also stimulates glutamine metabolism by cells, which can drive the citric acid cycle as an alternative substrate to glucose (Le et al., 2012). Hypoxic tumor conditions activate the transcription factor HIF-1alpha, which shares multiple targets with Myc, and thus collaborates in its metabolic effects.

One of the target genes of c-Myc is LDHA (lactate dehydrogenase A), which in tumor cells converts pyruvate into lactate. Interestingly, reduction of LDHA by siRNA or a small molecule inhibitor not only reduced lactate production, but also caused oxidative stress and increased cell death (Le et al., 2010). Similarly, growth of glioma cells that expressed a mutant isocitrate dehydrogenase and therefore relied on glutamine-derived alpha-ketoglutarate could be inhibited by reducing glutaminase activity (Seltzer et al., 2010). Together, these findings illustrate the potential of targeting cancer cells by modulating their altered metabolic characteristics.

Dr. Heales presented data on the metabolic interactions between glia cells and neurons, and how these can be related to neurodegeneration as occurring in various diseases, including brain LCH. A central hypothesis in this respect is that inflammatory cytokines stimulate increased formation of reactive nitrogen products, which cause neuronal mitochondrial damage, leading to neuronal death. The involvement of reactive nitrogen products in neuronal disease is supported, for example, by the observation that nitrite and nitrate concentrations are elevated in cerebrospinal fluid of MS patients. The effects of NO to the mitochondrial proteins involved in the electron transport chain are reversible. In contrast, irreversible

damage caused by peroxynitrite (ONOO) to cytochrome C oxidase is thought to play an important role in mitochondrial demise (Bolanos and Heales, 2010). While astrocytes have robust anti-oxidant mechanisms, neurons are much more susceptible to reactive species. Glutathione in reduced form is capable to scavenge reactive nitrogen and thus protect against mitochondrial damage (Heales and Bolanos, 2002). In vitro experiments using LPS+IFN-gamma stimulation of astrocytes indicated that especially enzymes in mitochondrial membrane complex IV are susceptible to NO-mediated damage, and to a lesser extent complex II and III. By increasing glycolysis at the expense of mitochondrial oxidative phosphorylation, astrocytes protect themselves against ATP depletion and induction of apoptosis. Interestingly, neurons contain significantly increased levels of glutathione upon co-culture with astrocytes. This putative neuroprotective mechanism is probably mediated via glutamate-induced release of glutathione from astrocytes, which can be taken up by neurons. Failure of this astrocyte-to-neuron glutathione trafficking might thus be an essential factor in neurodegeneration.

Molecular pathogenesis and therapeutic approaches of LCH

Recent work by **Dr. Rollins'** group has generated much excitement in the field of LCH research, since they could, finally, show consistent genetic changes in affected cells from the majority of patients with LCH (Badalian-Very et al., 2010; Badalian-Very et al., 2012). At the symposium, Dr. Rollins elaborated on these findings and discussed recent follow-up. The breakthrough findings were made using a newly developed mass spectrometric-based genotyping technology, called Oncomap, that allowed the profiling of cancer gene mutations in routine formalin-fixed, paraffin-embedded clinical samples (MacConaill et al., 2009). By multiplexing, nearly 1000 alleles in 115 different cancer-related genes could be probed simultaneously. Using this technology to assess 61 LCH samples, in 35 cases (57%) an oncogenic mutation V600E could be shown in the *BRAF* gene. This pathogenic mutation is frequently found in melanoma, thyroid cancer or colorectal cancer, but unique among histiocytoses, since no *BRAF* mutations were found in cases of dermatopathic lymphadenopathy, JXG or Rosai-Dorfman disease. Laser-capture microdissection enabled isolation of LCH cells followed by pyrosequencing, and this showed the mutation frequency correlated closely with approximately 50% of pathogenic LC abundance, consistent with the dominant behavior of this mutation in other contexts.

BRAF is a serine/threonine kinase functionally involved with the signaling cascade that typically is initiated by growth factor binding to a receptor tyrosine kinase, which subsequently activates Ras, Raf, MEK and ERK by phosphorylation (Montagut and Settleman, 2009). Interestingly, elevated levels of pMEK and pERK were found in all LCH cases, not only in those with the BRAF mutation. The cause of activation of the pathway in these non-mutated cases is unknown, but might be linked to overexpression of a receptor tyrosine kinase or its ligand. Dr. Rollins reported the absence of BRAF gene duplication in LCH, which could be another mechanism of pathway activation. The currently available clinical data on the investigated cases did not show a clear correlation between the presence of the V600E mutation and clinical behavior, such as extensiveness or location of the disease. However, the presence of the mutation did correlate with young age. The mutation was also found in 40% of pulmonary cases, comparable to its occurrence in patients of similar age with LCH in other locations. This is surprising, since pulmonary LCH is considered a polyclonal disease in many cases, in contrast to the monoclonal nature of non-pulmonary cases (Yousem et al., 2001).

To investigate whether BRAF V600E is causing the disease or just a marker, targeted expression in an animal model should be informative. Driving mutated Braf expression in a Langerhans cell-specific manner using the Langerin promoter generated mice that weighed less and showed slightly increased numbers of CD11c+ cells in the spleen, but showed no signs of LCH-like disease. In an alternative approach, mutated Braf was expressed in a poly-IC/IFN-inducible manner, using a modification of a previously generated model developed to study V600E Braf pathogenic potential (Mercer et al., 2005). Intriguingly, from these mice, approximately 50% have disease manifestations, which closely resemble human LCH. Extensive mixed histiocytic infiltrates can be found, appearing as early as 4 weeks of age in skin, spleen, liver, thymus, bone marrow, lung as well as GI tract. The affected cells have LC-like morphology and express CD11c, while lesions have an inflammatory appearance and frequently contain multinucleated giant cells. The difference in disease development between the two mouse models suggests that the Braf mutation is pathogenic in myeloid precursor cells, but not immediately in Langerhans cells. Independent of the cellu-

lar origin, the generation of a mouse model now allows the testing of existing and novel blocking agents of the pathogenic V600E BRAF molecule.

Dr. Allen, who previously contributed important findings on the gene expression profiling of LCH cells (Allen et al., 2010), presented data that fully confirmed the presence of the V600E BRAF mutation in approximately half of LCH biopsy samples (17/32; 52%). The mutation was specific for LCH cells, since epidermal CD207-expressing Langerhans cells, or lesional or tonsillar T-cells did not contain it. In two cases of recurrent disease, BRAF status was consistent in the presenting and the relapse CD207+ cells: wild-type BRAF in one case and V600E BRAF in another. Also in this independent patient cohort, mutation status did not correlate significantly with age, extent of disease, or future recurrent/refractory disease. To determine the impact of the BRAF mutation on global gene expression, the transcriptomes of CD207+ cells isolated from LCH lesions with and without the mutation were compared. Enigmatically, using standard statistical analysis in this pilot series, there were no genes identified as significantly up- or down-regulated as a result of the V600E mutation. This probably can be explained by the generally activated Ras pathway in LCH cells, also independent of BRAF mutational status. If this finding is confirmed in larger series, it suggests that alternative routes exist that result in the same molecular signaling, and thus cell behavioral outcome.

To study differences in environmental factors, associated with disease condition or mutational status, several patient groups were compared using plasma proteomic approaches. This showed considerable variability within and among clinically defined risk groups. Nonetheless, in the clinically most affected group of pediatric patients with multisystem disease and risk organ involvement significantly elevated levels were observed in the following inflammation-related molecules: TNF-alpha, sTNFRII, IL-8, CCL15, CCL19, CCL20, CXCL6 and OPN, compared to controls and other LCH patient groups. However, in contrast to previous reports (Coury et al., 2008), no elevated levels of IL-17 protein or mRNA could be observed (Peters et al., 2011).

The identification of involved molecular pathways in proliferative diseases enables targeted therapeutic approaches. This was illustrated by **Dr. Grupp**, who focused on the mTOR-regulated pathway in malignant and non-malignant lymphoproliferative disease (Teachey et al., 2009). mTOR is a cytoplasmic kinase that functions as an integrator of external and internal cellular signals regarding energy and nutrient status, growth factor stimulation and stress. Growth factor receptor triggering often signals to mTOR via PI3 kinase/Akt and/or Ras. Stimulation of the mTOR pathway then fuels protein synthesis and cell cycling by various routes, in particular by enhancing ribosome biosynthesis and activation. Thus, the mTOR pathway is an attractive target in proliferative diseases. Rapamycin (sirolimus) and derivatives are important inhibitors of mTOR and are long known to have strong immunosuppressive effects. A central question in Dr. Grupp's research is whether it can also be used effectively to treat lymphoproliferations, such as ALL. In a xenograft model, patient ALL cells were transplanted in NOD/SCID mice. Animals treated with the mTOR inhibitor CCI-779 showed a significant decrease in peripheral blood blasts and in splenomegaly, in contrast to the expanding human ALL cells in untreated animals (Teachey et al., 2006b). The efficacy of mTOR signaling inhibition was indicated by the finding that ribosomal phospho-S6 protein was strongly diminished in xenografted human ALL exposed to CCI-779. Importantly, mTOR inhibitor synergizes with methotrexate (MTX) in its therapeutic effect (Teachey et al., 2008). This is explained by the increased sensitivity of ALL cells for MTX, which is induced by mTOR inhibitor via increased degradation of cyclin D1 causing reduced dihydrofolate reductase (DHFR) synthesis. High levels of DHFR are characteristic of MTX-resistant leukemic cells.

Also in non-malignant proliferations mTOR inhibitor can be therapeutically effective. Autoimmune lymphoproliferative syndrome (ALPS) can be caused by different defects in the Fas apoptotic pathway, causing chronic lymphoproliferation with autoimmune manifestations and secondary malignancies. Characteristic for ALPS is the expansion of atypical CD4- and CD8-double negative T lymphocytes. In mouse models of ALPS, mTOR inhbition appeared to be highly effective: a dramatic and statistically significant decrease was observed in lymphocyte numbers, lymphadenopathy, splenomegaly, and autoantibodies compared with controls (Teachey et al., 2006a). Also in children with ALPS, treatment with sirolimus appears to be effective, leading to complete remission in most, but not all cases.

A scientific representative of GlaxoSmithKline, **Dr. Portnoy** discussed the initiation of a phase IIa clinical study aimed at testing an oral AKT (PKB) inhibitor, GSK2110183, for its efficacy and safety in adult and adolescent patients with LCH. As indicated above, the PI3K - AKT signaling route plays an important role in regulating vital cellular processes, including protein translation (via mTOR), cell cycle and

metabolism, and apoptosis. The rationale for such a trial stems from the findings that (i) pAKT and other active intermediates in the pathway are increased in LCH (Brown, 2005), that (ii) the AKT pathway stimulates proliferation and survival in human myeloid DC, and that (iii) experimental treatment with GSK2110183 of an LCH patient with chemo-refractory LCH showed a favorable clinical response. This drug has been extremely well tolerated in an initial study in patients with multiple myeloma, and showed mild to moderate gastrointestinal adverse events as predominant side effects. An expansion cohort at the maximum tolerated dose identified from the first time in human study is in progress to evaluate hematologic malignancies. For application in adult patients with LCH there is a particular need for alternative therapeutic approaches, since adults frequently have difficulty tolerating available agents due to high rates of neuropathy when treated with vinblastine and high rates of clinically significant neutropenia when treated with cladribine.

The primary endpoints of this phase II trial are objective disease response at 3 and 6 months and proportion of subjects experiencing ≥ grade 3 neutropenia, microbial infections, or newly diagnosed or worsening neuropathy. Secondary endpoints are time to disease progression, adverse event and laboratory safety testing, and pharmacokinetic parameters. A Bayesian sequential analysis (i.e. data evaluation during collection) will be used to determine the likelihood that GSK2110183 efficacy is comparable to standard of care. This phase IIa study will be open to adult patients with either treatment-naïve or refractory/reactivation disease and to adolescents (aged 12-17) only with refractory/reactivation disease who are over 40 kg of weight and Tanner stage >2. Eligible subjects must have a confirmed diagnosis of LCH or be willing to undergo biopsy, and their disease must require systemic treatment.

In addition, Dr. Portnoy discussed other compounds that could be potentially used for treatment of LCH. These include a selective BRAF inhibitor (GSK2110846) that is currently in phase III testing in different malignancies, including metastatic melanoma. Furthermore, a MEK inhibitor (GSK1120212), which is currently also in phase III testing, may also be promising as it is active in melanoma with mutant BRAF.

Summation and conclusions

In the summation session, **Dr. Beverley** initiated the discussion on the insights gained and remaining questions in LCH. Beyond doubt, the identification of the frequent occurrence of the V600E BRAF mutation in LCH is a major breakthrough that will guide further scientific and clinical developments in this disease. Although V600E BRAF and activation of the Ras pathway may be common denominators, they cannot be the only etiological factors, since the mutation is present in only 50-60% of cases. Furthermore, a striking feature of LCH is its clinical heterogeneity in terms of extent, aggressiveness and location of the lesions. As there is no clear association between the presence of the mutation and clinical appearance of the disease, the origin of this heterogeneity is unclear and requires further study.

Clinical studies in LCH have indicated that disease recurrences and late sequelae pose a major challenge. Better tools are needed to enable stratification of patients with regard to their prognosis and to which treatment can be adapted. Valuable information that might identify prognostic factors might be obtained in the upcoming LCH-IV clinical trial (discussed in last year's Nikolas Symposium) if patient samples could be collected and sent, as paraffin slides, to a reference center able to test for the presence of the V600E BRAF mutation. Although this approach would provide much needed information, there are obvious financial, logistic and regulatory issues related to shipping patient material. Another practical challenge is to identify the best way to perform actionable genetic analysis in patient samples. A balance needs to be found between the extent of genetic information and its cost and manageability. Technical developments will likely guide this process, but it is safe to predict that these will be based on direct sequencing, rather than on array technology. Whole exome sequencing, for instance, could be a feasible approach.

An important genetic question to be addressed is what other molecular lesions can be found in LCH beyond V600E. Other members of the Ras-Raf-MEK-ERK pathway, as well as upstream receptors or down-stream molecules are candidates and should be scrutinized. This pathway, like other signaling cascades is strongly regulated by microRNAs. Therefore, microRNA profiles should be determined also and might provide important clues to pathogenesis.

In general, the choice of control cells and -tissues is important in these studies, since those provide the reference values to which LCH cells are compared., However, the normal counterparts of LCH cells have not been definitively identified. Various factors, including LCH cell tissue distribution and the prom-

iscuity of Langerin expression by myeloid cells of different origins, question whether LCH cells are directly related to epidermal Langerhans cells. In addition, the more recently identified Langerin-expressing dermal DC may not be the normal counterpart. The latter cells are dependent on Flt3-ligand for their development, while LCH cells may belong to the M-CSF-dependent lineage, whether or not maintained in peripheral tissues independently from bone marrow precursor cells (Merad et al., 2008; Schulz et al., 2012). In this regard, it would be very informative to trace the V600E mutation in bone marrow and blood of patients with peripheral disease. Especially in multi-system LCH, it is highly likely that affected progenitors can be found in the circulation. More information in this area could also shed light on the enigmatic circulating DC precursor, which has been predicted and is likely a known cell, but so far has not been identified (Mortellaro et al., 2010). Taking all these data together, current notions suggest that LCH results from misguided myeloid DC precursor development, and the characteristic Langerin expression may well be induced in these cells by environmental conditions, rather than being an indication of lineage identity.

The unambiguous identification of the dysregulated Ras-Raf pathway in LCH opens new therapeutic opportunities with a rational basis. Specific BRAF inhibitors as well as inhibitors of other central regulators such as AKT or mTOR hold much promise for clinical application. However, it is felt that currently used conventional therapies have proven their therapeutic value and should not be dismissed too easily. As argued above, the BRAF mutation and Ras pathway activation are unlikely to be the only driving forces behind LCH etiopathogenesis. This is also supported by the inability to propagate LCH cells *in vitro* or *in vivo* in humanized mouse models, despite many efforts. The mutated mouse models that are currently being developed, carrying targeted defects in the Ras-Raf pathway, should be promising tools to learn more about additional requirements that lead to LCH development and pathogenesis.

Taken together, long-sought molecular defects have finally been identified that are fundamental to DC deviations underlying LCH. Although many questions remain, for instance concerning the origin of the clinical heterogeneity of the disease, this provides a strong basis for further research efforts and rational therapy development in LCH. With its 21st edition, the Nikolas Symposium has surely grown mature!

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