Meeting Proceedings - Nikolas XII

"Dendritic Cell Differentiation: Signals, Signaling and Functional Consequences: Clues to possible therapy"

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Kim E. Nichols, M.D., R. Maarten Egeler, M.D., Ph.D., V. Hugh Perry, Ph.D. and Robert Arceci, M.D., Ph.D.

Kim E. Nichols, MD
Department of Pediatric Oncology
Children's Hospital of Philadelphia
ARC 907C
3615 Civic Center Boulevard
Philadelphia, PA 19104
(215) 590-0159, voice mail
(215) 746-5542, lab
(215) 590-3770, fax
nicholsk@email.chop.edu

INTRODUCTION:
Langerhans cell histiocytosis (LCH) is a rare hematological disorder characterized by the accumulation of Langerhans cells (LCs) in one or more organs of the body. Within LCH tumors, abnormal LCs are found in association with other types of white blood cells including monocytes, macrophages, T lymphocytes and eosinophils. The accumulation of LCs and other white blood cells in affected organs leads to tissue destruction due to the secretion of chemical mediators of inflammation including chemokines and cytokines. Despite recent advances in the treatment of LCH patients, our understanding of the etiology underlying this disease remains unclear. To further improve the treatment of future patients, it is imperative that we clarify the epidemiological and biological factors contributing to LCH.

By bringing together basic scientists with expertise in the LC biology, and clinicians familiar with the management of LCH patients, the Nikolas Symposium fosters an exchange of ideas with the overall goal of increasing our understanding of this rare disorder. Ultimately, it is anticipated that new research explaining the differentiation and functional activation of either normal or "lesional" LCs, as well as the complex interrelationships that exist between LCs and environmental, genetic and host susceptibility factors, may facilitate development of a "rational" cure for future children with LCH. This is particularly important in regards to the small subset of patients that do poorly despite current treatment approaches. Moreover, this information will benefit a larger number of children who are cured of LCH, but subsequently develop late consequences of the disease including endocrine abnormalities, lung and liver fibrosis, central nervous system abnormalities, bone and dental problems and learning difficulties.

This year's symposium (Nikolas XII) explored the molecular mechanisms regulating the differentiation and function of dendritic cells (DCs), the general class of white blood cell to which LCs belong. Clarifying the signals that orchestrate the terminal maturation of normal LCs, and understanding how these signals are perturbed in the LCs found in LCH tumors, may facilitate the development of new treatments for LCH that are aimed at promoting lesional LC differentiation and correcting functional abnormalities. Indeed, similar approaches have recently proven successful in the treatment of specific types of cancers including acute promyeloctyic leukemia and neuroblastoma. The discussions comprising this year's meeting may be grouped into four broad categories, including: 1) an overview of normal DC and LC development; 2) regulation of DC activation and function; 3) pathology studies of LCH tumors, and 4) a discussion of clinical aspects of LCH, including new epidemiological studies and treatment approaches. This review will summarize the presentations given by meeting participants, and will place each discussion into one of these four general areas.

1) Normal DC and LC development: DCs coordinate important aspects of the normal immune response to infection and other challenges. They arise in the bone marrow and subsequently home into different tissues, where they form the first line of defense against foreign pathogens. Previous work has shown that numerous DC subsets exist. There is still debate, however, regarding the pathways that govern the development of DCs and, among these cells, the development of LCs. In this respect, the laboratory of **Dr. Jean Claude Gluckman** (Institut Universitaire d'Hematologie, Hopital Saint-Louis, Paris, France) has shown that under the influence of specific combinations of growth factors, DCs can be generated from diverse hematopoietic cells, including immature progenitors, cells committed to the lymphoid or the myeloid lineages, as well as from terminally differentiated monocytes. Using human cord blood progenitor cells, his group has found that LCs can differentiate either directly from lymphoid-committed progenitors, or from myeloid progenitors or precursors, but then upon additional signals such as transforming growth factor (TGF)-B1[1]. This may be taken as an example of a "constitutive" as opposed to "activation-induced" DC differentiation pathway. He hypothesizes that DCs do not belong to a particular hematopoietic lineage, but that a recently evolved "lineage-

independent developmental program" controls their differentiation from an array of white blood cells: once switched on, either constitutively or upon a “danger signal”, this program elicits these leukocytes to transform into “professional" antigen-presenting cells, i.e. DCs[2, 3].

The signals that drive hematopoietic precursor cells to mature into DCs are complex and involve the action of numerous cellular proteins. Drawing parallels from his work investigating human megakaryocyte differentiation, **Dr. Frederick Racke** (Johns Hopkins University, Baltimore MD) discussed the role played by protein kinase C (PKC), a family of serine/threonine kinases, during blood cell differentiation. Activation of PKC using phorbol esters or other chemical analogues stimulates peripheral blood stem cells to undergo cell cycle arrest and differentiation along a DC pathway. Conversely, blockade of PKC signaling leads to inhibition of DC development. Dr. Racke hypothesizes that medications that modulate the activity of PKC, or its downstream intermediates, may prove beneficial in the treatment of patients with dendritic cell disorders, such as LCH. In addition to PKC, additional molecules acting downstream of PKC may provide targets for potential therapeutic manipulation. For example, recent studies of dendritic cell differentiation suggest that the Extracellular signal Regulated Kinase (ERK) inhibits the terminal maturation of dendritic cells, whereas another Mitogen Activated Protein kinase family member, p38, promotes maturation. Pharmacologic inhibitors of ERK promote dendritic cell maturation, suggesting this class of drugs may facilitate growth arrest and terminal differentiation of LCH lesional cells. Dr. Racke also discussed integrin-mediated signaling during regulation of dendritic and/or Langerhans cell differentiation. Others have previously shown that ligation of E-cadherin inhibits the terminal differentiation of Langerhans cells *in vitro*[4]. Perhaps dysregulated E-cadherin-mediated signaling contributes to the maturation arrest that is characteristic of lesional LCs within LCH tumors.

Hematopoietic stem cell differentiation is a tightly regulated process that leads to cell cycle arrest and expression of lineage-specific genes. The Notch receptor, a cell surface molecule expressed by hematological precursor cells, has previously been shown to play a critical role during lymphoid cell fate decisions, with dysregulated Notch-mediated signaling leading to increased T cell differentiation at the expense of B cell development. **Dr. Irwin Bernstein** (Fred Hutchinson Cancer Research Center, Seattle WA) discussed the role of Notch signaling during murine and human hematopoietic stem cell development. By manipulating Notch signaling using an immobilized and engineered version of the Notch ligand Delta, Dr. Bernstein’s laboratory can inhibit cellular differentiation, while stimulating an expansion of precursor cells that are capable of short-term lymphoid and myeloid repopulation. In the context of specific growth factors, increased Notch signaling permits dendritic cell differentiation, but does not allow differentiation down a monocyte/macrophage pathway. From these studies, Dr. Bernstein concludes that Notch, and the notch ligand Delta, may play important roles during the development of stem cells, as well as common lymphoid and common myeloid progenitor cells.

Biological studies of LCH have been hampered by the rarity of this disease and by the paucity of LCs that can be purified from clinical samples. **Dr. Frederick Geissmann** (Skirball Institute, New York University NY) discussed experiments in which he isolated LCs from LCH lesions and evaluated functional capacity. Dr. Geissmann has found that lesional LCs appear to be immature. However, once they are isolated from tumors, lesional LCs retain their capacity to mature when activated in the laboratory via the CD40 co-stimulatory receptor. Interestingly, previous work has shown that, within LCH lesions, abnormal LCs express abundant CD40 receptor, and lesional T lymphocytes express the endogenous CD40 ligand CD40L[5]. In normal immune responses, engagement of the CD40 receptor on LCs leads to their activation, resulting in increased expression of co-stimulatory molecules, heightened proliferation and increased secretion of pro-inflammatory cytokines[6]. Dr. Geissmann hypothesizes that, in contrast to the normal immune response, lesional LCs may be maintained in an immature state due to secretion of the inhibitory cytokine interleukin 10 (IL-10) by macrophages present within LCH lesions. He

has used this information to develop a laboratory system to study the effects of the differentiating agent retinoic acid on the maturation and function of myeloid-derived DCs.

II) Dendritic cell activation and function:

Following the ingestion of bacterial products, or contact with inflammatory stimuli, immature dendritic cells become activated and migrate to regional lymph nodes where they contact T and B lymphocytes and initiate the "adaptive" arm of the immune response. This branch of the normal immune response leads to the recruitment of additional inflammatory cells from the bloodstream to the site of infection, and to the generation of immunological "memory" that provides protection from similar infections in the future.

Dr. Evangelos Andreakos (Kennedy Institute of Rheumatology, Imperial College School of Medicine, London, United Kingdom) has developed a laboratory system involving adenoviral gene transfer to study the biochemical signaling pathways regulating the function of monocyte-derived DCs. He has focused on the pathways involving NF-κB, a nuclear transcription factor that is central in regulating the genes that govern immune cell activation. Through his work, Dr. Andreakos has found that inhibition of NF-κB by overexpression of IκBα or a kinase-defective form of IκB kinase 2 (IKK2) but not NF-κB-inducing kinase (NIK) down-regulates the antigen presenting capacity of DCs, while activation of NF-κB, both in the laboratory and in an animal model of genetic immunization, enhances DC activation[7, 8].

Dr. Eugene Maraskovsky's laboratory (Ludwig Institute for Cancer Research, Heidelberg Australia) is interested in using normal human DCs to heighten the immune response against tumors as a new approach to anti-cancer treatment. In order to optimize this approach, one must understand the functional capacity of normal human DC subsets. Towards this end, Dr. Maraskovsky has investigated the ability of two types of human DCs known as monocyte-derived DCs (MoDCs) and FLT-3 mobilized peripheral blood DCs (PBDCs) to respond to different classes of physiologic stimuli (including pro-inflammatory mediators such as TNF-a, IFN-g, PGE_2; T cell-derived signals including ligation of the CD40 receptor; and pathogen derived signals such as intact bacteria or lipopolysaccharide). Using a panel of functional assays that include immunophenotypic analysis, evaluation of antigen uptake, migration to chemokines, cytokine production, and T cell stimulatory capacity, Dr. Maraskovsky has shown that individual DC subsets respond differently to various stimuli, leading to a spectrum of down stream T lymphocyte responses. Interestingly, MoDCs can express two functional fates following stimulation with particular combinations of stimuli. Migratory-type MoDC are generated in response to PGE_2-containing stimuli, whereas cytokine secreting MoDC that are poorly migratory are induced with either CD40 ligand or bacterial stimuli in the absence of PGE_2. In contrast, PBDC appear to only express a migratory-type profile, emphasizing that MoDC and PBDC are not functionally equivalent and may provide distinct functional roles *in vivo*. In addition to the type of stimulating agent, the sequence in which immature DC encounter inflammatory stimuli regulates their subsequent functional activation. These findings suggest that not all DC are destined to migrate to lymphoid organs and that the sequence in which stimuli are encountered significantly affects which functions are expressed. Thus, certain immature DC subsets recruited from the resting precursor pool may have multiple functional fates that play distinct roles during the induction and effector phases of the immune response. These findings have important implications for the clinical utility of DC in immunotherapy.

III) Pathology studies of LCH tumors:

The evaluation of previously obtained "banked" tumor samples provides an important avenue for increasing our understanding of LCH. Using an immunohistochemical approach, **Dr. Ronald Jaffe** (Children's Hospital of Pittsburgh, Pittsburgh PA) has examined affected lymph nodes from LCH patients. Lesional LCs appear to follow the usual pattern of migration within the lymph node. Although lesional LCs begin to mature as they migrate, they never complete the full maturation process (a similar finding was noted by Dr. Frederick Geissmann, please see above).

Dr. Jaffe discussed the relationship between LCH and hemophagocytic lymphohistiocytosis (HLH), a disorder of the macrophage lineage, and juvenile xanthogranuloma (JXG), a disorder of interstitial dendritic cells. Although HLH and JXG are occasionally observed together with LCH in rare patients, it is not known whether these disorders result because the dendritic precursors in LCH patients modulate between different DC phenotypes, or because normal macrophages or interstitial DCs are simply responding to abnormal inflammatory stimuli resulting from the underlying LCH[9].

Dr. Pancras Hogendoorn (Leiden University Medical Center, Leiden, the Netherlands) has examined LCH bone and skin lesions for expression of genes that regulate cell division and survival. In all cases, immunohistochemical staining of lesional LCs reveals expression of Ki-67, a marker of cellular proliferation, indicating that lesional LCs is continuously dividing. In addition, most samples concurrently express genes that arrest cell division, such as the tumor suppressor proteins p53 and pRB, the cell cycle inhibitors p21 and p16, and the growth suppressive receptors TGF-B types I and II. Dr. Hogendoorn hypothesizes that despite expression of these inhibitory genes, signals favoring cell division must predominate in LCH tumors. This may be due to abnormal environmental factors such as upregulated secretion of growth and/or survival factors, or to genetic alterations within lesional cells that allow them to escape from normal growth inhibitory pathways.

E-cadherin is an important surface adhesion molecule that signals to the cell via a variety of down stream molecules including the cytoskeletal protein actin and the transcription factor B-catenin. E-cadherin is expressed on the surface of normal LCs, where it mediates the interaction of dermal LCs with surrounding skin cells. Based on the hypothesis that the abnormal LCs in LCH might demonstrate defects in the E-cadherin signaling pathway, **Dr. Pieter Leenen** (Erasmus University, Rotterdam, The Netherlands) evaluated a series of LCH lesions for expression of this molecule and its downstream partner B-catenin. Although E-cadherin protein levels were low in most of the samples studied, the majority of samples expressed detectable B-catenin. The upregulated expression of B-catenin suggests that aberrant signaling through this pathway may contribute to the pathogenesis of LCH.

Chemokines are chemical regulators of leukocyte recruitment and activation that mediate normal inflammatory responses[10, 11]. This year's Artemis Fellow, **Dr Nicola Annels** (Leiden University Medical Center, Leiden, The Netherlands) is examining the expression of chemokines, and their cognate receptors, within the cells comprising LCH tumors. Using a combination of approaches including immunohistochemistry, immunofluorescence and confocal microscopy, Dr. Annels has found that both lesional LCs and associated T lymphocytes express a variety of these molecules. It is possible that use of medications to pharmacologically manipulate chemokine or chemokine receptor expression and/or function may prove beneficial for the treatment of LCH patients.

Using a more global approach, **Dr. Kenneth McClain** (Texas Children's Clinical Care Center, Houston TX) is using gene expression profiling to examine the pattern of genes expressed by lesional LCs within LCH tumors. For these studies, he is using laser microdissection to isolate individual LCs, and from these isolated LCs, cellular RNA is purified, and cDNA is prepared. Using T7 RNA polymerase, additional RNA molecules are synthesized and hybridized with slides containing immunologically relevant genes. The profile of genes expressed by lesional LCs (i.e. their genetic "signature") is then compared with the profile of genes expressed by dermal LCs isolated from normal human foreskin. Dr. McClain has examined one patient sample to date, and has found an increased expression of a number of genes, including the TGF-B receptor, IL-1a, G-CSF, CD40, GM-CSF, IL-1R, TNF receptor p55, among others. Dr. McClain plans to investigate a larger series of samples to identify the genes important for LCH, and to correlate genetic "signatures" with prognosis.

IV) Potential of Differentiation Therapy for LCH:

The observations that the LCH lesional Langerhans cell may be in an immature stage of maturation, but can be induced in vitro to undergo further maturation, is quite reminiscent of observations in acute promyelocytic leukemia and neuroblastoma with retinoic acid derivatives. Thus, the concluding part of the symposium was focused on the potential role of using differentiating agents in patients with LCH.

Dr. Jean Donadieu (Hospital Trousseau, Paris France) presented the preliminary results of a phase II open label non-randomized study using the medication All Trans-Retinoic Acid (ATRA) for patients with LCH and end-organ dysfunction. This trial was based on pre-clinical data showing that ATRA induces differentiation and/or cell death of normal as well as lesional LCs. Although the final results of this trial are forthcoming, the drug was shown to be safe, and to possibly stabilize MRI and clinical scores in a limited number of children with LCH and central nervous system involvement

Dr. C. Patrick Reynolds (Children's Hospital Los Angeles, Los Angeles CA) presented his work using fenretinide (4-HPR), a synthetic molecule that is similar to ATRA. By inducing cell death through a pathway involving the pro-apoptotic protein ceramide, 4HPR is cytotoxic against a variety of tumor cell lines, many of which are resistant to ATRA or the related retinoid 13-cis-RA. Pre-clinical data demonstrate that a ceramide modulator (safingol) enhances 4-HPR activity against neuroblastoma cell lines in culture and against xenograft tumors in mice. Clinical testing of 4-HPR is ongoing, and studies of new formulations of 4-HPR and 4-HPR in combination with safingol are planned. Dr. Reynolds suggests that 4-HPR, either alone, or in combination with other ceramide modulators, may have activity against LCH.

REMANING QUESTIONS:

Despite past advances, this year's Nikolas Symposium highlights several important questions regarding LCH that remain unanswered. First what are the epidemiological factors leading to the development of this rare disease? As aptly pointed out by **Dr. Louis Parker** (Sir James Spence Institute, Newcastle upon Tyne, England), the true incidence and epidemiologic associations that have been reported concerning LCH are far from definitively proven. Second, why is LCH such a clinically heterogeneous disease? LCH demonstrates remarkable variability in presenting features, sensitivity to treatment, and natural history. It remains possible that the expression of LCH within a given individual reflects the combined effect of environmental factors, such as exposure to infectious agents (viruses, bacteria, etc) or to chemical substances (cigarette smoking), along with host genetic differences. Third, what is the meaning of clonality? Prior investigations have shown that in nearly all cases studied, LCH is a clonal disorder (i.e. the abnormal LCs comprising lesions all appear to have arisen from a single precursor cell), suggesting that a possible genetic abnormality resulted in the accumulation of these cells in pathologic lesions. However, at this time, no definitive somatic mutations have been identified, although a significant amount of work is being done in this area of research. In addition, it remains unknown whether all pediatric cases of LCH are clonal, and whether all lesions within given patients are derived from the same clone or from different clonal expansions. Fourth, what causes the formation of LCH tumors? Are the defects in cell cycle progression, LC recruitment to lesions, and inhibition of terminal maturation due to primary defects within the abnormal LCs, "environmental" defects within the affected individual, or both? Identification of the genetic abnormalities associated with LCH, either by evaluation of gene expression or by large-scale genome-wide approaches, may facilitate our understanding of this disease. Last, how can we translate information regarding disease biology to improved treatments for future patients? This remains a particularly important question because of the rare and heterogeneous nature of this disease.

SUMMARY:

Much work remains to be done to more completely understand LCH and to develop improved strategies for treatment. These efforts will require collaboration between clinical and basic investigators to collect patients and tissue specimens, and to perform critical research investigations. The work presented in this year's Nikolas Symposium provides an important framework for future investigations of LCH. Novel approaches to LCH treatment are likely to include the use of clinically available differentiating agents such as retinoic acid or 4-HRA. It also remains possible that modulation of gene expression using demethylating agents, or alteration of biochemical signaling pathways involving NF-kB or PKC will drive differentiation of immature lesional LCs. Last, pharmacological manipulation of apoptotic pathways in favor of cell death may enhance the elimination of atypical LCs from lesions. Studies using clinical samples from human LCH patients, as well as mouse models of dendritic cell biology, will increase our understanding of the pathogenesis underlying LCH, and may provide insights into the mechanisms controlling dendritic cell differentiation and function.

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