

Langerhans Cell Histiocytosis: Bystander cells, interactions and pathophysiology

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The Nikolas Symposium

The mission of the annually held Nikolas Symposium is to find a rational cure for Langerhans Cell Histiocytosis (LCH). It 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 related to the disease, and attempt to apply this information towards an improved understanding and treatment of LCH. A particular focus is the biology of the dendritic cells 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 also our understanding of normal dendritic cells as well as other disorders in which dendritic cells are involved.

Introduction

Langerhans cell histiocytosis (LCH) is a rare disease in which an uncontrolled accrual of cells with Langerhans cell (LC) characteristics occurs (Arceci et al., 2002; Laman et al., 2003). LCH is of unknown etiology and occurs in various clinical forms in a broad age range from the newborn to the elderly but peaking between 1-4 years of age. The annual incidence in the pediatric age range has been estimated at 2-5 per million per year. A central question is whether LCH cells develop owing to an intrinsic proliferative defect of LCs, or whether the disease is reactive, resulting from environmental triggers (e.g. smoking) and leading to aberrancies in dendritic cells (DC). This question was extensively discussed during the 13th Nikolas Symposium (2003), which was summarized for the current participants by **Dr. Perry**. However, this question remains genuine as no definitive answer has been provided yet.

LCH primarily presents as a lesional disease, either with single or multifocal lesions in different organs. Skin and bone are most frequently affected. In these lesions, LCH cells are invariably present, which are characterized by CD1a, Langerin and S-100 expression and the presence of Birbeck granules (Favara et al., 1997; Geissmann et al., 2001). However, other cell types also contribute to the LCH lesions and are thought to have a profound influence on the biological behavior of the LCH cells. Therefore, the purpose of this 14th Nikolas Symposium was to highlight the "other" lesional cell types and their putative interactions with LCH cells. In particular, macrophages, multi-nucleated giant cells, eosinophilic granulocytes, stromal cells, natural killer cells and T-lymphocytes were discussed.

Although rare, LCH is the most frequent of the DC histiocytoses. In his introduction to the pathology of the DC histiocytoses, **Dr. Jaffe** indicated that the myeloid DC precursors may give rise to accumulations that can range from benign to sarcomatous in clinical and/or cytological appearance. In addition to LCH, there is the interstitial, dermal DC juvenile xanthogranuloma family (JXG), or those myeloid DC proliferations that lack the specific LC features (such as CD1a/Langerin expression) in other non-LC histiocytoses (Weitzman and Jaffe, 2005). Despite the fact that pathological distinction of the different histiocytoses is the basis for both clinical treatment and research, it should also be noted that there is plasticity in the system. On rare occasions, intermediate forms have been observed between LCH and

non-LCH histiocytosis, like JXG, occurring at different points in time or in different lesions in the same patient. Similarly, it is found on rare occasion that LCH cells found in a patient increase in malignant appearance over time. Conversely, histiocytic skin lesions have been found as a more mature tissue representation of underlying acute monocytic leukemia (Klemke et al., 2003). Together, these findings reinforce the notion that, in both normal and pathological conditions, the DC and their precursors constitute a continuum that considerably overlaps with macrophages.

Dendritic cells

Research in the last decades has indicated that DC are pivotal for the initiation of immune responses. **Dr. Steinman** pointed out that, more recently, it has become clear that these cells are also crucial for the induction and maintenance of tolerance towards self-antigens and harmless environmental antigens. Since clinical manipulation of DC function may be desirable as a therapeutic application in conditions such as autoimmunity, allergy or (tumor) vaccination, recent research has focused on (1) increasing the efficiency of targeting antigens to DC, and (2) influencing the DC maturation status. The stage of DC maturation is thought to determine the outcome of the immune response resulting from DC-T cell interaction: in general, immature DC induce tolerance, while mature DC induce immunity by stimulating effector T cell responses. In different experimental systems in mice, Dr. Steinman showed the validity of these notions. Extremely efficient DC targeting of antigens could be accomplished via monoclonal antibodies directed against cell surface molecules, in particular CD205, a membrane lectin on a major subset of DC (Bonifaz et al., 2004). Alternatively, injection of dying cells loaded with antigen primarily targeted a subset of CD8⁺CD205⁺ DC. In both instances, tolerance was induced if no additional DC maturation stimuli were given. A potent stimulus involved the activation of NKT cells via a lipid derivative, α -galactosyl ceramide, which is presented by CD1d molecules on DC and other antigen-presenting cells. This pathway of DC maturation is mediated via TNF- α , IFN- γ and CD40-CD40L interaction. Stimulating DC via CD40 appeared to be essential for the induction of immunity (Fujii et al., 2004); high level expression of MHC and co-stimulatory molecules was a prerequisite, but not sufficient to induce effective immune responses. However, the molecular nature of the signals expressed by DC upon CD40-stimulation that determine the outcome, i.e. immunity vs. tolerance, are as yet unknown.

This year's Artemis fellow, **Dr. Tudor**, discussed the features of a potential animal model for LCH, a mouse transgenically expressing thymic stromal cell-derived lymphopoietin (TSLP). Ubiquitous expression of this cytokine strongly induced accumulation of myeloid cells in multiple organs, including spleen, bone marrow, liver, lung and lymph nodes, while lymphopoiesis was impaired in these animals (Osborn et al., 2004). Serum levels of Th2 cytokines, such as IL-4, -5, -6 and -10, were elevated. Lung involvement in TSLP-transgenic mice appeared to be the primary cause of their premature death. The morphological and phenotypic features of the accumulating cells, such as large cell size with prominent nucleoli and expression of CD11c, F4/80 and Gr-1 surface molecules, suggested that the affected cells belong to the myeloid DC lineage. In support of these findings, recent studies in human indicate that TSLP primarily influences monocytes and DC, rather than lymphoid cells (Reche et al., 2001). Specific involvement of the LC lineage, however, was not shown in the human studies, nor in the TSLP transgenic mice. While systemic TSLP expression led to strong aberrancies in multiple organs, it was felt that conditional induction of locally

increased TSLP levels, for instance in the skin, might induce more subtle effects on cells of the DC lineage, and thus lead to a promising model more closely mimicking human LCH.

Multinucleated giant cells

The presence of multinucleated giant cells (MGC) in LCH lesions has long been recognized. Since these cells are thought to play an important pathogenic role in osteolytic lesions, **Dr. Egeler** initiated studies on the role and origin of these MGC in LCH lesions of bone, skin and lymph nodes (recently published in (da Costa et al., 2005)). It appears that in bone and lymph node lesions, MGC have multiple phenotypic characteristics of osteoclasts, including expression of CD68, TRAP (tartrate-resistant alkaline phosphatase), VNR (vitronectin receptor), cathepsin K and MMP-9 (matrix metalloproteinase-9). In addition, MGC in some skin or lymph node lesions, but not bone lesions, appeared to be CD1a-positive. This phenotype is atypical for osteoclasts and suggests the involvement of LCH cells in their formation. Interestingly, the key cytokines involved in osteoclast formation from precursor cells, M-CSF and RANK-L, were found in the majority of LCH lesions, expressed by CD1a⁺ LCH cells and activated CD4⁺ T-cells. Moreover, lesional CD1a⁺ cells appeared to express the cognate receptor, RANK, triggering of which not only allows osteoclast differentiation, but generally increases DC survival (Cremer et al., 2002). Therefore, it is likely that the lesional environment induces the local formation of the osteoclast-like MGCs even in unusual sites, such as these nonostotic LCH sites.

Macrophages

In LCH lesions, macrophages comprise an important bystander population. **Dr. Gordon** emphasized their extensive ability to interact with their environment by virtue of the expression of a wide range of non-opsonic pattern recognition receptors. These innate receptors enable the specific recognition of neighboring cells, matrix components, soluble molecules as well as microbial determinants (for a recent overview see (Taylor et al., 2005)). In particular, 4 types of receptors were highlighted: (i) F4/80 and other members of the EGF-Tm7 family, (ii) scavenger receptors, (iii) β -glucan receptor (Dectin-1 in mouse), (iv) macrophage mannose receptor (MR). The EGF-Tm7 family members are involved with adhesion, migration, cell-cell and cell-matrix interactions. Furthermore, F4/80 - homologous to human EMR1 - appears to be essential in peripheral tolerance induction, as demonstrated in knock-out mice. The scavenger R family includes a variety of molecules, such as CD36 (SR-B), CD68 (SR-D) and different SR-A members. In general, these are involved in clearance of (altered) self molecules, but also interact with micro-organisms like *Neisseria*. Finally, dectin-1 and macrophage mannose R are examples of lectins that interact with carbohydrate moieties on micro-organisms, but also appear to have endogenous ligands that may facilitate cell-cell interactions.

The generation and development of macrophages is strictly regulated by different cytokines, among which M-CSF or CSF-1 plays a predominant role. This was illustrated by **Dr. Stanley**, who elaborated on the severe deficiencies observed in mice that lack CSF-1 activity, either by absence of the cytokine itself (in natural mutant *op/op* or in CSF-1 ^{-/-} mice), or by lack of receptor expression in CSF-1R gene-deleted mice (see for a recent review (Pixley and Stanley, 2004)). Such mice suffer from osteopetrosis and toothlessness due to the absence of osteoclasts. Furthermore, they lack many, but not all macrophage populations in the various organs, causing decreased dermal thickness, female and male reproductive defects, and decreased sensory processing as most prominent features. CSF-1 occurs in three isoforms: as

secreted glycoprotein, secreted proteoglycan, or as membrane-spanning cell surface protein. Studies in transgenic mice, expressing only one of these isoforms in an otherwise CSF-1-deficient background indicated that these isoforms have broadly but not fully overlapping roles in the differentiation and activation of different macrophage populations. Examples of the involvement of CSF-1 in pathogenesis was provided in models of atherosclerosis and glomerulonephritis, in which lack of CSF-1 strongly ameliorated the disease. In addition, autocrine or paracrine signaling via CSF-1 and its receptor was implicated in a radiation-induced leukemia model in mice, and also appeared to be associated with poor prognosis in various solid tumors in humans, especially those of the female reproductive tract.

Eosinophilic granulocytes

The much-used alternative name "eosinophilic granuloma" for LCH occurring in bone already indicates that eosinophilic granulocytes are frequently observed in LCH lesions. This is particularly, but not exclusively the case at bone sites. **Dr. Keane-Myers** highlighted the general features of eosinophils as granulocytes which development is specifically stimulated by IL-5, and which are attracted to tissue sites by the chemokines eotaxin-1, and -2. Activated eosinophils are major producers of soluble mediators, including membrane-derived lipids (prostaglandins, leukotrienes and PAF), cytokines (esp. those involved in fibrogenesis such as TGF- β and TNF), granule-derived cationic proteins (MBP) and chemokines (eotaxin). They appear in large numbers in allergic diseases and in helminth infections, which are both characterized by a Th2-skewed immune response. Removal of the eosinophils in such disease models improves the health status, and thus suggests a role of these cells in pathogenesis. To study the regulation of eosinophil responses, Dr. Keane-Myers group combined helminth infection with allergic sensitization and challenge in a mouse model of conjunctivitis. The results clearly indicate that acute helminth infection increases allergen-induced eosinophil-mediated pathology, while chronic infection reduces the response by stimulation of regulatory T cells.

Dr. Robinson discussed the roles of eosinophils as pro-fibrotic repair cells vs. pro-inflammatory effector cells, in particular in the asthmatic allergic response. The central role of IL-5 in stimulating eosinophil accumulation and activity has inspired to use anti-IL-5 antibody as a therapeutic modality. In preclinical animal models, this strongly reduced both airway eosinophilia and bronchial hyper-responsiveness. In asthmatic volunteers, anti-IL-5 therapy led to a significant decrease ($\geq 90\%$) of peripheral blood eosinophil numbers, but to only about 50% reduction in bronchial mucosal and bone marrow eosinophils. Moreover, no clinical effect was observed.

In addition to their acute inflammatory function, eosinophils are also thought to be important in airway remodeling related to asthma (Kay et al., 2004). Growth factors such as TGF- β , produced by eosinophils, stimulate airway smooth muscle hyperplasia, myofibroblast infiltration and deposition of extracellular matrix components. In line with this, a reduction of extracellular matrix deposition was observed in the anti-IL-5-treated asthmatics, along with the reduced eosinophil numbers and TGF- β deposition.

Taken together, a specific role for eosinophils in LCH remains to be established. Given their potential to secrete large amounts of soluble mediators, they might be involved in both the maintenance of the cytokine storm in the LCH lesions, as well as in the late sequelae, especially the induction of fibrosis at the lesion site.

Stromal cells

While in LCH fibroblasts are specifically known for their pathological involvement in the late sequelae, they are in general increasingly recognized as key regulator cells of inflammatory responses. **Dr. Buckley** illustrated how important fibroblasts are in regulating the switch from acute, resolving to chronic, persistent inflammation, thus from a physiological to a pathological response. This relates to the concept that fibroblasts from different body locations have different phenotypes and functions, possibly caused by the differential expression of homeobox (Hox-) genes. Thus, by site-specific expression of cytokines, chemokines and adhesion molecules, fibroblasts define a stromal tissue address, which determines the tissue-specific response of inflammatory leukocytes (Parsonage et al., 2005). These leukocytes may be locally activated and undergo phenotypic changes, including their profile of chemokine receptors. In turn, cytokine-mediated stimulation of tissue fibroblasts may alter their topical identity, causing tissue-aberrant responses. In addition, production of type 1 IFNs by activated fibroblasts contributes to the increased survival of T cells and neutrophils. In conjunction with specific chemokine expression by these fibroblasts, this may cause the retention and survival of excess inflammatory leukocytes. Thus a vicious circle is created, leading to chronic, persistent inflammation.

NK cells

Natural killer (NK) cells are not prominently present in LCH lesions. However, they are of prime interest in the histiocytosis field because of (i) their functional deficiencies in the hemophagocytic histiocytic syndromes, and (ii) their increasingly recognized interaction with DC.

Dr. Moretta outlined the regulation of NK cell activity by inhibitory NK-receptors (e.g. KIR, LIR, CD94) which recognize different HLA molecules and activating NK-receptors (e.g. NKp46, NKp30, NKp44 and 2B4), which bind various ligands, identified only in part. Thus, it appears that activation of NK cells is regulated by different check points involving both activating and inhibitory interactions (Moretta, 2004). For example, acute myeloid leukemia cells may be killed by allogeneic, but not by autologous NK cells. This reflects the fact that AML blasts express ligands recognized by the activating receptors NKp46 and NKp30, although these receptors are profoundly downregulated in NK cells from most AML patients. Remarkably, optimal AML killing by allogeneic NK cells also requires mismatch between allogeneic inhibitory KIR expressed by NK cells and leukemic HLA-class I alleles, thus resulting in a net activating signal.

NK cells and DC have an intriguing relationship, leading to mutual stimulation, particularly when mature DC are involved. This is based on the production of cytokines such as IL-12p70 and IL-15 by DC, and IFN- γ and TNF- α by NK cells. However, immature DC (iDC) may be preferentially killed by activated NK cells, because iDC minimally express HLA-E molecules, causing a failure to inhibit NK activity. This may be viewed as selection of maturing DC for optimal HLA expression and may have interesting therapeutic implications in LCH, namely for the possible establishment of an NK cell-based adoptive immunotherapy.

T lymphocytes

The role of T lymphocytes in LCH lesions remains enigmatic, but these might be crucial to the expansion and survival of LCH cells given the known interactions of T cells with DC. **Dr. Abbas** emphasized the mutual regulation of T cells and antigen presenting cells, and the

consequences of dysregulation as observed in both human disease and mutant mouse models. While previous views explained aberrant accumulation of immune cells by exaggerated stimulation, currently, defects in the inherent down-regulation of the immune system are increasingly recognized as causes of such anomalies (Abbas et al., 2004). For example, familial hemophagocytic lymphohistiocytosis (FHL) appears to be related to the failure of T-cell- and/or NK-cell control of macrophage activity. Mutations in four major inhibitory pathways have been identified as causes of abnormal immune cell accumulation. These comprise (i) the death receptor Fas and the cognate Fas-ligand, (ii) the inhibitory CD28-family member CTLA-4, (iii) the cytokine IL-2 and its α and β receptor chains, and (iv) the FoxP3 transcription factor important in regulatory T cell generation. IL-2 appears to play a dual role in immune homeostasis: it stimulates clonal expansion of effector T cells in the initiation of the immune response, but IL-2 is also important in the stimulation of regulatory T cells and the induction of death of activated T cells.

Dr. Powell discussed some aspects of the conditions that determine the outcome of the decision whether T cells become activated or tolerized upon antigen encounter. An important role in this decision appears to be played by mTOR (mammalian target of rapamycin), a PI-3-like kinase that integrates signals from different exogenous sources, such as growth factors and nutrients. If mTOR is activated, TCR engagement leads to T cell activation; inactive mTOR with coincident TCR stimulation leads to tolerance. Thus, inhibiting mTOR has led to successful induction of long term tolerance in transplant models, without the need for long term immunosuppression. As such, mTOR may provide a potential target for therapy. Similarly, adenosine signaling through the A2a receptor plays an important role in negatively regulating immune responses. In the case of inflammation, this serves as a negative feedback loop controlling excessive tissue damage. However, under suboptimal activation conditions, A2a receptor engagement on T cells was shown to promote T cell tolerance. This observation raises the possibility that high local adenosine concentrations as found in a tumor microenvironment could promote tumor-specific T cell tolerance.

The inhibitory regulation of T cell responses was further elaborated by **Dr. Wraith**. He delineated different types of homeostatic regulation of T cells, namely competition for soluble (growth) factors and MHC peptide ligands, and inhibition via Fas-induced death or regulatory T cells. Absence of competitive inhibition offers an explanation for the expansion of self-reactive T cells, which often occurs under lymphopenic conditions. Different types of regulatory T cells can be discerned (O'Neill et al., 2004), most extensively characterized in the mouse. Naturally occurring regulatory T cells develop as such in the thymus and are characterized by expression of FoxP3 and relatively high levels of CD25. In contrast, induced regulatory T cells develop in the periphery after tolerogenic application of antigen, such as peptide administration via the nasal route. Such peptide-induced Treg suppressed *in vitro* in an IL-10-independent, but *in vivo* in an IL-10-dependent manner. They differ from naturally occurring Treg by the absence of high levels of CD25, and lack FoxP3. Excess IL-2 reverses their anergy and inhibitory function, which returns when exogenous IL-2 levels decrease. In humans, a unique subset of regulatory T cells was identified that also lacks CD25 and FoxP3, but does express IL-10 and CTLA-4. They induce effector T cell division arrest through an as yet unidentified mechanism.

Dr. Beverley explored the kinetics of the different naïve and memory T cell populations in humans (Beverley, 2004), providing a hitherto unprecedented insight into human T cell dynamics and a putative example for human studies in LCH cell dynamics. Naïve T cells appeared to have a much slower proliferation rate (0.5%/day) than memory T cells (2-5%/day). However, the disappearance rates from the circulation were similar for both T cell subsets (7-12%/day). Thus, once activated, the majority of T cells persist as a cycling population. Furthermore, T cells that have recently divided are at a higher risk of dying compared to non-cycling T cells. Among the memory T cells, central memory cells (CCR7⁺) have a proliferation rate more like naïve T cells (1.5%/day), in contrast to CCR7⁻ effector memory cells (4.7%/day).

A factor limiting clonal proliferation is chromosomal telomere shortening, which can be compensated for by telomerase activity. In acute infectious mononucleosis patients, the clonally expanded CD8⁺CD45RO⁺ memory T cells appeared to have high telomerase activity, coinciding with relatively long telomeres. In contrast, telomerase activity is much less upregulated in IL-7- or IL-15-stimulated proliferation of memory CD8⁺ cells, thus causing telomere shortening. This indicates that clonal lifespan of T cells - and possibly also of LCH cells - can be greatly influenced by the delivered external signals.

Epilogue

In the summation of this 14th Nikolas Symposium, **Dr. Abbas** and **Dr. Beverley** put forward diverse areas in which progress of different degrees has been made over the last few years. Furthermore, they identified additional areas for exploration that might provide new insights into the etio-pathogenesis of LCH, and thus help the future development of a rational cure.

It was felt that progress has been made concerning: (i) the role of cytokines in LCH, (ii) the identification of the clonal relationship between affected cells, (iii) the use of tissue banks, (iv) the generation of animal models, and (v) the relationship between pathology and disease biology in different organs, in particular the brain. However, at the same time, much still needs to be learned in each of these areas. Especially the implication of the clonality of LCH cells in virtually all locations, except lung, remains unclear as appropriate controls (normal LC from different locations and taken at different time points) are lacking.

Important areas of potential future development might include:

- (i) gene expression profiling of lesional LCH cells using gene array, which should provide more insight into the (potentially deviant) molecular pathways operative in LCH cells, regulating cell proliferation and survival,
- (ii) development of more sophisticated mouse models, using gene targeting of Langerhans cells via the Langerin-promoter,
- (iii) therapy development on the basis of breaking the maturation block in LCH cells,
- (iv) therapeutic application of phage antibody-derived anti-CD1a.

Regarding the role of bystander cells in LCH - the topic of this meeting - consensus was that these cells are as important for lesional development as the LCH cells themselves. In particular, the lesional T cells may play a hitherto underestimated role in stimulating the proliferation and survival of LCH cells. In addition, the bystander cells are a potential target for therapy, not only to reduce clinical symptoms, but also to control the LCH cells .

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