Mechanisms and therapeutic targets of inflammatory disease of the CNS

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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 Konto-yannis 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

Langerhans cell histiocytosis (LCH) is a rare disease that is characterized by the uncontrolled accumulation 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 is a sporadic disease of unknown etiology and occurs in various clinical forms 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. LCH primarily presents with single or multifocal lesions in different organs. Skin and bone are most frequently affected. More severe forms of LCH, in which multiple organ systems are affected, may resemble acute leukemia and occur especially in younger children. In LCH lesions of both single and multisystem disease, LCH cells are invariably present but other cell types also contribute and are thought to have a profound influence on the biological behavior of the LCH cells.

A central question in LCH research for several years 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 response to environmental triggers. Interestingly, supportive evidence for both scenarios exists (summarized in (Egeler et al., 2010)). Briefly, the clonal relationship of LCH cells in non-pulmonary cases, their maturation arrest, cell cycle dysregulation, shortened telomeres and sometimes aggressive clinical behavior as well as the high disease concordance rates observed in twin studies argue in favor of a genetic origin. Although an extensive search observed no consistent genomic aberrations in LCH cells (da Costa et al., 2009), a recent study has identified a mutation of the BRAF protein, which is part of the RAS - MAPK signaling cascade, in 35 of 61 studied LCH cases (Badalian-Very et al., 2010). In contrast, suggestive evidence for a reactive immune condition are the cytologically benign appearance of the cells, their non-clonal relationship in pulmonary cases. Furthermore, LCH disease activity and severity appear to correlate with the levels of growth factors regulating normal DC homeostasis, namely M-CSF and Flt-3L (Rolland et al., 2005). The granulomatous, inflammatory nature of the LCH lesion has been linked to abnormal production of IL-17A, in part by LCH cells themselves, but these findings have not been confirmed by others (Coury et al., 2008; Allen and McClain, 2009). Thus, the underlying cause of abnormal DC accumulation in LCH still remains to be definitively established.

LCH lesions may occur in virtually all organ systems and, besides skin and bone, the central nervous system (CNS) is relatively frequently involved (reviewed recently by the Histiocyte Society CNS LCH group (Grois et al., 2010)). Approximately 25% of all patients with LCH have diabetes insipidus (DI) as a manifestation of infiltration of the hypothalamic pituitary region. A significant fraction of these patients additionally suffers from other endocrinological problems due to anterior pituitary hormone loss. Furthermore, in some LCH cases symptoms of progressive neurodegeneration are observed. These may give rise to motor problems, cognitive deficits as well as behavioral issues. Clearly, these signs of CNS involvement present a significant and long term burden for patients and their environment. Therefore, the steering committee of the Nikolas Symposium decided to focus the meeting of this anniversary year on various aspects of CNS disease in LCH.

Dr. Malone introduced the pathology of the various forms of histiocytosis. These disorders are

divided into (pathologically) benign DC- or macrophage-related diseases and malignant forms. LCH is the most frequent DC-related histiocytosis, while macrophage-type disorders mostly present as hemophagocytic lymphohistiocytosis (HLH) or macrophage activation syndrome. The distinction between the DC and macrophage lineages in these diseases is made on the basis of cellular morphology and phenotypic markers, such as CD1a, S100a and CD68, but often cases with intermediate marker profiles are observed. Phenotypically, LCH cells are characteristic with a round morphology and large cytoplasm with perinuclear hof and indented nucleus. These cells express CD1a and Langerin as characteristic markers, although some discordance between these can be found. The racket-shaped Birbeck granules are characteristic of LC, but may be absent in LCH cells, especially in liver lesions. Together with LCH cells, a variety of other cell types is found in LCH lesions, such as eosinophils, T-cells, macrophages and multinucleated cells. Depending on the stage and activity of the disease, fibrosis can be detected, sometimes as the only remainder.

The developmental plasticity of the histiocytic cells often complicates the pathological diagnosis. This is illustrated by the (infrequent) finding of co-existence or sequential development of LCH and juvenile xanthogranuloma (JXG), which is characterized by accumulation of cells with a distinctive FXIIIa+ CD1a– S100– phenotype (Hoeger et al., 2001). Furthermore, LCH diagnosis may be complicated by coexisting macrophage activation syndrome (Favara et al., 2002) and accumulation of histiocytes in lymph node sinuses, also known as Rosai Dorfman disease (Chikwava and Jaffe, 2004). In the CNS, LCH lesions can be found in the hypothalamic pituitary region, in the meninges, the choroid plexus or in the brain parenchyma. Frequently, however, brain lesions detected in MRI result from gliosis rather than from granulomatous accumulation of LCH cells.

Brain inflammation: local and systemic aspects

Dr. Perry highlighted in his contribution the role of microglia in CNS disease and the impact of systemic inflammation on the brain. In steady state, microglia, the macrophages of the CNS, are maintained in a quiescent state by inhibitory signals, such as CD200 and CD47, provided by neurons (reviewed in (Perry et al., 2010)). Upon inflammatory triggering, this inhibition is removed and microglia become activated. As sentinels responding to altering local conditions, microglia can occur in different stages of activation. In a CNS condition such as Alzheimer's disease, increased numbers of activated microglia are observed, coinciding with increased levels of pro-inflammatory cytokines such as IL-1beta, TNF-alpha and IL-6. This can be modeled in mice by injecting prions, which causes fatal progressive neurodegenerative disease, characterized by increasing amyloid deposits and microglia activation, resulting in neuronal loss and multiple behavioral deficits. In the early phase of clinical prion disease, microglial cells show an activated (less ramified) morphology. However, these cells do not produce pro-inflammatory cytokines, and rather show significant levels of anti-inflammatory cytokines such as TGF-beta and prostaglandins (Cunningham et al., 2005). The resemblance to alternatively activated (M2-type) macrophages, which phenotype can be induced by phagocytosis of apoptotic cells, is striking. However, in this type of inflammation in the CNS, it is thought that this phenotype is induced by the endocytosis of synapses that are lost in a process called 'synaptic stripping', rather than of whole cells. Albeit microglia have an anti-inflammatory profile in these conditions, they are primed to respond in a pro-inflammatory manner upon subsequent triggering by stimuli such as LPS or IL-1. This is similar in aged microglia. As such, microglia are thought to play an important role in induction of sickness behavior, which is mediated by the effects of peripheral and central pro-inflammatory cytokines on the brain (Cunningham et al., 2005; Teeling and Perry, 2009). This mechanism likely plays an important role in the long term deteriorating effects of peripheral inflammation on the cognitive functions of patients with Alzheimer's disease (Perry et al., 2007; Holmes et al., 2009). Therefore, rapid anti-inflammatory treatment is indicated in these patients, and in the elderly in general, as this may help to slow down the neurodegenerative process.

In his presentation, **Dr. Prinz** elaborated on the origin of microglia and other myeloid cell populations in the brain and their contribution to damage induced by inflammation. The macrophage nature of microglia has long been established, but it has been an area of extensive scientific debate whether these cells are maintained in the steady state by proliferation of precursors that have seeded the brain during embryogenesis, or by influx of bone marrow-derived precursors (discussed recently in (Prinz and Mildner, 2011)). This question was previously approached by several authors, in particular by using BM transplant models. It appears that preconditioning of the animals to enable peripheral engraftment is

crucial for the outcome: protection of the brain during irradiation precluded the engraftment of microglia by circulating precursors (Mildner et al., 2007). This is explained by the effect of irradiation on the integrity of the blood-brain barrier (BBB), as microglia engraftment by circulating precursors also did not occur in CNS pathologies that were not associated with overt BBB disruption. In full agreement with this, Ginhoux and colleagues recently showed that adult steady-state microglia in the mouse derive from primitive myeloid progenitors that arise early in embryonic development (Ginhoux et al., 2010).

The existence of different populations of circulating monocytes (summarized in (Ziegler-Heitbrock et al., 2010)) raised the question which of these were the primary circulating precursors of microglia in pathological conditions. Using mouse bone marrow chimeras and adoptive transfer, it was shown that so-called classical Ly-6Chi-CCR2+ monocytes, and not the non-classical Ly-6Clo CX3CR1hi monocytes, were preferentially recruited to lesioned brain and differentiated into microglia (Mildner et al., 2007). A central role in this immigration was established for the chemokine receptor CCR2 (Prinz and Priller, 2010). In transfer experiments it was shown that disease severity in an EAE model was reduced in the absence of CCR2 on either the radio-resistant stromal cells or the hematopoietic cells, especially monocytes. The latter appeared to be the main culprits of CNS pathogenesis in this model, which developed into TNF-alpha- and iNOS-producing DC, so-called TipDC.

Dr. Ransohoff further elaborated on the origin of microglia and the roles of the different subsets of monocytes and resident microglia in CNS inflammation. He presented a new mouse model, in which the fluorescent markers GFP and RFP were expressed from the CX3CR1- and CCR2-promoters, respectively (Saederup et al., 2010). Thus, CX3CR1+ and CCR2+ monocytes in heterozygous mice express functional chemokine receptors as well as marker proteins, while these cells in homozygous mice lack the chemokine receptors, but can be traced by fluorescence. In this model, it was apparent that microglia precursors expressing both chemokine receptors could be found adjacent to developing CNS in embryos as early as embryonic day 8.5 (E8.5), hence before the onset of fetal hematopoiesis or vascularization (Ransohoff and Cardona, 2010). Coincident with CNS vascularization (around E10.5), microglia, which expressed CX3CR1 but not CCR2, started to colonize the CNS. Thus, these studies confirm findings mentioned before that microglia precursors seed the CNS very early in development, before monocytes are generated (Ginhoux et al., 2010).

The same gene-targeted mice were also used to study the role of the respective chemokine receptor-expressing monocyte subsets in an EAE model. In the absence of functional CCR2, the disease onset appeared to be delayed significantly, but reached the same intensity (Saederup et al., 2010). While the total number of infiltrating cells was not altered, recruitment of classical Ly-6Chi monocytes was completely abolished in these mice, indicating their dependence on CCR2 for vascular egress and CNS entry in this model. Interestingly, neutrophils now replaced the monocytes in the inflammatory process.

While functional CX3CR1 does not contribute to the influx of inflammatory monocytes into the CNS, it is known that this fractalkine receptor mediates anti-inflammatory signals on the resident microglia (Cardona et al., 2006). This was illustrated in a model of neurodegeneration induced by hyperphosphorylated tau protein. Absence of microglial CX3CR1 in these mice caused increased CNS pathology, coincident with increased inflammatory MAPK-signaling and associated cognitive impairment (Bhaskar et al., 2010). Thus, the extent of brain inflammation is regulated at the level of both the infiltrating leukocytes as well as the resident microglial cells.

Epidemiologic observations of meningitis in humans show that *Listeria monocytogenes* invades the CNS much more readily than other Gram-positive pathogens. **Dr. Drevets** discussed the background of this neurotropism, and the role of monocytes in this process using a mouse model of infection with L. monocytogenes. Bacterial invasion of the brain may occur via different pathways, but the influx of parasitized monocytes, the so-called Trojan horses, is thought to be the most prominent route (Drevets and Bronze, 2008). Interestingly, the classical Ly-6Chi monocytes are the major transporters of bacteria into the CNS (Drevets et al., 2004). Monocyte recruitment actually preceded brain infection and was found to be highly dependent on peripheral IFN-gamma production. It was enigmatic, however, why monocytes were now effectively parasitized instead of performing their known anti-bacterial function. This appeared to relate to the bacterial burden, and was strikingly different between sublethal and lethal infection. In lethal infection, large numbers of monocytes were generated in the bone marrow, but the highly inflammatory conditions induced a remarkably different phenotype and function in these cells (Drevets et al., 2010b). These monocytes had greatly diminished levels of the mononuclear phagocyte hallmark molecule M-CSFR (CD115), while they had obtained significant expression of the IL-1 decoy receptor CD121b and the anti-

inflammatory signaling proteins SOCS-1, -3 and IRAK-M. Furthermore, bone marrow monocytes in lethal infection showed an increased capacity to phagocytose bacteria compared to cells in steady state. Nonetheless, intracellular bacteria replicated in the cells and there was no concomitant increase in their ability to produce reactive oxygen intermediates. Together, these features provide a likely explanation for the parasitism of newly generated monocytes in severe infection. Then, why do these cells preferentially migrate into the CNS? CCR2-binding chemokines appeared to be the major determinants in other models of monocyte CNS invasion as discussed above. These chemokines were indeed expressed at high levels in the CNS upon peripheral *L. monocytogenes* infection, but also non-CCR2-binding chemokines were induced (Drevets et al., 2010a). Although release of monocytes from the bone marrow was delayed in CCR2-deficient mice, the influx of these cells into the CNS reached the same levels as in controls. Moreover, similarly unaffected monocyte influx was found in mice lacking other chemokines or -receptors, in particular CXCR3, CCL2, CCR1, CCR5, and CX3CR1. There-fore, these studies show that IFN-gamma is critical for triggering brain influxes of classical Ly-6Chi monocytes during systemic infection with *L. monocytogenes*. Remarkably, however, this initial burst of monocyte migration appears to be largely independent of individual chemokine receptors.

Dr. Laman focused in his presentation on the immunological mechanisms that are involved with the initiation and progression of autoimmunity as occurring in multiple sclerosis (MS). These concerned the fundamental aspects of how CNS antigens reach the immune system and which antigen-presenting cell (APC) subsets in the CNS could be involved with antigen capture and local presentation. The pathology of MS is characterized by the presence of inflammatory lesions especially in white matter, which lead to demyelination and axonal loss. This destructive process is mediated by locally activated Th1 and Th17 cells specific for myelin components as well as by activated microglia and autoantibodies (reviewed in ('t Hart et al., 2009; Ransohoff and Perry, 2009)). Lesional APC, characterized by MHC class II expression, are especially observed at the interface of normal and myelin-depleted areas in the brain, while phagocytes filled with myelin-derived lipids are present throughout the lesions. The latter are thought to play an important role in the initiation of the autoimmune response by emigration from the CNS towards cervical and lumbar lymph nodes and thus providing the source of auto-antigen in an immune-stimulatory environment (van Zwam et al., 2009b). In a marmoset model of EAE, it was found that immunization with a small myelin (MOG34-56) peptide in incomplete Freund's adjuvant was sufficient to induce demyelinating disease, thus disproving the dogma that microbial ligands must be present to break T-cell tolerance (Jagessar et al., 2010). In the draining lymph nodes of these primates, as well as in those of MS patients, various neuronal antigens could be demonstrated, indicating significant damage and antigen transport to the periphery. Interestingly, removal of the cervical lymph nodes in a mouse model of chronic EAE delayed the onset of a secondary relapse and reduced disease severity, indicating that these lymph nodes not only play a crucial role in the induction of autoimmunity, but also contribute to flare-ups of the disease (van Zwam et al., 2009a).

Under steady state conditions the CNS is thought to lack DC as genuine APC, besides some DC residing in meninges and choroid plexus (Ransohoff and Perry, 2009). Using non immune-challenged CD11c-GFP mice, however, it was found that predilection areas of MS lesions were preferentially populated by CD11c+ cells (Prodinger et al., 2011). Most CD11c+ cells were located within the juxtavascular parenchyma rather than the perivascular spaces and expressed typical myeloid markers. However, MHC class II expression was restricted to DC in the choroid plexus. Cellular processes projected into the glia limitans, suggesting the possible transport of antigens or presentation to extravasated T cells in perivascular spaces. Interestingly, a similar population of DC was observed in human brains using anti-DC-SIGN (CD209) antibodies.

Trafficking of immune cells in general as well as into the CNS in particular was further discussed by **Dr. Sallusto**. She emphasized that functionally distinct T-cell subsets are characterized by the expression of different chemokine receptors, and thereby have unique migratory capacities (Sallusto and Lanzavecchia, 2009). For instance, IL-17-producing Th17 cells are uniformly CCR6-positive, while IL-22 is primarily produced by skin-homing cells that express, besides CCR6, also CCR10 and the homing receptor cutaneous leukocyte antigen (CLA). For a long time, the low frequency of T-cells with selective antigen specificities has been a major obstacle in investigating T-cell functionality. Dr. Sallusto presented a recently developed approach that allowed the analysis of naïve and memory repertoires of human T-cells using libraries of *in vitro* amplified T-cells that were screened by stimulation with antigen-loaded autologous monocytes (Geiger et al., 2009). These analyses showed that T-cell responses against recall anti-

gens are in general very focused and lead to the activation of a limited set of T-cell subtypes. For instance, Candida albicans-specific T-cells are primarily Th17, while PPD-specific T-cells are Th1 cells with the characteristic expression of the CCR6 chemokine receptor (indicated as Th1*). CCR6, mediating the response to CCL20 (LARC/ MIP-3alpha), is thought to be involved with homing of T-cells to the CNS. In accordance, CCR6 appeared to be essential for the induction of EAE as CCR6-deficient mice were highly EAE-resistant (Reboldi et al., 2009). The actual immune response leading to EAE pathogenesis involved the CCR6-dependent entry of Th17 cells into the CNS, occurring at the choroid plexus. At this location, CCL20 is expressed by epithelial cells in both mice and humans in the steady state, suggesting that this route may be a constitutive port of CNS entry for CCR6+ T-cells. Only after this selective immigration of Th17 cells and inflammatory triggering, subsequent massive recruitment of effector cells could be observed in the parenchyma - a process that can occur independently of CCR6 - causing full EAE development. This proposes a two-step mechanism for MS pathogenesis, and in accordance with these findings, elevated levels of CD4+CCR6+ cells were observed in blood and CSF of MS patients.

In clinical diagnostics, imaging techniques are increasingly important, and this is especially the case in CNS disease because of the inaccessibility of the organ. **Dr. Pomper** provided a broad overview of the characteristics of the various methods, and, in particular, their application to infection and inflammatory disease. In the last decades, imaging has rapidly evolved from mere anatomic (e.g. CT, MRI) to functional (e.g. CT angiography, fMRI) technologies and further into true molecular imaging (reviewed in (Peterson et al., 2011)). At this latter level, biological processes are not only visualized at cellular and molecular levels, but also quantitative information can be obtained from living individuals in a non-invasive manner. The specific features of infection and inflammation, such as the increased vascular permeability and leukocyte immigration into tissues, provide several possibilities for molecular imaging,. Other targets include endothelial cell activation (e.g. detection of E-selectin), the micro-organism itself and increased metabolism, for instance detected by [^{18}F]FDG and PET.

Dr. Pomper showed several examples of the various techniques. A recent development is the use of imaging in bacteriolytic tumor therapy. This is based on the lysis of tumor cells by anaerobic bacteria (*Clostridium novyi* type A) in the hypoxic center of a solid tumor. Since the local conditions impose a quiescent phenotype upon these tumor cells, they remain relatively resistant to conventional therapy. After tumor reduction by bacteria, these can be imaged by treatment with radiolabeled FIAU (fialuridine), a nucleoside analog that freely enters and exits cells as well as serving as a substrate for cellular transporters. Phosphorylation by thymidine kinase, however, traps the radiolabel inside pro- and eukaryotic cells. Therefore, it can be used to visualize bacterial infection. Radiolabeled FIAU, can also be used to image virus-related cancer, such as EBV-positive tumors, since the virus expresses elevated levels of functional thymidine kinase once activated. Pharmacological agents such as bortezomib induce in tumor cells the switch from latent to lytic infection, and thus function therapeutically. This state of EBV lytic gene expression *in vivo* can be visualized using radiolabeled FIAU (Fu et al., 2008). For imaging of CNS inflammation, the translocator protein (TSPO) present in microglial mitochondria is a useful target (Endres et al., 2009). Upon activation of microglia these cells show a significantly increased expression of TSPO and this can be visualized by radiolabeled TSPO ligands. In this manner, inflammatory lesions of various CNS diseases such as MS, Alzheimer and HIV dementia have been visualized with high sensitivity using PET scanning.

Paraneoplastic CNS disease

In a group of CNS diseases, which are collectively termed "autoimmune channelopathies" or "autoimmune encephalitides", autoantibodies play an important pathogenic role (Vincent, 2010). **Dr. Vincent** illustrated the diversity of clinical presentations of these diseases related to their molecular background. In general, these neurological and neuro-muscular disorders can present at any age and are characterized by their responsiveness to immunotherapy, such as corticosteroids, plasma exchange, intravenous immunoglobulins or other immune suppression. The best known example is myasthenia gravis, in which autoantibodies against the acetylcholine receptor located in the neuromuscular junction block skeletal muscle fiber triggering. It is now increasingly recognized that similar pathogeneses underlie CNS diseases. The autoantibodies involved are directed to ion channels such as voltage-gated potassium channels, which regulate neurotransmitter release, or to postsynaptic neurotransmitter receptors expressed by muscle cells or neurons. The diseases may be monophasic or more chronic, and it is remarkable that the limbic system

is a usual target of these autoimmune disorders. A possible explanation is that the blood-brain barrier in the hippocampal area is somewhat more leaky than in other areas. The clinical symptoms caused by a single type of autoantibodies may be very diverse, as was illustrated in a case of Morvan's syndrome (Spinazzi et al., 2008). This disease is characterized by antibodies against voltage-gated potassium channels. This case was initially misdiagnosed as schizophrenia, and besides behavioral problems, the patient suffered from cramps and pain, sensory loss and sleep disorders. In some patients, the pathogenic autoantibodies develop as a consequence of an anti-tumor response. Malignancies such as small cell lung carcinoma, breast- or testicular carcinoma may express high levels of the surface molecules that function as autoimmune targets in the autoimmune channelopathies. Another recently identified example is provided by encephalitis that is caused by anti-NMDA-receptor antibodies, often but not always elicited in the context of ovarian teratoma (Irani et al., 2010). The antibodies trigger a syndrome with characteristic features that include a psychiatric onset followed by movement abnormalities. Besides antibodies, leukocyte accumulation is observed in the CNS consisting of CD68+ activated microglia, perivascular T cells and plasma cells (Tuzun et al., 2009). Finally, it is interesting to note that the vast majority of these autoimmune channelopathies have only been identified in the past decade, suggesting that this may be only the tip of the iceberg; other, currently enigmatic CNS conditions and some forms of more common conditions, such as epilepsy or psychosis, may have a similar pathogenesis.

Dr. Blachere further elaborated on the paraneoplastic neurological disorders (PND), in which antigens expressed by tumor cells as well as neurons are recognized by the immune system as foreign. This results in tumor immunity, but also in the immune-mediated destruction of neurons in the CNS. Although these disorders are rare, they afflict patients with a variety of malignancies, in particular lung, breast, and ovarian cancers. Previously, Dr. Blachere's lab has demonstrated that antigens expressed by neurons are targets of both the cellular and humoral arms of the immune system, i.e. that antigen presenting cells, T cells and antibodies are hallmarks of the development of PND (Albert et al., 1998; Santomasso et al., 2007). In paraneoplastic cerebellar degeneration, for instance, antibody reactivities as well as cytotoxic T-cell responses have been shown against the cdr2 antigen. Cdr2 is is expressed by breast- and ovarian cancer cells as well as Purkinje neurons in the cerebellum. Intracellular expression of cdr2 results in peptides being presented in an immunogenic fashion on the MHC class I molecules of tumor cells and targeted by cytotoxic T cells in the periphery. The recruitment of T cells into the CNS in these patients is facilitated by high levels of CXCL10 in the cerebrospinal fluid (CSF).

Using a mouse model of graft-versus-host disease (GvHD) induced by anti-minor histocompatibility antigen reactivity, the question was approached whether peripherally activated autoreactive T cells could affect antigen processing and presentation in the parenchyma of the brain. The GvH response in these mice was associated with an influx of donor CD4+ Th1- and CD8+ cytotoxic T cells into the brains as well as activation of microglia mediated by IFN-gamma. Interestingly, these microglia now appeared to be potent APC for both memory CD4+ and CD8+ T-cells, despite the lack of costimulatory molecules. In a different mouse model, expression of the lacZ molecule as neoantigen was induced by the CNS-specific NOVA2 promoter. Cytotoxic T cells appeared to be tolerant to immunization with lacZ-expressing adenovirus. This tolerance, however, was broken by triggers that resulted in activation of microglia, which led to clinical symptoms of CNS disease similar to PND. Together, findings in these models suggest that activation of microglia, for instance by peripheral inflammation, could give rise exacerbation of PND once T cells recognizing neuronal antigens are initiated in the periphery.

Langerin-expressing DC and LCH

One of this year's Jon Pritchard fellows, **Dr. Bigley**, focused on the origin of LCH cells. This was inspired by the finding in mouse models that Langerin expression is not confined to epidermal LC, but also present in a small subset of DC located at other tissue sites including the dermis (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007). In her presentation, she defended the hypothesis that LCH cells would be at least as closely related to Langerin-positive dermal DC (LDC) as to LC. To that end, the APC composition of human dermis was studied in detail. Different populations of CD1a+ and CD14+ DC, with low levels of autofluorescence, were identified, as well as highly autofluorescent dermal macrophages (Haniffa et al., 2009). In addition, the human dermis contained a small population of LDC, which differed clearly from LC by their lower level of CD1a and absence of EpCAM expression. Furthermore, LDC showed significantly higher levels of CD11b, CD11c, CD13 and CD31 than LC. Cells with a similar phenotype appeared to be present in human lung tissue as well. Interestingly, following hematopoietic

stem cell transplantation, donor-derived LDC repopulated the skin earlier than LC , similarly to the LDC and LC repopulation kinetics observed in the mouse. This indicates that LDC, as a population, behave independently of LC. The notion that Langerin expression may reflect an inducible state in DC, rather than a distinct DC lineage, was reinforced by the induction of Langerin expression *in vitro* in hematopoieitic stem cell-derived DC by different culture conditions, which gave rise to DC with different phenotypes but sharing Langerin. When the immunophenotype of isolated LCH cells was compared to that of LDC or LC, it was apparent that LCH cells showed a marker profile closely resembling LDC ($CD1a^{int}$, $CD11c^{+}$, $Langerin^{int}$, $EpCAM^{neg}$, $CD86^{low}$) rather than LC. This difference is also in accordance with the gene expression microarray data published recently in which LCH profiles were compared with isolated LC (Allen et al., 2010). Together, these findings suggest that LCH cells are closely related to LDC and that expression of Langerin by LCH cells may reflect their activation in an inflammatory lesional environment.

The other Jon Pritchard fellow, **Dr. Quispel**, highlighted the contribution of a special type of bystander cells in the LCH lesions, the FoxP3+ CD25+ T-lymphocytes. A previous study has indicated that this population of cells, which bear characteristics of regulatory T cells (Treg), is expanded in LCH lesions as well as in the blood of patients with active disease (Senechal et al., 2007). Extending this analysis Dr. Quispel showed that the frequency of CD3+CD25+FoxP3+ Treg was higher in LCH lesions in lymph nodes and skin than in bone, although the number of lesions analyzed so far was limited. Notably, some FoxP3+ T cells appeared to be CD8+ rather than CD4+. Different subsets of human Treg have been identified, which differentially express the costimulatory molecule ICOS (Ito et al., 2008). These cells differ functionally as ICOS+ Treg inhibit DC function via IL-10 secretion, while ICOS- Treg do so via TGF-beta, which can be demonstrated in an inactive proform as latency-associated peptide (LAP). T cells in LCH lesions, both FoxP3+ and FoxP3-, express ICOS rather uniformly. Furthermore, it was demonstrated that ICOSL is expressed in LCH lesions, allowing functional interaction with ICOS+ T cells. Associating ICOS and cytokine expression, a significant degree of variability in IL-10 and LAP expression was observed between lesions in different tissues, and even among different samples from the same tissue. Collectively, these observations underline the heterogeneity of LCH lesions and suggest that Treg could affect, directly or indirectly, the maturation status of LCH cells.

Dr. Imashuku discussed in detail the state-of-the-art in clinical research and management of CNS disease in patients with LCH. The most frequent CNS manifestations are diabetes insipidus as a consequence of posterior pituitary involvement and neurodegenerative disease, which may be initiated by perivascular LCH lesions and responding glial cell proliferation. Typically, in LCH patients who develop CNS disease this becomes apparent mostly as a sequel to the primary episode, and often only years after diagnosis. Patients at high risk to develop CNS disease are those with multisystem LCH with involvement of specific skull and facial bones (Imashuku et al., 2008a; Imashuku et al., 2008b; Imashuku et al., 2009; Grois et al., 2010). To identify patients with increased risk more specifically, in order to provide them with treatment to prevent CNS-LCH, better predictive biomarkers are required. Concentrations in serum of specific cytokines and chemokines, such as M-CSF, IL-10, HGF, CCL2, CXCL10, OPG and sRANKL, clearly associate with disease severity (Ishii et al., 2006; Morimoto et al., 2010). Therefore, assessing the levels of these factors in CSF of risk patients at disease onset, in combination with brain MRI, might be a good indicator for preventive treatment. Therapeutics that could be applied are intravenous immunoglobulin (IVIg) and cladribine (2-CDA). IVIg is thought to function as immunomodulating agent in inflammatory responses and has been tested for use in LCH (Imashuku et al., 2008a; Imashuku et al., 2008b; Imashuku, 2009). 2CDA might be beneficial in preventing clinical CNS disease since it effectively penetrates the BBB. Currently, efforts are being made to evaluate these measures in high-risk CNS-LCH patients in clinical trials.

Dr. Rodriguez-Galindo provided an overview and update of the clinical trials that have been performed in LCH patients. Since 1991 the Histiocyte Society has initiated and coordinated three completed large-scale, international, prospective therapeutic studies (LCH-I to III) for multisystem LCH (MS-LCH)(www.histiocytesociety.org) and a fourth trial (LCH-IV) is in preparation (Minkov, 2011). The prospect of MS-LCH patients is significantly worse compared to those in which only a single organ system is affected. In particular patients with involvement of hematopoietic organs, spleen, liver and/or lung are at risk of dying of the disease. Therefore, three groups of patients were identified and included in LCH-III: (1) MS-LCH with risk organ involvement, (2) MS-LCH without risk organ involvement, and (3) patients

with poliostotic or CNS-risk lesions. In the first, high risk group the question was evaluated whether addition of methotrexate (MTX) to the regular induction and maintenance protocol would increase survival. Evaluation of 204 patients that were equally distributed among two treatment arms showed that survival in both groups was not significantly different (87 and 80% without or with MTX, resp.) Also the rate of reactivation of the disease (approximately 30% after 3 years) did not differ. Interestingly, in LCH-III the survival rate had improved and reactivation rates were lower compared to the outcome with similar treatment protocols applied in LCH-I and -II. The reasons for this difference are not clear, and probably are multifactorial. In the MS-LCH patient group without risk organ involvement the question was addressed whether prolonged maintenance therapy with vinblastine and prednisolone (12 vs. 6 months) would reduce the reactivation rate of the disease. This indeed appeared to be the case as 55% of patients with the shorter treatment showed reappearance of the disease within 3 years, compared to 42% of patients with longer treatment. Although beneficial, this alteration in therapeutic approach clearly requires further improvement. Therefore, an important aim of LCH-IV will be to decrease reactivations and permanent consequences by further prolongation of therapy and addition of 6-mercaptopurine. In addition, it is aimed to decrease mortality by early identification of patients at risk and their switch to a salvage protocol. A third aim is to investigate CNS-LCH in the included patients in more depth. The quality of life of patients and their environment is significantly influenced by CNS disease and especially the causes of neurodegenerative disease are poorly understood (Grois et al., 2010). In some patients, neurodegenerative lesions are only radiologically detectable, but the vast majority with MRI-detectable masses larger than 4 mm eventually develops clinical neurodegenerative disease. Therefore, focus on these aspects is highly warranted.

Summation and conclusions

In the summation session, chaired by **Drs. Perry and Merad**, the various topics presented at the meeting were discussed in view of the etio-pathogenesis of CNS-LCH and improvement of diagnosis and therapy of the disease. The existence of a prolonged preclinical stage of CNS-LCH in a significant fraction of LCH patients urges the development of improved diagnostic tools, based on tests that assess mood, motivation and cognition, in addition to the more routinely applied endocrine tests. Possibly, psychological specialists with experience in fragile X syndrome could be of help in this area.

Despite many years of research, the current knowledge of the neuropathology of LCH is scant and based on the analysis of a relatively small number of samples. Clearly, different forms of CNS-LCH can be distinguished, based on the presence of mass lesions in the brain parenchyma, meningeal lesions on the surface of the brain, or neurodegenerative-type lesions. The latter may reflect paraneoplastic-like lesions characterized by activated microglia and, predominantly, CD8+ T-cells as LCH cells are typically absent from neurodegenerative lesions.

A key question in CNS-LCH is: What drives the disease in the brain? Is it the LCH cells themselves, the accompanying T cells, or the dysregulated inflammation in which these and other cells participate? Since the precursors of the LCH cells are probably found among the Langerin+ DC fraction related to the dermal LDC, and less likely among the epithelial tissue-bound Langerhans cells, this offers a great opportunity to approach these cells by studying circulating myelomonocytic cells in LCH patients. The sampling of only minimal amounts of peripheral blood is sufficient for detailed phenotypic and molecular analysis that could lead to the identification of putative aberrations in the precursor LCH cells.

Since CD1a+ DC are thought to be foreign to the steady state CNS parenchyma, another question is: what mechanisms are responsible for the migration of LCH cells into the brain? The link between peripheral inflammation and recruitment and activation of cells in the CNS, observed in other diseases, raises the possibility that peripheral conditions drive inflammatory cells, including LCH cell precursors and T cells into the CNS. This process requires no antigen specificity, inflammatory conditions suffice. Chemokine receptors, like CCR2 and CCR6 that have been shown to be important in CNS migration of myeloid cells and T cells, might mediate this migration, although different mechanisms could apply in the different form of CNS-LCH.

An interesting finding in MRI analysis is that parenchymal CNS lesions are often found to be present in a symmetric fashion. A possible explanation might be that a focal CNS lesion causes the initiation of an adaptive immune response, leading to the formation of antibodies to CNS antigens, which could give rise to acellular, symmetric lesions associated with inflammatory cytokine production at nearby perivascular sites. Down-regulation of fractalkine signaling on microglia could possibly play an activating role in this process. Thus, besides (paraffin-embedded) LCH lesional tissue, blood leukocytes and serum, also

CSF obtained from patients with shown or suspected CNS disease would be an important resource to investigate the putative origins of CNS-LCH. The focus in LCH-IV on CNS disease would be an opportunity to implement this. In particular, finding determinants that differ between patients that reactivate and those that do not would be a significant step forward in the understanding and treatment of the disease.

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