Viral, autoimmune and neoplastic mechanisms of granuloma formation and possible relevance to LCH

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The Nikolas Symposia

The mission of the annually held Nikolas Symposium is to find a rational cure for Langerhans Cell Histiocytosis (LCH) (Beverley et al., 2005). This meeting is sponsored by Paul and Elizabeth Kontoyannis whose son Nikolas developed LCH in infancy but has survived his battle with this disease. The symposium is an interactive forum of basic scientists and clinicians who discuss different aspects related to 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 Langerhans cells (LC) (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 as a lesional disease, either with single or multifocal lesions in different organs. Skin and bone are most frequently affected. More severe forms of LCH occur especially in younger children, and these cases of multisystem disease tend to have a clinically malignant character. 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.

Since long, LCH investigators have tried to answer the question whether abnormal LCH cells develop due to an intrinsic defect or as a response to environmental triggers such as viral infection. Either cause may lead to aberrant regulation of dendritic cells (DC) and thus result in their accumulation. Interestingly, supportive evidence for both scenarios exists (summarized in (Egeler et al., 2010)). Briefly, the clonal relationship of LCH cells, their maturation arrest and aggressive clinical behavior in some cases, and the high disease concordance rates observed in twin studies argue in favor of genetic dysregulation. However, no consistent genomic aberrations have been observed in an extensive recent study (da Costa et al., 2009) and the cells appear cytologically benign. 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). Thus, the underlying cause of DC accumulation in LCH still remains to be determined.

The granulomatous nature of the LCH lesions, comprising the aberrant LCH cells and a mixture of bystander cells, might implicate that a hitherto unidentified cause drives a localized, chronic inflammation leading to the characteristic lesional composition. Extensive microscopic analysis has ruled out infection with bacteria or larger microbial organisms as etiological factors, but other causes such as viral infection or autoimmune phenomena might play an important role. Therefore, this year's symposium focused on the various factors that contribute to granuloma formation to discuss such possibilities in more depth.

The pathology of the various forms of histiocytosis was introduced by **Dr. Malone**. Related to the main cell types involved, the disorders can be divided into DC or macrophage anomalies, although the distinction between the two lineages is far from absolute. LCH is the most frequent DC-related histiocytosis, while macrophage-type disorders mostly present as hemophagocytic lymphohistiocytosis (HLH) or macrophage activation syndrome. Both involve pathologically non-malignant cells, but the clinical pictures can be very severe. Phenotypically, LCH cells are characteristic with a round morphology and large cytoplasm and indented nucleus. These cells typically express CD1a and Langerin as characteristic markers, although some discordance between these can be found. Together with LCH cells, a variety of other cell types is found in LCH lesions, such as eosinophils, T-cells, macrophages and multinucleated cells. The accumulations are often called granulomatous, although the classic, walled-off granuloma structure is lacking. 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) (Hoeger et al., 2001). Cells characterizing the latter disease have a distinctive phenotype (FXIIIa$^+$ CD1a$^-$ S100$^-$) and were previously designated as dermal DC. Recent studies suggest, however, that FXIIIa expression typifies dermal macrophages rather than dermal DC (Haniffa et al., 2009). LCH diagnosis may be further complicated by coexisting macrophage activation syndrome (Favara et al., 2002) and lymph node sinus histiocytosis, also known as Rosai Dorfman disease (Chikwava and Jaffe, 2004).

DC subsets

In his presentation on human DC surface markers, **Dr. Hart** highlighted various diagnostic and therapeutic aspects. Since peripheral blood DC are most readily accessible in human but not in mice, the homologies between the DC subtypes in both species have not been fully elucidated. One of the at least five identified human blood DC subtypes (MacDonald et al., 2002), the CD114$^+$ (a.k.a. BDCA3$^+$ or thrombomodulin$^+$) DC, appears to represent the homolog of mouse CD8alpha$^+$ DC. This is based on molecular profiling and the shared expression of Clec9a, TLR3, and transcription factor IRF8. In addition, these CD114$^+$ DC, which are present in blood and secondary lymphoid organs, can produce IFN-beta and IL-12 at high level and have the unique capacity to cross-present exogenous antigens on MHC class I. Interestingly, the activating lectin receptor Clec9a expressed by these cells provides an efficient target for vaccination purposes (Sancho et al., 2009). Another family of molecules that might be very important in regulating DC activation is the CD300 family, which comprises both activating and inhibitory members (Clark et al., 2009). Triggering of CD300a/c, for instance, increases production of IFN-alpha, but decreases TNF-alpha and IL-6. Also cellular antigen uptake, processing and migration are regulated by CD300 molecules.

Targeting DC to establish selective immune suppression is an important goal in the transplantation setting in order to prevent graft-versus-host disease (GvHD), but simultaneously maintain responses against infectious agents and tumor cells. Presence of the DC activation marker detected by CMRF-44 mAb on peripheral blood DC appeared to predict acute GvHD accurately (Lau et al., 2007). Preventive treatment using the DC maturation marker CD83 was established in a SCID mouse model, in which transplantation of human peripheral blood cells caused GvHD in a DC-dependent manner (Wilson et al., 2009). Application of CD83 antibody prevented GvHD but, unlike conventional immunosuppressants, did not prevent engraftment of human T cells, including cytotoxic T lymphocytes (CTL) responsive to viruses and malignant cells. Immunization of CD83 antibody-treated hu-SCID mice with irradiated human leukemic cell lines induced allo-responsive CTL effectors against leukemic cells *in vivo* that lysed ^{51}Cr-labeled target cells *in vitro* without further stimulation. Together, these findings indicate that antibodies that target activated DC are a promising new therapeutic approach to control GvHD.

Dr. Collin outlined in detail the characteristics of different subpopulations of DC and macrophages in the human skin. In contrast with the situation previously described for mouse skin, it was found that patients that had undergone bone marrow transplantation did not retain recipient LC for long intervals even when transplanted with T-cell-depleted bone marrow and reduced intensity conditioning protocols (Collin et al., 2006). Active GvHD, however, could be shown to contribute to increased skin engraftment with donor-type LC, in line with findings in the mouse where LC were replenished by circulating monocytes under inflammatory conditions (reviewed in (Merad et al., 2008)). The independence of the LC population from circulating monocytes in non-transplanted steady state was illustrated by the finding that a patient with a rare condition of monocytopenia showed a normal LC network in the skin, while monocytes and DC in the blood as well as dermal DC and macrophages were strongly diminished. Delineating the dermal cells after isolation, three main populations of cells could be distinguished: macrophages, characterized by high autofluorescence, and two distinct populations of DC, namely CD14$^+$ DC and CD1a$^+$ DC (Haniffa et al., 2009). The level of CD1a expression by the latter cells is typically lower than that of migrating LC. The population of CD1a$^+$ dermal DC contained a minor subset (~ 5%) of dermal Langerin$^+$ DC, which are the presumed counterparts of the LC-independent Langerin$^+$ DC population identified in the mouse (Ginhoux et al., 2007). Also the level of Langerin expression by the human dermal cells was significantly lower compared to genuine LC. Like in the mouse, this population of Langerin$^+$ DC is not restricted to the skin, but was also found in human lung parenchyma. In both tissue sites, a small percentage of cells appeared to be proliferating, as did epidermal LC. Interestingly, the morphology and phenotype of dermal Langerin$^+$ DC showed much more agreement with that of LCH cells than epidermal LC.

This included the round morphology, with more or less round nucleus, the absence of EpCAM expression and the lower level of CD1a. Therefore, it is tempting to speculate that LCH cells are more closely related to the connective tissue-type of Langerin-expressing DC, rather than the epidermal LC.

Beta-catenin-mediated survival and maturation signals in macrophages and DC

Dr. Zahner was one of this year's Pritchard fellows. She discussed the characteristics of intriguing *in vivo* models in which E-cadherin or beta-catenin molecules were targeted in a DC-specific manner. E-cadherin is a component of adherens junctions used by immature LC to adhere to the keratinocytes. This molecule is down-regulated during LC emigration from the epidermis. Likewise, beta-catenin contributes to adherens junctions in a complex with E-cadherin while, as a central component of the canonical WNT signaling pathway, it controls gene expression as a transcriptional co-activator. Previously, a role for this pathway was hypothesized in LCH, based on the involvement in epithelial proliferative disorders and the atypical absence of E-cadherin in LCH cells (Leenen and Egeler, 1999). Furthermore, Mellman and collaborators have more recently shown in *in vitro* studies that activation of the beta-catenin pathway in DC stimulated their maturation to tolerogenic cells (Jiang et al., 2007). The mouse models were produced by crossing CD11c-Cre-recombinase-expressing mice with mice in which the E-cadherin or beta-catenin gene or exon 3 of beta-catenin was flanked by *loxP* sites. Thus, E-cadherin or beta-catenin was deleted in CD11c-expressing cells, or, in case of the lack of exon 3, beta-catenin was constitutively activated in DC. Unexpectedly, the LC network in all three DC-specific mutants was still intact and indistinguishable from wildtype mice. The beta-catenin stabilization mutant showed the most significant functional changes, as it essentially failed to mount a contact hypersensitivity response. Moreover, this mutant also did not develop airway hyper-responsiveness and eosinophilia in a mouse model for allergic asthma. Intriguingly, the steady state percentage of CD4+Foxp3+ regulatory T-cells (Treg) was significantly increased in these mice. Together, these results strongly suggest a crucial contribution of beta-catenin signals to the generation of a regulatory DC phenotype *in vivo*.

Interestingly, **Dr. Colonna**'s recent findings on the function of the activating receptor DAP12 in macrophage proliferation and survival induced by M-CSF also involved beta-catenin activity (Otero et al., 2009). DAP12 is a transmembrane adaptor protein, which signals via an activating ITAM motif. Mutations in the DAP12 gene, or its putative receptor TREM2, underlie the human disorder Nasu-Hakola disease (NHD). This disease is characterized by brain demyelination and gliosis as well as polycystic osteodysplasia, causing spontaneous fractures. DAP12 is expressed in microglia and osteoclasts, suggesting that NHD may be caused by defective development or function of these cells. Indeed, NHD patient blood monocytes fail to fuse and develop bone resorptive activity when stimulated *in vitro* with M-CSF and RANKL to form osteoclasts. Similarly, in DAP12-deficient mice, osteoclast differentiation is defective, but microglial involvement is unclear in the different mouse strains. *In vitro* generation of macrophages from precursor cells by M-CSF stimulation indicated that DAP12-deficiency has a significant deleterious effect only when M-CSF is limiting. A similar condition may occur *in vivo* when mice age and experience decreased M-CSFR signaling. This may explain the observed decrease in microglia in specific brain areas in aged DAP12-deficient mice (Otero et al., 2009). Multiple intracellular pathways are known to be linked to M-CSFR triggering, including PI-3K / Akt for cell survival and ERK for proliferation, but involvement of DAP12 was not known so far. Mechanistically, M-CSFR stimulation appeared to activate DAP12, which initiated a cascade involving Pyk2 activation, followed by beta-catenin stabilization via tyrosine phosphorylation. Subsequent nuclear translocation of beta-catenin then activated genes involved in cell proliferation and survival. It remains remarkable, however, that beta-catenin activation has apparently distinct effects in macrophages and DC, i.e. involving proliferation and survival in macrophages, while inducing maturation towards a tolerogenic function in DC.

Regulation of T-cell homeostasis and tolerance

Maintaining the balance between effective responses against pathogenic triggers and tolerance against innocuous and endogenous substances is key to the proper functioning of the immune system. This is in particular regulated at the level of antigen-presenting cells and T-cells. Recent studies presented by **Dr. Li** have identified the regulatory cytokine transforming growth factor-beta1 (TGF-beta1) as a pivotal regulator of T-cell responses. The direct impact of TGF-beta1 signaling on T-cells was investigated in a strain of mice with T-cell-specific deletion of TGF-beta receptor II (Tgfbr2) gene (Li et al., 2006). These experiments indicated that TGF-beta1 signaling was important for the survival of low-affinity T-cells, while tol-

erizing high-affinity autoreactive T-cells. TGF-beta1 is produced by multiple cells, including T-cells themselves, in a latent form. To study cell-type specific function of TGF-beta1 itself, mice were generated with a floxed allele of the Tgfb1 gene. T-cell-specific deletion of TGF-beta1 resulted in immunopathology in multiple organs and the development of inflammatory bowel disease (IBD), which was associated with enhanced Th1 cell differentiation (Li et al., 2007). On the other hand, T-cell-produced TGF-beta1 was also required for Th17 cell differentiation and the induction of experimental autoimmune encephalomyelitis (EAE). Therefore, these findings demonstrate opposing roles for T-cell-produced TGF-beta1 in Th1 and Th17 cell differentiation. Using a Langerhans cell (LC)-specific Cre transgenic mice, we found LC-produced TGF-beta1 is essential for the differentiation and/or maintenance of LC (Kaplan et al., 2007). Therefore, TGF-beta1 regulates immune cell differentiation and activation largely via an autocrine/paracrine mechanism.

Another factor in T-cell tolerance identified by Dr. Li and collaborators is Foxo1, a member of the Forkhead box O family of transcription factors. Foxo1 is highly expressed in naïve T-cells and down-regulated upon activation (Ouyang et al., 2009). T-cell-specific deletion of the Foxo1 gene in mice led to spontaneous T-cell activation, effector T-cell differentiation, autoantibody production, and the induction of IBD in a transfer model. In addition, Foxo1 was critical for the maintenance of naïve T-cells in the peripheral lymphoid organs. Gene expression analyses of T-cells identified Foxo1-regulated genes encoding, among others, cell surface molecules, signaling proteins, and nuclear factors that control gene expression. Functional studies validated interleukin-7 receptor-alpha as a Foxo1 target gene essential for Foxo1 maintenance of naïve T-cells. Together, these findings reveal crucial functions of Foxo1-dependent transcription in control of T-cell homeostasis and tolerance.

Dr. Belkaid discussed the balance of T-cell responses in conjunction with microbial exposure, focusing on the intestines. In various forms of chronic infection, large numbers of Treg have been identified that suppress excessive immune activation, but simultaneously create a niche for pathogens by preventing their full eradication (reviewed in (Belkaid and Tarbell, 2009)). Especially in the intestines, generation of $Foxp3^+$ Treg takes place by conversion of naïve effector T cells. A specific subpopulation of $CD103^+CD11b^{hi}$ DC located in the lamina propria underneath the gut epithelium plays an important role in the induction of these adaptive Treg (Sun et al., 2007). The lamina propria DC express high levels of retinoic acid (RA-) synthesizing enzymes, and it appears that Treg conversion is dependent in RA-receptor-alpha signals. In addition, RA induces alpha4-beta7 integrin and CCR9 expression and thus stimulates a gut-homing phenotype in T-cells. RA also inhibits cytokine production by T-cells, but stimulates IL-2 production thus favoring Treg expansion. Omission of the precursor molecule of RA, vitamin A, from the diet inhibits Treg conversion in mice. Interestingly, this diet also has significant consequences for the lamina propria DC, as the $CD103^+$ population decreases significantly, while Langerin expression is strongly up-regulated.

An important question concerning the intestinal immune balance is what role is played by the microbial gut flora. It appears that DNA from commensal bacteria, which contains CpG motifs that trigger TLR9 activation, is a potent inhibitor of Treg induction (reviewed in (Hand and Belkaid, 2010)). Conversely, antibiotic treatment is associated with enhanced Treg and inhibition of intestinal effector cell responses, while CpG-containing bacterial DNA restores these. Interestingly, DNA from probiotic bacteria such as *Lactobacillus sp.* failed to restore immune responses in this regard. Thus, it appears that manipulation of the balance between DC-induced adaptive Treg and bacterial DNA-stimulated effector T-cells plays an important role in the outcome of gut immune responses. In case of infection with the intestinal pathogen Toxoplasma gondii, it was found that lethal infection induces expression of the Th1-associated transcription factor Tbet in intestinal $Foxp3^+$ cells, with concomitant IFN-gamma production (Oldenhove et al., 2009). This was associated with exhaustion of IL-2 sources, which is essential for Treg maintenance. Together these notions indicate that environmental cues, in particular of microbiotic nature, are crucial in maintaining a proper T-cell balance in the intestines.

IL-17A in granuloma formation and LCH

In her contribution, **Dr. Delprat** discussed the role of IL-17A in Mycobacterium-induced granuloma formation and its relevance for LCH. Granulomas, as structured collections of mononuclear phagocytes including epithelioid cells and multinucleated giant cells, are typically formed in chronic cell-mediated immune responses, although T-cells are not always required for their formation. The trigger for granuloma formation is often infectious, but - as is the case in LCH - is sometimes unknown. Previous findings have suggested that IL-17A might be important in granuloma generation, as it induced fusion of immature

monocyte-derived DC (moDC) (Coury et al., 2008), while granuloma formation was impaired in *M. bovis* BCG-infected IL-17A-deficient mice. (Umemura et al., 2007). In LCH patients, disease activity was found to be associated with the IL-17A-dependent fusion-inducing activity in the serum, although not with IL-17A levels directly. The observed variable anti-IL-17A response may play a role in this (Coury et al., 2008). The LCH cells themselves appeared to be a major local source of IL-17A, rather than the Th17 cells that occur in other IL-17A-associated diseases. Investigating the effect of IL-17A on moDC in more detail, Dr. Delprat and colleagues observed that IL-17A induced increased survival of the cells to apoptosis-inducing signals, including oxidative stress. Transcriptome analysis indicated that IL-17A and BCG induced similar profiles in monocyte-derived DC. Interestingly, IL-17A induced an altered lipid metabolism in moDC. In addition, IL-17A induced expression of chemokines, including CCL20, which mediated clustering of moDC thus enabling fusion of the cells. Regarding their functional capacities, IL-17A-stimulated moDC were strongly activated as indicated by their high capacity to phagocytose, to stimulate alloresponses, as well as to produce TRAIL when facing a viral stimulation, cathepsin D, oxygen radicals and MMP12. Thus, the finding of IL-17A in LCH lesions and in serum and the significant metabolic and functional impact of this cytokine on monocyte-related cells might provide a novel route for therapeutic approach in LCH.

Dr. Belot, also a Pritchard fellow, presented data on the association between IL-17A and pulmonary LCH. In contrast to other forms of LCH, the affected cells in the pulmonary lesions are in general not clonal, indicative of the reactive nature of the disease, which typically occurs in young adult smokers. Also in these cases, significant numbers of IL-17A-expressing LCH cells were observed. Interestingly, consistently more lesional $CD1a^+$ than $Langerin^+$ cells were observed. Most, but not all, IL-17A-secreting DC appeared to be $CD1a^+$ $Langerin^-$, ranging from 2 to 52% (mean 15%) of the $CD1a^+$ DC. These findings are in line with the previous demonstration in childhood LCH where many $CD3^-$ $Langerin^-$ IL-17A-expressing DC were detected, especially in bone lesions (Coury et al., 2008). In agreement with these data, no IL-17A-producing T-cells were observed. The lesional pulmonary LCH cells as well as the lung epithelial cells expressed CCL20. This chemokine may underlie the accumulation of the $CCR6^+$ DC and is presumably induced by IL-17A exposure as the levels of IL-17A were similar as those observed before in childhood LCH (Coury et al., 2008). Therefore, despite the possibly distinct etiopathogenic nature of adult pulmonary LCH, the involvement of IL-17A is strikingly similar and may be causative in the loss of tissue integrity.

In marked contrast to the previous contributions, findings by **Dr. Allen** and collaborators in LCH could not confirm the central role of IL-17. In PCR analyses of $Langerin^+$ and $CD3^+$ cells sorted from lesions of 14 LCH patients, as well as from 2 unsorted lesions, no IL-17A mRNA could be found (Allen and McClain, 2009). In addition, ELISA analyses on plasma samples from 36 LCH patients, including patients with high-risk multisystem disease, low-risk multisystem disease, and single lesion disease, failed to demonstrate the presence of elevated levels of IL-17A, although technical and biological controls showed the expected results.

Dr. Allen furthermore elaborated on the results of genome-wide expression profiling studies on $Langerin^+$ and $CD3^+$ cells isolated from LCH lesions. Profiles from $Langerin^+$ LCH cells were compared to freshly isolated LC from control skin and $CD3^+$ lesional T-cells were compared to peripheral blood $CD3^+$ cells from the same patient and with control tonsil $CD3^+$ cells. Interestingly, the global expression patterns of LCH cells and LC showed a relatively low level of correlation and 2113 genes were found to be differentially expressed ($p<0.01$)(Allen et al., 2010). Of these, 520 genes were up-regulated, while 1593 genes showed decreased expression. A surprising finding was that expression of many genes previously associated with LCH, including cell-cycle regulators, pro-inflammatory cytokines and chemokines was not significantly different from control LC. However, the study revealed increased expression of several interesting genes not previously associated with LCH, including genes involved in regulation of cell cycle (CDC2A, AFF3, SMYD3, HOXB7), apoptosis (BAX, BCL2L1, CFLAR) signal transduction (DUSP4, JAK3, PRKCA, TLR2, TLR4, SOCS3, JAG2), tumor invasion and metastasis/tissue invasion (CEACAM6, MMP1, TGF-beta1), early myeloid cell maturation (CD1d, CD13, CD14, CD33, ITGA2B, ITGAX, ITGAM, CD300LF) and lymphocyte trafficking (SPP1, VNN1, NRP1, CCR1). Decreased or absent expression in LCH cells was found for a large number of genes involved in cell-cell adhesion, but also an apoptosis effector, PERP, was strongly down-regulated in LCH cells. Compared to the peripheral $CD3^+$ T-cells, the expression profile of the CD3+ T-cells isolated from the lesions is consistent with an activated regulatory T cell phenotype, including increased expression of FOXP3 and CTLA4. SPP1 (encoding osteopontin) had the highest relative expression in both LCH lesion CD207+ and CD3+ cells.

Taken together, the LCH cell gene expression profile observed in these studies is not consistent with the prevailing model in which LCH cells are deviant epidermal LC that have acquired an activated-immature DC phenotype and independently elaborate a 'cytokine storm'. Instead, dr. Allen proposed a model in which LCH lesions are generated by an as yet unknown trigger causing the accumulation of BM-derived immature myeloid DC. These recruit activated T-cells and, in collaboration, contribute to tissue destruction and tumor progression.

Viruses: interaction with DC and identification of novel species

Although bacteria and eukaryotic micro-organisms have been excluded as initiators of LCH lesion formation, viruses remain a possible trigger. In his presentation, **Dr. Soumelis** discussed how human papillomaviruses (HPV), of which many subtypes with different degrees of pathological behavior are known, influence the epithelial tissue microenvironment of DC. His particular focus was on the impact of virus-affected epithelium on the migratory capacities of DC, since these are an important determinant for the interaction with adaptive immunity in the tissue-draining lymph nodes. Histological examination of HPV-infected tissue showed that epidermal LC are largely depleted from these sites, despite the fact that pro-inflammatory cytokines like IL-1 and TNF, which typically induce LC migration, are virtually absent from HPV-induced lesions. However, epithelial cells in HPV-infected sites express high levels of the cytokine TSLP (thymic stromal lymphopoietin), and these levels correlated with the absence of LC. Previous work has indicated that epithelium-derived TSLP contributes to DC maturation (Bogiatzi et al., 2007). In addition, Dr. Soumelis now showed that TSLP strongly stimulated the polarization of DC as well as their spontaneous, chemokine-independent migration. Both TSLP-induced polarization and migration appeared to be dependent on the interaction between actin polymers and myosin II and not just on actin polymerization itself. Together, these findings show that TSLP is a major player in determining the functional interaction between virus-infected epithelium and DC.

Subsequently, **dr. Wang** illustrated how novel technology can help in identifying hitherto undetected viral pathogens. In many diseases, including respiratory and intestinal disorders, but also cancers and autoimmune diseases the pathogenic contribution of viruses is suspected, but not proven. Existing discovery methods like culture, electron microscopy, immunoassays and PCR have clear limitations. More sophisticated technology is based on high throughput, unbiased methods that rely on nucleic acid-based comparison with sequences from known viruses. To this end, the so-called Virochip has been developed, a microarray containing the most highly conserved 70-mer sequences from every reference viral genome in GenBank (Wang et al., 2002). On the basis of hybridization profiles on this chip, identity or phylogenetic relationships of a suspected pathogen with known viruses can be established. Using this approach, various novel viruses have been discovered recently, including SARS. In addition, a complementary approach of high throughput sequencing has lead to the discovery of novel viruses such as KI and WU polyomaviruses. The latter virus was recovered from patients with respiratory infection and appeared to be widespread (Gaynor et al., 2007). By five years of age more than 70% of people is seropositive for this virus. The high frequency with which novel, widely spread viruses are discovered illustrates our relative ignorance of the viral environment. The current availability of 'deep sequencing' methods that allow up to giga-base determinations in single runs greatly facilitate the hunt for novel viruses. For novel pathogen discovery, the rational strategy should thus include screening of specimens for candidate agents by the molecular methods mentioned above, and then assess their relevance by determining epidemiological association and using standard virological methods to fulfill Koch's postulates to prove a pathogen's involvement.

Dr. Moore specifically drew the attention to the involvement of viral agents in the pathogenesis of human cancer. Estimates are that approximately 20% of human cancers are infection-related, of which 10-15% concern viruses. The microbial influence on the tumorigenic process can be either indirect, i.e. stimulated by chronic inflammatory triggers, are direct, when viral oncogenes are introduced into the human genome. Although different genes are involved, the general profile is that oncogenic viruses influence control mechanisms of cell cycle, apoptosis as well as immune modulation. As outlined above, discovery of foreign agent nucleic acids in human tumors is greatly helped by recent advances in high-throughput sequencing and genomic databases. Dr. Moore and colleagues developed digital transcriptome subtraction (DTS) as a means to identify exogenous viral sequences in tumors that are suspected to have a direct viral carcinogen (Feng et al., 2007). To investigate a putative viral involvement in Merkel cell carcinoma (MCC), DTS was performed on 400,000 cDNA from four MCC libraries. This resulted in the isolation from a

single tumor library of two unique cDNAs that correspond to a novel human polyomavirus large T antigen. Using these as lead, the Merkel cell polyomavirus (MCV) could be identified (Feng et al., 2008). This appeared to be integrated into host genome in a monoclonal pattern in ~70-80% of MCC. Tumor cells express MCV T antigen and maintain virus genome at an average of ~5 copies per cell. Interestingly, T antigen sequences derived from tumors, but not from non-tumor sources, have truncation mutations that eliminate the T antigen's viral genome replication capacity. Thus, both viral integration and T antigen truncation mutations prevent MCV from being a passenger virus of this tumor. Using virus-like particle (VLP) enzyme-immune assays (EIA) MCV was shown to be a common infection of adults (Tolstov et al., 2009). Persons with MCV-positive MCC, however, have extremely elevated levels of MCV IgG but not IgM antibodies. Taken together, these data indicate that MCV causes most but not all Merkel cell carcinomas. These results also demonstrate that DTS has potential to identify new tumor viruses. The application to LCH, however, needs serious contemplation since crucial to the method is the comparison of sequences from tumor tissue to those derived from control tissue. The cellular heterogeneity of LCH samples thus adds a level of complexity.

Summation and conclusions

In the summation session, chaired by **Drs. Beverley** and **Merad**, the various topics presented at the meeting were placed in the perspective of applicability for understanding the etiology of LCH and for improving diagnosis and therapy of the disease.

Concerning the cell of origin of LCH cells, the view is changing and several aspects in this year's contributions add to that. The notion that LCH cells derive from epidermal LC was broadly accepted since the discovery of Birbeck granules in these cells (Nezelof et al., 1973). However, various findings now question this view. The gene expression profiling studies by Allen and colleagues have indicated that LCH cells express higher levels of early myeloid maturation markers than control LC(Allen et al., 2010). Since these markers are typically down-regulated when LC mature, this suggests that LCH cells represent a more immature stage, rather than a subsequently activated stage, compared with steady state epidermal LC. Furthermore, the discovery of the BM-derived non-epidermal lineage of Langerin-expressing DC, initially in mice but now confirmed in humans, provides a potential alternative cellular origin of LCH cells. The morphological and phenotypic similarities between isolated dermal Langerin$^+$ DC and LCH cells speak in favor of such a notion. Finally, the observed incomplete overlap between CD1a and Langerin expression on LCH cells on the one hand, and the presumed up-regulation of Langerin expression in DC under retinoic acid-limiting conditions, indicate more heterogeneity and suggest inducibility of a diverse LC-like phenotype in DC precursors. Together, this leads to the still hypothetical view that myelomonocytic precursor cells may accumulate in LCH lesions and adopt an LC-like phenotype under influence of local microenvironmental conditions. Vitamin A metabolites, or lack thereof, and cytokines like TGF-beta or activin, which are known to stimulate LC development, certainly deserve (re-) evaluation concerning a putative role in LCH etiopathogenesis.

The cytokine IL-17A remains an important point of focus as a potential driving factor in LCH pathogenesis. IL17A was initially reported to be secreted by circulating monocytes in LCH patients and to play a role in the formation of giant cells in LCH lesions (Coury et al., 2008). It is intriguing that Allen et al. failed to identify elevated IL-17A in the profiling of LCH lesions (Allen and McClain, 2009). The discrepancy between the results obtained by Delprat and Allen may relate to potential artifacts caused by the anti-IL-17 Abs utilized or the heterogeneity of LCH lesions studied in these studies. In this regard, it may be important that Allen and colleagues used isolated Langerin$^+$ cells for their profiling studies, while IL-17A expression was primarily found in CD1a$^+$Langerin$^-$ cells, in particular in bone and pulmonary lesions (Coury et al., 2008). Osteopontin, which is strongly expressed by LCH cells (Prasse et al., 2009; Allen et al., 2010), plays an important role in the induction of Th17 cells or Th1 cells, depending on the molecular form of osteopontin present (Cantor and Shinohara, 2009). The role of IL-17 and osteopontin in LCH lesions remains to be scrutinized as the associations between IL-17, osteopontin and granulomatous disease are strongly suggested by recent literature (Umemura et al., 2007; Cantor and Shinohara, 2009).

The gene profiling studies of isolated lesional T-cells confirm the earlier findings that LCH lesions have elevated numbers of Foxp3$^+$ T-cells with presumed regulatory function (Senechal et al., 2007; Allen et al., 2010). In addition, signs of activation of T-cells were observed. Since Foxp3 expression is induced by TGF-beta, but does not necessarily imply a regulatory function of human T-cells (Tran et al., 2007), elucidation of the role of T-cells in LCH lesions is clearly warranted. In line with this, the functional and developmental plasticity of different Th cell subsets (Zhou et al., 2009) may provide a platform for therapeutic intervention in LCH.

Additional therapeutic possibilities that were discussed for potential development concern targeting of molecules expressed by activated DC. Antibody-mediated therapy directed at cell surface molecules becomes clinical practice more and more. Besides CD1a as an obvious target, other molecules expressed by activated DC may be considered. A good example in this respect is CD300F. This is expressed at high levels by LCH cells and cross-linking stimulates cytokine release. Thus, preventing CD300F from interaction with its natural ligand might down-regulate the inflammatory activity of LCH cells. Furthermore, adaptor- and other signaling molecules in activated myeloid cells, such as DAP12 or beta-catenin, could be targets for therapy development using small molecule modulators. It remains important, however, to establish a well-founded understanding of the nature and origin of LCH cells, since molecular regulation of distinct cell types is essentially different.

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