# CHAPTER 7

## General Discussion

**LCH cell: intrinsic defect or external conditions leading to pathologic phenotype?**

New findings in the field of DC biology are essential to get further insight into the pathological mechanism of LCH. LCH cells have the phenotype of immature, activated LCs (*2*). In the lesions, however, several cytokines, such as TNF-alpha, IL-1beta, as well as CD40L, a member of the TNF superfamily, are present which are able to drive LCs to maturation in a healthy situation (*4*). *Could there be an intrinsic abnormality in LCH cells that inhibits further development to a mature, antigen-presenting phenotype?* LCH cells are found to be devoid of the transcriptionally active form of beta-catenin **(Chapter 2)**. Beta-catenin is, as transcriptional co-activator in the canonical Wnt-pathway responsible for the transcription of genes that are involved in differentiation and maturation (Figure 1). As blood-derived monocytes are one of the few possible origins of LCH cells, beta-catenin involvement in their differentiation and maturation towards mo-DCs was studied, to determine a possible role for beta-catenin in the healthy situation **(Chapter 2)**. Transcriptionally active beta-catenin was found to be required early in the differentiation process, since blockade of beta-catenin signaling in the first few days inhibited the cells from acquiring a mature phenotype. This finding suggests that the absence of transcriptionally active beta-catenin in LCH cells, which might be caused by an intrinsic defect, is keeping the cells in an immature state. *What could then be this intrinsic defect?* Aberrancies in the beta-catenin pathway would be most obvious. Abnormal production of proteins involved in the beta-catenin pathway due to a gene defect, leading to an upregulated activity of this pathway, is known to be the cause for some types of cancer. For example, a mutation in the tumor suppressor gene APC is believed to be responsible for approximately 80% of all colon cancers and mutations in beta-catenin have been found in several types of malignancies, including melanoma, pilomatricomas, hepatocellular carcinoma, and hepatoblastomas (*5*). A role for inactivated beta-catenin signaling in oncogenesis has, on the other hand, also been described. Cai et al. investigated osteosarcoma tissue and cell lines (*6*). They found that the beta-catenin pathway is inactivated in this malignant bone tumor. Activation of beta-catenin signaling resulted in the inhibition of cell proliferation and promoted cell differentiation. Dickkopf-1, an inhibitor of the Wnt-pathway, is relatively highly expressed in osteosarcoma and might,

therefore, contribute to inactivation of beta-catenin signaling. Also in the pathogenesis of malignant fibrous histiocytoma, inactivation of the beta-catenin pathway has been described (*7*). In LCH, a mutation causing a downregulation of transcriptionally active beta-catenin might be present. In theory, a mutation in a gene leading to an upregulated expression of a protein of the so called scaffolding complex, which is involved in the ubiquitination of beta-catenin such as GSK3-β, Axin2, and APC, or upregulation of Wnt antagonists such as proteins of the Dickkopf family could be present in LCH. A mutation in a Wnt receptor at the cell surface of LCH cells also belongs to the possibilities.

A single mutational event, however, is unlikely to be the only cause of LCH. Additional (epi-) genetic hits are, in general, necessary for tumor formation. Of interest is the study of Badalian-Very et al., in which they showed evidence that LCH is a neoplasm (*8*). Using high-throughput genotyping in search for oncogene mutations in LCH, they found the BRAF V600E mutation in more than half of the investigated specimens. LCH cells specifically stained for phospho-MEK and phospho-ERK, indicating active BRAF pathway signaling. This signaling was, however, not correlated with the mutational status of BRAF, meaning that other mutations are most likely present as well. The concurrence of aberrant beta-catenin signaling and BRAF activation has been found in several neoplasms. In serrated adenomas, abnormal nuclear labeling of beta-catenin always occurred in cells that also harbored the BRAF V600E mutation (*9*). In melanomas, inhibition of BRAF lead to decreased expression of nuclear receptors resulting in upregulation of genes that are known to function as negative regulators of beta-catenin signaling (*10*). Together, it is tempting to speculate that in LCH activation of the MAPK pathway by the BRAF V600E mutation and absent beta-catenin signaling together underlie its pathogenesis.

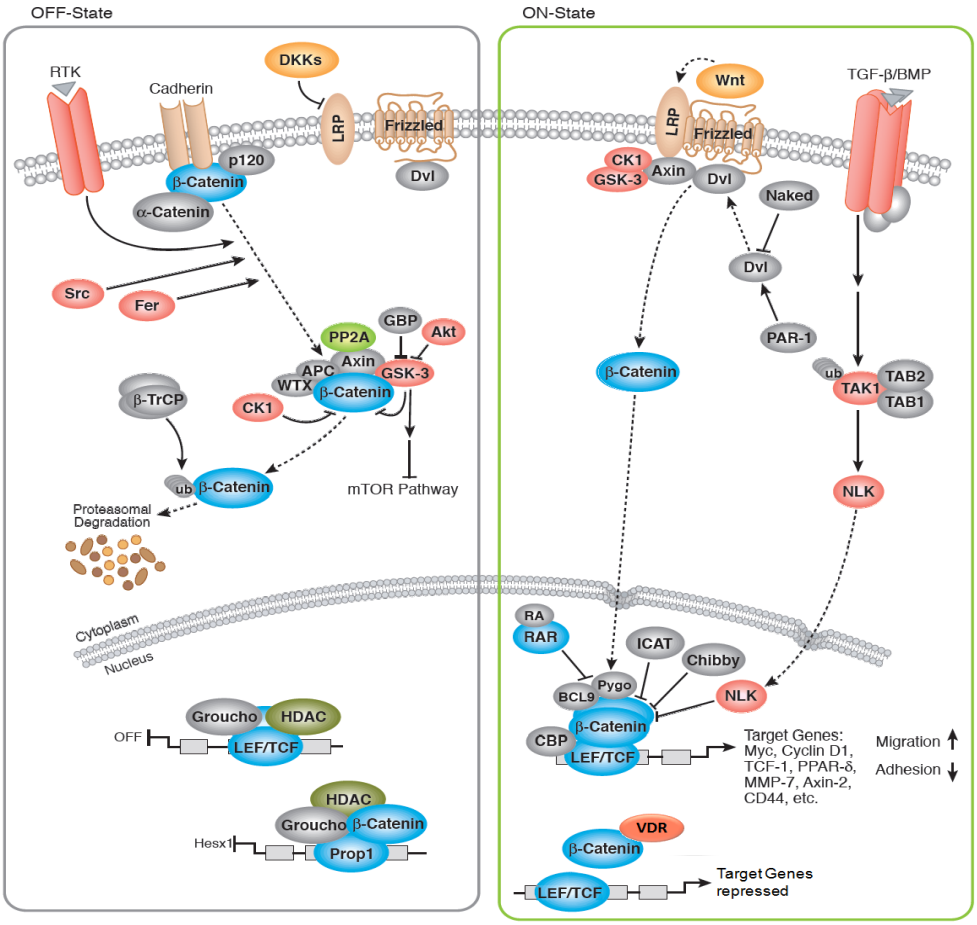


**Figure 1. Beta-catenin signaling in the Wnt pathway.**

In the presence of Wnt proteins, beta-catenin is phosphorylated by a destruction complex and subsequently degraded in the proteasome. In the presence of Wnt proteins the destruction complex is inactivated, enabling beta-catenin to translocate to the nucleus in its non-phosphorylated form, where it displaces groucho-related repressor from TCF/LEF transcription factor. TCF/LEF is then able to regulate expression of Wnt target genes that are involved in differentiation, proliferation, and maturation. TFG-β activates TAK1, which stimulates NLK activity that in turn downregulates transcriptional activation mediated by beta-catenin. VDR inhibits beta-catenin from binding to TCF, and has been found to promote nuclear transport of beta-catenin to the plasma membrane leading to an increased production of E-cadherin. Adapted from Cell signaling technology.

*What if there is no intrinsic defect present in LCH cells that inhibits their maturation process?* There is substantial evidence that the microenvironment within the LCH lesions contains substances that have an inhibiting effect on the differentiation of LCH cells. For example, Geissmann et al. have shown that LCH cells isolated from lesions were able to obtain a mature phenotype upon addition of CD40L *in vitro*. An explanation for the fact that CD40L present on T cells in LCH lesions is unable to achieve a similar effect *in vivo*, could be an imbalance between cytokines produced by the LCH cells themselves as well as by bystander cells that stimulate maturation, such as TNF-α and IL-1β, and cytokines that inhibit maturation including IL-10 and TGF-β (*4*). In relation to the latter, it is of interest to note that TFG-β is involved in the activation of TGF-β Activated Kinase 1 (TAK1). TAK1 in turn stimulates Nemo Like Kinase (NLK) activity which downregulates transcriptional activation mediated by beta-catenin (*11*). TGF-β has also been found to rapidly induce vitamin D receptor (VDR) during LC lineage specification in $CD14^+$ monocytic precursors (*12*). VDR is an antagonist of the transcriptionally active form of beta-catenin in that it inhibits beta-catenin from binding to the transcription factor TCF. In colon cancer, it has also been observed that VDR promotes nuclear transport of beta-catenin to the plasma membrane leading to an increased production of E-cadherin (*13*). In this respect, a high level of TGF-β might be an important microenvironmental factor within the LCH lesion, keeping the LCH cells in an immature state by blocking the beta-catenin pathway.

The imbalance of Notch ligands could also have an inhibiting effect on LCH cell-maturation. Cheng et al. demonstrated in mice that the presence of either Jagged-1 or Delta-1 in stroma had different effects on the differentiation of DC precursors to terminally differentiated DCs. The presence of Jagged-1, found in bone marrow stroma, led to the accumulation of DC precursors, while Delta-1, found in spleen stroma, stimulated DC precursors to differentiate into mature DCs (*14*). In the human situation similar results were found, but more importantly, the results were extended with the finding that Delta-1 induced the upregulation of Frizzled receptors on the cell surface. Wnt proteins bind to Frizzled receptors, leading to an activation of the canonical Wnt-pathway, which includes the upregulation of beta-catenin transcriptional activity (*15*). Hoshino et al. demonstrated that human $CD14^+$ monocytes could differentiate into phenotypically mature LCs in the presence of Delta-1, GM-CSF, and TGF-beta, indicating involvement of the Notch pathway in this

differentiation process (*16*). Together, these findings demonstrate the critical link between the Notch and Wnt/beta-catenin pathways in DC/LC differentiation and maturation, which should be further explored in light of LCH.

The protein sequence of beta-catenin is highly conserved between mammals. Studies on the function of beta-catenin in the immune system of mice can, therefore, provide valuable information on the human situation. The *in vivo* role of beta-catenin in DCs and LCs is currently under investigation. The lab of Bjorn Clausen has developed 2 mouse models, one in which beta-catenin is not expressed in $CD11c^+$ cells, and one in which transcriptionally active beta-catenin is constitutively expressed in $CD11c^+$ cells. In both models, an intact LC network is present in the epidermis. Mice that are devoid of beta-catenin in $CD11c^+$ cells lack any sign of pathology, suggesting that the development of DCs and LCs is not altered in this model. Mice with constitutively active beta-catenin in $CD11c^+$ cells do not show a cutaneous hypersensitivity response upon sensitization and subsequent challenge with haptens, they fail to induce an allergic response in an allergic asthma model, and also do not show the appropriate response to experimentally induced autoimmune encephalomyelitis. These mice have increased numbers of $CD4^+$ $CD25^{high}$ T regulatory cells in lymphoid tissues (*17*). These observations suggest that transcriptionally active beta-catenin plays a role in the tolerogenicity of DCs (Figure 2a,b), which is in line with the findings of Jiang et al., who showed that beta-catenin activation in immature mouse DCs *in vitro* leads to the development of mature DCs which are capable of mediating tolerance (*18*). In regard to LCH, in which we see an absence of transcriptionally active beta-catenin in the pathological LCs, it could be hypothesized that a loss of tolerogenicity plays a role in the pathological mechanism. In theory, a relatively harmless factor, which could be of self or foreign origin, could be the initiating trigger for the recruitment of monocytes from the blood that transform into DCs with a LC phenotype as well as other inflammatory cells. Microenvironmental conditions leading to aberrant beta-catenin expression, such as discussed earlier, transform the LCs into LCH cells that have an immunogenic instead of a tolerogenic phenotype, leading to the overproduction of pro-inflammatory cytokines and chemokines and ultimately to the formation of LCH lesions.

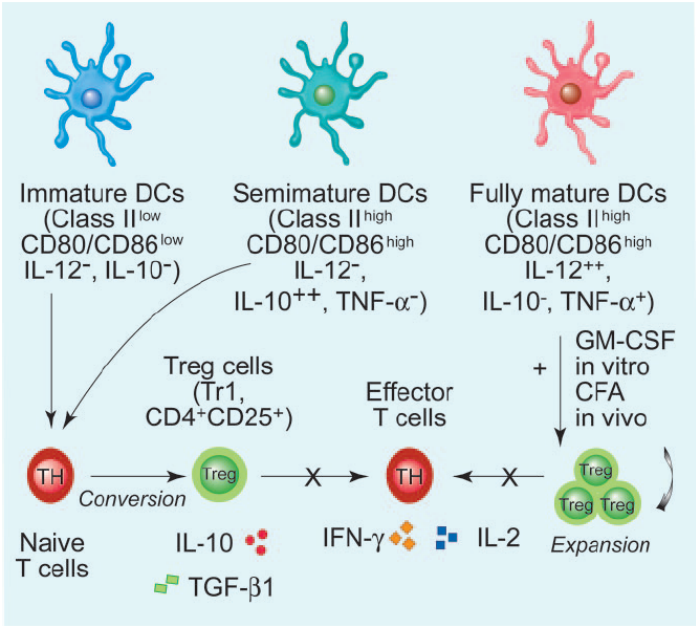


**Figure 2a. DC maturation stages in tolerance induction.**

Presently, it is believed that DCs integrate a variety of incoming signals and decide whether protective immunity or antigen-specific unresponsiveness develops. Current experimental evidence supports the concept that cytokine-modulated semi-mature DCs promote the conversion of naive T cells into adaptive regulatory T cells and/or CD4-CD25- regulatory T cells that inhibit effector T-cell responses via secreted or membrane-bound immunosuppressive cytokines. The inability to produce detectable amounts of bioactive IL- 12p70 coupled with enhanced IL-10 release are unique functional features of cytokine-modulated, semi-mature tolerogenic DCs. On the contrary, mature DCs might be crucially involved in the expansion of preformed regulatory cells. Adapted from reference (*3*).

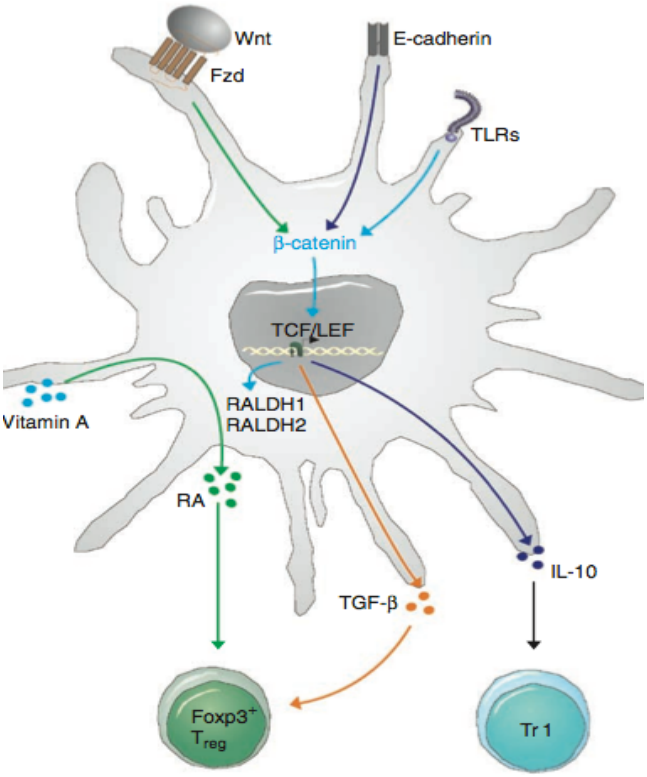


**Figure 2b. β-catenin signaling pathways in programming regulatory DCs.**

Activation of β-catenin pathway by E-cadherin, Toll-like receptors or Wnt ligands in DCs promotes induction of anti-inflammatory factors such vitamin A, IL-10 and TGF-β that are critical for promoting T regulatory response and limiting inflammatory responses. Fzd, the Wnt receptor Frizzled; TLRs, Toll-like receptors. Adapted from reference (1).

**How do LCH cells proliferate and survive?**

In the normal situation, LCs proliferate when necessary. For instance, healthy mouse epidermis is populated by $CX_3CR1^+$ $CD45^+$ $Langerin^-$ LC precursors between embryonic day 18 and postnatal day 2. These precursors then differentiate into $CX_3CR1^{low}$ $CD45^+$ $Langerin^+$ $MHCII^+$ $CD11c^+$ LCs. Between 4-7 days after birth, the $Langerin^+$ LCs proliferate and establish an extensive LC network in the epidermis by increasing their number more than 10-fold (*19*). Also in adult mouse epidermis LCs are found to continue to proliferate, as determined by the presence of the proliferation marker Ki-67, in the steady state in order to maintain the LC network (*19, 20*). In case of skin inflammation, keratinocytes produce factors, activating VDR, that induce a massive proliferation of LCs.

In the human situation, a specific bone marrow precursor for LCs has not yet been identified. Studies in patients who received hematopoietic stem cell transplantation showed, however, that most epidermal LCs were of donor origin a few months after treatment (*21, 22*). Human epidermal LCs also have the potential to proliferate in situ, which was demonstrated by their cell cycling activity in the steady state (*23, 24*). More evidence for the proliferative capacity of human LCs came from the observation that human skin engrafted onto immunodeficient mice contained donor LCs indefinitely (*25*), and that donor LCs were identified in human limb graft years after transplantation (*26*). In addition, recipient LCs were found to be resistible to radiation-based transplantation regimens (*21*). During inflammatory conditions LCs proliferate as well, as was demonstrated in skin of patients with atopic dermatitis (*19*).

LCH cells show a heterogeneous overexpression of gene products that regulate proliferation and apoptosis, such as TGF-β receptor I and II, MDM2, p53, p21, p16, Rb, and Bcl2 and are capable of proliferation as an elevated expression of Ki-67 was reported (*27-29*). The combination with the observation that LCH cells are of clonal origin (*30, 31*), with the exception of the pulmonary form (*32*) suggests that LCH cells have the properties of neoplastic cells.

Altered telomere length is also a characteristic of malignant and premalignant disorders. Short telomeres in combination with the failure of protective mechanisms, such as functional tumor-suppressor genes (*33*), lead to chromosomal instability which in turn can lead to cancer. Attrition of telomeres can be overcome by the enzyme telomerase, which catalyzes DNA synthesis to maintain telomere length.

This way, cells with active telomerase can proliferate indefinitely (*33*), which is the case in malignant cells. Insight into the telomere length of LCH cells could reveal clues in the search for the etiology of LCH as well as for the mechanism behind the proliferation and survival of these pathological cells. We determined the telomere lengths of LCH cells and lymphocytes in the local, multisystem and systemic forms of the disease and compared these to telomere lengths of LCs in reactive lymph nodes, to rule out the possible influence of the inflammatory microenvironment on the telomeres, as well with LCs in healthy skin **(Chapter 5)**. Our results showed that LCH cells have borderline significant shorter telomeres in the local form and truly significant shorter telomeres in the multisystem and systemic forms of disease, compared to the control cells. The telomere shortening is most likely exclusive to the LCH cells as lymphocytes in the studied LCH samples had telomere lengths comparable to lymphocytes in reactive lymph nodes. These results suggest that telomere length shortening is an intrinsic characteristic of LCH cells, and it might be an early molecular change leading to additional, as yet unknown, molecular changes as found in neoplasias. *How can LCH cells survive with such short telomeres?* So far, although the presence of p53 (*27, 29, 34*) and an upregulation of Bcl-2 (*27*) are reported in LCH, no genetic defects are found to date that could explain these observations (*35*). *Is it possible that LCH cells are capable of maintaining telomere length to prevent critical shortening leading to chromosomal instability and apoptosis?* Da Costa et al. reported on the presence and activity of telomerase in all restricted skin and multisystem lesions studied. Of the solitary bone lesions, only 24% were positive for telomerase, however. These results indicate that LCH cells in some lesions indeed are capable of maintaining their telomere lengths. Telomere length assessment in 1 multisystem lesion with telomerase-positive LCH cells showed homogeneously longer telomeres compared to those of control cells obtained from dermatopathic lymphadenitis samples. Telomerase-negative cells from a bone lesion showed heterogeneously shorter telomeres. These findings support the idea that LCH is a heterogeneous disease in terms of clinical presentation and course. The severe, multisystem, form contains LCH cells with neoplastic characteristics such as telomerase activity while the less aggressive form, which requires minimal to no treatment, has no active mechanism for maintaining telomere length (*36*). Although the finding of telomerase activation in LCH cells could be an explanation for the lack of chromosomal abberancies and apoptotic

mechanisms in LCH, the discrepancy on telomere lengths of LCH cells between the 2 studies makes it difficult to interpret. Preferably, telomerase activity would be determined in our samples with shorter telomeres and telomere length would be assessed in more samples from the study of da Costa et al., to obtain a larger sample size from which conclusions could be drawn.

**Role for a virus in the etiology of LCH?**

Viral infection has been proposed to play a role in the etiology and/or pathogenesis of LCH. It is known that viruses such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are able to infect monocytes, DCs, and LCs (*37-40*) and that cells of the myeloid lineage are a target for human herpes virus type 6 (HHV-6) (*41, 42*). Several research groups have, therefore, investigated LCH patient samples for the presence of these viruses. A couple of studies have reported on EBV expression in LCH tissues. Chen et al. published a case report on identical twins which both suffered from LCH after an EBV infection (*43*), and Sakata et al. published a case report on a 2 year old child which developed LCH in association with a chronic EBV infection (*44*). Shimakage et al. detected EBV mRNA and protein in LCH cells in 17/17 cases studied (*45*). There are also reports, however, that contradict these findings. For instance, McClain et al. studied 56 patient samples for the presence of EBV, but found none to be positive (*46*). Jeziorski et al. found 3/19 LCH samples to be positive for EBV, but the affected cells appeared to be bystander $CD20^+$ $CD79a^+$ B lymphocytes, which may thrive well in LCH lesions due to the immunosuppressive environment present (*47*).

To investigate a potential role for CMV in LCH, Kawakubo et al. studied 27 LCH samples, and found CMV to be present in the nuclei and/or cytoplasm of 9 samples (*48*). Again, other studies contradict these findings by showing that either CMV was undetectable in LCH lesional tissues (*46*) or that LCH patients as well as controls had anti CMV IgG titers in combination with undetectable virus in the serum and in biopsy samples (*47*).

HHV-6 has been reported to be detected in LCH lesions, but the viral titer was too low to be of any significance to the etiology or pathogenesis of LCH (*47*). The finding that the prevalence of HHV-6 in control samples was equal to that of LCH patients (*49*) is in line with this thought. Other viruses that have been studied in relation to

LCH, but have found to be not associated with its etiology or pathogenesis are adenovirus, herpes simplex virus (types 1 and 2), human immunodeficiency virus, human T-cell leukemia virus types I and II, and parvovirus (*46*). Although the most obvious viruses do not seem to be associated with the etiology and/or pathogenesis of LCH, *in vivo* models have shown that Langerin$^{+}$ cells can be infected by viral agents, leading to forms of histiocytosis. In the MHSV model, BALB/c and DDD mice were infected with a recombinant virus of Harvey murine sarcoma virus and Friend Mink Cell Focus-forming virus, called MHSV. Besides erythroblasts, myeloid precursor cells, and macrophages, also CD8alpha Langerin$^{+}$ DCs were found to be infected **(Chapter 3)**. These virally transformed cells were able to metastasize to various organs, causing a substantial tumor burden in terminal stage animals. Furthermore, we found that cell lines generated from isolated tumor cells were able to adapt phenotypically to their microenvironment upon injection into healthy mice. This observation first indicates that viral transformation of cells of the mononuclear phagocyte system (MPS) can lead to an aggressive disease with accumulation of these infected cells. Secondly, it underlines the plasticity of the cells of the MPS.

A more direct link between viral infection and histiocytosis has been shown by Steiner et al. This group generated a transgenic mouse expressing the SV40 large T antigen, which is an oncogene derived from the polyomavirus SV40 (*50*), under the control of the CD11c promoter. Depending on the levels of transgene expression, mice developed an early to late-onset infiltrative disease in spleen, liver, bone marrow, thymus, and mesenteric lymph node, resembling multisystem LCH. The affected cell in this model is interestingly the non-LC-related Langerin$^{+}$ CD8alpha$^{+}$ CD11b$^{-/lo}$ CD205$^{+}$ lymphoid tissue DC. Besides the virally-induced histiocytic diseases that became apparent from these mouse models, the observation that non-LC-related DCs with LC-specific markers are affected, support the idea that LCH is caused by such cells rather than epidermal LCs.

**How far are we from a rational treatment for LCH?**

As described in the introduction of this thesis, several well-defined and well-controlled clinical trials as well as anecdotal therapies have been published in regard to LCH. Undeniably, the clinical trials have significantly improved the outcomes of

LCH patients. Unfortunately, however, the negative side-effects of therapy, especially for the multisystem form of LCH, cannot be overlooked. *After having initiated the evaluation of a completely human anti-human CD1a antibody for its potential use as a therapeutic agent for LCH, where do we stand now in terms of developing a rational therapy for LCH?* Important to state is that CD1a is indeed a rational therapeutic target in LCH. First, CD1a is expressed on all LCH cells and is therefore used as a diagnostic marker. Secondly, in the human body CD1a is expressed on cortical thymocytes, epidermal LCs, and some DC subsets. These CD1a expressing cells originate from $CD1a^-$ precursors, and CD1a-targeted therapy is therefore not expected to cause a substantial, long-term deficit of these cells. In **Chapter 4**, the properties of CR2113, a human anti-human CD1a monoclonal antibody, have been described. CR2113 has been tested on various types of CD1a-expressing and control cell lines and has proven to be highly specific for the CD1a protein. The binding affinity of CR2113 is comparable to that of high affinity antibodies (*51*), and is higher than that of the mouse anti-human CD1a monoclonal antibody NA1/34, which could already successfully localize lesional tissues in LCH patients (*52*). Furthermore, CR2113 bound to CD1a could be internalized, which is a very useful property in view of the delivery of toxins inside the target cell. CR2113 induced a moderate level of complement-dependent cytotoxicity (CDC) and is therefore able to activate the complement cascade (*53*). The ability of CR2113 to mobilize immune cells to kill the CD1a-expressing cells was tested in antibody-dependent cell-mediated cytotoxicity experiments. CR2113 could efficiently recruit NK-cells to kill high percentages of the target cells. These data indicate that further testing of CR2113 is worthwhile in view of development of CD1a-targeted therapy in LCH. Well-established monoclonal antibodies that have been in use as agents in cancer therapies, such as anti-CD20, anti-VEGF, and anti-ErbB2, could serve as an example for preclinical testing of CR2113. Ofatumumab, a human anti-CD20 monoclonal antibody, for instance, has recently been approved by the Unites States (USA) Food and Drug Administration (FDA) for treatment of chronic lymphocytic leukemia (CLL) in patients who are resistant to fludarabine and anti-CD52 mAb therapy (*54*). The preclinical evaluation consisted of determination of the mode of action of this monoclonal antibody by *in vitro* experiments as we also performed with CR2113, such as CDC, ADCC, and apoptosis. From experience with monoclonal antibody therapy, it has become evident that target cells are able to upregulate the

expression of membrane-bound Complement Regulatory Proteins (mCRPs), such as CD46, CD55, CD59, and CD35 (CR1), and can thereby inhibit CDC (*55*). Hernandez-Campo et al. have shown that monocytes and several DC subset in peripheral blood of healthy subjects express CD55 and CD59 (*56*). It is therefore plausible that LCH cells also express or will be able to upregulate the expression of mCRPs. This has to be determined when studies with CR2113 and isolated LCH cells will be performed. To continue with ofatumumab as our example, *in vivo* efficacy was tested in CLL tumor mouse models and pharmacokinetic and toxicological profiles at different dose levels were tested in cynomolgus monkeys (*57*). Evaluation of the efficacy of CR2113 in a mouse model of human histiocytosis is not a realistic option, since we are still far from such a model. Instead, a transgenic mouse expressing human CD1a under its endogenous human promoter (Felio, JEM 2009) could be used as an *in vivo* model. As cynomolgus monkeys have been found to express CD1a and Yoshino et al. have reported that NA1/34 recognizes CD1a in these animals (*58*), it is expected that CR2113 will also bind to monkey-CD1a.

Evaluation of the clinical activity of monoclonal antibodies like ofatumumab, is performed according to the regulations of official authorities such as the FDA. Phase I and Phase II clinical trials are the first to be pursued. LCH is designated to be a rare disease according to the United States' National Institutes of Health Office of Rare Diseases, since it affects less than 200,000 people in the USA (*59*). CR2113, therefore, qualifies to receive the designation of orphan drug (*59*). To encourage the pharmaceutical industry to invest in the development of therapies for rare diseases, sponsors of orphan drug development are entitled to receive various benefits according to the Orphan Drug Act, including marketing exclusivity for 7 years, tax credits for clinical research, and grant funding. In spite all the benefits, the legal requirements for orphan drug investigation are similar to other drugs, meaning that well-controlled clinical trials are needed to make sure that the orphan drug is safe for use in patients (*59*). Performing such studies can be very difficult in case of rare diseases, since conventionally used clinical studies often require large patient numbers to obtain sufficient amounts of data. There are examples of trial designs with orphan drugs, however, that were performed with a small number of patients, such as pegademase for severe combined immunodeficiency (*60*). For CR2113, a similar study could be envisaged to determine its efficacy in patients.

*Can we make a realistic estimation of how much time it will take before CR2113 can be used in patients?* Successful drug development depends on multiple variables. The most obvious variable is the quality of the drug. Furthermore, in case of orphan drugs it has been found that the most important predictor of market authorization, at least in Europe, is the experience of a company of successfully launching an orphan drug to the market (*61*). Thus, for further development of CR2113, it would be best to find a pharmaceutical company that has relevant and successful experience with monoclonal antibody therapy development. The typical development timeline for drugs is on average 8 years for drug discovery, which includes the evaluation of the drug in *in vitro* and *in vivo* systems, and 7 years for clinical development and approval by authorities (*62*). A similar timeframe is most likely also applicable to the development of CR2113.

**Future Directions**

Looking back on 6 years of research on different aspects of LCH, as a senior researcher I would pursue to explore the following as the next step in LCH research:
The finding of the significant role of beta-catenin in the differentiation and maturation of human and mouse DCs and the apparent lack of this protein in LCH cells should be further investigated. Ideally, LCH cells would be isolated from fresh biopsy tissues and maintained *in vitro*. Stimulators of the Wnt/beta-catenin pathway, such as Wnt3a and GSK3beta-inhibitors, would then be added to determine whether stimulation of the transcriptional activity of beta-catenin would lead to phenotypical maturation of the LCH cells. If LCH cells would retain their immature, activated phenotype, experiments to investigate abberancies in the Wnt/beta-catenin pathway should be planned. RQ-PCR could be performed to determine the gene expression levels of proteins involved in this pathway. Levels of protein expression could be determined by Western Blotting. If maturation of LCH cells would indeed occur, the conclusion that the Wnt/beta-catenin pathway is intact and that microenvironmental conditions are causing the absence of transcriptional active beta-catenin could be suggested. LCH tissues could in this case be investigated for the presence or absence of certain microenvironmental factors that have been linked to the maturation process of DCs and LCs, such as the Notch ligands Jagged-1 and Delta-1, by immunohistochemistry or immunofluorescence. If the results would indicate an abnormal expression of one

or more factors, *in vitro* experiments for LCH cell stimulation with these factors could be designed. Another way to manipulate the Wnt/beta-catenin pathway is to transduce LCH cells with constructs that lead to overexpression of beta-catenin and to monitor the effect on their phenotypic appearance.

Göbel et al. described that vitamin D3 receptor (VDR) is downstream of TGF-β1 during LC differentiation from $CD34^+CD45RA^+CD19^-$ myeloid progenitor cells (*12*). VDR expression is repressed during GM-CSF/IL-4-dependent generation of moDCs from myeloid progenitors, which play an important immunogenic role in inflammatory sites. Ectopic VDR expression, however, has an inhibitory effect on moDC differentiation. Is it possible that LCH cells express increased amounts of VDR, leading to their development from circulating myeloid precursors and a LC-phenotype? Determination of the presence of VDR and its quantity in LCH cells compared to LCs generated from myeloid progenitor cells and LCs isolated from healthy human epidermis, by immunofluorescence and western blotting, might give us information on whether this receptor has a role in the development of LCH cells. In case VDR would be found to be overexpressed in LCH cells, it could be speculated that in LCH an appropriate immune response against a pathogen cannot take place, because VDR overexpression prevents myeloid progenitors from developing into immunogenic moDCs. Instead, cells with a LC phenotype that are not able to handle the pathogen are found at the site of infection, leading to a hyperactive immune response with overproduction of cytokines and chemokines and attraction of other cells of the immune system.

Unfortunately, LCH cells are still very difficult to maintain *in vitro*, making it practically not feasible to perform experiments with these cells. Until better methods are developed to study pathways and protein expression in LCH cells, it would be interesting to perform the experiments described as in Chapter 3 in LCs generated from monocytes and LCs isolated from healthy donor skin. Pilot experiments with the latter showed that beta-catenin is present in immature LCs and increases during spontaneous maturation.

In LCH lesions $CD1a^+$ $Langerin^+$ cells as well as $CD1a^+$ $Langerin^-$ cells are reported to be present (*63*). This observation suggests that the CD1a antigen might possibly have a critical role in the pathology of LCH. CD1a expression has been reported, besides on human Langerhans cells and dermal dendritic cells, on human myeloid DCs derived either from blood monocytes or $CD34^+$ precursors. In the latter subtype,

its expression was found to be dependent on the absence of serum lipoproteins and PPARγ. Upon stimulation, $CD1a^+$ DCs produce IL-12p70 and they are able to polarize naive $CD4^+$ T cells to a Th1 phenotype, comparable to the murine $CD8\alpha^+$ DC subset (*64, 65*). Functionally, CD1a is involved in antigen presentation to T cells. These antigens include mycobacterial phospholipids, glycolipids, and lipopeptide components of intracellular pathogens (*66*) as well as sulfatide, which is a self glycolipid that is highly expressed in neuronal cells, kidney, and pancreas (*67-69*). Sulfatide synthesis is also upregulated in DCs upon bacterial infection (*70*). In light of the immature activated phenotype of LCH cells it is interesting to note that microbial lipid presentation by CD1 molecules does not require DC maturation, enabling the immune system to quickly respond to certain invading pathogens (*71*). The observation of IL-17A production in LCH lesions is notable in regard to CD1a expression, since IL-17A is also found to be involved in infections with mycobacteria (*72, 73*). In the ongoing search of the etiology of LCH, it might be important to investigate LCH cells and other cells in LCH lesions for the presence of antigens (self or of (myco) bacterial origin) specifically presented by CD1a.

Further pre-clinical testing of the fully human anti-CD1a antibody CR2113 should be continued in order to investigate its potential as a diagnostic and therapeutic tool in LCH. The CD1a-expressing B16 mouse melanoma cell line has been very valuable in *in vitro* experiments performed with CR2113. The tumor mouse model developed with this cell line, however, shows a very rapidly increasing tumor load and is, therefore, not an optimal model for *in vivo* testing of our antibody. An alternative model, with a less aggressive tumor phenotype could be developed by injecting mice with C1R, a human B-cell lymphoblastoid line, transfected with cDNA encoding human CD1a. If the anti-CD1a antibody proofs to match the promising results as found in *in vitro* experiments *in vivo* in a mouse model, the next step would be to test CR2113 in a non-human primate model since they are very suitable for preclinical testing of biopharmaceutics due to ancestral proximity and developmental similarities with humans (*74*). In case CR2113 shows potent activity and specificity in the latter animal model, following steps according to the orphan drug regulation might be pursued.

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