Langerhans cell histiocytosis: A reactive or neoplastic disease?

Costa, C.E.T. da

1

General Introduction

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The histiocytoses, a group of disorders that most often occur in children and adolescents, are characterised by an abnormal accumulation of cells of the mononuclear phagocytic system and dendritic cell system, commonly called "histiocytes". The histiocytic disorders include, among others, haemophagocytic lymphohistiocytosis, characterised by the involvement of mononuclear phagocytes or macrophages, and Langerhans cell histiocytosis (LCH), characterised by the accumulation of Langerhans cells (LCs; Table 1). Whereas in haemophagocytic lymphohistiocytosis syndromes perforin and MUNC13-4 gene mutations have been implicated in the pathogenesis of most cases, in LCH the cause is yet unknown. LCH has an estimated annual incidence of 2-5 per one million children in the age range of 0-15 years. It occurs in different forms, namely as a single system or as a multi-system disease. Thus, in children under 2 years of age it is often presenting as a multi-system disease; in children between 2 and 5 years of age it often occurs as multifocal single system disease; and above the age of 5 years it appears in 50% of unifocal bone disease cases.

Table 1. Classification of histiocytic disorders*

Disorders of varying biologic behaviour	*Dendritic cell – related:*	**- Langerhans cell histiocytosis** - Secondary dendritic cell disorders - Juvenile xantogranuloma and related disorders
	Macrophage - related:	- Hemophagocytic lymphohistiocytosis (familial and sporadic) - Secondary hemophagocytic lymphohistiocytosis o Infection – associated o Malignancy - associated o Other - Sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease) - Solitary histiocytoma of macrophage phenotype
Malignant disorders	Dendritic cell – related:	- Histiocytic sarcoma (localized or disseminated) o Follicular dendritic cell o Interdigitating dendritic cell o Other
	Macrophage – related:	- Macrophage – related histiocytic sarcoma
	Monocyte – related:	- Leukaemias o Monocyte leukaemia o Acute myelomonocytic leukaemia o Chronic myelomonocytic leukaemia - Extramedullary monocytic tumors or sarcoma

*Adapted from reference (1)

Historical hallmarks in LCH

The nomenclature of LCH is closely linked to the evolving discoveries concerning the biology of the LCs, which were firstly described in 1868 by Paul Langerhans (2,3). Thus, in 1961 Birbeck introduced a dis-

tinctive recognition marker in the LCs, characteristic granules, which were termed after his name (4). In the meantime, Lichtenstein had grouped Hand-Schuller disease, Letterer-Siwe disease and eosinophilic granuloma of bone as being part of the same disorder which he called histiocytosis X (5). However, it was only in 1973 that Christian Nezelof proposed that the LC was the cell of origin for all forms of what was still named histiocytosis X, as he showed the presence of Birbeck granules in these cells (Table 2) (6).

Table 2. Important hallmarks in the history of LCH

Dates	*Historical facts*
400-450 BC	Hippocrates describes a non-fatal disease associated with painful skull lesions (7).
1865	Smith described for the first time a patient with impetigo and holes in the cranium (8).
1868	Langerhans published a manuscript describing the non-pigmentary DCs in the epidermis (LCs) (2,3).
1893	Hand described a child with polyuria and exophthalmos, which he attributed to tuberculosis (9).
1915/1920	Schuller and Christian, respectively, described similar patients with skull defects, exophthalmos and diabetes insipidus, and thus the eponym Hand-Schuller-Christian disease was attributed to the clinical condition characterised by the triad of exophthalmos, skull lesions and diabetes insipidus (10,11).
1933	Siwe grouped previously reported cases (including one by Letterer in 1924) of organomegaly, lymphadenopathy, localized tumours in bone, secondary anemia, a hemorrhagic tendency and hyperplasia of non-lipid storing macrophages, into the disease that later became known as Letterer-Siwe disease (12,13).
1941	Farber noted that Hand-Schuller disease and Letterer-Siwe disease, plus the newly diagnosed eosinophilic granuloma of bone, described in the previous year in two separate articles, represented variations of the same disease process (14).
1953	Lichtenstein introduced the concept that the three entities were part of a spectrum of the same disorder which he called histiocytosis X (5).
1961	Birbeck described the characteristic granules seen on electron microscope, thus introducing a distinctive recognition marker. In the next few years several investigators described the finding of Birbeck granules in different forms of LCH (4).
1973	Nezelof published a report showing that histiocytosis X was the result of proliferation of pathologic LCs (6).
1983	Risdall suggested that the name histiocytosis X should be changed to Langerhans cell histiocytosis, in recognition of the key role of LCs in all forms of LCH (15).
1985	D'Angio convened the first workshop on histiocytosis leading to the formation of the Histiocyte Society, an international society dedicated to the understanding of all aspects of the histiocytic disorders (16).
1987	The Writing Group of the Histiocyte Society outlined the morphologic, immunohistochemical and clinical criteria required for the diagnosis of LCH (16).
1980's-present	The Toughill family formed the parent support group, now the very successful Histiocytosis Association of America (1985), which together with homologous groups from other countries, is a major supporter of research, as well as parent and patient support. The Kontoyannis family supports the annual "brain storm" meeting called the "Nikolas Symposium", at which basic researchers and clinicians interested in these diseases are brought together to formulate new ideas to find a rationale for a cure for LCH.

Diagnosis, classification and treatment of LCH

The diagnosis of the histiocytoses relies on combined clinical, pathological and radiological criteria. In LCH, the diagnosis is based on the expression of CD1a and S-100 by the cells in the lesions together with their morphology. LCH cells are round instead of dendritic-shaped cells (Figure 1 A), typically having a moderate amount of homogeneous, pink, granular cytoplasm and distinct cell margins. The nucleus shows folding and nucleoli are indistinct (Figure 1 B) (1,17,18). Expression of Langerin (CD207) and the presence of cytoplasmic Birbeck granules in LCH cells complete the picture, but they are not always used as diagnostic markers in routine practice.

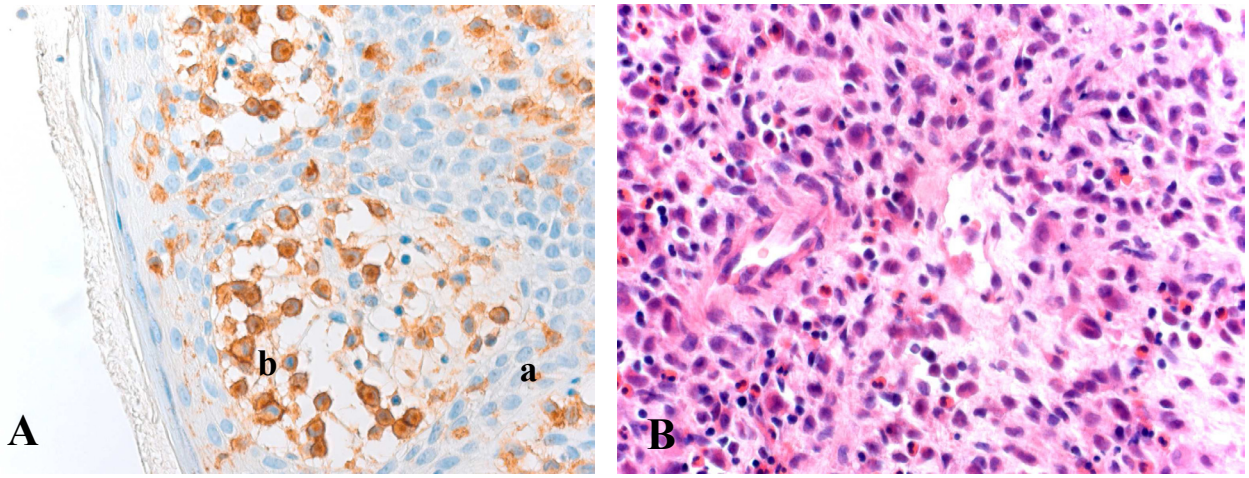

Figure 1. Phenotypic and morphologic characteristics of LCH cells. A. Normal CD1a+ Langerhans cells with typical dendrites (a), and pathologic CD1a+ LCH cells with a typical round shape (b). B. The characteristic nucleus folding and indistinct nucleoli in an H/E stained section of a LCH lesion.

LCH can be divided in three groups, according to the number of sites and types of tissues/organs involved and the presence or absence of involved organ failure. LCH in childhood is very diverse, presenting as a single system disease or as a disseminated form. Single system involvement is observed in two thirds of patients and occurs mostly in bone. In this case the clinico-radiological presentation may show overlap with Ewings sarcoma and osteomyelitis. The multi-system form of LCH in childhood may or may not be accompanied by organ failure and occurs often in young infants. Finally, pulmonary LCH, involving the lungs only, is thought to be a different form of this disease, as it is often present in adults and it is considered to be a reactive condition due to smoking (Table 3) (19).
The treatment of LCH depends on the number and types of organs involved, such as single system LCH involving e.g. bone, skin, and multi-system LCH with or without involvement of visceral organs. Although always biopsied, many of the patients with single system involvement require minimal to no treatment. If treatment is considered, this includes the use of corticosteroids applied locally

on skin lesions or intralesionally in bone. Despite the lower severity, there still may be persisting, mainly orthopaedic, consequences in cases of bone involvement (20). In addition, these patients may show organ-restricted recurrences. On the other hand, in this form of LCH there are still cases of spontaneous clinical remissions and obviously then the need for treatment is overcome.

Table 3. Classification of Langerhans cell histiocytosis*

Forms of LCH	Characteristics
Extensive or disseminated	- Highest incidence in infants (particularly but not exclusively in children younger than 2 years of age) - Visceral organ involvement, with or without bone lesions, diabetes insipidus, adjacent lymph node involvement and/or skin rash, with or without signs of organ dysfunction of any of lung, liver, gastrointestinal tract or haematopoietic system
Restricted or localized	- Mostly diagnosed between the age of 5-15 years - Biopsy proven skin rash (scaly, erythematous, seborrhea-like brown to red papules) without any other site of involvement. Monostotic and/or polyostotic lesions (painfull swelling; irregularly marginated lytic lesions of bone), with or without diabetes insipidus (polyuria and polydipsia), adjacent lymph node involvement or skin rash
Pulmonary	- Most commonly during the third decade (can, however, occur at any age as part of extensive LCH). Strong but not absolute link with smoking - Localized lung involvement with in the extreme form emphysematous changes with interstitial fibrosis

* Adapted from reference (21)

In multi-system LCH, the treatment is systemic with corticosteroids in combination with chemo-therapeutic agents, mostly vinblastine (VBL)- and/or etoposide (VP-16)-based (22). This form of treatment is held after the comparison of the LCH-I international clinical trial with the multicentric clinical trials DAL HX-83/90 results, that showed a clear superiority of combination therapy (DAL HX-83/90) given for 1 year with respect to initial response and rate of reactivation as compared to monotherapy for six months (22,23). In the following international clinical trial, LCH-II, the goal was to match the results of the DAL HX studies and to confirm whether addition of VP-16 to prednisone (PDN) and VBL would be beneficial. The results in the “low risk” LCH were satisfying. However, the “high risk” LCH population still presented the same probability of survival, which shows that about 20% of the multi-system patients cannot be rescued with standard treatment including VBL and PDN with or without VP-16 (22,24). Thus, this patient group is being subject to the use of new agents in the initial treatment and alternative salvage strategies. As for the ongoing international trial LCH-III, patients are categorized in three groups: single system multifocal bone disease or localized special site involvement, and multisystem risk and low risk patients. Treatment of patients with multifocal bone disease or special-site involvement includes PDN and VBL for 24 weeks; low risk patients are treated with these steroids during 6 to 12 months; and risk patients receive or not metothexatre in addition

to the standard PDN, VBL and mercaptopurine. Recently the Histiocyte Society developed a treatment regime for non-responding patients to LCH III, entitled LCH-Salvage. This treatment is based on the use of cladribine (2CDA) and Cytarabine (Ara-C). In extreme cases, stem cell transplantation combined with myeloablative therapy has been performed but with low rates of success. Sequelae and permanent consequences are common in multi-system patients and include small stature due to growth-hormone deficiency, diabetes insipidus, cerebellar ataxia, deafness, orthodontic problems, lung fibrosis, liver cirrhosis, malabsorption due to gastrointestinal involvement and neuropsychological problems (25,26).

In the case of pulmonary LCH, smoking cessation is often sufficient for remission to occur (27). First line therapy is corticosteroids and in cases of severe disease, lung transplantation may be an option.

Biology and immunology of normal Langerhans cells

Origin and differentiation of dendritic cells

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs) *in vitro* and *in vivo*. They are bone marrow-derived leukocytes which have a wide distribution within the body. This enables them to fulfil their role as sentinels of the organism since their main function is to initiate and modulate the immune response (28,29). As the pathologic cells in LCH are Langerhans DCs, it is important to understand the classification and biology of DCs in general.

The characterization of DCs began only 25 years ago with the work of Steinman and Cohn, who purified and identified DCs in peripheral lymphoid organs of mice (30). Today, several subsets of DCs have been described that, depending on their local microenvironment, mediate different types of immune responses. These subsets include interstitial DCs (intDCs), Langerhans DCs (LCs), conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (29,31).

Although DCs were originally considered to have a strict myeloid origin, subsequent studies have shown that they can also develop from lymphoid-committed progenitors (32-34). The common feature of the progenitors capable of developing into DCs is the surface expression of Flt3 receptor. Among other studies on this subject, Inaba *et al*. showed that mouse bone marrow myeloid precursors had a capacity to produce DCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (35). A similar finding was described in the studies of human cells where a CD34+ bone marrow-derived precursor differentiated into, besides other cell types, a bi-potential precursor population with the ability to produce mature DCs when cultured in the presence of GM-CSF and tumour necrosis factor alpha (TNF-a), or macrophages when cultured in the presence of macrophages- colony stimulating factor (M-CSF) (36,37). On the other hand, early studies on the lymphoid tissue resident

DCs demonstrated that thymic cDCs and subpopulations of cDCs in mouse spleen and lymph nodes express markers associated with lymphoid cells. One such case is the pDC, originally suggested to have a lymphoid origin based on the expression of many lymphoid markers, such as IgK and pre-T cell receptor, and the absence of myeloid markers (38,39). However, more recent studies revealed that Flt3+ cells within either common lymphoid or myeloid precursors could differentiate into both cDC and pDC in cultures and in vivo (40-42). Finally, interstitial DCs and Langerhans cells (LCs), other types of DCs, were shown to differentiate from bone marrow precursors in mice models and in vitro human models (Figure 2) (43,44).

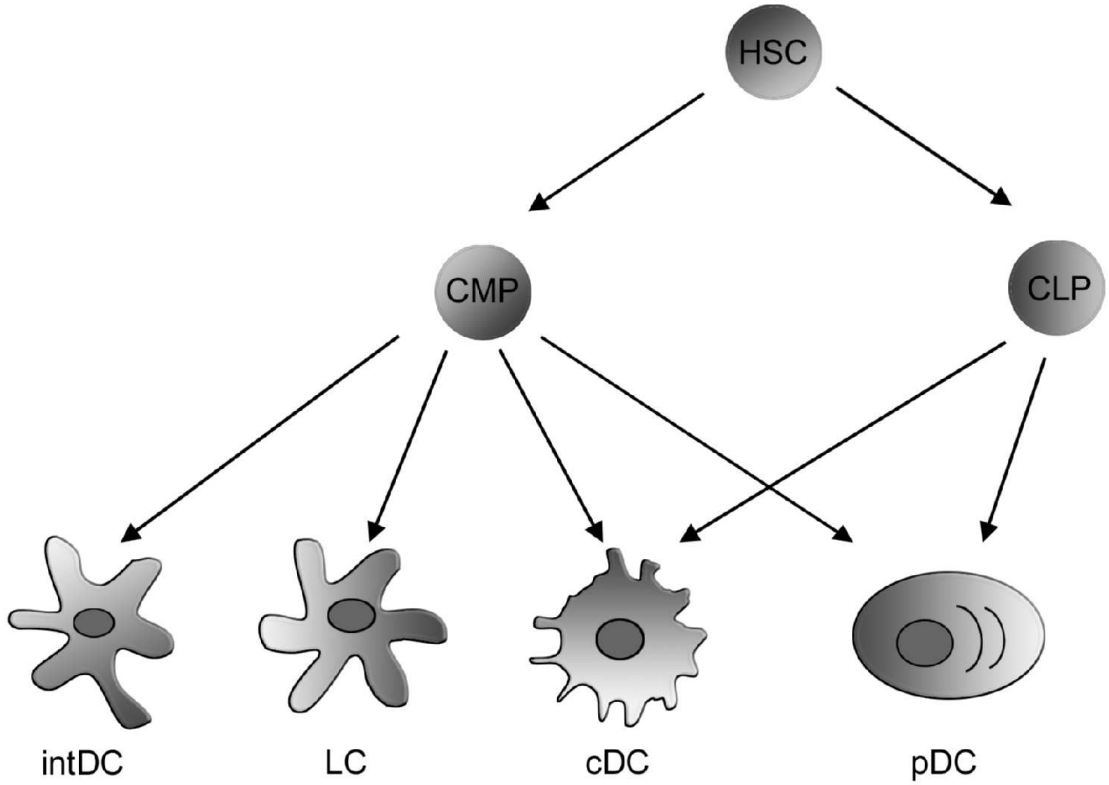

Figure 2. The origin of human DCs. Human stem cells (HSC) differentiate into common myeloid precursors (CMP) and common lymphoid precursors (CLP). CLP develop into conventional DCs (cDC) and plasmacytoid DCs (pDCs). Similarly, CMP develop into cDC and pDC, but they are also able to differentiate into interstitial DC (intDC) and Langerhans cells (LC).

Origin and phenotype of LCs

LCs were first described by Paul Langerhans 140 years ago at the dermal/epidermal junction of the skin (2). Only in 1977 were LCs recognized within the immunology field after reports of the expression of molecules involved in immune interactions, such as Fc receptors, complement receptors and MHC class II molecules on these cells (45-47). Subsequently, they were considered the most efficient APC of the DC family.

Katz *et al.* (48) and Frelinger *et al.* (49) in the mouse and Perreault *et al.* (50) and Volc-Platzer *et*

al. (51) in humans have shown that LCs originate from bone marrow-derived monocyte precursors. LC precursors were found in two types of blood cells: myeloid DCs and monocytes (52, 53). In fact, Ginhoux *et al.* (44) demonstrated *in vivo* that monocytes are direct precursors of LCs, by showing that mice deficient in the receptor for colony stimulating factor-1 (CSF-1), lack LCs in the steady state and that bone marrow progenitors from these mice are unable to reconstitute both LCs and macrophages in an inflammatory state. During ontogeny, LC precursors populate the epidermis (Figure 3) and acquire immunologically important molecules, which allow them to be distinguished from all the other cells.

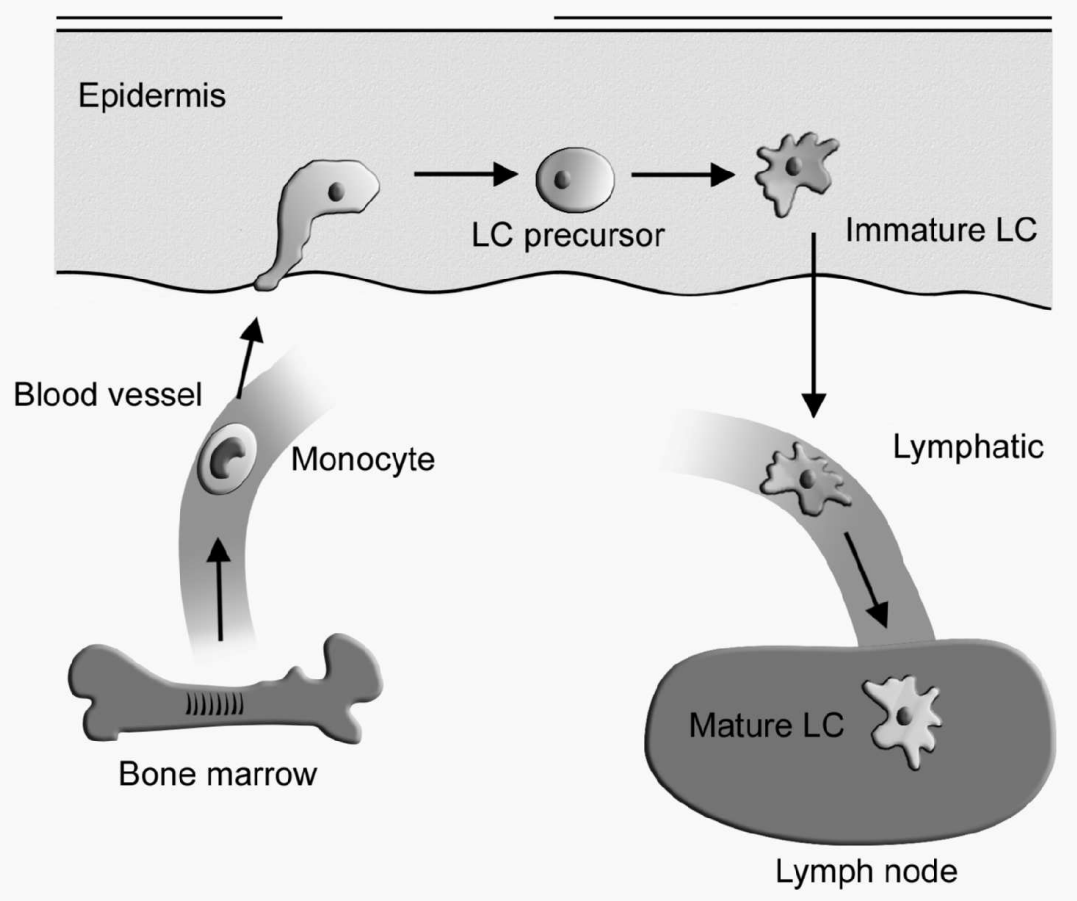

Figure 3. The life cycle of Langerhans cells (LCs). LC precursors originate in the bone marrow, from where they travel through the bloodstream to the epidermis as monocytes. This accounts for inflammatory and possible non-inflammatory situations during early development. In the epidermis, LC precursors differentiate into immature LCs, maintained locally in the steady state. They travel to the draining lymph nodes in a low level, steady state efflux manner (adapted from reference (54)).

These molecules include Langerin/CD207, expressed in the tennis-racket shaped Birbeck granules characteristic of LCs (Figure 4 B) (55). In addition, CD1a, which is involved in the presentation of microbial lipidic antigens, is abundantly present on LCs (Figure 1 A) but low or absent on intDCs (56). Finally, in healthy epidermis, the expression of MHC class II molecules is specific for LCs, whereas in the dermis MHC II is expressed not only on intDCs but also on a variety of other cells such

as macrophages and endothelial cells of blood vessels.

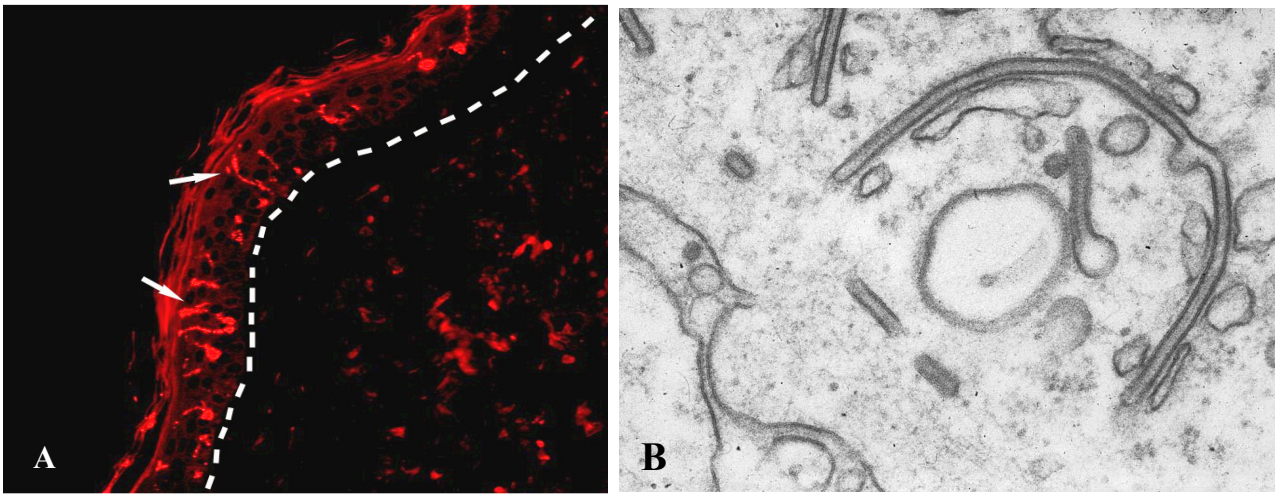

Figure 4. Normal Langerhans cells (LCs) in the epidermis of a human skin biopsy and their characteristic marker: the Birbeck granule. Immunofluorescent detection of LCs in normal human skin epidermis with CD1a antibody (red; A). Rod- and tennis-racket shaped Birbeck granules present in a LC (B).

LC function

Together with macrophages, LCs are responsible for the first line of defense in the body, namely the skin, epithelial surfaces of airways and gastrointestinal tract, which are constantly exposed to microbes. In the skin LCs are uniquely present in the epidermis and in multilayered Malpighian epithelia in mucous membranes.

Due to their peripheral, sentinel location, they are able to survey the epidermal environment and to initiate an immune response against microbial threats. After taking up antigen and becoming activated, they migrate to the draining lymph nodes. Kissenpfennig *et al.* (57) showed that LCs migrate preferentially to the T cell areas, where they secrete chemokines that allow the attraction of naïve T cells and induce their proliferation and differentiation into helper and cytotoxic T effector cells (Figure 5).

In vitro models have shown that LCs possess outstanding immunostimulatory capacities. Firstly, a few studies showed that LCs were immunostimulatory in the allogeneic mixed leukocyte reaction and induced cytotoxic T lymphocytes (59,60). Then, Steinman's group analysed the immunostimulatory properties, such as the antigen processing and T cell stimulatory capacities of LCs in relation to their maturation status (61,62). The main finding is that LCs undergo a process of maturation during which they strongly up-regulate their capacity to stimulate resting T cells but down-regulate their ability to process antigens in the context of MHC class II. On the other hand, intDCs migrate to B cell follicles, where they induce the differentiation of naïve B cells and the generation of immunoglobulin-secreting

plasma cells. These two DC subsets also differ in the cytokines they secrete, as only intDCs produce IL-10. Finally, recently it was also shown that LCs are capable of cross-presentation, a mechanism whereby extracellular protein antigens can be processed by, in this case, the LCs and presented in the context of MHC class I molecules to CD8+ T cells (63).

In vivo, there are not many studies on LC immunity compared to the numerous data available from *in vitro* experiments. However, there are a few reports including two from the group of Streilein and Bergstresser who showed that LCs are critical in the induction of contact hypersensitivity reactions (64,65). In these studies, they demonstrated that under all circumstances of LC depletion there was diminished contact hypersensitivity reaction or prolonged skin graft survival.

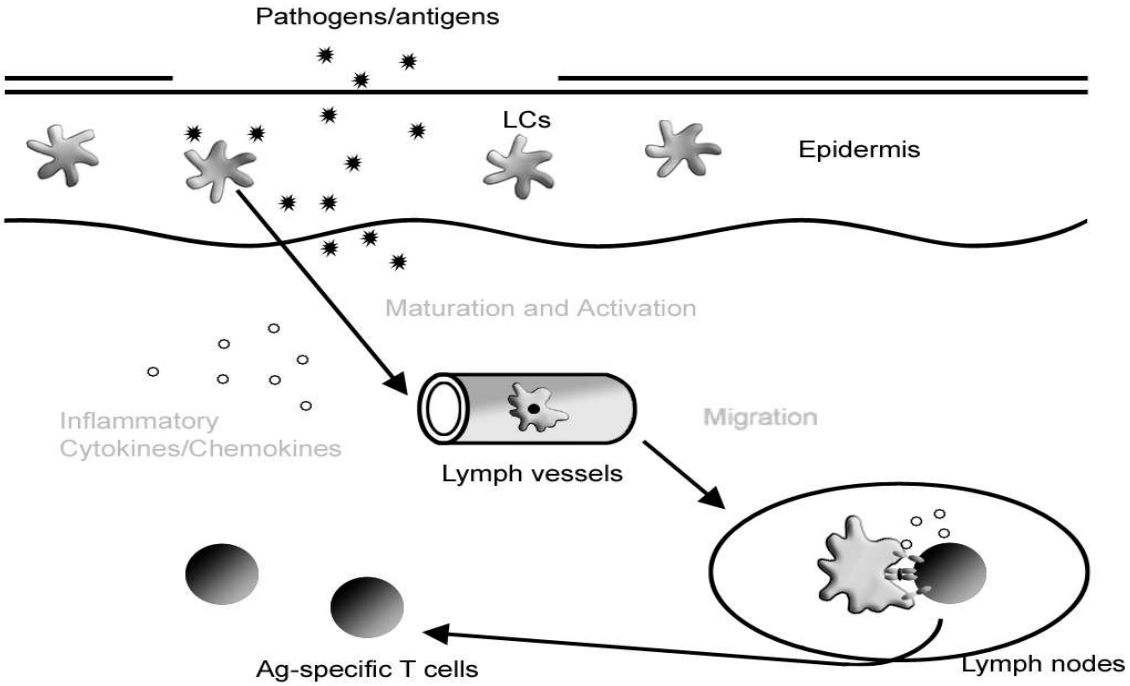

Figure 5. Langerhans cells (LCs) in immunity against pathogens. LCs undergo maturation either by direct interaction with a microbe or in response to products of surrounding cells and migrate through lymphatic vessels into T cell zones of draining lymph nodes, where they secrete chemokines that attract naïve T cells and induce their proliferation and differentiation (adapted from reference (58)).

The tolerogenic role and homeostasis of LCs

Besides their immunogenic role, LCs, like other DCs, are also able to maintain tolerance. Thus, in the absence of inflammation and pathogenic stimuli via Toll-like receptors, LCs induce peripheral tolerance *in vivo* by migrating to the lymph nodes and presenting the antigen in the steady state (66). Hemmi *et al*. (67) and Yoshino *et al*. (68) showed that LCs carry self antigens such as melanin granules to the lymph nodes in the steady state. Migration under steady state conditions seems to be differently regulated than migration under inflammatory conditions. However, there is no distinct phenotype, by

which one could distinguish tolerogenic from immunogenic LCs. Ohl *et al*. (69) showed that both require expression of CCR7 for migration and the only slight difference in phenotypic markers is that more IL-12 is produced by immunogenic LCs.

The homeostasis of LCs seems to be regulated differently from that of other DC subsets. This was demonstrated by Merad *et al.* (70) who showed that in a bone marrow transplant model host DCs other than LCs are replenished by donor DCs within approximately two months of transplantation. Host LCs, however, persist much longer and can be detected even 18 months after transplantation because of their ability to renew in situ and their resistance to g-radiation. In addition, their high longevity is also due to their low level of cell division (71).

Normal Langerhans cells versus LCH CD1a+ cells

Knowledge of the biology of LCs can help in identifying the abnormal mechanisms in LCH cells, whilst at the same time understanding the pathogenesis of LCH may help elucidate basic immunological mechanisms involving DC dysregulation in humans. Thus, it is of interest to compare pathologic LCH cells with their normal counterparts.

Like normal epidermal LCs, lesional LCs in LCH show typical staining with CD1a and Langerin (table 4). During LC maturation, cells down-regulate their ability to acquire and process antigens and start to express molecules associated with antigen presentation. Remarkably, CD40, CD80 and CD86 are expressed in high amounts, or exclusively, by the LCs in LCH in comparison with normal epidermal LCs (72,73). However, LCs in LCH are not completely activated. They show greater proliferation and lower antigen presenting capacity than mature LCs, and a certain capacity for migration, which suggests a maturation arrest at an activated state (74). LCs in LCH also express CCR6, a chemokine receptor normally expressed by skin LCs and down-regulated upon LC activation (75).

Table 4. Features of LCH cells compared to normal resting LCs and activated lymph node DCs.

	LCH cells	Resting epidermal LCs	Activated LN DCs
Immature DC markers			
CD1a	+	+	-
Langerin (CD207)	+	+	-
Birbeck granules	+	+	-
CD14	+	+	-
CD68	+	±	-
CLA	+	+	-
DC-SIGN (CD209)	-	-	-
CCR6	+	+	-
Mature DC markers			
CD83	- or ±	-	+
Fascin	- or +	-	+
DC-LAMP (CD208)	-	-	+
CCR7	-	-	+
Cell activation			
IL-2R (CD25)	+	-	+
GM-SCFR (CD116)	+	±	+
Antigen presentation			
MHC class II	+	±	++
CD40	++	+	++
CD80 and CD86	± or +	-	+
APC function	- or ±	±	++
Adhesion			
E-cadherin	- or ±	++	- or ±
CD49d	+	-	+
CD54	+ or ++	±	++
CD2-CD58	+ or ++	- or ±	++

*Adapted from reference (21)

Brief summary of previous research in LCH

Although the research in LCH has been relatively scarce since the first reports of LCH as a disease, the last 20 years have produced important knowledge regarding LCH pathophysiology. Initial studies looked at the phenotype of LCH cells. As indicated in the previous section, these studies showed that the LCH cell phenotype corresponds to an early-activated stage of DC maturation, combining an immature phenotype of DCs with a high level of cytokine expression. These findings suggested that the pathogenesis of LCH might be due to a blockage in the normal maturation pathway of the CD1a+ LCH cells and that this blockage could be overcome *in vitro* by stimulation with CD40L. However, it is still unclear the reason why LCH cells are retained in the different lesional sites.

Besides the accumulation of LCH cells in different lesional sites, the lesional microenvironment in LCH is characterised by the production of many cytokines. A few studies described a "cytokine storm" in the LCH lesional microenvironment, the source of which was found to be the LCH cells

themselves as well as T cells and macrophages that typically characterise LCH lesions (76,77). In order to better understand the relation of this "cytokine storm" and the pathogenesis of LCH it is still important to clarify whether these cytokines are inducing the attraction or in situ formation of other cell types in the lesions, which could help explain the clinical symptoms of LCH.

The aberrant immunity observed in LCH was also leading researchers to hypothesize that a viral trigger could be behind the aetiology of this disease (78-80). However, these studies showed no evidence for viral genomes in any of the LCH samples analysed. At the same time, reports on clonality of LCH cells were emerging (81,82), which started raising the idea that LCH could actually be a neoplasm. In addition to this finding, other studies reported that the cell cycle of LCH cells is disrupted (83-85) and many other reports have tried to assess the genetic characteristics of LCH, in order to understand the nature of this disease (86-88). However, none of these has carried out an extensive analysis at the genetic level which would allow a clear conclusion. The studies that have addressed all these issues are reviewed in chapter 2.

Thus, there are still many unanswered questions in LCH, crucial for understanding the pathogenesis of this disease. The studies in this thesis aimed to elucidate many of the important outstanding questions in LCH, in particular why LCH cells are aberrantly present in many body sites, how do these cells and others characteristic of the cellular composition of the lesions originate in these sites, whether by recruitment or by in situ origin and the definitive answer to what is causing LCH, either a reactive trigger or a neoplastic defect.

Outline of this thesis

This thesis set out to try to address these unanswered immunological and genetic aspects of LCH, which are important for increasing the knowledge of the pathogenesis of this disease. The lack of LCH cell lines and animal models led us to carry out a range of immunohistochemical techniques and develop new techniques to enable us to perform our investigations on LCH biopsies.

Chapter 2 outlines the research current at the time of writing on immunological aspects of LCH. Here the unique phenotype and immune function of LCH cells, the cytokine-rich lesional microenvironment and data on the expression of chemokines in LCH lesions are described. Furthermore, genetic and functional alterations related to cell-cycle regulation and proliferation, are also recapitulated in this chapter. Finally, an overview of the studies that looked at clonal aspects is included as well as the controversial aspects on the aetiology of LCH.

The abnormal retention and proliferation of LCH cells in many sites within the body is yet enigmatic. Due to the integral role that migration plays in normal function and distribution of Langerhans cells,

it is possible that deregulation of chemokine production and/or chemokine receptor expression plays a role in LCH. **Chapter 3** analyses the presence of chemokines in LCH lesions as well as chemokine receptors by the pathological lesional CD1a+ cells and by other cells in the lesions, and their consequences on the migration and attraction processes. For this purpose several immunohistochemistry techniques were used, including the optimization of an immunogold staining combined with immunofluorescence. **Chapter 4** analyses the presence of these chemokine receptors by the lesional CD1a+ cells in pulmonary LCH lesions, where LCH cells are thought to be more mature and the disease has been shown to be polyclonal.

Besides the abnormal retention of LCH cells in several sites in the body, another feature that may help in elucidating the clinical symptoms in LCH is the presence of cytokines in these lesions and their consequence for the disease. Thus, in **chapter 5** the origin and role of multinucleated giant cells, one of the other cell types characteristic of LCH lesions, is analysed. This is performed using an immunohistochemical approach analysing cytokines and receptors involved in the differentiation of osteoclasts (which are also multinucleated giant cells) present in the lesions. In addition, the expression by these cells of markers for osteoclasts, such as enzymes and adhesion molecules, is studied. Also the aberrant presence of these cells in non-ostotic lesions is analysed in this chapter.

The abnormal migration of LCH cells described in chapter 3, combined with evidence for survival of LCH cells in the lesions, provide evidence that these cells are defective. In order to help understand these observations, it is hypothesized in **chapter 6** that LCH is a neoplastic disease. The possible presence of a telomere maintenance mechanism by LCH cells, in particular the presence of telomerase, an enzyme present in a high percentage of cancers, is investigated. For this purpose, immunohistochemistry for the catalytic subunit of this enzyme is performed in single system as well as multi-system LCH lesions. A TRAP assay is also carried out to assess the activity of the enzyme. Telomerase positive cells are known for having a homogeneous and short telomere length (89). Thus, a method to isolate the CD1a+ LCH cells from frozen biopsies is developed, with the aim of extracting the genomic DNA from these cells for measuring the telomere length. In addition, FISH is carried out for detection of the telomeres in combination with immunofluorescence for detection of a protein which is a hallmark of the alternative lengthening of telomeres.

Despite the significance of the previous findings for LCH, it is still necessary to understand the exact cause of this disease. **Chapter 7** investigates the possible existence of consistent genetic abnormalities in LCH cells which may provide evidence that LCH is a neoplastic disease. Two different types of state-of-the-art array platforms, such as aCGH and SNPs, are used to screen the whole genome of the isolated CD1a+ LCH cells. In addition, ploidy, karyotype and p53 mutation analyses are also carried out on these cells.

In **Chapter 8** all results from the above mentioned studies are discussed and a hypotetical model of

LCH disease onset is provided. In addition, suggestions for future research directions in LCH are given.

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