Universiteit
Leiden
The Netherlands

# Langerhans cell histiocytosis: A reactive or neoplastic disease?

Costa, C.E.T. da

**Citation**
Costa, C. E. T. da. (2008, November 10). *Langerhans cell histiocytosis: A reactive or neoplastic disease?*. Retrieved from https://hdl.handle.net/1887/13235

| | |
|---|---|
| Version: | Corrected Publisher's Version |
| License: | Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden |
| Downloaded from: | https://hdl.handle.net/1887/13235 |

**Note:** To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

Leiden University Repository

The handle http://hdl.handle.net/1887/13235 holds various files of this Leiden University dissertation.

**Author**: Costa, C.E.T. da
**Title**: Langerhans cell histiocytosis: A reactive or neoplastic disease?
**Issue date**: 2008-11-10

# Langerhans cell histiocytosis: a reactive or neoplastic disease?

# Langerhans cell histiocytosis: a reactive or neoplastic disease?

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. P. F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op maandag 10 november 2008
klokke 13.45 uur

door
Cristiana Elizabete Teixeira da Costa
geboren te Braga (Portugal)
in 1979

# Promotiecommissie

| | |
|---|---|
| Promotores: | Prof. Dr. R. M. Egeler<br>Prof. Dr. P. C. W. Hogendoorn |
| Co-Promotor: | Dr. N. E. Annels<br>(University of Surrey, UK) |
| Referent: | Prof. Dr. J. D. Laman<br>(Erasmus Universiteit Rotterdam) |
| Overige commissieleden: | Prof. Dr. H. A. Delemarre-van der Waal<br>Prof. Dr. A. H. M. Taminiau<br>Prof. Dr. F. Koning<br>Prof. Dr. K. F. Rabe |

The research described in this thesis was performed at the Immunology Laboratory, Department of Paediatrics of Leiden University Medical Center, Leiden, The Netherlands.

The studies described in this thesis were supported by grants from the Fundação para a Ciência e a Tecnologia through its correspondent financing program, POCI 2010 and FCE (SFRH/BD/12393/2003), the Histiocytosis Associations of America and Canada and Stichting Kinderen Kankervrij (KIKA).


*“There’s more to the truth than just the facts”*

*Author unknown*

Aos meus pais e mano

Voor Massi

© 2008, Cristiana Elizabete Teixeira da Costa, Leiden, The Netherlands
All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without prior written permission of the author.

ISBN: 978-90-9023605-6

Printed by: Gildeprint B.V. te Enschede, The Netherlands

The printing of this thesis was kindly financially supported by Fundação para a Ciência e a Tecnologia, Portugal, and by Stichting LCH Nederland.

Cover picture: Confocal microscopy image of a bone Langerhans cell histiocytosis lesion, with the lesional Langerhans cells stained in red, and the multinucleated giant cells and macrophages stained in light blue.

## Table of Contents

# 1

# General Introduction

## General Introduction

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

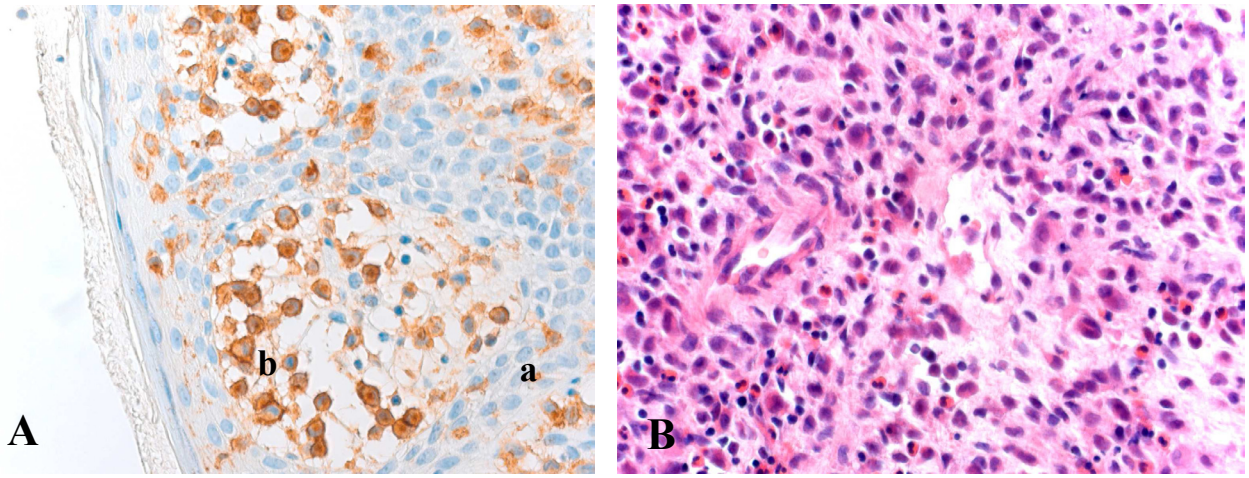


**Figure 1.** Phenotypic and morphologic characteristics of LCH cells. A. Normal CD1a+ Langerhans cells with typical dendrites (a), and pathologic CD1a+ LCH cells with a typical round shape (b). B. The characteristic nucleus folding and indistinct nucleoli in an H/E stained section of a LCH lesion.

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)<br>- 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<br>- 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).<br>Strong but not absolute link with smoking<br>- 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).

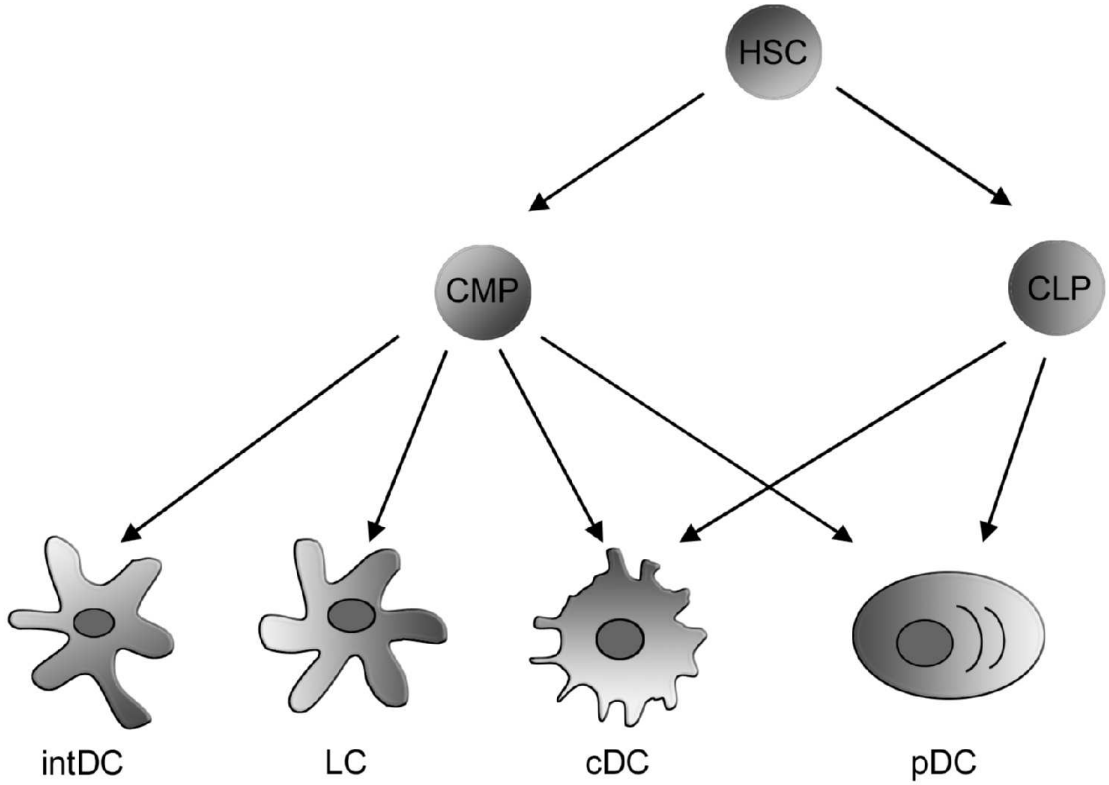


**Figure 2.** The origin of human DCs. Human stem cells (HSC) differentiate into common myeloid precursors (CMP) and common lymphoid precursors (CLP). CLP develop into conventional DCs (cDC) and plasmacytoid DCs (pDCs). Similarly, CMP develop into cDC and pDC, but they are also able to differentiate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

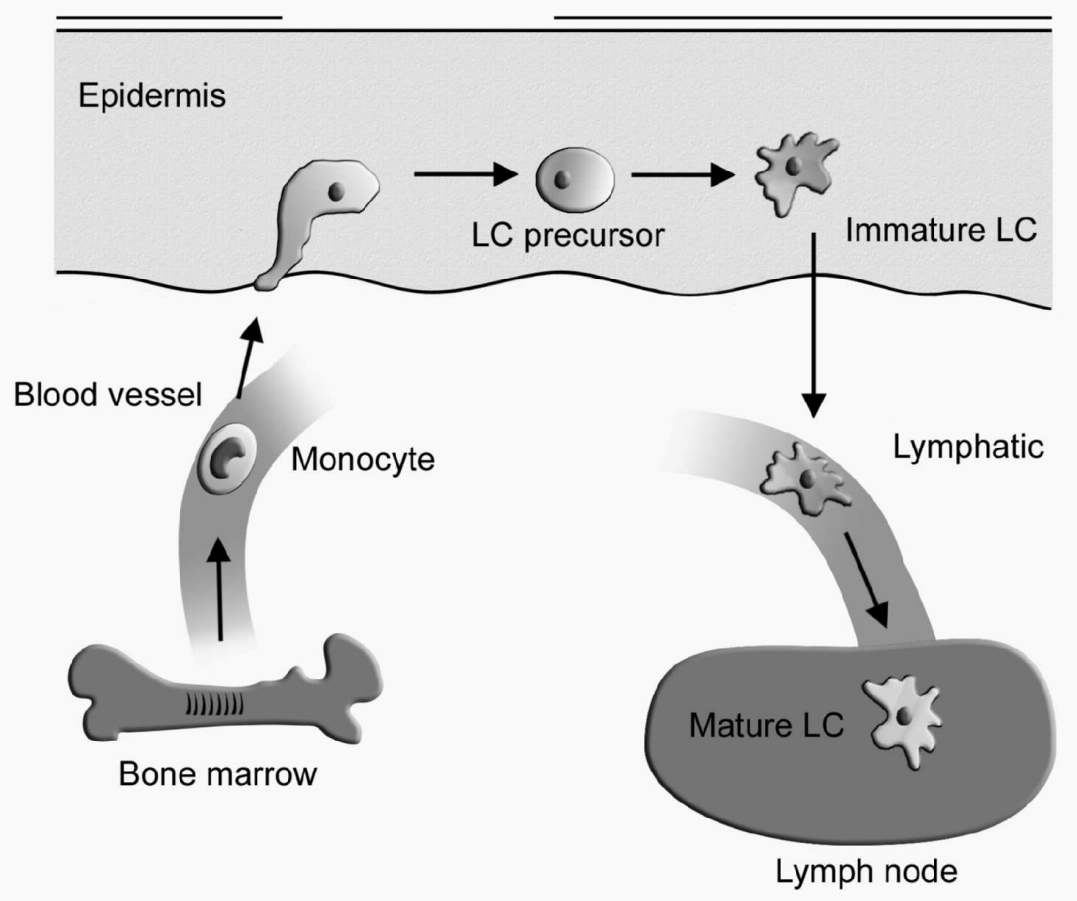


**Figure 3.** The life cycle of Langerhans cells (LCs). LC precursors originate in the bone marrow, from where they travel through the bloodstream to the epidermis as monocytes. This accounts for inflammatory and possible non-inflammatory situations during early development. In the epidermis, LC precursors 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.

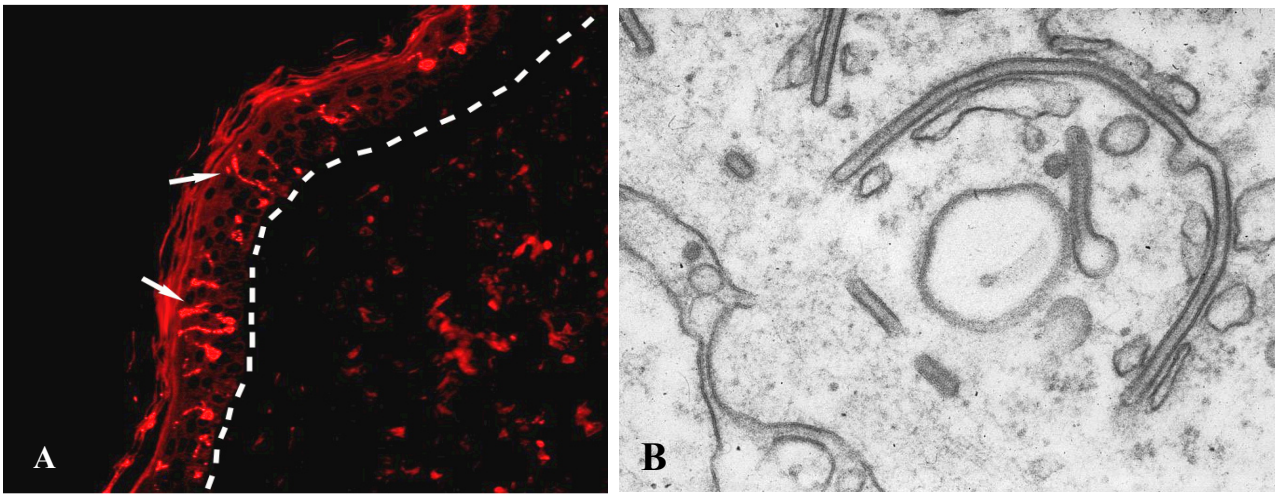


**Figure 4.** Normal Langerhans cells (LCs) in the epidermis of a human skin biopsy and their characteristic marker: the Birbeck granule. Immunofluorescent detection of LCs in normal human skin epidermis with CD1a antibody (red; A). Rod- and tennis-racket shaped Birbeck granules present in a LC (B).

***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.

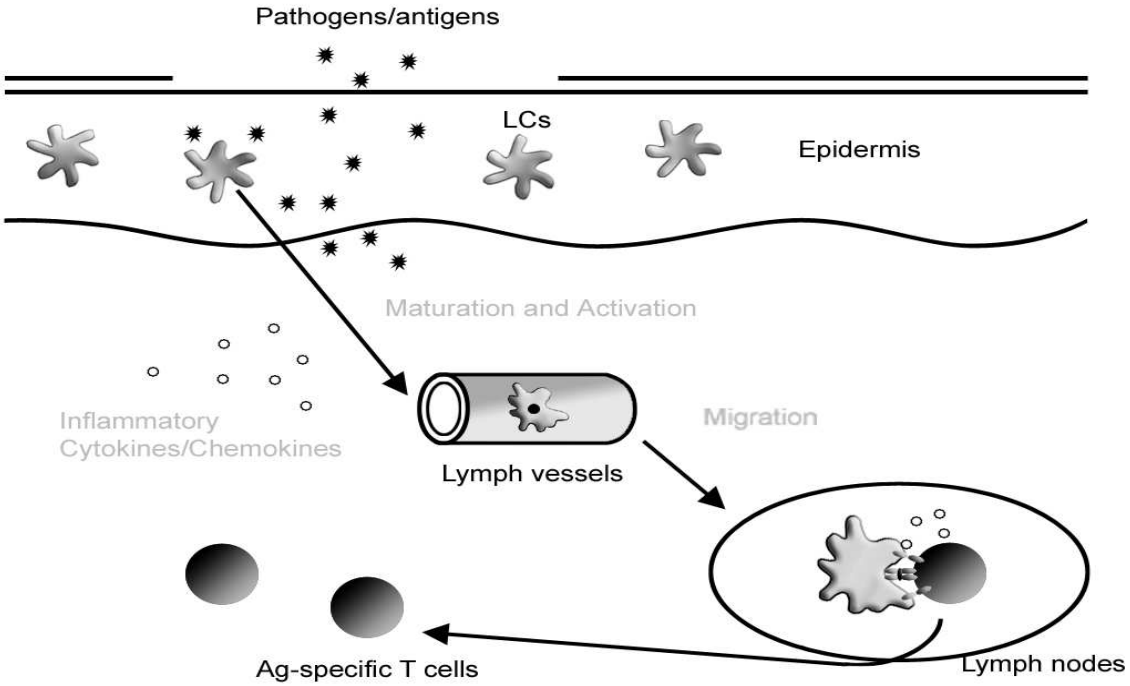


**Figure 5.** Langerhans cells (LCs) in immunity against pathogens. LCs undergo maturation either by direct interaction with a microbe or in response to products of surrounding cells and migrate through lymphatic vessels into T cell zones of draining lymph nodes, where they secrete chemokines that attract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

**References**


(1) Favara BE, Feller AC, Pauli M, Jaffe ES, Weiss LM, Arico M *et al*. Contemporary classification of histiocytic disorders. The WHO Committee On Histiocytic/Reticulum Cell Proliferations. Reclassifi cation Working Group of the Histiocyte Society. Med Pediatr Oncol 1997; 29(3):157-166.

(2) Langerhans P. Uber die Nerven der menschlichen Haut. Virchows Arch [A] 1868; 44:325-337.

(3) Wolff K. The fascinating story that began in 1868. In: Schuler G, editor. Epidermal Langerhans Cells. Boca Raton FL:CRC Press Inc., 1991:1-21.

(4) Birbeck MS, Breathnach AS and Everall JD. An electron microscopy study of basal melanocytes and high-level clear cells (Langerhans cells) in vitiligo. J Invest Dermatol 1961; 37:51-63.

(5) Lichtenstein L. Histiocytosis X; integration of eosinophilic granuloma of bone, Letterer-Siwe dis ease, and Schuller-Christian disease as related manifestations of a single nosologic entity. AMA Arch Pathol 1953;56(1):84-102.

(6) Nezelof C, Basset F, Rousseau MF. Histiocytosis X histogenetic arguments for a Langerhans cell ori gin. Biomedicine 1973; 18(5):365-371.

(7) Donadieu J and Pritchard J. Langerhans cell histiocytosis – 400BC? Med pediatr Oncol 1999;3:520.

(8) Coppes-Zantinga A, Egeler RM. The Langerhans cell histiocytosis X files revealed. Br J Haematol 2002;116(1):3-9.

(9) Hand A. Polyuria and tuberculosis. Arch Pediatr 1893; 10:673-675.

(10) Schuller A. Uber eigenartige Schadeldefekte im jugendalter. Fortschritte Auf der Gebiete Rontgenst rahlen 1915; 23:12-18.

(11) Christian H. Defects in membranous bones, exophtalmos and diabetes insipidus: an unusual syn drome of dyspituitarism. Med Clin North Am 1920; 3:849-871.

(12) Siwe S. Die Reticuloendotheliose - ein neues krankheitsbild unter den hepatosplenomegalien. Zeitschrift fur kinderheilkunde 1933; 55:212-247.

(13) Letterer E.. Aleukamische retikulose (ein Beitrag zu den proliferativen Erkrankungen des reticuloen dothelial apparates). Frankfurter Zeitschritte Pathol 1924; 30:377-394.

(14) Farber S. The nature of solitary or eosinophilic granuloma of bone. Am J Pathol 1941; 17:625-629.

(15) Risdall RJ, Dohner LP, Duray P, Kobrinsky N, Robison L and Nesbit ME Jr. Histiocytosis X (Langer hans cell histiocytosis). Prognostic role of histopathology. Arch Pathol Lab Med 1983; 107:50-63.

(16) Chu T, D'Angio GJ, Favara BE, Ladisch S, Nesbit M, Pritchard J. Histiocytosis syndromes in chil dren. Lancet 1987; 2(8549):41-42.

(17) Emile JF, Wechsler J, Brousse N, Boulland ML, Cologon R, Fraitag S *et al*. Langerhans' cell histiocy tosis. Definitive diagnosis with the use of monoclonal antibody O10 on routinely paraffin-embedded samples. Am J Surg Pathol 1995; 19(6):636-641.

(18) Geissmann F, Lepelletier Y, Fraitag S, Valladeau J, Bodemer C, Debre M *et al*. Differentiation of Langerhans cells in Langerhans cell histiocytosis. Blood 2001; 97(5):1241-1248.

(19) Arico M, Egeler RM. Clinical aspects of Langerhans cell histiocytosis. Hematol Oncol Clin North Am 1998;
12(2):247-258.

(20) TitgemeyerC, Grois N, Minkov M, Flucher-Wolfram B, Gatterer-Menz I, Gadner H. Pattern and course of single-system disease in Langerhans cell histiocytosis data from the DAL-HX 83- and 90-study. Med Pediatr Oncol 2001;37(2):108-114.

(21) Laman JD, Leenen PJ, Annels NE, Hogendoorn PC, Egeler RM. Langerhans-cell histiocytosis 'in

sight into DC biology'. Trends Immunol 2003; 24(4):190-196.
(22) Gadner H, Grois N, Arico M, Broadbent V, Ceci A, Jakobson A, Komp D, Michaelis J, Nicholson S, Pötschger U, Pritchard J, Ladisch S; Histiocyte Society. A randomized trial of treatment for multisys tem Langerhans' cell histiocytosis. J Pediatr 2001; 138(5):728-34.
(23) Gadner H, Heitger A, Grois N, Gatterer-Menz I, Ladisch S. Treatment strategy for disseminated Langerhans cell histiocytosis. DAL HX-83 Study Group. Med Pediatr Oncol 1994; 23(2):72-80.
(24) Gadner H, Grois N, Potschger U, Minkov M, Arico M, Braier J, Broadbent V, Donadieu J, Henter J-I, McCarter R, Ladisch S. Improved outcome in multisystem Langerhans cell histiocytosis is associ ated with therapy intensification. Blood 2008
(25) Haupt R, Nanduri V, Calevo MG, Bernstrand C, Braier JL, Broadbent V, Rey G, McClain KL, Janka-Schaub G, Egeler RM. Permanent consequences in Langerhans cell histiocytosis patients: a pilot study from the Histiocyte Society-Late Effects Study Group. Pediatr Blood Cancer 2004; 42(5):438-444.
(26) Grois N, Tsunematsu Y, Barkovich AJ, Favara BE. Central nervous system disease in Langerhans cell histiocytosis. Br J Cancer Suppl 1994; 23:S24-S28.
(27) Vassallo R, Ryu JH, Schroeder DR, Decker PA, Limper AH. Clinical outcomes of pulmonary Langer hans'-cell histiocytosis in adults. N Engl J Med 2002; 346(7):484-490.
(28) Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 2(6673):245-252.
(29) Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ *et al*. Immunobiology of dendritic cells. Annu Rev Immunol 2000; 18:767-811.
(30) Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Func tional properties *in vitro*. J Exp Med 1974; 139(2):380-397.
(31) Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precur sors. Annu Rev Immunol 2005; 23:275-306.
(32) Wu L, D'Amico A, Hochrein H, O'Keeffe M, Shortman K, and Lucas K. Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. Blood 2001; 98:3376-3382.
(33) Manz MG, Traver D, Miyamoto T, Weissman IL, and Akashi K. Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood 2001; 97:3333-3341.
(34) Izon D, Rudd K, DeMuth W, Pear WS, Clendenin C, Lindsley RC, and Allman D. A common path way for dendritic cell and early B cell development. J.Immunol 2001; 167:1387-1392.
(35) Inaba K, Inaba M, Deguchi M, Hagi K, Yasumizu R, Ikehara S *et al*. Granulocytes, macrophages, and den dritic cells arise from a common major histocompatibility complex class II-negative progeni tor in mouse bone marrow. Proc Natl Acad Sci U S A 1993; 90(7):3038-3042.
(36) Reid CD, Stackpoole A, Meager A, Tikerpae J. Interactions of tumor necrosis factor with granulo cyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth *in vitro* from early bipotent CD34+ progenitors in human bone marrow. J Immunol 1992; 149(8):2681-2688.
(37) Caux C, Vanbervliet B, Massacrier C, Dezutter-Dambuyant C, Saint-Vis B, Jacquet C *et al*. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. J Exp Med 1996; 184(2):695-706.
(38) Vremec D, Zorbas M, Scollay R, Saunders DJ, Ardavin CF, Wu L *et al*. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a sub population of dendritic cells. J Exp Med 1992; 176(1):47-58.
(39) Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J Exp Med 1997; 185(6):1101-1111.
(40) Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal MR *et al*. Reciprocal control of T helper cell and dendritic cell differentiation. Science 1999; 283(5405):1183-1186.

(41) Chicha L, Jarrossay D, Manz MG. Clonal type I interferon-producing and dendritic cell precursors are con tained in both human lymphoid and myeloid progenitor populations. J Exp Med 2004; 200(11):1519-1524.
(42) Shigematsu H, Reizis B, Iwasaki H, Mizuno S, Hu D, Traver D *et al*. Plasmacytoid dendritic cells activate lymphoid-specific genetic programs irrespective of their cellular origin. Immunity 2004; 21(1):43-53.
(43) Randolph GJ, Beaulieu S, Lebecque S, Steinman RM, Muller WA. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. Science 1998; 282(5388):480-483.
(44) Ginhoux F, Tacke F, Angeli V, Bogunovic M, Loubeau M, Dai XM *et al*. Langerhans cells arise from monocytes *in vivo*. Nat Immunol 2006; 7(3):265-273.
(45) Rowden G, Lewis MG, Sullivan AK. Ia antigen expression on human epidermal Langerhans cells. Nature 1977;268(5617):247-248.
(46) Klareskog L, Tjernlund U, Forsum U, Peterson PA. Epidermal Langerhans cells express Ia antigens. Nature 1977; 268(5617):248-250.
(47) Stingl G, Wolff-Schreiner EC, Pichler WJ, Gschnait F, Knapp W, Wolff K. Epidermal Langerhans cells bear Fc and C3 receptors. Nature 1977; 268(5617):245-246.
(48) Katz SI, Tamaki K, Sachs DH. Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature 1979; 282(5736):324-326.
(49) Frelinger JG, Hood L, Hill S, Frelinger JA. Mouse epidermal Ia molecules have a bone marrow ori gin. Nature 1979;282(5736):321-323.
(50) Perreault C, Pelletier M, Landry D, Gyger M. Study of Langerhans cells after allogeneic bone mar row transplantation. Blood 1984; 63(4):807-811.
(51) Volc-Platzer B, Stingl G, Wolff K, Hinterberg W, Schnedl W. Cytogenetic identification of allogeneic epidermal Langerhans cells in a bone-marrow-graft recipient. N Engl J Med 1984; 310(17):1123-1124.
(52) Ito T, Inaba M, Inaba K, Toki J, Sogo S, Iguchi T *et al*. A CD1a+/CD11c+ subset of human blood den dritic cells is a direct precursor of Langerhans cells. J Immunol 1999; 163(3):1409-1419.
(53) Valladeau J, Saeland S. Cutaneous dendritic cells. Semin Immunol 2005; 17(4):273-283.
(54) Shortman K and Naik SH. Steady-state and inflammatory dendritic-cell development. Nat Rev Im munol 2007; 7:19-30.
(55) Hunger RE, Sieling PA, Ochoa MT, Sugaya M, Burdick AE, Rea TH *et al*. Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. J Clin Invest 2004; 113(5):701-708.
(56) Lenz A, Heine M, Schuler G, Romani N. Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization. J Clin Invest 1993; 92(6):2587-2596.
(57) Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N *et al*. Dynamics and function of Langerhans cells *in vivo*: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity 2005; 22(5):643-654.
(58) Asahina A and Tamaki K. Role of Langerhans cells in cutaneous protective immunity: is the reap praisal necessary? J.Dermatol.Sci 2006; 44:1-9.
(59) Stingl G, Katz SI, Clement L, Green I, Shevach EM. Immunologic functions of Ia-bearing epidermal Langerhans cells. J Immunol 1978; 121(5):2005-2013.
(60) Pehamberger H, Stingl LA, Pogantsch S, Steiner G, Wolff K, Stingl G. Epidermal cell-induced gen eration of cytotoxic T-lymphocyte responses against alloantigens or TNP-modified syngeneic cells: requirement for Ia-positive Langerhans cells. J Invest Dermatol 1983; 81(3):208-211.
(61) Schuler G, Steinman RM. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. J Exp Med 1985; 161(3):526-546.
(62) Romani N, Koide S, Crowley M, Witmer-Pack M, Livingstone AM, Fathman CG *et al*. Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by

immature, epidermal Langerhans cells. J Exp Med 1989; 169(3):1169-1178.
(63) Stoitzner P, Tripp CH, Eberhart A, Price KM, Jung JY, Bursch L *et al*. Langerhans cells cross-present antigen derived from skin. Proc Natl Acad Sci U S A 2006; 103(20):7783-7788.
(64) Toews GB, Bergstresser PR, Streilein JW. Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. J Immunol 1980; 124(1):445-453.
(65) Streilein JW, Lonsberry LW, Bergstresser PR. Depletion of epidermal langerhans cells and Ia immu nogenicity from tape-stripped mouse skin. J Exp Med 1982; 155(3):863-871.
(66) Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol 2003; 21:685-711.
(67) Hemmi H, Yoshino M, Yamazaki H, Naito M, Iyoda T, Omatsu Y *et al*. Skin antigens in the steady state are trafficked to regional lymph nodes by transforming growth factor-beta1-dependent cells. Int Immunol 2001;13(5):695-704.
(68) Yoshino M, Yamazaki H, Nakano H, Kakiuchi T, Ryoke K, Kunisada T *et al*. Distinct antigen traf ficking from skin in the steady and active states. Int Immunol 2003; 15(6):773-779.
(69) Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J *et al*. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. Immunity 2004; 21(2):279-288.
(70) Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I *et al*. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol 2002; 3(12):1135-1141.
(71) Kamath AT, Henri S, Battye F, Tough DF, Shortman K. Developmental kinetics and lifespan of den dritic cells in mouse lymphoid organs. Blood 2002; 100(5):1734-1741.
(72) Egeler RM, Favara BE, Laman JD, Claassen E. Abundant expression of CD40 and CD40-ligand (CD154) in paediatric Langerhans cell histiocytosis lesions. Eur J Cancer 2000; 36(16):2105-2110.
(73) Holter W, Ressmann G, Grois N, Lehner M, Parolini O, Gadner H. Normal monocyte-derived den dritic cell function in patients with Langerhans-cell-histiocytosis. Med Pediatr Oncol 2002; 39(3):181-186.
(74) Schouten B, Egeler RM, Leenen PJ, Taminiau AH, van den Broek LJ, Hogendoorn PC. Expression of cell cycle-related gene products in Langerhans cell histiocytosis. J Pediatr Hematol Oncol 2002; 24(9):727-732.
(75) Annels NE, da Costa CE, Prins FA, Willemze A, Hogendoorn PC, Egeler RM. Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. J Exp Med 2003; 197(10):1385-1390.
(76) de Graaf JH, Tamminga RY, Dam-Meiring A, Kamps WA, and Timens W. The presence of cytokines in Langerhans' cell histiocytosis. J.Pathol 1996; 180:400-406.
(77) Egeler RM, Favara BE, van Meurs M, Laman JD, and Claassen E. Differential in situ cytokine pro files of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. Blood 1999; 94:4195-4201.
(78) McClain K, Jin H, Gresik V, and Favara B. Langerhans cell histiocytosis: lack of a viral etiology. Am.J.Hematol 1994; 47:16-20.
(79) Glotzbecker MP, Carpentieri DF, and Dormans JP. Langerhans cell histiocytosis: a primary viral in fection of bone? Human herpes virus 6 latent protein detected in lymphocytes from tissue of chil dren. J.Pediatr.Orthop 2004; 24:123-129.
(80) Leahy MA, Krejci SM, Friednash M, Stockert SS, Wilson H, Huff JC, Weston WL, and Brice SL. Human herpesvirus 6 is present in lesions of Langerhans cell histiocytosis. J.Invest Dermatol 1993; 101:642-645.
(81) Willman CL, Busque L, Griffith BB, Favara BE, McClain KL, Duncan MH, and Gilliland DG. Lang erhans'-cell histiocytosis (histiocytosis X) - a clonal proliferative disease. N.Engl.J.Med 1994; 331:154-160.
(82) Yu RC, Chu C, Buluwela L, and Chu AC. Clonal proliferation of Langerhans cells in Langerhans

cell histiocytosis. Lancet 1994; 343:767-768.
(83) Schouten B, Egeler RM, Leenen PJ, Taminiau AH, van den Broek LJ, and Hogendoorn PCW Expres sion of cell cycle-related gene products in Langerhans cell histiocytosis. J.Pediatr.Hematol.Oncol 2002; 24:727-732.
(84) Bank MI, Rengtved P, Carstensen H, and Petersen BL. Langerhans cell histiocytosis: an evaluation of histopathological parameters, demonstration of proliferation by Ki-67 and mitotic bodies. APMIS 2003; 111:300-308.
(85) Bank MI, Gudbrand C, Rengtved P, Carstensen H, Fadeel B, Henter JI, and Petersen BL. Immunohis tochemical detection of the apoptosis-related proteins FADD, FLICE, and FLIP in Langerhans cell histiocytosis. J.Pediatr.Hematol.Oncol 2005; 27:301-306.
(86) Betts DR, Leibundgut KE, Feldges A, Pluss HJ, and Niggli FK. Cytogenetic abnormalities in Langer hans cell histiocytosis. Br.J.Cancer 1998; 77:552-555.
(87) Chikwava KR, Hunt JL, Mantha GS, Murphy JE, and Jaffe R. Analysis of loss of heterozygosity in single-system and multisystem Langerhans' cell histiocytosis. Pediatr.Dev.Pathol 2007; 10:18-24.
(88) Murakami I, Gogusev J, Fournet JC, Glorion C, and Jaubert F. Detection of molecular cytogenetic aberrations in langerhans cell histiocytosis of bone. Hum.Pathol 2002; 33:555-560.
(89) Broccoli D, Young JW, and de Lange T. Telomerase activity in normal and malignant hematopoietic cells. Proc.Natl.Acad.Sci.U.S.A 1995; 92:9082-9086.

# 2

# The immunological basis of Langerhans cell histiocytosis

Cristiana ET da Costa
Nicola E Annels
R Maarten Egeler

In: S Weitzman, RM Egeler (eds)

Histiocytic disorders of children and adults, Ed.1
*Cambridge press United Kingdom* 2005; 66-82

## The immunological basis of Langerhans cell histiocytosis

Although Langerhans cell histiocytosis (LCH) was first described a century ago, the aetiology is still not understood. Recent studies on the role of cytokines, chemokines, immunologic dysfunction, cell surface antigen expression, clonality and cell cycle regulation have provided new insights into the pathogenesis of LCH. Much of the data from these studies points to the Langerhans cell (LC) being intrinsically abnormal in LCH. Studies have shown that there is a proliferation of clonal LCs in the lesions of LCH. Furthermore, these LCH cells not only have differences in cytoplasmic and surface markers compared to the normal LC but also show abnormalities in cytokine production and antigen presentation. The recent progress in LCH research has provoked much discussion on whether LCH is a reactive disease resulting from environmental triggers, or a neoplastic process (Arceci *et al.*, 2002; Egeler *et al.*, 2004; Nezelof & Basset, 2004). Unfortunately, the described features of LCH have not as yet provided a clear answer.

Continuing progress in the field of dendritic cell biology has allowed us to gain an increased understanding of the phenotype and function of LCH cells as well as their interaction with their microenvironment and hence the pathophysiology of this disease. Conversely, LCH, as an *in vivo* example of a dendritic cell abnormality, may serve as a "lesson" to dendritic cell biologists (Laman *et al.*, 2003). This chapter summarises some of the most recent studies investigating the immunological basis of LCH.

### Phenotypic characterisation of LCH cells

Typically, the LCH cell phenotype corresponds to the early-activated stage of DC maturation, combining an immature phenotype with high-level cytokine expression. LCH cells have many features in common with early-activated dendritic cells (DC) that develop from the stage of immature DC on contact with bacterial products (Ricciardi-Castagnoli & Granucci, 2002). However, in contrast to early activated DC, LCH cells have rounded morphology, and show high expression of some co-stimulatory molecules that are known to drive interactions with T-cells. One such co-stimulatory molecule, CD40 is highly expressed by the LCH cells in lesional sites (Egeler *et al.*, 2000). There is also prominent expression of CD40L by T-cells in LCH lesions suggesting potential interactions between these cell types. The CD40-CD40L interaction leads to up-regulation of two other co-stimulatory molecules involved in antigen presentation, CD80 and CD86 (B7-2), also expressed by LCH cells (Emile *et al*., 1994a; Egeler *et al.*, 2000). However, another study showed that although CD80 expression was frequently detected, CD86 was not present on the majority of LCH cells in most bone and some

skin lesions (Geissmann *et al.*, 2001). This suggests that the functional ability of LCH cells may differ between restricted skin lesions and disseminated ostotic lesions. Whether productive antigen presentation to T-cells is occurring at all, is questionable as MHC class II expression is only moderate (Geissmann *et al.*, 2001).

Although LCH cells have been described as undergoing a 'maturation block' (Annels *et al.*, 2003; Laman *et al.*, 2003), several reports have observed expression of more mature DC markers by LCH cells. In one such study, DC-LAMP and CD83, markers of mature DCs, were found to be expressed by scattered LCH cells in LCH lesions. Interestingly, cases of skin-only LCH showed higher expression of CD83 and DC-LAMP than bone lesions. Moreover, the highest DC-LAMP positivity was evident in the spontaneously regressive form of the disease, suggesting that these cells had overcome any potential blockade in their maturation resulting in resolution of the disease (Geissmann *et al.*, 2001).

Another marker of LC activation is fascin, a highly selective marker for DCs of lymphoid tissues and peripheral blood, and completely absent from normal epidermal LCs. This actin bundling protein is involved in the formation of dendritic processes in maturing epidermal LCs. Fascin expression has been shown to correlate with dendritic morphology, cell differentiation and antigen-presenting activity of normal DCs (Ross *et al.*, 1998; Ross *et al.*, 2000; Al-Alwan *et al.*, 2001). In contrast, despite being positive for fascin, LCH cells have been shown to be functionally defective in antigen presentation *in vitro* (Geissmann *et al.*, 2001). Thus, the fascin positivity of LCH cells represents another aberration in their phenotype (Pinkus *et al.*, 2002).

One of the abnormal features of LCH cells is their occurrence at sites where normal LCs do not usually reside, e.g. bone. Aberrant migration and homing of LCs resulting from the expression of cellular adhesion molecules may play a role in the pathogenesis of LCH. Several investigations have shown that LCH cells indeed express different adhesion molecules from normal LCs (Ruco *et al.*, 1993; de Graaf *et al.*, 1994, 1995). CD54 (ICAM-1), CD58 (LFA-3), and the β1 integrin α4, adhesion molecules that are expressed during activation of normal LCs, were shown to be up-regulated in LCH lesions. In addition, adhesion molecules not found on normal LCs such as CD2, CD11a and CD11b, could be demonstrated on LCH cells in a number of cases (de Graaf *et al.*, 1995). The aberrant expression of these molecules may result in homotypic adhesion of LCH cells through ligand binding of CD2 to CD58 or CD11a to CD54. Another molecule found to be aberrantly expressed by LCH cells was CD62L. CD62L is only found in LCs in normal skin and is shed from monocytes after activation (Stibenz & Buhrer, 1994). Thus, the expression of CD62L by LCH cells is peculiar, as these cells are thought to be in an early activated state. One can speculate that the aberrant activation of LCH cells results in failure to shed CD62L (de Graaf *et al.*, 1995).

Normal LCs express high levels of the homophilic adhesion molecule E-cadherin and undergo E-cadherin-dependent adhesion with epidermal keratinocytes (Tang *et al.*, 1993). E-cadherin expression

is markedly down-regulated upon the migration and maturation of epidermal LCs (Borkowski *et al.*, 1994). A study carried out to investigate whether or not E-cadherin expression correlates with the clinical outcome of LCH showed that the LCH cells of seven children with skin-only involvement were positive for E-cadherin, whereas seven children who developed disseminated LCH displayed negative or low expression (Geissmann *et al.*, 1997). It has already been observed that E-cadherin down-regulation in many carcinomas correlates with tumour cell metastasis. Thus, one can speculate that down-regulation of E-cadherin surface expression by LCH cells, similarly correlates with the occurrence of dissemination of the disease.

Based on the phenotypic studies carried out to date, it is clear that LCH cells display an abnormal phenotype that includes characteristics of both normal epidermal LCs and activated LCs. This phenotype is indicative of LCH cells being in an arrested state of activation and/or differentiation. Whether this phenotype is due to a dysregulated immune response to an antigenic stimulus, or is a reflection of an intrinsic defect of LCH cells, remains unknown.

**The immune function of LCH cells**

Since LCH cells display such an abnormal phenotype, in which immature markers coexist with adhesion molecules and antigen presentation markers, it seems logical that these cells also display defective functional capabilities. The presence of many T-cells within LCH lesions raises the question of whether there is an immune interaction between these cells and LCH cells. To date only a few studies have assessed the functional activity of LCH cells, due to the paucity of fresh material.

Normal LCs are potent antigen-presenting cells (APCs) and activators of T cells (Banchereau & Steinman, 1998). In order to determine whether indeed LCH cells function as APCs, an early study used highly purified LCH cells as stimulator cells in an allogeneic mixed cell reaction (Yu *et al.*, 1995). This study, of three LCH patients, showed that the antigen-presenting capacity of LCH cells, derived from four different organs affected by LCH namely skin, lymph node, bone and gum, was greatly reduced when compared to normal epidermal LCs. This result was in keeping with an earlier study from the same group, in which lesional LCH cells from a fatal case of LCH also displayed poor alloantigen-presenting activity *in vitro* (Yu *et al.*, 1992).

Several receptors and their ligands are involved in dendritic cell/T-cell interaction. The CD40-CD40L pathway is an integral part of this bi-directional communication. The ligation of CD40 expressed by DCs, is an early and pivotal signal for the up-regulation of antigen-presenting functions by these cells (Laman *et al.*, 1996; Van Kooten & Banchereau, 1996). In addition, ligation of CD40L expressed by activated CD4+ T cells is crucial to T cell priming and cytokine production (Peng *et al.*, 1996).

In a more recent study, LCH cells under the stimulation of CD40L were shown to be able to mature *in vitro* and acquire potent immunostimulatory characteristics (Geissmann *et al.*, 2001). In this study, sorted CD1a+ LCH cells and control immature DCs, were cultured with CD40L or CD32-transfected fibroblasts for two days before being added to allogeneic lymphocytes. Although LCH cells and immature DCs cultured with CD32-transfected cells retained an immature phenotype and stimulated lymphocyte proliferation equally poorly, both LCH cells and the control cells stimulated with CD40L, expressed high membrane MHC class II and CD86, and showed strong capacity to stimulate lymphocytes. Interestingly, in the spontaneously regressive form of the disease, including self-healing cutaneous lesions, LCH cells frequently exhibit the expression of mature markers such as CD86 and DC-LAMP in situ, suggesting that they represent more mature DCs (Geissmann *et al.*, 2001).

It is still unclear why LCH cells *in vivo* appear to have reduced functional activity. The fact that this defect appears to be confined to LCH cells and does not affect all cells of the DC lineage in LCH patients (Holter *et al.*, 2002), plus the ability of LCH cells to mature *in vitro*, suggest that the lesional microenvironment may be having a suppressive effect on the LCH cells. The detection of IL-10, a cytokine capable of down-regulating the expression of B7 molecules and class II antigens by DCs, in bone and lymph node lesions, but not in skin lesions from patients with limited or self-healing disease (Geissmann *et al.*, 2001), perhaps gives further evidence that the cytokine environment is the extrinsic factor affecting the differentiation and functional capabilities of LCH cells *in vivo*.

**The role of cytokines in LCH**

A central feature of normal immunologic regulation involves the production and local action of cytokines. This action is normally short-lived however. In cases of immunological dysregulation, as is thought to occur in LCH, the over-production of cytokines can lead to pathological consequences. Indeed, LCH is characterized by a lesional 'cytokine storm', a term referring to both the high level and diversity of cytokines produced locally (Egeler *et al.*, 1999).

In LCH lesions, the predominant sources of this 'cytokine storm' are the T cells and LCH cells. LCH cells produce high levels of a range of cytokines, including pro-inflammatory (interleukin-1α (IL-1α)) and interferon-γ (IFN-γ)), anti-inflammatory (IL-10), and growth factors (e.g.GM-CSF).

The pro-inflammatory cytokine IFN-γ is known to be produced by activated NK cells and in larger amounts by effector T cells, and thus appears mainly after the induction of an adaptive immune response. Elevated levels of IFN-γ are also expressed by T lymphocytes and LCH cells in LCH lesions. In fact, IFN-γ has been reported to be a marker of LCH cells in the skin (Neumann *et al.*,

1988). However, IFN-γ+ LCH cells were also found in bone and lymph node lesions, suggesting that IFN-γ is not a specific marker of skin LCH cells (de Graaf *et al.*, 1996). The fact that IFN-γ was found to be highly expressed in LCH lesions suggests that it possibly plays a stimulatory role on LCH cells such as enhancing their IL-1 secretory capacity (Arenzana-Seisdedos *et al.*, 1986).

Indeed, IL-1α, IL-1β and another pro-inflammatory cytokine, TNF-α, were shown to be present in high amounts in LCH lesions (de Graaf *et al.*, 1996; Egeler *et al.*, 1999). This finding may help explain the osteolytic capacity of LCH cells, as these cytokines are known to activate osteoclastic bone resorption (Kudo *et al.*, 2002). Multinucleated giant cells resembling osteoclasts, one of the cell types described in LCH lesions, may originate from macrophage activation under such IL-1 and TNF-α influence. Besides the expression of IL-1 in LCH lesions, a study showed elevated levels of IL-1 receptor antagonist (IL-1Ra), a naturally occurring IL-1 antagonist, in LCH patients. Whereas the IL-1/IL-1R complex triggers several inflammatory events, such as cyclooxigenase-2 induction and the production of prostaglandin E2 (Arend *et al.*, 1998), the IL-1Ra/IL-R complex on the cell membrane does not induce any response. The role of IL-1Ra in the pathophysiology of LCH is however unknown. Two main hypotheses have been proposed: IL-1Ra is a primary product of abnormal DCs, or it is produced by normal cells in an attempt to cope with LCH and its manifestations (Rosso *et al.*, 2003).

Another pro-inflammatory cytokine highly expressed in LCH lesions is interleukin-2 (IL-2). IL-2 is involved in the interaction of antigen-presenting LCs with T lymphocytes, being involved in T-cell activation as well as in programmed cell death. A study has shown that the IL-2 receptor (IL-2R) is expressed by LCH cells, suggesting that LCs are activated and induced to proliferate in LCH lesions (Barbey *et al.*, 1987; Emile *et al.*, 1994a). Furthermore, elevated amounts of soluble IL-2R (sIL-2R) were found in the sera of seven children with various forms of LCH (Schultz *et al.*, 1998). sIL-2R is capable of binding to IL-2, potentially inhibiting the normal immune response by occupying the binding region of this T cell derived cytokine.

In contrast to the pro-inflammatory cytokines, anti-inflammatory cytokines such as TGF-β or IL-10 may prevent LC maturation. In particular, IL-10 is a cytokine capable of down-regulating the expression of B7 molecules and class II antigens by DCs *in vitro* (Ozawa *et al.*, 1996). Two recent studies report conflicting results regarding the expression of IL-10 in LCH. In one study, both LCH cells and macrophages appeared to be the source of the IL-10 in 9 of 11 bone and lymph node biopsies (Egeler *et al.*, 1999). However, in a second study, IL-10-expressing cells in eosinophilic granuloma were predominantly large-sized CD3-, Langerin-, CD68+ cells, and therefore were neither LCH cells nor T cells, but macrophages (Geissmann *et al.*, 2001). In another study of adult lung patients, IL-10 could not be detected in five of five LCH lesions in which the LCH cells expressed CD86 (Tazi *et al.*, 1999). This difference between pulmonary and bone LCH lesions suggests that the different clinical picture characteristic of pulmonary LCH, where LCH cells present a more mature phenotype,

may be a consequence of the absence of this anti-inflammatory cytokine. Further evidence to support this hypothesis comes from the observation that macrophages are very rare in skin lesions from patients with limited or self-healing disease, and there are consistently no IL-10+ cells (Geissmann *et al.*, 2001). Another anti-inflammatory cytokine, TGF-β, has been shown to be present in LCH lesions. TGF-β is known to be involved in LC differentiation (Jaksits *et al.*, 1999). It has also been identified as the major player producing tissue fibrosis (Border & Noble, 1994), therefore explaining this outcome in LCH lesions.

Cytokines are also known to induce proliferation, differentiation and activation of normal LCs. One such cytokine is the growth factor GM-CSF. In three studies (Emile *et al.*, 1993, 1994b, 1995) GM-CSF was detected within the cytoplasm of all the LCH cells but not other cell types within the lesions. Children with disseminated active LCH but not localized (e.g. bone) LCH, had an elevated serum GM-CSF level. Additionally, LCH cells from all the samples stained positively with GM-CSFR-antibody. This suggests that GM-CSF may be a growth factor for LCH cells and that the GM-CSF level is related to the extent and activity of LCH.

The presence of this 'cytokine storm' probably explains the abnormal phenotype and function of LCH cells and may provide these cells with an optimal microenvironment to prolong their viability, possibly by creating autocrine loops. Thus it is highly likely that cytokines play a prominent role in the pathogenesis of LCH and may explain common phenomena such as osteolysis and fibrosis and the recruitment of typical inflammatory infiltrates.

**The role of chemokines in LCH**

Chemokines are chemoattractant molecules that determine the tissue distribution of many cell types. LCH lesions may be present in the skin or lymph nodes where one expects LCs, but also in many other sites. This inappropriate accumulation of LCs at various sites in LCH suggests an abnormality of cell trafficking. Several studies have demonstrated that the movement of LCs from the site of antigen-capture to the draining lymphoid organs involves selective chemokines which act on maturing LCs through particular chemokine receptors (Dieu *et al.*, 1998; Sozzani *et al.*, 1998).

Immature DCs respond to many chemokines, in particular CCL20, which to date appears to be the most powerful chemokine to induce migration of $CD34^{+}$-derived immature DCs (Dieu *et al.*, 1998). CCL20 mRNA expression seems to be restricted to epithelium and is upregulated by inflammation (Rossi *et al.*, 1997). Thus it is thought that during pathogen invasion, immature LCs expressing CCR6, the major functional CCL20 receptor, would be attracted to the site of inflammation through the local production of chemokines such as CCL20. After antigen uptake, the maturation of DC

results in a complete reprogramming of the cell, with down-regulation of endocytic activity (Sallusto *et al.*, 1995), up-regulation of MHC, adhesion and costimulatory molecules (Cella *et al.*, 1997b) as well as a striking switch in chemokine receptor usage (Sozzani *et al.*, 1998). The response to a set of chemokines, in particular to CCL20, is rapidly lost due to the down-regulation of CCR6 expression, thereby enabling LCs to escape the local gradient of CCL20. At the same time, maturing LCs start to express CCR7, resulting in attraction of these cells to CCL19 and CCL21 which are expressed in the T-cell zones of lymph nodes (Gunn *et al.*, 1998). Because these two chemokines can attract mature DC and lymphocytes, they are likely to play a key role in helping antigen-loaded DC to encounter specific naive T-cells.

Through our own work on LCH, we have demonstrated that lesional LCs are indeed in an immature state as defined by their expression of the chemokine receptor CCR6. Conversely, CCR7 expression appears to be absent on these cells, in keeping with the fact that LCH cells are hardly ever found in lymph nodes that drain the lesional sites. Thus, despite various inflammatory stimuli such as TNF-α, which should induce LC maturation, the lesional CD1a+ cells do not lose their expression of CCR6 and do not up-regulate CCR7 (Annels *et al.*, 2003). In contrast, other work has shown that the lesional CD1a+ cells have the intrinsic capability to fully differentiate and mature once removed from the lesion. (Geissmann *et al.*, 2001). The fact that they do not properly differentiate in situ, indicates that a factor/factors in LCH lesions prevents full differentiation. This, together with the high expression of the CCR6 ligand CCL20, in LCH lesions, probably prevents lesional CD1a+ cells from leaving their peripheral tissue sites and instead enhances their accumulation.

In contrast to our findings, Fleming recently reported coincident expression of the chemokine receptors CCR6 and CCR7 by pathologic LCH cells (Fleming *et al.*, 2003). One possibility for the discrepancy could be the type of lesion that was studied. It has been reported that in some LCH cases *in vivo*, most notably in self-healing cutaneous lesions, a more mature phenotype can be observed and LCH cells appear to down-regulate CD14 and up-regulate CD86 and DC-LAMP (Geissmann *et al.*, 2001). It may be that in these lesions, the LCH cells have partially overcome the maturation blockade and are able to downregulate CCR6 and up-regulate CCR7 as they mature. This would in turn release these cells from the local control of CCL20, and allow them to follow the normal lymphoid drainage pathways, thus explaining the spontaneous resolution which may be seen.

In our study of the chemokine/chemokine receptor interactions involved in LCH (Annels *et al.*, 2003), we showed that lesional CD1a+ cells express not only CCL20 but other inflammatory chemokines such as CCL5 and CXCL11. These chemokines are likely responsible for the recruitment of other inflammatory cell types, characteristic of LCH lesions. Indeed, as well as expressing CXCR3, the receptor for CXCL11, the infiltrating T-cells in LCH lesions also expressed CCR6. This finding along with the finding that CCL20 specifically attracts the memory subset of T-cells *in vitro* (Liao *et al.*,

1999), strongly implicates CCL20 as an important chemo-attractant responsible for T-cell recruitment in LCH lesions. In another study, CCL22 positive DCs were shown to be present in LCH lesions (Vulcano *et al.*, 2001). CCL22 is a constitutively produced DC chemokine known to be chemotactic for DCs, IL-2-activated NK cells and chronically activated T-lymphocytes (Godiska *et al.*, 1997. This finding suggests a role for CCL22 in co-localization and interaction of lesional CD1a+ cells and T-cells in LCH lesions. Thus, lesional CD1a+ cells, through their production of various chemokines, may not only be causing their own recruitment and retention, but that of other inflammatory cells as well.

In future studies it will be interesting to see if the same spectrum of chemokines and their receptors are expressed in spontaneously resolving lesions and in adult pulmonary LCH lesions.

**Cell cycle regulation and proliferation in LCH**

It is often assumed that the massive accumulation of LCs at the sites of LCH lesions results not only from aberrant chemoattraction, but also from abnormal local proliferation of these cells. This uncontrolled proliferation could be due to a neoplastic transformation of the LCH cells or to locally secreted cytokines (de Graaf *et al.*, 1996; Egeler *et al.*, 1999), perhaps as part of a dysregulated immune response. In order to address this, genetic and functional alterations, essential for tumorigenic growth (controlling proliferation and apoptosis), have been investigated.

Two recent studies measured the proliferative activity of LCs in LCH by studying the expression of the proliferation marker Ki-67 (Schouten *et al.*, 2002; Bank *et al.*, 2003). In both studies, and in agreement with an earlier study (Hage *et al.*, 1993), Ki-67 nuclear-positive LCs were found in all the lesions examined, ranging from a small number of clusters to the majority of the cells. As Ki-67 is expressed during all active phases of the cell cycle, but not in resting cells, this suggests that the cell cycle is not terminated in LCH cells. In addition to this finding, mitotic figures were observed in 34 of 61 evaluated specimens (Bank *et al.*, 2003), in keeping with a number of earlier reports (Shamoto, 1977; Pierard *et al.*, 1982; Risdall *et al.*, 1982; Ruco *et al.*, 1993), giving further support to the interpretation that LCH infiltrates have local proliferative activity.

Another element critically involved in the cellular pathways controlling proliferation, DNA repair, or apoptosis is the transcription factor p53. p53 regulates the normal cell cycle by activating transcription of genes that control progression through the cycle, and of other genes that cause arrest in G1 when the genome is damaged. In some cell types, p53 can also promote apoptosis (Lane, 1992). In normal cells, the p53 gene product is expressed at very low levels, undetectable by immunohistochemical methods because of rapid turnover. In contrast, mutant p53 proteins have a stable conformation,

resulting in the accumulation of the protein to detectable levels (Reich *et al.*, 1983). Several groups have investigated the expression of p53 (Bank *et al.*, 2002; Schouten *et al.*, 2002) as well as p53 gene mutations in LCH cells (Weintraub *et al.*, 1998). In all the studies p53 protein was detectable by immunohistochemistry specifically localized to the LCs in LCH lesions. Furthermore, p53 expression occurred in all cases of LCH studied, including patients with localized bone lesions and multi-system disease, indicating that this abnormal p53 expression is not limited to the most severe forms of LCH, but is also found in the mildest, frequently self-resolving forms. Mutations in the single copy p53 gene are the most frequent genetic changes associated with human cancers. However, by PCR/SSCP (polymerase chain reaction/single stranded conformational polymorphism), Weintraub *et al.* found no mutations in exons 4 to 11 of the p53 gene in LCH cells. Another mechanism that can cause abnormal expression of the p53 protein is stabilization of the protein as a result of binding of p53 to other proteins. One such protein is mdm2, an oncogene product, which binds to the transactivation domain of p53 and down-regulates its ability to activate transcription. Following DNA damage, the p53 protein induces the transcription of the mdm2 oncogene that in turn inhibits p53-dependent transcriptional activation, creating a feedback loop resulting in down-regulation of p53 activity. The study by Schouten *et al.* found heterogeneous over-expression of mdm2 by lesional LCs, probably induced by p53, and reflecting the existence of the auto-regulatory feedback loop between p53 and mdm2 (Schouten *et al.*, 2002).

Only one study so far has investigated the expression of a number of other key factors that control proliferation and apoptosis, namely p21, p16 and Rb. p53 activates p21 in response to DNA damage; p21 inhibits cell cycle progression at both G1 and G2 checkpoints (El Deiry *et al.*, 1994). Another important pathway for detecting DNA damage and inducing cell cycle arrest is the p16-Rb pathway. Both the p21 and p16-Rb pathways were found to be active in virtually all LCH cases studied (Schouten *et al.*, 2002).

As well as abnormal proliferation, the accumulation of LCH cells may also be due to disturbances in normal apoptosis (programmed cell death). The product of the Bcl-2 gene is an important regulator of apoptosis (Tsujimoto *et al.*, 1984), now recognized as a survival factor for many cell types. In LCH, over-expression of the Bcl-2 protein has been found (Savell *et al.*, 1998; Schouten *et al.*, 2002), possibly playing a role in the activation of p53 and p16 and subsequent arrest of apoptosis.

Correlating the results of these studies leaves a fairly conflicting picture of the cell proliferation and apoptotic pathways that may be involved in LCH. Indeed, both stimulatory and inhibitory pathways of cell proliferation and apoptosis appear to be upregulated. Survival and proliferation are probably supported by several mechanisms active in LCH. Firstly, proliferation of LCH cells is probably due to various cytokines present in LCH lesions. Secondly, the high-level of expression of Bcl-2 will inhibit apoptosis and thirdly, the observed mdm2 expression will inhibit p53 suppressive activity. Despite

the fact that these stimulation pathways result in a high level of cellular proliferation, there are also several counter-regulatory pathways active in LCH. The suppressive cytokine TGFβ is present, as are the TGFβ receptors (Schouten *et al.*, 2002) and downstream, the inhibitory p53-p21 and p16-Rb pathways are also activated. As the outcome is enhanced LCH cell proliferation and survival, despite the presence of negative regulators, it appears that the counter-regulatory pathways are unable or insufficient to keep the cells in check.

**LCH: A clonal proliferative disease**

Several studies that showed that LCH cells are intrinsically proliferative, (Hage *et al.*, 1993; Schouten *et al.*, 2002; Bank *et al.*, 2003) led to the question of whether LCH is a reactive polyclonal disorder or due to proliferation of a single LCH cell resulting in a clonal histiocytic disease. Studies that can detect clonal or polyclonal X chromosome inactivation patterns in female tissues, have been performed in LCH. These studies have unequivocally shown that the LCs from single system and multisystem LCH lesions are clonal (Willman *et al.*, 1994b). In a further study, clonality was shown in CD1a-positive cells, FACS sorted from lesions of females with multi-system disease (Yu *et al.*, 1994). The fact that clonal histiocytes were found in all forms of LCH, including clinically benign disease, led to the opinion that LCH is most likely a clonal neoplastic disease with highly variable biologic behaviour (Willman *et al.*, 1994a). The number of patients studied is small however, and it remains a controversial point. In future studies, it would be interesting to determine whether multiple samples from different sites, or from a single patient over-time, show identical clonality.
When clonality was assessed in adult pulmonary LCH, a unique form of the disease commonly affecting smokers in the third to fifth decades, the disease was found to be non-clonal (Yousem *et al.*, 2001). Thus, adult pulmonary LCH appears to be primarily a reactive process, possibly due to tobacco smoke-driven hyperplasia of LCH cells in which occasional clones may arise.

**LCH: A role for viruses?**

Regardless of whether the disease is monoclonal or polyclonal, the proliferation of LCH cells may be induced by some intrinsic mutation or by external factors such as a virus. Immature DCs typically respond to pathogen exposure by undergoing a maturation process that facilitates induction of further innate and adaptive immune responses (Banchereau *et al.*, 2000). Maturation is induced when immature DCs are exposed to pathogens such as Escherichia coli, Candida, and influenza virus or

to inflammatory cytokines (Banchereau *et al.*, 2000; Huang Q *et al.*, 2001). However, some viruses, such as HIV, vaccinia, measles and dengue viruses, interfere with DC function and maturation in order to evade immune surveillance (Grosjean *et al.*, 1997; Engelmayer *et al.*, 1999; Tortorella *et al.*, 2000; Izmailova *et al.*, 2003). In addition, viral infection of DCs can induce aberrant or uncontrolled cytokine production, a major feature of LCH (Moss, 1996; Braun *et al.*, 1997; Smith *et al.*, 1997). Thus, it is highly plausible that a viral infection may be a pathogenic factor causing the immunological abnormality in LCH.

In an early study nucleic acid extracts of biopsy specimens from LCH patients were injected into mice. Cell suspensions were subsequently made from the lungs of these animals and assayed for cytopathic effects on primary human embryo cultures. The presence of syncytia and nuclear changes were interpreted as evidence of a transmissible agent (Nastac *et al.*, 1970). Since then studies have detected the presence of HHV-6 (Leahy *et al.*, 1993) and CMV (Kawakubo *et al.*, 1999) in the LCH cells of LCH lesions. However, just as many published studies refute these findings (McClain *et al.*, 1994; McClain & Weiss, 1994; Jenson *et al.*, 2000). In one study, the presence of nine different viruses which commonly infect children, namely adenovirus, CMV, EBV, herpes simplex virus, HHV6, parvovirus, human T-cell viruses type I and II and HIV, was investigated. In situ hybridisation (ISH) and PCR performed on 56 cases of LCH did not result in consistent evidence of any viral genome and the study concluded that none of the viruses tested was responsible for LCH (McClain *et al.*, 1994).

In a recent publication, the presence of viral particles as well as DNA from the HHV-6B variant, which has been associated with disease in humans, was detected by immunohistochemistry and ISH in a large number of patients with LCH, again raising the possibility of a viral trigger (Glotzbecker *et al.*, 2004). However, in the same study a high prevalence of HHV-6 was also found in control tissues, thus the presence of a virus alone does not establish a causal role in LCH.

Viral infection as a causative factor of LCH has still not been proven. However, even if a virus is not the causative factor in LCH, this does not preclude the involvement of other microbial agents.

**LCH: A reactive or neoplastic disease?**

For decades it has been thought that LCH is a reactive rather than a neoplastic process. This was based on the absence of aneuploidy (Rabkin *et al.*, 1988), the failure to identify consistent cytogenetic alterations, and the occurrence of spontaneous clinical regression. However, the finding of proliferation of LCs in LCH lesions, has renewed the arguments that LCH may indeed be a neoplasm. This has been supported by the clonality studies quoted above. Despite the fact that clonality is widely considered to be characteristic of a neoplastic disease, there are several circumstances in which clonal

populations may be detected over several years without the development of a malignancy (Weiss *et al.*, 1986; Kurahashi *et al.*, 1991). Furthermore, adaptive immune responses are associated with clonal expansions that may be detected over many years and are certainly not malignant (Maini *et al.*, 1999). The fact that LCH cells are clonal however, raises the possibility that these cells may acquire somatic mutations in a gene or genes that regulate cell growth, survival, or proliferation. Indeed, there is now evidence of increased chromosomal breakage in patients with LCH, from investigations using comparative genomic hybridization (CGH) and other molecular methods (Betts *et al.*, 1998; Scappaticci *et al.*, 2000; Murakami *et al.*, 2002). Besides chromosomal instability, abnormal clones showing a t(7;12)(q11.2;p13) translocation have been observed (Betts *et al.*, 1998). These data strongly suggest that there is a component of genetic instability in LCH, as observed in some types of neoplasms and myelodysplastic disorders. Furthermore, as described, the cell-cycle regulation of LCH cells is severely disrupted (Schouten *et al.*, 2002; Bank *et al.*, 2002), probably as a result of a combination of both external signals (growth factors and cytokines), and intrinsic factors from as yet unidentified DNA changes. To test the hypothesis of malignancy, more rigorous studies are needed into the expression of oncogenes and antioncogenes by LCH cells. To date, only one such study has been carried out in which it was reported that the c-myc and H-ras proto-oncogenes were expressed in the active terminal phases of the disease, but not in the quiescent phase (Abdelatif *et al.*, 1990). However, now that the pathways and molecules involved in oncogenesis are much better defined, it is known that the expression of these oncogenes is associated with proliferative activity of any kind of cell.

Once the role and expression of oncogenes and their pathways in LCH has been fully assessed, the argument about a reactive *versus* a neoplastic disorder can finally be laid to rest.

**References**


- Abdelatif, O.M., Chandler, F.W, Pantazis, C.G., *et al*. (1990). Enhanced expression of c-myc and H-ras oncogenes in Letterer-Siwe disease. *Arch Pathol Lab Med*. **114**, 1254-1260.
- Al-Alwan, M.M., Rowden, G., Lee, T.D., *et al*. (2001). Fascin is involved in the antigen presentation activity of mature dendritic cells. *J Immunol*. **166**, 338-345.
- Annels, N.E., da Costa, C.E.T., Prins, F.A., *et al*. (2003). Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. *J Exp Med.* **197**, 1385-1390.
- Arceci, R.J., Longley, J. & Emanuel, P.D. (2002). Atypical cellular disorders. *Hematol*. 297-314.
- Arend, W.P., Malyak, M., Guthridge, C.J., *et al*. (1998). Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol*. **16**, 27-55.
- Arenzana-Seisdedos, F., Marbey, S., Virelizier, J.L., *et al*. (1986). Histiocytosis X. Purified (T6+) cells from bone granuloma produce interleukin 1 and prostaglandin E2 in culture. *J Clin Invest*. **77**, 326-329.
- Banchereau, J. & Steinman, R. (1998). Dendritic cells and the control of immunity. *Nature*. **392**, 245-252.
- Banchereau, J., Briere, F., Caux, C., *et al*. (2000). Immunobiology of dendritic cells. *Annu Rev Immunol*.

**18**, 767-811.
- Bank, M.I., Rengtved, P., Carstensen, H., *et al*. (2002). p53 expression in biopsies from children with Langerhans cell histiocytosis. *J Pediatr Hematol Oncol*. **24**, 733-738
- Bank, M.I., Rengtved, P., Carstensen, H., *et al*. (2003). An evaluation of histopathological parameters, demonstration of proliferation by Ki-67 and mitotic bodies. *Acta Pathol, Microbiol Immunol Scand*. **111**, 300-308.
- Barbey, S., Gane, P., Pelletier, O.L., *et al*. (1987). Histiocytosis X Langerhans cells react with antiinterleukin-2 receptor monoclonal antibody. *Pediatr Pathol*. 7, 569-574.
- Betts, D.R., Leibundgut, K.E., Feldges, A., *et al*. (1998). Cytogenetic abnormalities in Langerhans cell histiocytosis. *Br J Cancer*. **77**, 552-555.
- Border, W.A. & Noble, N.A. (1994). Transforming growth factor β in tissue fibrosis. *N Engl J Med*. **331**, 1286-1292.
- Borkowski, T.A., Van Dyke, B.J., Schwarzenberger, K., *et al*. (1994). Expression of E-cadherin by murine dendritic cells: E-cadherin as a dendritic cell differentiation antigen characteristic of epidermal Langerhans cells and related cells. *Eur J Immunol*. **24**, 2767-2774.
- Braun, D.K., Dominiquez, G.D. & Pellett, P.E. (1997). Human herpes virus 6. *Clin Microbiol Rev*. **10**, 521-567.
- Cella, M., Sallusto, F. & Lanzavecchia, A. (1997a). Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol*. **9**, 10-16.
- De Graaf, J.H., Tamminga, R.Y., Dam-Meiring, A., *et al*. (1996). The presence of cytokines in Langerhans' cell histiocytosis. J Pathol. 180, 400-406.
- De Graaf, J.H., Tamminga, R.Y.J., Kamps, W.A., *et al*. (1994) Langerhans cell histiocytosis: expression of leukocyte cellular adhesion molecules suggests abnormal homing and differentiation. *Am J Pathol*. **144**, 466-472.
- De Graaf, J.H., Tamminga, R.Y.J., Kamps, W.A., *et al*. (1995). Expression of cellular adhesion molecules in Langerhans cell histiocytosis and normal Langerhans cells. *Am J Pathol***. 147**, 1161-1171.
- Dieu, M.C., Vanbervliet, B., Vicari, A., *et al*. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med*. **188**, 373-386.
- Egeler, R.M., Annels, N.E. & Hogendoorn, P.C. (2004). Langerhans cell histiocytosis: a pathologic combination of oncogenesis and immune dysregulation. *Pediatr Blood Cancer*. **42**, 401-403.
- Egeler, R.M., Favara, B.E., Laman, J.D., *et al*. (2000). Abundant expression of CD40 and CD40-ligand (CD154) in paediatric Langerhans cell histiocytosis lesions. *Eur J Cancer*. **36**, 2105-2110
- Egeler, R.M., Favara, B.E., van Meurs, M., *et al*. (1999). Differential in situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. *Blood.* **94**, 4195-4201.
- El Deiry, W.S., Harper, J.W., O'Connor, P.M., *et al*. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res*. 54, 1169-1174.
- Emile, J.F., Fraitag, S., Leborgne, M., *et al*. (1994a). Langerhans cell histiocytosis cells are activated Langerhans cells. *J Pathol*. **174**, 71-76.
- Emile, J.F., Fraitag, S., Pascale, A., *et al*. (1995). Expression of GM-CSF receptor by Langerhans cell histiocytosis cells. *Virchows Arch*. **427**, 125-129.
- Emile, J.F., Peuchmaur, M., Fraitag, S., *et al*. (1993). Immunohistochemical detection of granulocyte/ macrophage colony-stimulting factor in Langerhans cell histiocytosis. *Histopathol*. 23, 327-332.
- Emile, J.F., Tartour, E., Brugieres, L., *et al*. (1994b). Detection of GM-CSF in the sera of children with Langerhans cell histiocytosis. *Pediatr Allergy Immunol*. **5**,162-163.
- Engelmayer, J., Larsson, M., Subklewe, M., *et al*. (1999). Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J Immunol*. **163**, 6762-6768.
- Fleming, M.D., Pinkus, J.L., Alexander, S.W., *et al*. (2003). Coincident expression of the chemokine receptors CCR6 and CCR7 by pathologic Langerhans cells in Langerhans cell histiocytosis. Blood. 101, 2473-2475.

- Geissmann, F., Emile, J.F., Andry, P., *et al*. (1997). Lack of expression of E-cadherin is associated with dissemination of Langerhans cell histiocytosis and poor outcome. *J Pathol*. **181**, 301-304.
- Geissmann, F., Lepelletier, Y., Fraitag, S., *et al*. (2001). Differentiation of Langerhans cells in Langerhans cell histiocytosis. *Blood*. **97**, 1241-1248.
- Glotzbecker, M.P., Carpentieri, D.F. & Dormans, J.P. (2004). Langerhans cell histiocytosis: a primary viral infection of bone? Human herpesvirus 6 protein detected in lymphocytes from tissue of children. *J Pediatr Orthop*. **24**,123-129.
- Godiska, R., Chantry, D., Raport, C.J., *et al*. (1997). Human macrophage derived chemokine (MDC) a novel chemoattractant for monocytes, monocyte derived dendritic cells, and natural killer cells. *J Exp Med*. **185**, 1595-1604.
- Grosjean, I., Caux, C., Bella, C., *et al*. (1997). Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells. *J Exp Med*. **186**, 801-812.
- Gunn, M.D., Tangemann, K., Tam, C., *et al*. (1998). A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naïve T lymphocytes. *Proc Natl Acad Sci USA*. **95**, 258-263.
- Hage, C., Willman, C.L., Favara, B.E., *et al*. (1993). Langerhans cell histiocytosis (histiocytosis X): immunophenotype and growth fraction. *Hum Pathol*. **24**, 840-845.
- Holter, W., Ressmann, G., Grois, N., *et al*. (2002). Normal monocyte-derived dendritic cell function in patients with Langerhans-cell-histiocytosis. *Med Pediatr Oncol*. **39**, 181-186.
- Huang, Q., Liu, D., Majewski, P., *et al*. (2001). The plasticity of dendritic cell responses to pathogens and their components. *Science*. **294**, 870-875.
- Izmailova, E., Bertley, F.M.N., Huang, Q., *et al*. (2003). HIV-1 Tat reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages. *Nat Med*. **9**, 191-197.
- Jaksits, S., Kriehuber, E., Charbonnier, A.S., *et al*. (1999). CD34+ cell-derived CD14+ precursor cells develop into Langerhans cells in a TGF-beta 1-dependent manner. *J Immunol*. **163**, 4869-4877.
- Jenson, H.B., McClain, K.L., Leach, C.T., *et al*. (2000). Evaluation of human herpesvirus type 8 infection in childhood Langerhans cell histiocytosis. *Am J Hematol*. **64**, 237-241.
- Kawakubo, Y., Kishimoto, H., Sato, Y., *et al*. (1999). Human cytomegalovirus infection in foci of Langerhans cell histiocytosis. *Virchows Arch*. **434**, 109-115.
- Kudo, O., Fujikawa, Y., Itonaga, I., *et al*. (2002). Proinflammatory cytokine (TNF alpha/ IL-1 alpha) induction of human osteoclast formation. *J Pathol*. **198**, 220-227.
- Kurahashi, H., Hara, J. & Yumura-Yagi, K. (1991). Monoclonal nature of transient abnormal myelopoiesis in Down's syndrome. Blood. 77, 1161-1163.
- Laman, J.D., Claassen, E. & Noelle, R.J. (1996). Functions of CD40 and its ligand, gp39 (CD40L). *Crit Rev Immunol*. **16**, 59-108.
- Laman, J.D., Leenen, P.J., Annels, N.E., *et al*. (2003). Langerhans-cell histiocytosis 'insight into DC biology'. *Trends Immunol*. **24**, 190-196.
- Lane, D.P. (1992). Cancer. p53, guardian of the genome. *Nature*. **358**, 15-16.
- Leahy, M.A., Krejci, S.M., Friednash, M., *et al*. (1993). Human herpesvirus 6 is present in lesions of Langerhans cell histiocytosis. *J Invest Dermatol*. **101**, 642-645.
- Liao, F., Rabin, R.L., Smith, C.S., *et al*. (1999). CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. *J Immunol*. **162**, 186-194.
- Maini, M.K., Casorati, G., Dellabona, P., *et al*. (1999). T cell clonality in immune responses. *Immunol Today*. **20**, 262- 266.
- McClain, K., Jin, H., Gresik, V., *et al*. (1994). Langerhans cell histiocytosis: lack of a viral etiology. *Am J Hematol*. **47**, 16-20.
- McClain, K. & Weiss, R.A. (1994). Viruses and Langerhans cell histiocytosis: is there a link? *Br J Cancer*. **70**, S34-S36.
- Moss, B. (1996). Poxviridae: the viruses and their replication. In Fields Virology, 3rd Ed. B.N. Fields, D.M.

Knipe, and P.M.Howley, eds. Lipincott-Raven, Philadelphia, p.26-37.
- Murakami, I., Gogusev, J., Fournet, J.C., *et al*. (2002). Detection of molecular cytogenetic aberrations in langerhans cell histiocytosis of bone. *Hum Pathol*. **33,** 555-560.
- Nastac, E., Athanasiu, P., Lungu, M., *et al*. (1970). Experimental investigations in human eosinophilic granuloma. *Rev Roumaine Inframicrobiol*. **7,** 125-149.
- Neumann, C., Schaumberg-Lever, G., Dopfer, R., *et al*. (1988). Interferon gamma is a marker for histiocytosis X cells in the skin. *J Invest Dermatol*. **91**, 280-282.
- Nezelof, C. & Basset, F. (2004). An hypothesis. Langerhans cell histiocytosis: the failure of the immune system to switch from an adaptive mode. *Pediatr Blood Cancer*. **42**, 398-400.
- Ozawa, H., Aiba, S., Nakagawa, S., *et al*. (1996). Interferon-γ and interleukin-10 inhibit antigen presentation by Langerhans cells for T helper type 1 cells by suppressing their CD80 (B7-1) expression. *Eur J Immunol*. **26**, 648-652.
- Peng, X., Kasran, A., Warmerdam, P.A., *et al*. (1996). Accessory signalling by CD40 for T cell activation: induction of Th1 and Th2 cytokines and synergy with interleukin-12 for interferon-gamma production. *Eur J Immunol*. **26**, 1621-1627.
- Pierard, G.E., Franchimont, C. & Lapiere, C.M. (1982). Proliferation of the characteristic histiocyte of histiocytosis X in the skin. Am J Dermatopathol. 4, 215-221.
- Pinkus, G.S., Lones, M.A., Matsumura, F., *et al*. (2002). Immunohistochemical expression of fascin, a dendritic cell marker. *Hematopathol*. **118**, 335-343.
- Rabkin, M.S., Wittwer, C.T., Kjeldsberg, C.R., *et al*. (1988). Flow-cytometric DNA content of histiocytosis X (Langerhans cell histiocytosis). *Am J Pathol*. **131**, 283-289.
- Reich, N.C., Oren, M. & Levine, A.J. (1983). Two distinct mechanisms regulate the levels of a cellular tumour antigen, p53. *Mol Cell Biol*. **3**, 2143-2150.
- Ricciardi-Castagnoli, P. & Granucci, F. (2002). Interpretation of the complexity of innate immune responses by functional genomics. *Nat Rev Immunol*. **2**, 881-888.
- Risdall, R.J., Dehner, L.P., Duray, P., *et al*. (1982). Histiocytosis X (Langerhans cell histiocytosis): prognostic role of histopathology. *Arch Pathol Lab Med*. **107**, 59-63.
- Ross, R., Ross, X.L., Schwing, J., *et al*. (1998). The actin-bundling protein fascin is involved in the formation of dendritic processes in maturing epidermal Langerhans cells. *J Immunol*. **160**, 3776-3782.
- Ross, R., Jonileit, H., Bros, M., *et al*. (2000). Expression of the actin-bundling protein fascin in cultured human dendritic cells correlates with dendritic morphology and cell differentiation. *J Invest Dermatol*. **115**, 658-663.
- Rossi, D.L., Vicari, A.P., Franz-Bacon, K., *et al*. (1997). Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3alpha and MIP-3beta. *J Immunol*. **158**, 1033-1036.
- Rosso, D.A., Ripoli, M.F., Roy, A., *et al*. (2003). Serum levels of interleukin-1 receptor antagonist and tumor necrosis factor-alpha are elevated in children with Langerhans cell histiocytosis. *J Pediatr Hematol Oncol*. **25**, 480-483.
- Ruco, L.P., Stoppacciaro, A., Vitolo, D., *et al*. (1993). Expression of adhesion molecules in Langerhans cell histiocytosis. *Histopathol*. **23**, 29-37.
- Sallusto, F., Cella, M., Danieli, C., *et al*. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex II compartment: downregulation by cytokines and bacterial products. *J Exp Med*. **182**, 389-400.
- Savell, V.H. Jr., Sherman, T., Scheuermann, R.H., *et al*. (1998). Bcl-2 expression in Langerhans cell histiocytosis. *Pediatr Develop Pathol*. **1**, 210-215.
- Scappaticci, S., Danesino, C., Rossi, E., *et al*. (2000). Cytogenetic abnormalities in PHA-stimulated lymphocytes from patients with Langerhans cell histiocytosis. *Br J Hematol*. **111**, 258-262.
- Schouten, B., Egeler, R.M., Leenen, P.J.M., *et al*. (2002). Expression of cell cycle-related gene products in Langerhans cell histiocytosis. *J Pediatr Hematol Oncol*. **24**, 727-732.
- Schultz, C., Klouche, M., Friedrichsdorf, S., *et al*. (1998). Langerhans cell histiocytosis in children: does soluble interleukin-2-receptor correlate with both the disease extent and activity? *Med Pediatr Oncol*. **31**,

61-65.
- Shamoto, M. (1977). Mitotic histiocytes and intranuclear Langerhans cell granules in histiocytosis X. *Virchows Arch B: Cell Pathol*. **24**, 87-90.
- Smith, G.L., Symons, J.A., Khanna, A., *et al*. (1997). Vaccinia virus immune evasion. *Immunol Rev*. **159**, 137-154.
- Sozzani, S., Allavena, P., D'Amico, G., *et al*. (1998). Differential regulation of chemokine receptors during dendritic cell maturation: A model for their trafficking properties. *J Immunol*. **161**, 1083-1086.
- Stibenz, D. & Buhrer, C. (1994). Down-regulation of L-selectin surface expression by various leukocyte isolation procedures. *Scand J Immunol*. **39**, 59-63.
- Tang, A., Amagai, M., Granger, L.G., *et al*. (1993). Adhesion of epidermal Langerhans cells to keratinocytes mediated by e-cadherin. *Nature*. **361**, 82-85.
- Tazi, A., Moreau, J., Bergeron, A., *et al*. (1999). Evidence that Langerhans cells in adult pulmonary Langerhans cell histiocytosis are mature dendritic cells: importance of the cytokine microenvironment. *J Immunol*. **163**, 3511-3515.
- Tortorella, D., Gewurz, B.E., Furman, M.H., *et al*. (2000). Viral subversion of the immune system. *Ann Rev Immunol*. **18**, 861-926.
- Tsujimoto, Y., Yunis, J., Onorato-Showe, L., *et al*. (1984). Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukaemias with the t(11;14) chromosome translocation. *Science*. **224**, 1403-1406.
- Van Kooten, C. & Banchereau, J. (1996). CD40-CD40L ligand: a multifunctional receptor-ligand pair. *Adv Immunol*. **61**, 1-77.
- Vulcano, M., Albanesi, C., Stoppacciaro, A., *et al*. (2001). Dendritic cells as a major source of macrophage-derived chemokine/CCL22 *in vitro* and *in vivo*. *Eur J Immunol*. **31**, 812-822.
- Weintraub, M., Bhatia, K.G., Chandra, R.S., *et al*. (1998). p53 expression in Langerhans cell histiocytosis. *J Pediatr Hematol Oncol*. **20**, 12-17.
- Weiss, L.M., Wood, G.S. & Trela, M. (1986). Clonal T cell populations in lymphomatoid papulosis: evidence of a lymphoproliferative origin for a clinically benign disease. *N Engl J Med*. **315**, 475-479.
- Willman, C.L. (1994a) Detection of clonal histiocytes in Langerhans cell histiocytosis: biology and clinical significance. *Br J Cancer*. **23**, 29-33.
- Willman, C.L., Busque, L., Griffith, B.B., *et al*. (1994b). Langerhans'-cell histiocytosis (histiocytosis X): a clonal proliferative disease. *N Engl J Med*. **331**, 154-160.
- Yousem, S.A., Colby, T.V., Chen, Y-Y., *et al*. (2001). Pulmonary Langerhans'cell histiocytosis. Molecular analysis of clonality. *Am J Surg Pathol*. **25**, 630-636.
- Yu, R.C., Alaibac, M. & Chu, A.C. (1995). Functional defect in cells involved in Langerhans cell histiocytosis. *Arch Dermatol Res*. **287**, 627-631.
- Yu, R.C., Chu, C., Buluwela, L., *et al*. (1994). Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis. *Lancet*. **343**, 767-768.
- Yu, R.C., Morris, J.F., Pritchard, J., *et al*. (1992). Defective alloantigen-presenting capacity of 'Langerhans cell histiocytosis cells'. *Arch Dis Child*. **67**, 1370-1372.

# 3

# Aberrant Chemokine Receptor Expression and Chemokine Production by Langerhans Cells underlies the Pathogenesis of Langerhans Cell Histiocytosis

Nicola E Annels
Cristiana ET da Costa
Frans A Prins
Annemieke Willemze
Pancras CW Hogendoorn
R Maarten Egeler

*The Journal of Experimental Medicine*

2003; 197: 1385-1390

**Abstract**

Langerhans cell histiocytosis (LCH) is characterised by a clonal proliferation and retention of cells with a Langerhans cell (LC)-like phenotype at various sites within the body. Due to the important role that migration plays in the normal functioning and life cycle of dendritic cells, the present study set out to elucidate whether aberrant expression of chemokine receptors or dysregulation of chemokine production in LCH lesions could explain abnormal retention of these cells. Immunohistochemical analysis on 13 LCH biopsies of bone, skin and lymph node all expressed the immature dendritic cell (DC) marker CCR6 on the lesional LCs and absence of the mature DC marker CCR7. Furthermore, regardless of the tissue site, LCH lesions markedly overexpressed CCL20/MIP-3α, the ligand for CCR6. The lesional LCs themselves appeared to be the source of this CCL20/MIP-3α production as well as other inflammatory chemokines such as CCL5/RANTES and CXCL11/I-TAC. These may explain the recruitment of eosinophils and CD4+CD45RO+T cells commonly found in LCH lesions. The findings of this study emphasize that, despite the abundance of TNF-α, lesional LCs remain in an immature state and are induced to produce chemokines, which via autocrine and paracrine mechanisms cause not only the retention of the lesional LCs but also the recruitment and retention of other lesional cells. We postulate that the lesional LCs themselves control the persistence and progression of LCH.

**Introduction**

Langerhans Cell Histiocytosis (LCH), a rare disorder often presenting during childhood, is uniquely characterized by a clonal proliferation of CD1a+ dendritic Langerhans cells (LCs) (1). Signs and symptoms of LCH can be explained by the existence of the granulomatous lesions, not only present in skin or lymph node, where LCs normally reside, but also in many other sites like bone marrow, lung and liver (2). Other inflammatory cells may also accumulate within the lesions, such as eosinophils, T cells and macrophages. Particularly the described lesional ‘’cytokine storm’’ with LCH cells and T cells as major producers are accountable for the more systemic symptoms like fever, failure to thrive, as well as for the well-known sequellae like osteolysis and fibrosis leading to organ dysfunction (3). Despite the rarity of this disease, with an annual incidence in the pediatric age range estimated at 2-5 per $10^6$/ year, studies on LCH should help contribute to our understanding of human *in vivo* dendritic cell (DC) biology.

Due to the integral role that migration plays in the normal function and distribution of LCs as well as the other lesional cells, it seems possible that dysregulation of chemokine production and/or chemok-

ine receptor expression plays a role in LCH. Chemokines have already emerged as major regulators of DC migration (4-8). DC subsets express a distinct pattern of functional chemokine receptors at different stages of their maturation. Immature DC express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5, CCR6 and CXCR1 which enable the recruitment of immature DCs to sites of inflammation where cognate ligands are produced. Maturation of DCs is associated with the coordinated down-regulation of receptors for inflammatory chemokines and the up-regulation of receptors for constitutive chemokines such as CXCR4 and CCR7. This results in the responsiveness of these cells to lymphoid chemokines causing the migration of mature DCs to draining lymph nodes where they are effective at activating naïve and central memory T cells (9,10).

As well as responding to chemokines, DCs also produce both constitutive and inflammatory chemokines depending upon their stage of maturation. Immature DCs release the constitutive chemokines CCL22/MDC and CCL17/TARC (11). At early stages of maturation, DCs produce high levels of inflammatory chemokines such as CCL20/MIP-3α, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL8/IL-8 and CXCL10/IP-10. These chemokines will help to recruit both circulating immature DCs as well as other immune cell types to inflamed tissue (12). At later time points in DC maturation, constitutive chemokines are selectively up-regulated including CCL19/MIP-3β, CCL17/TARC and CCL22/MDC (13).

There is some evidence that the CD1a+ cells in LCH are in an arrested state of activation and/or differentiation and thus act like immature DCs (14-16). However, it remains to be determined whether this arrest is also reflected at the level of chemokine receptor expression. In addition, abnormal chemokine receptor expression could explain the aberrant accumulation of the LC-like cells in these lesions. Furthermore, dysregulated production of chemokines by the CD1a+ LCH cells might lie at the bottom of why various other inflammatory cell types accumulate in these lesions.

In the present study we show that all lesional CD1a+ LCH cells express CCR6 and not CCR7 confirming that LCH cells are indeed of an immature phenotype. In addition these CD1a+ cells appear to be a major source of CCL20/MIP-3α. Finally, evidence is presented that, although other chemokines are present as well, T cells may be recruited to and/or retained in the lesions using the same CCR6-CCL20/MIP-3α receptor-ligand pair.

## Materials and Methods

### *Tissue*

Paraffin blocks of tissues from 13 patients with LCH were identified by pathologists at Leiden University Medical Center, which acts as a reference centre for bone tumours. In all cases the diagnosis

was reviewed and confirmed by immunohistochemistry for S100 and CD1a. All biopsies showed the presence of characteristic lesions containing histiocytes, macrophages, lymphocytes, and eosinophil granulocytes. Nine of the specimens were from bone in cases of ostotic LCH, two were from skin biopsies in cases of isolated skin disease and two were from excisional lymph node biopsies from patients with solitary lymph node involvement.

*Reagents*

Secondary antibodies were from DakoCytomation, and substrate chemicals were from Vector Laboratories. Secondary immunofluorescent reagents were goat anti-mouse and goat anti-rabbit isotype specific Alexa Fluor antibodies (Molecular Probes, NL).

*Immunohistochemistry*

Paraffin sections were cut at 4 μm and placed onto aminopropyltriethoxysilane coated slides. The sections were dried overnight at 37°C, dewaxed, and rehydrated. Endogenous peroxidase was blocked using methanol/0.3% $H_20_2$ for 20 minutes. The sections were then subjected to heat mediated antigen retrieval in a microwave using either citrate buffer (10 mM, pH 6.0) or EDTA buffer (1 mM, pH 8.0).

Primary antibodies were diluted in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and incubated overnight at room temperature in a humidity chamber. The bound primary antibodies were detected using several approaches. Single staining with antibodies specific for chemokines was detected enzymatically using either MouseEnvision or a rabbit anti-goat-HRP antibody followed by VECTOR NovaRed detection. Double and triple staining with primary anti-chemokine receptors in combination with cell-specific markers was detected fluorescently using the relevant secondary goat anti-mouse or goat anti-rabbit isotype-specific Alexa Fluor 488, Alexa Fluor 647 or Alexa Fluor 546 antibodies. To test the specificity of immunostaining, primary antibodies were omitted or replaced by an isotype-matched control antibody. Under these conditions no positive cells were identified. In addition, sections of suitable tissues were used as positive controls (Table 1).

*Immunogold Labelling*

In order to carry out double staining of CD1a+ cells and chemokines, immunofluorescent staining of CCL20/MIP-3α was combined with immunogold labelling of CD1a+ cells. As both primary antibodies were mouse IgG1, the anti-human CCL20/MIP-3α was applied after direct labelling with Alexa Fluor 488 using a monoclonal antibody labelling kit (A-20181, Molecular Probes, NL). The first primary antibody, CD1a, was diluted in 0.1% cationic BSA (Aurion, NL) in PBS, and the incubation was performed overnight at room temperature in a humidity chamber. Prior to immunogold labelling,

an incubation step with 5% BSA (diluted in PBS) for 30 minutes was introduced to block non-specific labelling. The secondary immunoreagent, goat anti-mouse IgG coupled to ultra small colloidal gold particles (Aurion, NL) was diluted 1:50 in 0.1% cationic BSA in PBS and the conditions of incubation were 2 h at room temperature. After rinsing several times with PBS followed by several washes in MilliQ water silver enhancement was performed for 20 minutes at room temperature. Slides were then washed again with MilliQ water followed by several rinses in PBS. The second directly labelled fluorescent antibody, CCL20/MIP-3α, was diluted 1:25 in PBS and incubated overnight on the sections at room temperature. The sections were mounted using Mowiol and then analysed by confocal microscopy using a Carl Zeiss MicroImaging, Inc. LSM 510 confocal fluorescence microscope in fluorescence and brightfield mode.

**Table 1**. **Technical details of antibodies used in immunohistochemical study**

| Antibody | Clone | Species/Isotype | Source | Ag retrieval | Control |
|---|---|---|---|---|---|
| CCR6 | 53103.111 | Mouse IgG2b | R&D Systems | Citrate | Tonsil |
| CCR7 | 6B3 | Mouse IgG1 | EBioscience | EDTA | Lymph node |
| CCR7 | 2H4 | Mouse IgM | BD Biosciences | Citrate | Lymph node |
| CXCR3 | 1C6 | Mouse IgG1 | BD Biosciences | Citrate | Tonsil |
| CCL20 | 67310.111 | Mouse IgG1 | R&D Systems | Citrate | Tonsil |
| CCL5 | 21445.1 | Mouse IgG1 | R&D Systems | Citrate | Tonsil |
| CXCL11 | | Rabbit IgG | PeproTech | Citrate | Tonsil |
| CD3 | | Rabbit IgG | DakoCytomation | Citrate | Tonsil |
| CD4 | 1F6 | Mouse IgG1 | NovoCastra | Citrate | Tonsil |
| CD8 | 4B11 | Mouse IgG1 | NovoCastra | Citrate | Tonsil |
| CD45RO | UCHL1 | Mouse IgG2a | DakoCytomation | Citrate | Tonsil |
| CD1a | 1CA04 | Mouse IgG1 | Neomarkers | Citrate | Skin |

## Results

*Accumulation of CCR6-expressing CD1a+ cells in LCH lesions*

To investigate whether LCs in LCH lesions are in an arrested state of activation and/or differentiation, the expression of particular chemokine receptors known to be characteristic of different stages of DC maturation, namely CCR6 (marker of immature DC) and CCR7 (marker of mature DC) were studied. For this analysis double immunofluorescent staining of CD1a and CCR6 as well as CD1a and CCR7

was performed. In all the LCH tissues studied double staining of CCR6 and CD1a on the same cells was consistently found irrespectively of the site of the lesion (Figure 1 A). In contrast, expression of CCR7 was not observed on the CD1a+ cells in these lesions (Figure 1 B).

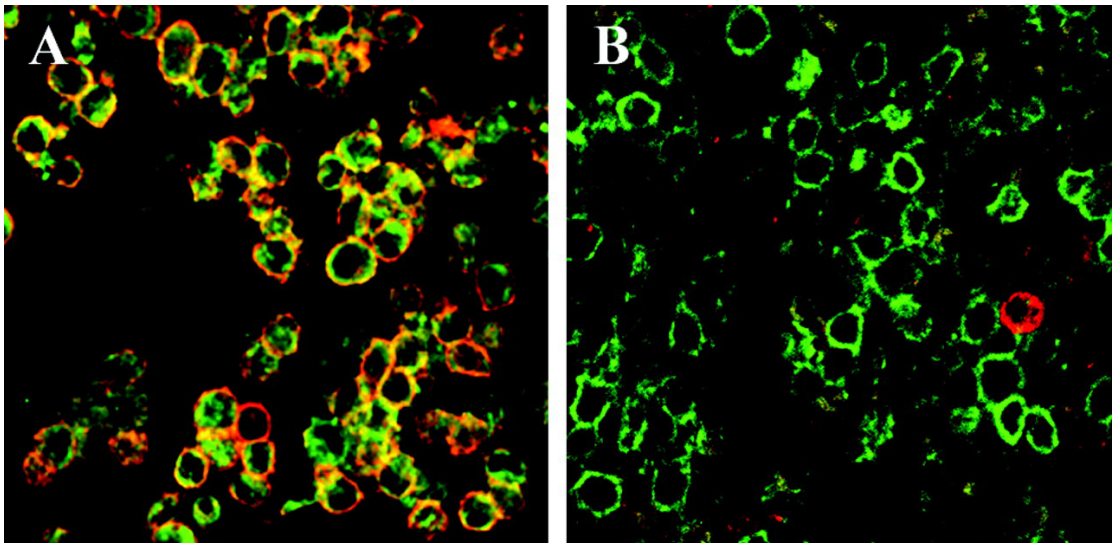


**Figure 1.** Expression of CCR6 but not CCR7 by LCH CD1a+ cells. Immunofluorescence staining of a representative LCH bone lesion using antibodies specific for CD1a (green), CCR6 (red) and CCR7 (red). Double immunofluorescent staining shows that all the CD1a+ cells are positive for CCR6, which appear yellow in the merged image (A). In contrast CCR7 is negative on the CD1a+ cells (B). Original magnification 400x.

*Expression of CCL20/MIP-3α by CD1a+ cells in LCH lesions*

Due to the expression of CCR6 by CD1a cells in LCH lesions the presence of its cognate ligand, CCL20/MIP-3α, in the affected tissues was investigated. First, single enzymatic staining for CCL20/ MIP-3α on normal control skin was carried out. As previously reported, the epidermis showed weak CCL20/MIP-3α expression by keratinocytes (Figure 2 A). However, the same staining procedure on LCH skin lesions revealed an increased level of CCL20/MIP-3α immunoreactivity, not only in the epidermis but also in the dermal region (Figure 2 B). This marked expression of CCL20/ MIP-3α staining was also consistently found in LCH bone and lymph node lesions (Figures 2 C and D).

The pattern of CCL20/MIP-3α staining displayed in LCH lesions appeared to closely match the distribution of the lesional CD1a+ cells. In order to evaluate CCL20/MIP-3α expression by the lesional CD1a+ cells, a double staining was performed using immunogold labelling followed by silver enhancement to detect the CD1a+ cells in combination with immunofluorescent staining for CCL20/ MIP-3α. As shown in Figure 3 this immunostaining of LCH lesions consistently showed expression of CCL20/MIP-3α by CD1a+ cells. Most of the CCL20/MIP-3α up-regulation could thus be attributed to the CD1a+ cells themselves.

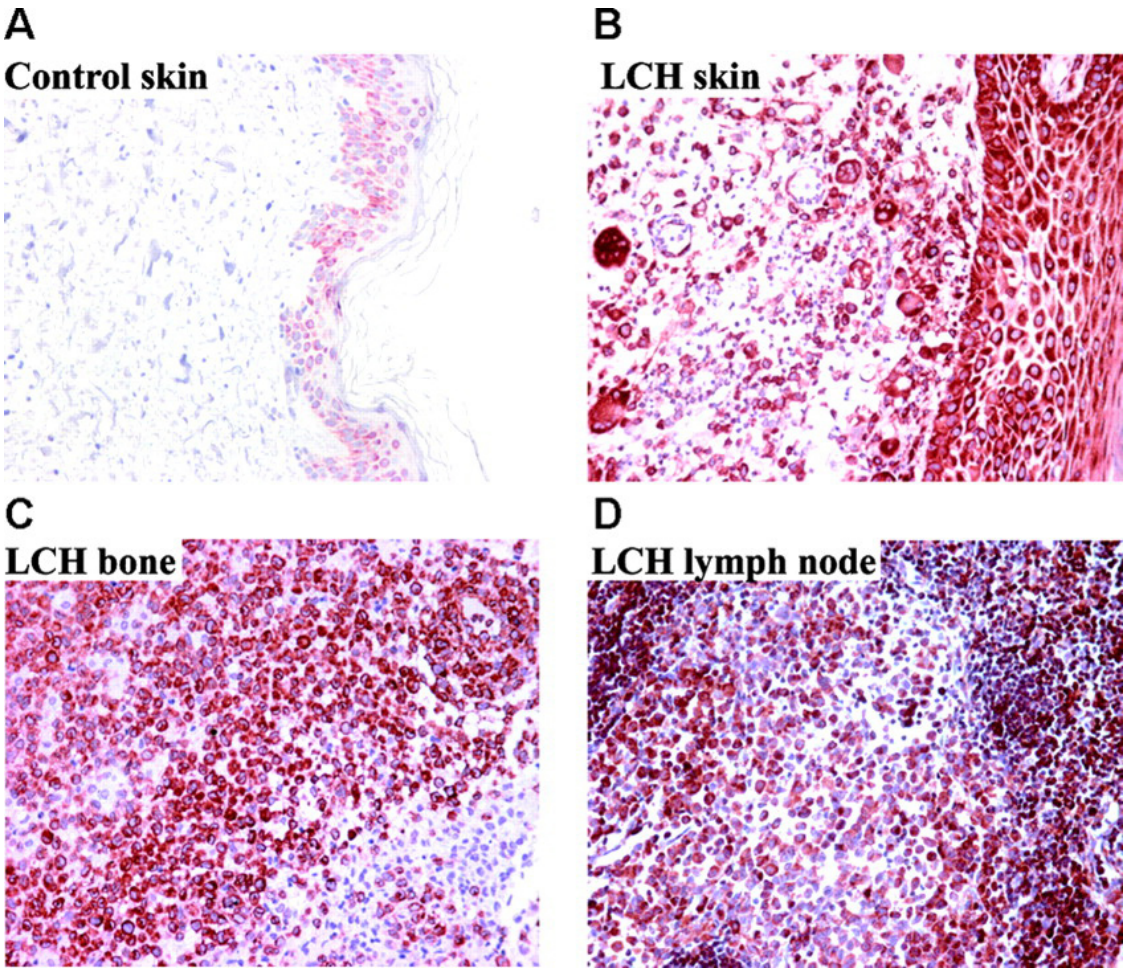


**Figure 2.** High expression level of CCL20/MIP-3α in LCH lesions. Immunohistochemistry was performed with an anti-hCCL20/MIP-3α monoclonal antibody and NovaRed detection. CCL20/MIP-3α was weakly expressed by epidermal keratinocytes in normal skin (A) in contrast to LCH skin lesions where CCL20/MIP-3α expression was greatly up-regulated both in the epidermis and by cells infiltrating the dermis (B). Similarly a high expression level of CCL20/MIP-3α was found in LCH bone and lymph node lesions (C and D repectively). Original magnification 250x.

*Expression of other inflammatory chemokines by lesional CD1a+ cells*

To determine whether the recruitment of various inflammatory cell types characteristic of LCH lesions could be explained by the production of chemokines by the CD1a+ cells, we investigated the expression of particular chemokines associated with the infiltration of other lesional cells. Besides CCL20/MIP-3α, prominent expression of the inflammatory chemokines CCL5/RANTES and CXCL11/I-TAC was found in all lesions studied. Similar to the CCL20/MIP-3α staining the pattern of CCL5/RANTES and CXCL11/I-TAC expression appeared to closely match the distribution of the lesional CD1a+ cells (unpublished data). As eosinophils are an important infiltrating population in LCH lesions, the expression of CCL5/RANTES seems relevant as this chemokine is known to be a potent activator of not only eosinophil chemotaxis but also eosinophil effector function. CXCL11/I-TAC on the other hand is a well-known chemotactic agent for IL-2 activated memory T cells expressing CXCR3.

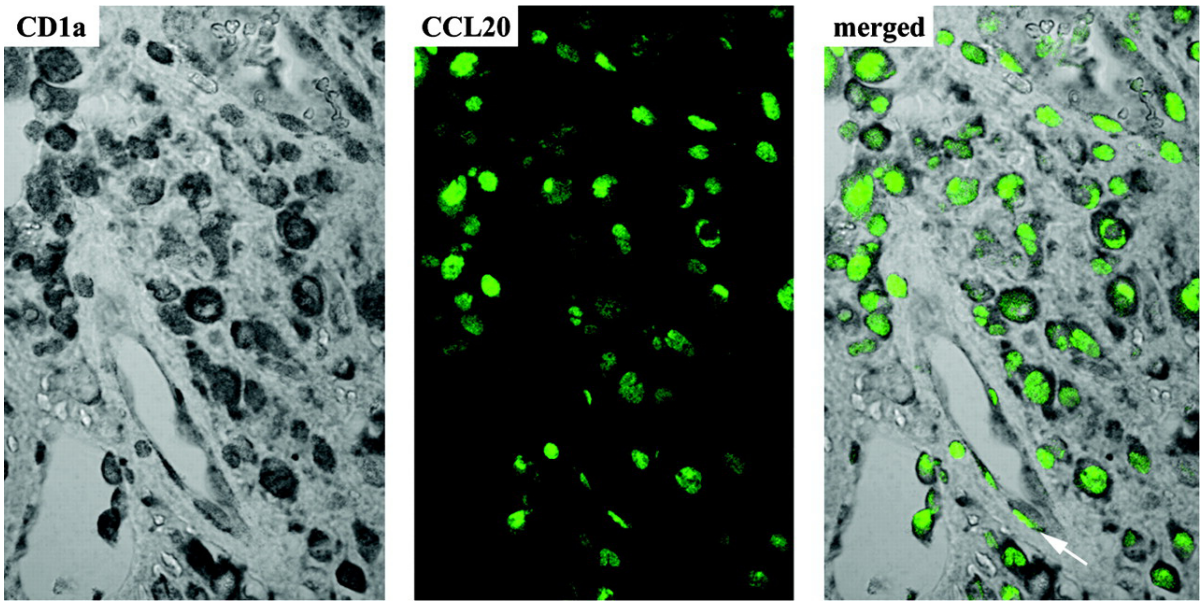


**Figure 3.** Expression of CCL20/MIP-3α by CD1a+ cells in LCH lesions. Immunohistochemistry was performed using antibodies specific for CD1a and CCL20/MIP-3α. The CD1a was detected by an immunogold/silver method (black) and the CCL20/MIP-3α by immunofluorescence (green). The merged image shows the same cells positive for CD1a and CCL20/MIP-3α. Note: the arrow points to endothelial cells expressing CCL20/MIP-3α. Original magnification 400x.

*Accumulation of CCR6-expressing CD4+ T cells in LCH lesions*

One other predominant cell type that infiltrates LCH lesions is the T cell. To characterise these cells further and to try to determine which of the prominently expressed chemoattractants could explain their presence, double and triple immunofluorescent staining was performed. All LCH lesions studied showed a predominance of CD4+ T cells which also displayed a memory /activated type as indicated by their CD45RO+ expression (unpublished data). In addition, a large majority of these T cells expressed CXCR3, a chemokine receptor specific for CXCL11/I-TAC, which is commonly expressed on activated T cells (unpublished data). Due to the enhanced expression of CCL20/MIP-3α in the LCH lesions, expression of its cognate receptor CCR6, on the infiltrating T cells was also investigated. Triple immunofluorescent staining of LCH lesions for CD3, CD4 and CCR6 clearly showed positive staining of CCR6 on the T cell infiltrate (Figure 4). Thus, both CXCR3 and CCR6 may explain the presence and retention of the lesional T cells through the aberrant up-regulation of CCL20/MIP-3α and CXCL11/I-TAC by the CD1a+ cells.

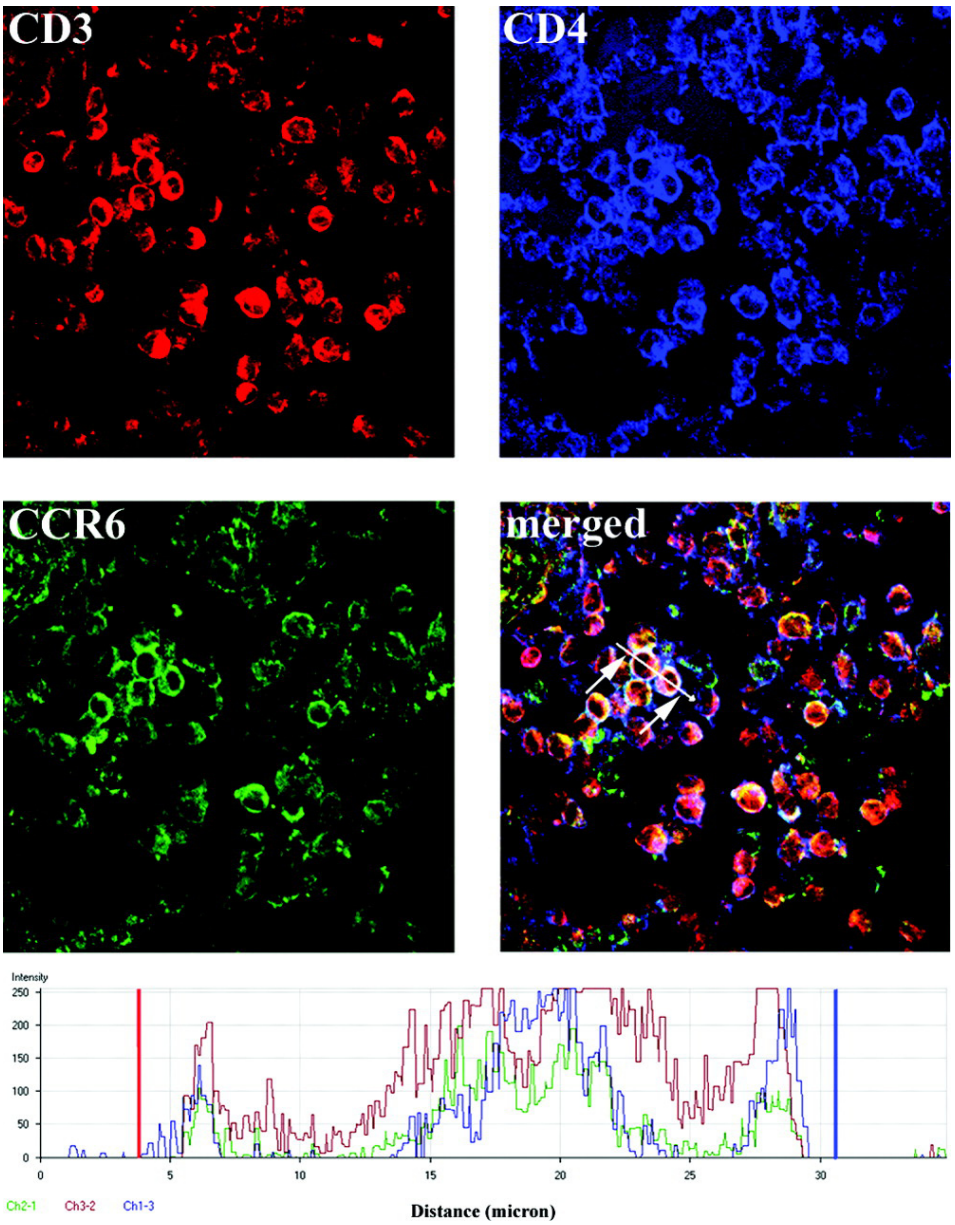


**Figure 4.** Lesional CD4+ T cells express CCR6. Triple immunofluorescent staining on a representative LCH bone lesion for CD3 (red), CD4 (blue) and CCR6 (green). The intensity profile measured between the arrows demonstrates on two representative cells the three different fluorescent labels. Original magnification 500x.

## Discussion

LCH is a disease characterized by the abnormal accumulation and retention of cells with a LC-like phenotype at various tissue sites. LCH cells do not acquire typical dendritic-like processes and their phenotype reflects only partial maturation when compared to the normal DC life cycle. Due to the integral role that migration plays in the normal functioning of DCs at their distinct stages of maturation we hypothesised that inappropriate expression and/or function of chemokine receptors on the lesional CD1a+ cells may help explain the pathophysiology of this disease. Since the presence of CD1a+ cells

uniquely define these lesions it can be speculated that the accumulation of other lesional cells is secondary to that of the presence of aberrant CD1a+ cells.

We demonstrate here that the lesional CD1a+ cells are indeed in an immature state as defined by their expression of the chemokine receptor CCR6. This finding is in keeping with a previous report by Geissmann *et al*. (16) who showed that LCH cells are immature LC-like DCs that express higher levels of CD68 and CD14 than normal LCs. Furthermore, they express intracellular MHC class II, are frequently negative for CD86 and DC-LAMP and have the same allostimulatory activity as immature normal DCs. Conversely, CCR7 expression, a chemokine receptor indicative of DC maturation which localizes DC in lymphoid organs by responding to CCR7 agonists, appeared to be absent on the lesional CD1a+ cells. Despite the various inflammatory stimuli present in LCH lesions, such as TNF-α, which should induce the maturation of the LCs, the CD1a cells do not lose their expression of CCR6 and do not up-regulate CCR7 (ref. 17). Thus it would appear from these findings that due to the fact that the lesional CD1a+ cells have the intrinsic inability to fully differentiate and mature they do not express the correct chemokine receptors. Thereby, the lesional CD1a+ cells are prevented from leaving their peripheral tissue sites and accumulate. Although we cannot provide functional data due to the unavailability of live lesional cells, we feel that lesional CD1a+ cells remain sensitive to the ligand, CCL20/MIP-3α. One reason for this is that the CCR6 expression levels remain high. Several mechanisms can occur which result in cellular desensitization to chemokines. However, DCs appear to regulate their reponsiveness mainly by up- and down-regulating their expression levels of chemokine receptors (18). Furthermore, there is evidence from the literature that in pancreatic cancer, the tumor cells also coexpress the CCR6 receptor and its ligand CCL20/MIP-3α. Although this is a very different cell system, here there is no indication that the receptor is desensitized (19).

Although it is now clear that lesional CD1a+ cells *in vivo* remain in an immature state, it has been shown that *in vitro* CD1a+ LCH cells could differentiate toward mature DCs in response to CD40 triggering (16). This raises the question then why *in vivo* are these CD1a+ cells not responding to inflammatory maturation signals, such as TNF-α which are abundantly expressed in LCH lesions? In the present study it was also shown that CD1a+ cells are the probable source of up-regulated CCL20/MIP-3α production in all LCH lesions studied. It is now known that CCL20/MIP-3α expression is under the direct control of TNF-α signalling (20, 21). Thus, these appear to be conflicting observations which will require *in vitro* experiments to elucidate whether the failure to up-regulate CCR7 is due to a signalling defect by inflammatory cytokines or due to conflicting cytokine signals e.g., IL-10 as suggested by Geissman *et al*. (16). Evidence showing signalling defects would be more supportive of an aberrant/malignant phenotype of the CD1a+ cells underlying the disease which has been suggested by groups who have shown clonality and proliferation to be present in LCH lesions (22-24). Alternatively, conflicting signalling would be more supportive of a reactive disease.

Although the etiology of LCH is not clear, certainly the CD1a+ cells are capable of maintaining and progressing the disease. In the present study it was shown that lesional CD1a+ cells express not only CCL20/MIP-3α but other inflammatory chemokines such as CCL5/RANTES and CXCL11/I-TAC. These chemokines are the likely factors responsible for the recruitment of other inflammatory cell types characteristic of LCH lesions. Although the presence of T cells in all LCH lesions is a striking feature, the mechanism by which these T cells are recruited has not so far been addressed. Most of the T cells surround the lesions in the reactive 'rim', however a few are present within most active lesions (25). To date there has been little information on the *in situ* characterisation of these T cells. The present study has now clearly shown that the T cells in LCH lesions mainly comprise CD4+ CD45RO+ T cells with very few CD8+ T cells. Furthermore, most of the lesional T cells express CXCR3 which is known to be closely related to cell-mediated immunity (Th1-type immune response). This memory/activated phenotype found on the lesional T cells fits with previous findings of CD154 on these cells (26). Immunohistochemical analysis consistently detected expression of the CCL20/MIP-3α receptor, CCR6, on the infiltrating T cells in LCH lesions. This finding along with the fact that it has already been shown that CCL20/MIP-3α specifically attracts the memory subset of T cells *in vitro (*27), strongly implicates CCL20/MIP-3α as an important chemoattractant responsible for T cell recruitment in LCH lesions. Thus the lesional CD1a+ cells, the probable source of CCL20/MIP-3α in the LCH lesions, are not only causing their own recruitment and retention but that of other inflammatory cells as well.

Although in the present study we concentrated on a limited set of chemokines and chemokine receptors, it is notable from our findings that regardless of the tissue site of the lesion, the same chemokine and chemokine receptor profile was found in bone, skin and lymph node LCH. However, in light of the role of some chemokine receptors as tissue-specific homing molecules, it will be interesting to determine whether other chemokine receptors expressed by the CD1a+ cells specifically determine the anatomical localization of LCH lesions. It will be important to study this not only in single isolated lesions but also in patients with disseminated LCH where multiple sites are affected. So far though our results indicate that any future intervention strategies based on chemokines or their receptors will be applicable to all LCH lesions.

**Acknowledgements**

This work was partly funded by The Histiocytosis Association of America.

We would like to thank Dr Maarten van Tol for his critical reading of this manuscript.

**References**


1. Broadbent V, Egeler RM, Nesbit ME Jr. 1994. Langerhans cell histiocytosis – Clinical and epidemio logical aspects. Br J Cancer. 70:S11-6.
2. Egeler RM, D'Angio GJ. 1995. Medical progress: Langerhans cell histiocytosis. J Pediatr.127:1-11.
3. Egeler RM, Favara BE, van Meurs M, Laman JD, Claassen E. 1999. Differential in situ cytokine pro files of Langerhans-like cells and T cells in Langerhans cell histiocytosis: Abundant expression of cytokines relevant to disease and treatment. Blood 94: 4195-4201.
4. Greaves DR, Wang W, Dairaghi DJ, Dieu MC, Saint-Vis B, Franz-Bacon K, Rossi D, Caux C, Mc Clanahan T, Gordon S, Zlotnik A, Schall TJ. 1997. CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3a and is highly expressed in human dendritic cells. J Exp Med. 186:837-844.
5. Dieu MC, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, Briere F, Zlotnik A, Leb ecque S, Caux C. 1998. Selective recruitment for immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J Exp Med. 188:373-386.
6. Sozzani S, Allavena P, D'Amico G, Luini W, Bianchi G, Kataura M, Imai T, Yoshie O, Bonecchi R, Mantovani A. 1998. Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. J Immunol. 161:1083-1086.
7. Delgado E, Finkel V, Baggiolini M, Mackay CR, Steinman RM, Granelli-Piperno A. 1998. Mature dendritic cells respond to SDF-1, but not to several beta-chemokines. Immunobiology 198:490-500.
8. Yanagihara S, Komura E, Nagafune J, Watarai H, Yamaguchi Y. 1998. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. J Immunol. 161:3096-3102.
9. Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD, Williams LT.1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naïve T lymphocytes. Proc Natl Acad Sci USA 95:258-263.
10. Ngo VN, Tang HL, Cyster JG. 1998. Epstein-Barr virus-induced molecule 1 ligand chemokine is ex pressed by dendritic cells in lymphoid tissues and strongly attracts naïve T cells and activated B cells. J Exp Med. 188:181-191.
11. Vulcano M, Albanesi C, Stoppacciaro A, Bagnati R, D'Amico G, Struyf S, Transidico P, Bonecchi R, Del Prete A, Allavena P, Ruco LP, Chiabrando C, Girolomoni G, Mantovani A, Sozzani S. 2001. Den dritic cells as a major source of macrophage-derived chemokine/CCL22 *in vitro* and *in vivo*. Eur J Im munol. 31:812-822.
12. Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I, Forster R, Burgstahler R, Lipp M, Lanzavecchia A. 1999. Distinct patterns and kinetics of chemokine production regulate den dritic cell function. Eur J Immunol. 29:1617-1625.
13. Vissers JLM, Hartgers FC, Lindhout E, Teunissen MBM, Figdor CG, Adema GJ. 2001. Quantitative analysis of chemokine expression by dendritic cell subsets *in vitro* and *in vivo*. J Leukoc Biol. 69:785-793.
14. Emile JF, Fraitag S, Leborgne M, de Prost Y, Brousse N. 1994. Langerhans cell histiocytosis cells are activated Langerhans cells. J Pathol. 174:71-76.
15. Yu RC, Alaibac M, Chu AC. 1995. Functional defect in cells involved in Langerhans cell histiocyto sis. Arch Dermatol Res. 287:627-631.
16. Geissmann F, Lepelletier, Fraitag S, Valladeau J, Bodemer C, Debre M, Leborgne M, Saeland S, Brousse N. 2001. Differentiation of Langerhans cells in Langerhans cell histiocytosis. Blood 97:1241-1248.
17. Kimber I, Cumberbatch K, Dearman RJ, Bhushan M, Criffiths CEM. 2000. Cytokines and chemok ines in the initiation and regulation of epidermal langerhans cell mobilization. Br J Dermatol. 142: 401-412.
18. Caux C, Ait-Yahia S, Chemin K, de Bouteiller O, Dieu-Nosjean MC, Homey B, Massacrier C, Van

bervliet B, Zlotnik A, Vicari A. 2000. Dendritic cell biology and regulation of dendritic cell traffick ing by chemokines. Springer Semin Immunopathol 22:345-369.
19. Kleeff J, Kusama T, Rossi DL, Ishiwata T, Maruyama H, Friess H, Buchler MW, Zlotnik A, Korc M. 1999. Detection and localization of MIP-3α/LARC/EXODUS, a macrophage proinflammatory chem okine, and its receptor in human pancreatic cancer. Int J Cancer. 81:650-657.
20. Sugita S, Kohno T, Yamamoto K, Imaizumi Y, Nakajima H, Ishimaru T, Matsuyama T. 2002. Induc tion of macrophage-inflammatory protein-3a gene expression by TNF-dependent NF-KB activation. J Immunol. 168:5621-5628.
21. Harant H, Eldershaw SA, Lindley IJD. 2001. Human macrophage inflammatory protein-3a/CCL20/ LARC/Exodus/SCYA20 is transcriptionally upregulated by tumor necrosis factor-a via a non-stand ard NF-KB site. FEBS Letters 509:439-445.
22. William CL, Busque L, Griffith BB, Favara BE, McClain KL, Duncan MH, Gilliland DG. 1994. Langerhans-cell histiocytosis (histiocytosis X)- a clonal proliferative disease. N Engl J Med 331: 154-160.
23. Yu RC, Chu C, Buluwela L, Chu AC. 1994. Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis. Lancet 343: 767-768.
24. Schouten B, Egeler RM, Leenen PJM, Taminiau AHM, Broek vd LJCM, Hogendoorn PCW. 2002. Expression of cell cycle-related gene products in Langerhans cell histiocytosis. J Pediatr Hematol Oncol 24: 727-732.
25. Favara BE and Jaffe R. 1994. The Histopathology of Langerhans Cell Histiocytosis. Br J Cancer 70: S17-S23.
26. Egeler RM, Favara BE, Laman JD, Claassen E. 2000. Abundant expression of CD40 and CD40-lig and CD154) in paediatric Langerhans cell histiocytosis lesions. Eur J Cancer 36: 2105-2110.
27. Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. 1999. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage in flammatory protein 3 alpha. J Immunol. 162:186-194.

# 4

# Differential expression of CCR6 by the lesional cells in pulmonary Langerhans cell histiocytosis

Cristiana ET da Costa
Abdellatif Tazi
Yuko Matsumoto
Nicola E Annels
R Maarten Egeler

*Submitted for publication*

**Abstract**

Solitary pulmonary Langerhans cell histiocytosis occurs predominantly in young adults, who are frequently heavy smokers. Besides the strong association to smoking, it differs from childhood Langerhans cell histiocytosis as well in that it is a polyclonal disorder, and the lesional Langerhans cells in this form of disease are reported to display mature markers. Thus, in this study we set out to analyse the chemokine receptor expression pattern of CCR6 and CCR7, chemokine receptors associated with immature or mature dendritic cells, respectively. This study is a follow up of a previous report that has shown that in childhood Langerhans cell histiocytosis CD1a LCH cells always express CCR6 and lack CCR7. The current study showed that there is a differential expression of CCR6 in pulmonary Langerhans cell histiocytosis lesions, ranging from lesions with all CD1a LCH cells expressing CCR6, to lesions where there is a partial population of CD1a LCH cells that express CCR6, to lesions where all CD1a LCH cells lack CCR6 expression. In addition, CCR7 was always absent on the CD1a cells even on those LCH cells that lacked CCR6. Thus, in contrast to childhood LCH lesions where LCH cells always express CCR6, pulmonary LCH lesions display a differential pattern of expression of chemokine receptor CCR6.

**Introduction**

Pulmonary involvement with Langerhans cell histiocytosis (LCH) can be observed in patients of any age. Multifocal and systemic forms of the disease are usually seen in infants and children, and pulmonary involvement is often not a prominent feature. In contrast, isolated pulmonary LCH (pLCH) occurs predominantly in young adults with a peak frequency between 20 and 40 years of age (1-3). The main epidemiological factor associated with pLCH is smoking: 90-100% of patients have been current smokers in almost all series and tend to be heavy smokers (4-6). Localized pLCH is actually the form most frequently encountered by specialists in pulmonary medicine. It has several unique clinical and epidemiological features that justify its classification as a distinct clinicopathological entity. The natural history of pLCH remains poorly defined and no treatment has been found to be efficacious. The characteristic lesion of pLCH is composed of activated Langerhans cells (LCs) organized into a loose granuloma and associated with lymphocytes and inflammatory cells, particularly eosinophils and macrophages (7-9). LCs in pLCH express a unique surface phenotype, which strongly suggests that these cells are activated. They express CD80, CD86 and CD40, which are not present on normal pulmonary LCs or other pulmonary lesions such as lung cancer, and are thus likely important in the pathogenesis of LCH (10). Under normal circumstances, upon exposure to antigens, LCs respond to

various stimuli, such as TNF-a, LPS, and migrate to the regional lymphoid organs, where they stimulate antigen-specific T cells (11, 12). This migration process is strongly influenced by the chemokine gradient between the afferent lymph ducts and the antigen introduction site. During migration, LCs lose their expression of Birbeck granules and switch their surface expression of the chemokine receptor CCR6, typically expressed by immature dendritic cells (DCs), to CCR7, typically expressed by mature DCs, and thus become responsive to the CCR7 ligands CCL19/MIP-3b and CCL21/6Ckine (13, 14). In a previous study it was shown that regardless of the tissue site of LCH lesions, CCR6 is expressed by CD1a+ LCs in bone, skin and lymph node LCH lesions (15). Due to the fact that pLCH is a different form of LCH as, among other factors, LCs here are described to be more mature, we set out to investigate the presence of CCR6 and CCR7 in pLCH lesions. For this purpose, we carried out a combined immunohistochemical analysis for CCR6 and CCR7 with the marker for LCH cells, CD1a, in several pLCH lesions. This will hopefully reveal any potential differences between this form of disease and childhood LCH, which may help corroborate the already described differences.

## Materials and methods

*Patients*

Paraffin biopsies from eleven pulmonary LCH patients were used in this study. This tissue was obtained from the pneumologie service, Paris, France. In all cases the diagnosis was reviewed and confirmed by immunohistochemistry for S-100 and CD1a.

*Immunohistochemistry analysis*

Paraffin sections were cut at 4 μm and double and triple immunofluorescence stainings were carried out, according to Annels *et al.* (15). Mouse monoclonal antibodies to CD1a (O10) from Neomarkers, CCR6 (53103.111) from R&D Systems and CD68 (514H12) from Serotec, and rabbit polyclonal antibody to CCR7 (E271) from AbCam, were used in this study.

## Results

*Differential expression of CCR6 in pLCH*

In order to analyse the expression of CCR6 in LCH lesions, triple immunofluorescence stainings were carried out on eleven pulmonary LCH cases. The CCR6 antibody was combined with CD1a, for detecting LCH cells, and CD68, for detecting macrophages. A differential expression of CCR6

by the CD1a LCH cells in the pLCH lesions was observed. In 4 out of 11 pLCH all CD1a LCH cells expressed CCR6 (Figure 1 A, Table 1), in 2 out of 11 cases we found that between 30 and 50% of the CD1a LCH cells expressed CCR6 and the remaining CD1a+ cells lacked the expression of this chemokine receptor (Figure 1 B), and finally in 5 out of 11 cases all CD1a LCH cells lacked the expression of CCR6 (Figure 1 C).

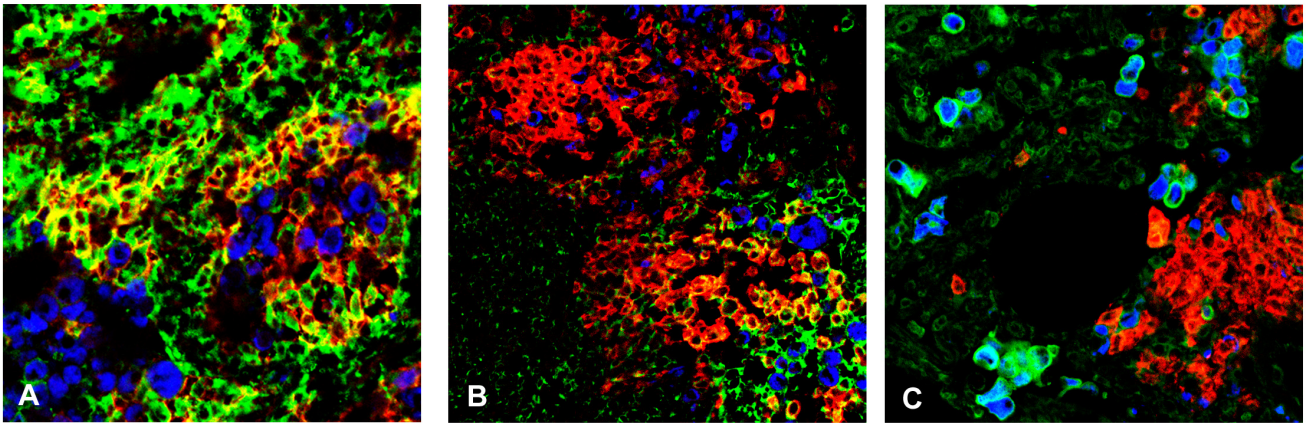


**Figure 1.** Differential expression of CCR6 in pulmonary Langerhans cell histiocytosis. Immunofluorescence staining of three representative lung LCH lesions using antibodies specific for CD1a (red), CCR6 (green) and CD68 (blue). Triple immunofluorescence staining shows that all the CD1a+ cells are positive for CCR6 which appear yellow in 4 out of 11 lesions analysed (A), between 30 and 50% of the CD1a+ cells are positive for CCR6 in 2 out of eleven cases analysed (B), and no CD1a+ cells are positive for CCR6 in 5 out of 11 cases analysed (C). Original magnification 400X.

Table 1. Clinical information and results of CCR6 and CCR7 expression on the pulmonary LCH patients used in this study.

| Patients* | Gender | Age (yr) | Treatment | Outcome | CCR6 expression | CCR7 expression |
|---|---|---|---|---|---|---|
| 1 | M | 47 | No | Alive | + | - |
| 2 | F | 27 | No | Alive | + | - |
| 3 | F | 28 | No | Lost of follow up | + | - |
| 4 | N.A. | N.A. | N.A. | N.A. | + | - |
| 5 | F | 16 | No | Alive | + and - | - |
| 6 | M | 46 | No | Lost of follow up | + and - | - |
| 7 | F | 37 | No | Alive | - | - |
| 8 | M | 31 | No | Alive (lung cancer) | - | - |
| 9 | F | 38 | No | Alive | - | - |
| 10 | F | 44 | N.A. | N.A. | - | - |

-, lack of expression; +, expression; + and -, partial expression; N.A, data not available.
*All patients were heavy smokers.

*Lack of CCR7 expression in pLCH lesions*

As it has previously been reported that LCH cells in pLCH lesions have a more mature phenotype and in the current study we observed that in at least 7 out of 11 cases of pLCH all or part of the CD1a LCH cells were lacking CCR6, we were interested to look at whether these cells had up-regulated CCR7. Interestingly, we found that all CD1a LCH cells that were lacking CCR6 also lacked CCR7 expression (Figure 2).

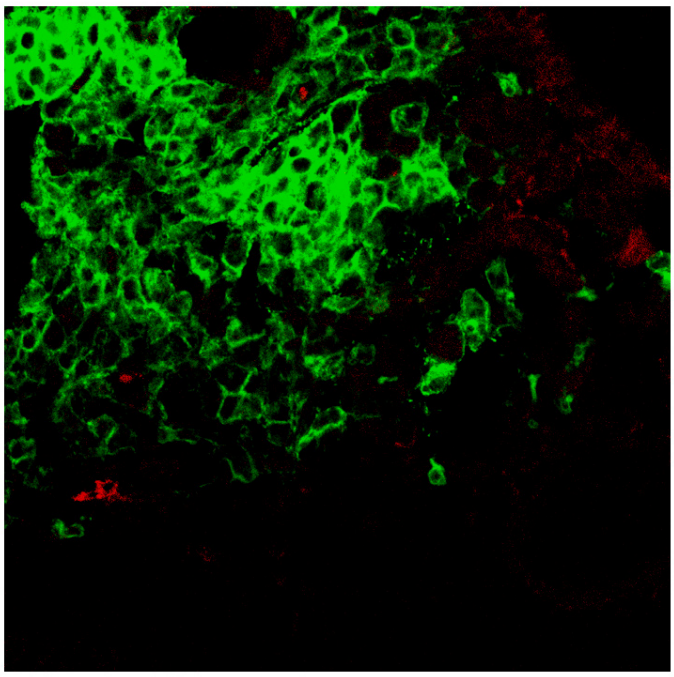

**Figure 2.** Lack of CCR7 expression in all pulmonary Langerhans cell histiocytosis lesions. Immunofluorescence staining on a pLCH lesion using antibodies specific for CD1a (green) and CCR7 (red). All CD1a+ cells in lung LCH lesions showed no expression of CCR7. It is possible to see that other cells in the lesion (arrow) are positive for CCR7. Original magnification 400X.

## Discussion

In childhood LCH lesions it has been shown that CD1a+ LCs always express CCR6, a chemokine receptor expressed by immature LCs (15). These findings contrast with the results from another study which showed that LCH cells co-express CCR6 and CCR7 (16). This difference may be due to different technical approaches. However, the results from the first study are in keeping with other reports that show additional evidence that these cells combine an early stage of activation with an immature phenotype. In contrast, in pulmonary LCH (pLCH), LCH cells were reported to express surface markers associated with activation, that are not present on normal pLCs and that are likely important in the pathogenesis of LCH (10). In light of the previous report by Annels *et al*. in which only CCR6 was observed by the pathologic LCH cells in childhood LCH and the report by Tazi *et al.* which demonstrated a more mature phenotype of the LCH cells in pLCH the current study set out to investigate the expression of CCR6 and CCR7 in pLCH. The results showed that in pLCH lesions there is a dif-

ferential expression of CCR6, ranging from the totality of CD1a LCH cells being CCR6 positive, to a mixture of CCR6 positive and CCR6 negative CD1a LCH cells, and finally to all of CD1a LCH cells being CCR6 negative.

These differences of expression of CCR6 by the CD1a+ LCH lesions may reflect the gradual stages of the disease, clinically observed by the destruction of the epithelium. In pLCH early lesions are responsible for eccentric infiltration of the walls of terminal and respiratory bronchioles, which undergo gradual destruction. LCs are abundant at this stage and form a compact central granuloma with a large number of lymphocytes located between the LCs and at the periphery of the lesion. Later in the process the LCs are less abundant and form clusters surrounded by lymphocytes and inflammatory cells. Finally, in advanced disease there is few or no LCs (17, 18).

LCH cells in pLCH lesions have been shown to display a more mature phenotype than LCH cells in childhood LCH, as they express B7-1 and B7-2 molecules (10). The microenvironment in which cells lie exerts a strong influence on dendritic cell function at all steps of the immune response and influences the elicitation of an efficient immune response or tolerance. In fact, the profile of cytokines expressed in pLCH lesions corresponds to one that has been shown by in vitro studies to induce the maturation of LCs into cells with strong lymphostimulatory activity (10, 19). Epithelial cells are able to produce a variety of cytokines including factors that influence the proliferation, survival and differentiation of LCs. In this regard, bronchiolar epithelial cells overlying early LCH granulomas produce greater amounts of GM-CSF than epithelial cells in adjacent uninvolved bronchioles (20-22). This supports the observation of greater amount of LCs observed in earlier LCH lesions in comparison to the lesser number of LCs observed in a later stage of pLCH (18). It may also explain the differential expression of CCR6 in the pLCH lesions.

Although LCs in pLCH lesions display a mature surface phenotype, no functional studies such as T cell stimulation have been performed to confirm the fact that these cells are fully mature cells,. Interestingly, in all the lesions studied CD1a LCH cells always lacked CCR7 expression, even in the lesions where CCR6 was totally or partially absent. This is quite surprising as once dendritic cells down-regulate CCR6 they up-regulate CCR7. Thus, it appears that, like in childhood LCH, there is a blockade in the up-regulation of CCR7 in pLCH. In fact, these cells may be similar to semi-mature DCs described by Steinman *et al*., which are actually tolerogenic DCs (23).

The clusters of LCH cells typically observed in pLCH lesions appears to be due to accumulation instead of local proliferation. In fact, LCs in pLCH lesions have a rather low rate of proliferation, considerably less than that of carcinoma cells (24).

The absence of CCR6 expression in some LCH lesions may suggest that another recruitment pathway (than CCL20-CCR6) is involved in the accumulation of CD1a cells in these specimens. In fact, CCR2 was shown to directly control the accumulation of DCs into allergic lungs (25). Likewise, Chiu *et*

*al*. has shown that CCR2 knockout mice confers an intrinsic DC activation defect (26). Thus, further studies on chemokine ligand and receptor profiles in pLCH lesions will confirm whether other sets of chemokines have an important role in the recruitment of DCs into the lungs in LCH lesions.

There is still much speculation concerning initiating triggers in pLCH. Tobacco smoke in itself induces an increase in LC numbers in the airway epithelium. However, the transition to unbridled accumulation of LCs likely requires additional factors, which could be genetic predisposition, acquired mutations such as allelic loss at the level of tumor suppressor genes, or maybe another environmental trigger, such as viral infection. In this study we aimed at analysing the CCR6 and CCR7 expression in pLCH lesions in order to gain further understanding of the pathogenesis of LCH by comparing with the expression of these chemokine receptors previously reported in childhood LCH. However, more studies need to be carried out in order to complete this knowledge.

**References**


1. Ha, S. Y., P. Helms, M. Fletcher, V. Broadbent, and J. Pritchard. 1992. Lung involvement in Langer hans' cell histiocytosis: prevalence, clinical features, and outcome. Pediatrics 89:466-469.
2. Baumgartner, I., A. von Hochstetter, B. Baumert, U. Luetolf, and F. Follath. 1997. Langerhans'-cell histiocytosis in adults. Med.Pediatr.Oncol. 28:9-14.
3. Vassallo, R., J. H. Ryu, T. V. Colby, T. Hartman, and A. H. Limper. 2000. Pulmonary Langerhans'-cell histiocytosis. N.Engl.J.Med. 342:1969-1978.
4. Hance, A. J., F. Basset, G. Saumon, C. Danel, D. Valeyre, J. P. Battesti, J. Chretien, and R. Georges. 1986. Smoking and interstitial lung disease. The effect of cigarette smoking on the incidence of pul monary histiocytosis X and sarcoidosis. Ann.N.Y.Acad.Sci. 465:643-656.
5. Bernstrand, C., K. Cederlund, L. Ashtrom, and J. I. Henter. 2000. Smoking preceded pulmonary involvement in adults with Langerhans cell histiocytosis diagnosed in childhood. Acta Paediatr. 89:1389-1392.
6. Howarth, D. M., G. S. Gilchrist, B. P. Mullan, G. A. Wiseman, J. H. Edmonson, and P. J. Schomberg. 1999. Langerhans cell histiocytosis: diagnosis, natural history, management, and outcome. Cancer 85:2278-2290.
7. Hance, A. J., J. Cadranel, P. Soler, *et al*. 1988. Pulmonary and extrapulmonary Langerhans cell granulomatosis (histiocytosis X). Semin. Respir. Med. 9:349-368.
8. Tazi, A., P. Soler, and A. J. Hance. 1996. Langerhans cell granulomatosis. In: Kradin R. L., B. W. S. Robinson, eds. Immunopathology of lung disease. Boston: Butterworth-Heinemann. 191-212.
9. Travis, W. D., Z. Borok, J. H. Roum, J. Zhang, I. Feuerstein, V. J. Ferrans, and R. G. Crystal. 1993. Pulmonary Langerhans cell granulomatosis (histiocytosis X). A clinicopathologic study of 48 cases. Am.J.Surg.Pathol. 17:971-986.
10. Tazi, A., J. Moreau, A. Bergeron, S. Dominique, A. J. Hance, and P. Soler. 1999. Evidence that Lang erhans cells in adult pulmonary Langerhans cell histiocytosis are mature dendritic cells: importance of the cytokine microenvironment. J.Immunol. 163:3511-3515.
11. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. Annu.Rev.Immunol. 18:767-811.
12. Berger, C. L., J. G. Vasquez, J. Shofner, K. Mariwalla, and R. L. Edelson. 2006. Langerhans cells: mediators of immunity and tolerance. Int.J.Biochem.Cell Biol. 38:1632-1636.
13. Dieu, M. C., B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik,

S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J.Exp.Med. 188:373-386.
14. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 99:23-33.
15. Annels, N. E., C. E. Da Costa, F. A. Prins, A. Willemze, P. C. Hogendoorn, and R. M. Egeler. 2003. Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. J.Exp.Med. 197:1385-1390.
16. Fleming, M. D., J. L. Pinkus, S. W. Alexander, C. Tam, M. Loda, S. E. Sallan, K.E. Nichols, D. F. Carpentieri, G. S. Pinkus, and Rollins B. J. 2003. Coincident expression of the chemokine receptors CCR6 and CCR7 by pathologic Langerhans cells in Langerhans cell histiocytosis. Blood. 101(7):2473-2475.
17. Kambouchner, M., F. Basset, J. Marchal, J. F. Uhl, A. J. Hance, and P. Soler. 2002. Three-dimension al characterization of pathologic lesions in pulmonary langerhans cell histiocytosis. Am.J.Respir.Crit Care Med. 166:1483-1490.
18. Tazi, A. 1998. Adult pulmonary Langerhans cell histiocytosis. Eur. Respir. J. 27:1272-1285.
19. Chang, C. H., M. Furue, and K. Tamaki. 1995. B7-1 expression of Langerhans cells is up-regulated by proinflammatory cytokines, and is down-regulated by interferon-gamma or by interleukin-10. Eur.J.Immunol. 25:394-398.
20. Banchereau, J. and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392:245-252.
21. Tazi, A., F. Bouchonnet, M. Grandsaigne, L. Boumsell, A. J. Hance, and P. Soler. 1993. Evidence that granulocyte macrophage-colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/Langerhans cells in human lung and lung cancers. J.Clin.Invest 91:566-576.
22. Tazi, A., M. Bonay, A. Bergeron, M. Grandsaigne, A. J. Hance, and P. Soler. 1996. Role of granulo cyte-macrophage colony stimulating factor (GM-CSF) in the pathogenesis of adult pulmonary histio cytosis X. Thorax 51:611-614.
23. Steinman, R.M., Hawiger, D., and M.C. Nussenzweig. 2003. Tolerogenic dendritic cells. Annu. Rev. Immunol. 21:685-711.
24. Brabencova, E., A. Tazi, M. Lorenzato, M. Bonay, M. Kambouchner, J. F. Emile, A. J. Hance, and P. Soler. 1998. Langerhans cells in Langerhans cell granulomatosis are not actively proliferating cells. Am.J.Pathol. 152:1143-1149.
25. Robays, L. J., T. Maes, S. Lebecque, S. A. Lira, W. A. Kuziel, G. G. Brusselle, G. F. Joos, and K. V. Vermaelen. 2007. Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pul monary dendritic cells during allergic airway inflammation. J. Immunol. 178:5305-5311.
26. Chiu, B. C., C. M. Freeman, V. R. Stolberg, J. S. Hu, K. Zeibecoglou, B. Lu, C. Gerard, I. F. Charo, S. A. Lira, and S. Chensue. Impaired lung dendritic cell activation in CCR2 knockout mice. 2004. Immunopathol. infect. dis. 165 (4):1199-1209.

# 5

# Presence of osteoclast-like multinucleated giant cells in the bone and nonostotic lesions of Langerhans cell histiocytosis

Cristiana ET da Costa
Nicola E Annels
Claudia MJM Faaij
Ramses G Forsyth
Pancras CW Hogendoorn
R Maarten Egeler

*The Journal of Experimental Medicine*

2005; 201(5): 687-693

**Abstract**

Langerhans cell histiocytosis (LCH) is a disease which can involve one or multiple organ systems characterized by an accumulation of CD1a+ Langerhans-like cells as well as several other myeloid cell types. The precise origin and role of one of these populations, the multinucleated giant cell (MGC), in this disease remains unknown. This work shows that in three different lesional tissues, bone, skin and lymph node, the MGCs expressed the characteristic osteoclast markers, tartrate-resistant acid phosphatase and vitronectin receptor, as well as the enzymes cathepsin K and matrix metalloproteinase-9. Although, in bone lesions, the osteoclast-like MGCs were only CD68+, in the nonostotic sites, they co-expressed CD1a. The presence of osteoclast-like MGCs may be explained by the production of osteoclast-inducing cytokines such as receptor activator of nuclear factor κB ligand and macrophage colony-stimulating factor by both the CD1a+ LCH cells and T cells in these lesions. As osteoclast-derived enzymes play a major role in tissue destruction, the osteoclast-like nature of MGCs in all LCH lesions makes them a potential target for the treatment of this disease.

**Introduction**

Langerhans cell (LC) histiocytosis (LCH) is a rare disease often present in childhood with a continuum of clinical entities ranging from a localized lytic lesion to a fatal disseminated myeloid-like leukemia and is associated with fibrosis and osteolysis, which leads to organ dysfunction (1). Although the pathophysiology is still obscure, at the cellular level, LCH is characterized by the clonal proliferation and retention of $CD1a^+$ dendritic LCs, commonly referred to as LCH cells. Together with LCH cells, other cell types have been shown to be present in LCH lesions, including lymphocytes, macrophages, eosinophils and multinucleated giant cells (MGCs) (2).

MGCs are thought to originate from the fusion of monocyte-macrophage lineage cells (3). Morphologically, they can be classified into Langhans' giant cells (normally found in infective granulomatous diseases, ref. 4), foreign body giant cells (commonly found in foreign body granulomas, ref. 5), or thirdly, osteoclasts, which are present in bone sites where they function in bone resorption (6). Although all these types of MGCs originate from a common precursor cell, they differ markedly in their association with disease states, location and prevalence in various tissues or organs, stimuli that induce their formation, and subsequent function.

It is unclear how monocyte fusion is induced *in vivo* and whether different mechanisms are involved in different pathological states. However, a number of papers have reported on how the formation of MGCs can be induced *in vitro*. Evidence has accumulated to show that the *in vitro* generation

of MGCs occurs as a result of cell fusion rather than cell division (7). In fact, the *in vitro* fusion of adherent macrophages from both humans and experimental animals is a normal event at a terminal stage of maturation (8). This phenomenon is enhanced, among other stimuli, by the addition of various cytokines. Indeed, the cytokines IL-4, IFN-γ, or IL-13 clearly play a prominent role in monocyte fusion and subsequently, in the generation of MGCs (5, 9). Furthermore, an appropriate cytokine environment can regulate the commitment of a cell towards one or another cell lineage. For example, osteoclast differentiation from monocyte/macrophage precursor cells occurs in the presence of cytokines, such as TNF-α and IL-1α (10) or receptor activator of NFκB ligand (RANKL), and growth factors, such as M-CSF (11). In contrast, although DCs originate from the same monocyte/macrophage precursor cells as osteoclasts, DCs are derived *in vitro* from circulating human monocytes after stimulation with GM-CSF, IFN-α and IL-4 (12, 13), or from human CD34+ myeloid progenitors in response to GM-CSF and TNF-α (14). These bone marrow progenitors were identified recently through their ability to differentiate into DCs or osteoclasts, depending on whether RANKL was present together with GM-CSF or M-CSF, respectively (15).

Thus, it is clear that the cellular environment plays a crucial role in cell differentiation. In this report, we demonstrate that the cytokine environment of LCH lesions may allow local formation rather than attraction of osteoclast-like MGCs. The local formation may explain the coexpression of CD1a observed on osteoclast-like MGCs in nonbone lesions as the normal osteoclast precursors are likely to be absent in these tissues. So, although the phenotype of the osteoclast-like MGCs was more normal in bone lesions, it seems likely that this population must contribute a large part of the chronic tissue destruction in all LCH lesions. Thus, the osteoclast-like nature of MGCs provides a rationale for the successful treatment of LCH patients with antiosteoclast therapy.

## Methods

*Tissue samples*

Representative specimens of formalin-fixed, paraffin embedded tissue from 26 patients with a diagnosis of LCH were identified by immunohistochemistry using S-100 and CD1a as markers. All biopsies showed characteristic CD1a+ LCs cells, CD68+ macrophages, CD3+ T cells, and eosinophils. Fifteen of the specimens were obtained from bone in cases of ostotic LCH, seven were obtained from skin biopsies in cases of isolated skin disease and four were obtained from excisional lymph node biopsies from patients with solitary lymph node involvement. Multinucleated giant cells were seen in 20 out of the 26 cases. One tissue each of Paget's disease, dermatopathic lymphadenopathy, and normal skin were used as methodological controls in order to avoid false positive or negative stains

due to technical flaws. Lesional tissue of Paget's disease of the bone, a disease characterised by the presence of activated osteoclasts, was used as positive control for the osteoclast-like MGCs of LCH lesions. Dermatopathic lymphadenopathy, a disease characterised by the accumulation of CD1a+ dendritic cells, but without MGCs and normal skin, were used as negative controls. Experiments were approved by the ethical committee of Leiden University Medical Center.

*Antibodies*

All staining was done in PBS with 1% BSA. Mouse monoclonal antibodies to CD1a (1CA04), MMP-9 (4A3) and CD31 (JC/70A) were obtained from Neomarkers (Fremont, California). Goat and rabbit polyclonal antibodies to Cathepsin K, M-CSF and GM-CSF respectively, were obtained from Santa Cruz Biotechnology, Inc. The mouse monoclonal to RANKL (70525) and the goat polyclonal to RANK were obtained from R&D Systems (Minneapolis, Minnesota). The rabbit polyclonal to CD3 and the mouse monoclonal to Ki-67 (MIB-1) were obtained from DakoCytomation (Glostrup, Denmark). The mouse monoclonals to VNR (CJ00) and ICAM-1 (23G12) were obtained from Novocastra (Newcastle upon Tyne, UK). The mouse monoclonal to CD68 (514H12) was obtained from Serotec (Oxford, UK). Secondary antibodies for enzymatic staining were obtained from DakoCytomation, and substrate chemicals were obtained from Vector Laboratories (Burlingame, California). Secondary immunofluorescent reagents were goat anti-mouse, goat anti-rabbit, and donkey anti-goat isotype-specific Alexa Fluor antibodies (Molecular Probes, NL).

*Immunohistochemistry*

The preparation of paraffin sections for staining was carried out as previously described (16). Double and triple stainings with primary antibodies anti-cytokine, cytokine receptors, or osteoclast markers in combination with cell-specific markers were detected fluorescently using the relevant secondary goat anti-mouse, goat anti-rabbit, or donkey anti-goat isotype specific Alexa Fluor 488, 647, or 546 secondary antibodies. Replacement of the primary antibodies by PBS/BSA 1% was used as a negative control. Results were analyzed by confocal microscopy using a confocal microscope in fluorescence and brightfield mode (LSM 510; Carl Zeiss MicroImaging, Inc.).

*Single enzymatic staining for tartrate-resistant acid phosphatase (TRAP)*

TRAP staining was performed using a combination of solutions that include naphtol-AS BI Phosphate, dimethylformamide, tartaric acid, acetate buffer, vermoalbuffer, sodium nitrite, and pararoseaniline. Tissue sections were deparaffinized, rehydrated, and incubated with the reactive solution for 20 min. After washing with distilled water, the tissue sections were counterstained with Mayer's hematoxylin and mounted using Histomount media (National Diagnostics, Atlanta, Georgia).

## Results and Discussion

*MGCs in LCH lesions phenotypically express osteoclast markers*

Although the different types of MGC all have a haematopoietic precursor, the osteoclast has very distinct functional and phenotypic characteristics (3). Thus, in order to clarify whether the MGCs observed in LCH lesions are indeed of an osteoclast-like phenotype, we carried out multicolour immunohistochemical analysis for the typical osteoclast markers, CD68, TRAP, vitronectin receptor (VNR), and enzymes, cathepsin K (CatK) and matrix metalloproteinase–9 (MMP-9) (Table 1 and Figure 1).

CD68, a marker of the monocyte-macrophage lineage cells, was used to detect MGCs in LCH lesions. CD68+ MGCs were observed in 13 out of the 15 LCH bone biopsies analyzed. Importantly, MGCs were also found in nonostotic LCH lesional sites, namely the lymph node (4/4) and the skin (3/7). Five out of seven nonostotic lesions that contained MGCs stained positive for TRAP (Figure 1 A, right), an enzyme present in osteoclastic vesicles that fuse with endocytic vesicles containing the bone matrix degradation products. This enzyme induces the release of reactive oxygen species that destroy the matrix components of the bone (17). Nine out of the 13 bone lesions also showed TRAP positivity on the CD68+ MGCs (Figure 1 A, left). Triple immunofluorescent staining for CD68, VNR, and CatK showed that all the bone (Figure 1 B, left) and lymph node lesions (not depicted) with MGCs were VNR+ and CatK+. In contrast, one out of three skin lesions containing MGCs was positive for VNR and CatK (Figure 1 B, right). A further enzyme characteristic of osteoclasts, MMP-9, was also present on the CD68+ MGCs in all bone lesions (Figure 1 C). Moreover, MMP-9 was also expressed in the MGCs of skin (1/3) and lymph node lesions (4/4). CatK and MMP-9 are proteases involved in the degradation of organic components from the bone matrix, such as type I collagen and other matrix proteins (18, 19). VNR is a receptor for the integrin vitronectin commonly found in osteoclasts and likely to be involved in the interaction between the osteoclast and the bone matrix (20). Thus, the expression of typical osteoclast markers as well as characteristic osteoclast-secreted enzymes by the MGCs in LCH lesions confirms that these cells are indeed osteoclast-like MGCs.

*Possible origin of MGCs in LCH lesions*

The presence of these osteoclast-like giant cells in LCH bone lesions is perhaps not that unusual as this is the normal tissue site for osteoclasts, which, through their resorbing activity, help to maintain the normal homeostasis of the bone (6). However, even in the ostotic LCH lesions these osteoclast-like cells were present in relatively higher numbers than in normal bone and appeared to be “floating” within the cellular infiltrate of the lesion, whereas normally close contact with bone would be expected. In contrast, the finding of osteoclast-like cells in nonostotic LCH sites raises the question

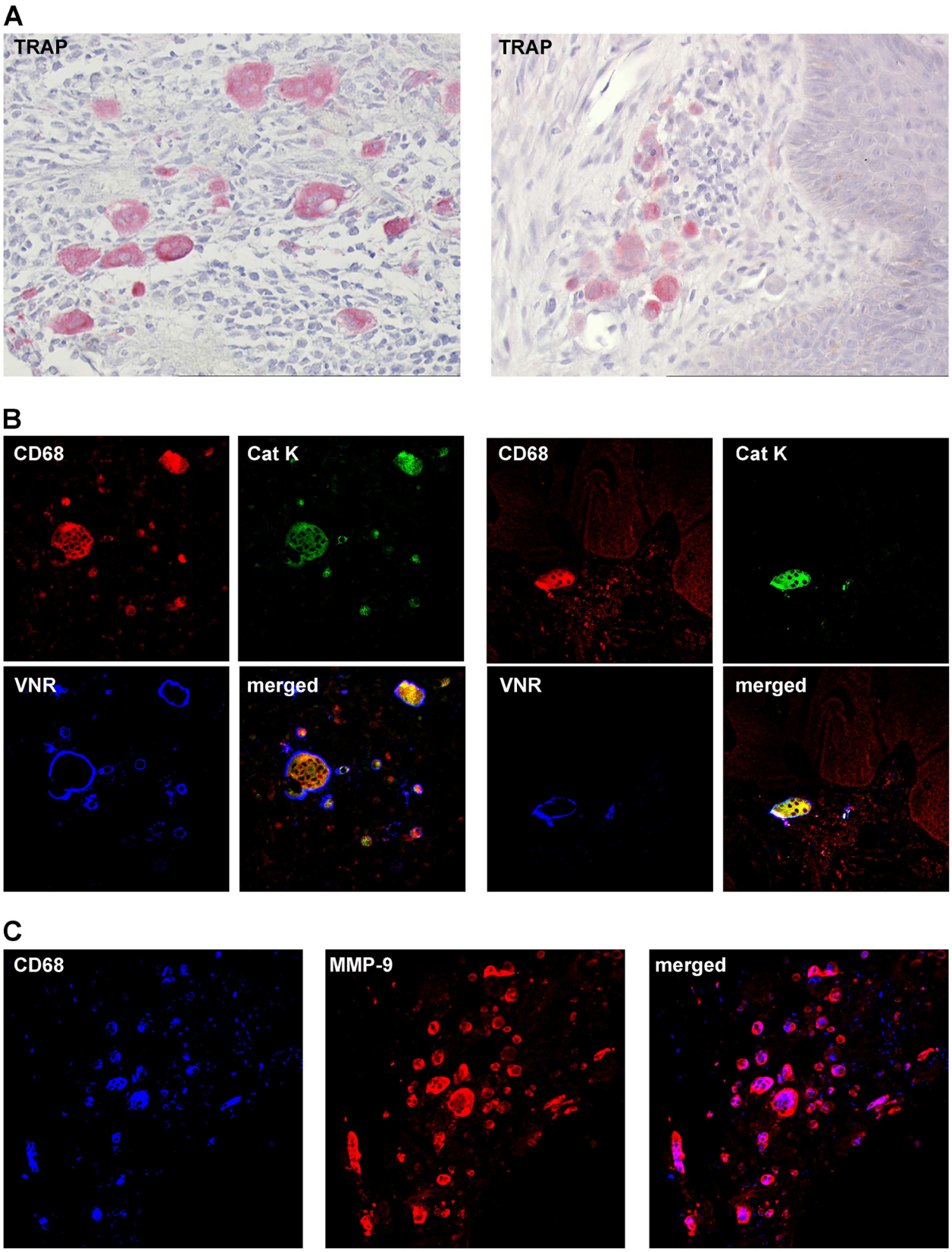


**Figure 1.** Phenotypic characterisation of MGCs in LCH lesions for osteoclast markers. (A) MGCs in bone (left) and skin (right) LCH lesions were TRAP+. (B) Triple colour immunofluorescent staining for CD68, Cat K and VNR in an LCH bone lesion (left) and a skin lesion (right). (C) Double immunofluorescent staining for CD68 and another osteoclast marker, MMP-9, in an LCH bone lesion. Original magnifications: (A) 220X; (B) 290X; and (C) 270X.

**Table 1.** Characterisation of MGCs in LCH lesions for osteoclast markers, osteoclast-secreted enzymes, and osteoclast-inducing environment.

| MGCs in lesion | | | | | | | Lesional environment | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lesional site | | Phenotypic markers of osteoclasts | | | Osteoclast-secreting enzymes | | Lesional site | Osteoclast-inducing cytokines and receptor | | |
| | CD1a | CD68 | TRAP | VNR | CatK | MMP-9 | | M-CSF | RANKL | RANK |
| *Bone* | | | | | | | *Bone* | | | |
| b1 | - | ++ | ++ | ++ | ++ | ++ | b1 | ✓ | ✓ | ✓ |
| b2 | - | ++ | - | ++ | ++ | ++ | b2 | - | ✓ | ✓ |
| b3 | - | - | - | - | - | - | b3 | ✓ | ✓ | ✓ |
| b4 | - | + | - | + | + | + | b4 | ✓ | ✓ | ✓ |
| b5 | - | ++ | ++ | ++ | + | ++ | b5 | - | ✓ | ✓ |
| b6 | - | +++ | +++ | +++ | + | ++ | b6 | ✓ | ✓ | ✓ |
| b7 | - | ++ | ++ | ++ | ++ | ++ | b7 | ✓ | - | ✓ |
| b8 | - | - | - | - | - | - | b8 | ✓ | ✓ | ✓ |
| b9 | - | ++ | - | + | + | ++ | b9 | ✓ | - | ✓ |
| b10 | - | ++ | ++ | + | + | ++ | b10 | ✓ | - | ✓ |
| b11 | - | + | + | + | + | + | b11 | ✓ | ✓ | ✓ |
| b12 | - | +++ | +++ | +++ | +++ | +++ | b12 | ✓ | - | ✓ |
| b13 | - | ++ | - | + | + | ++ | b13 | - | ✓ | ✓ |
| b14 | - | +++ | +++ | ++ | ++ | ++ | b14 | ✓ | ✓ | ✓ |
| b15 | - | + | + | + | + | +++ | b15 | - | ✓ | ✓ |
| *Skin* | | | | | | | *Skin* | | | |
| s1 | ++ | ++ | ++ | ++ | ++ | + | S1 | ✓ | ✓ | ✓ |
| s2 | - | - | - | - | - | - | S2 | ✓ | - | ✓ |
| s3 | - | - | - | - | - | - | S3 | n.d. | - | ✓ |
| s4 | - | - | - | - | - | - | S4 | n.d. | n.d. | - |
| s5 | - | + | + | - | - | - | S5 | n.d. | n.d. | n.d. |
| s6 | - | + | - | - | - | - | S6 | - | ✓ | ✓ |
| s7 | - | - | - | - | - | - | s7 | - | - | - |
| *Lymph node* | | | | | | | *Lymph node* | | | |
| l1 | ++ | ++ | ++ | ++ | ++ | ++ | l1 | ✓ | ✓ | n.d. |
| l2 | n.d. | + | + | + | + | + | l2 | ✓ | ✓ | ✓ |
| l3 | - | +++ | - | +++ | +++ | +++ | l3 | ✓ | ✓ | ✓ |
| l4 | + | + | + | + | + | + | l4 | - | ✓ | ✓ |

Scoring: (-) indicates absence of expression;
(+) indicates 0-30% expression by the MGCs;
(++) indicates 30-70% expression by the MGCs;
(+++) indicates 70-100% expression by the MGCs;
n.d.: not done due to lack of tissue.

Scoring: (-)indicates absence of expression;
✓indicates expression
n.d.: not done due to lack of tissue

of their origin. In order to investigate this we carried out triple immunofluorescent stainings for the Langerhans cell marker, CD1a, the macrophage marker, CD68, and CatK to more clearly identify the MGCs. In all bone lesions the CatK+ osteoclast-like cells coexpressed the macrophage marker CD68. In none of the ostotic lesions did these osteoclast-like MGCs express CD1a (Table 1 and Figure 2 A). This finding suggests that the MGCs in bone LCH display the features of a normal osteoclast. In contrast, in one out of three skin and two out of four lymph node lesions that contained osteoclast-like

cells, the MGCs expressed both CD68 and CD1a (Table 1 and Figure 2 B). Hence, although both the osteoclast-like giant cells in bone as well as in nonbone lesions expressed CD68, only the giant cells in skin and lymph node coexpressed CD1a. This unusual phenotype of these osteoclast-like giant cells in skin LCH has been reported before in a single case without any further characterisation (21). The majority of nonostotic lesions studied were in fact from patients without additional bone lesions. This excludes the possibility that the MGCs were derived from bone lesions. Therefore, it is likely that the lesional environment induces the local formation of the osteoclast-like MGCs even in unusual sites, such as these nonostotic LCH sites. This, together with the fact that the normal precursors of osteoclasts are likely to be absent from these sites, may result in osteoclast-like MGCs derived from a different origin (e.g., CD1a+ cells). Alternatively, the CD1a+ expression by MGCs in these sites may be due to induced expression of CD1a at a later stage.

In order to better understand the likely mechanisms of MGC formation in LCH lesions we looked at the expression of intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule expressed by monocytes upon fusion to form MGCs (22), and Ki-67, a nuclear protein associated with somatic cell proliferation (23). We found that in all LCH lesions the MGCs displayed strong membrane staining for ICAM-1. In contrast, the MGCs were consistently negative for the proliferative marker Ki67 (unpublished data). However, there was a high expression of Ki-67 in other cells in the lesions that we and others have previously shown to be largely due to the CD1a+ LCH cells (24, 25). These findings suggest that the osteoclast-like MGCs present in LCH lesions may be formed by the fusion of resident monocytes-macrophages rather than by cell division. Cytokines such as IFN-γ, which has previously been shown to be expressed in LCH lesions (26), are well-known inducers of ICAM-1 expression and, thus, may initiate the fusion of monocytes and macrophages to form MGCs. Thus, MGCs seem to be intrinsic to LCH lesions and specific factors within the well-characterized “cytokine storm” in LCH lesions are responsible for their formation.

*The osteoclast-inducing cytokines RANKL and M-CSF are highly expressed in LCH lesions*

As shown by *in vitro* studies, the environment in which the mononuclear cells are present determines their differentiation into the various mononuclear phagocyte system-derived cells. Similarly, the tissue site and environment may have a large influence on the cellular composition of LCH lesions. Previous work by our group and others has clearly shown the presence of a cytokine storm in LCH lesions (26). For instance, factors involved in osteoclastogenesis like IL-1, IL-6 and TNFα are highly expressed. In the present study, we have extended the analysis of cytokines to those specifically involved in the induction of osteoclast differentiation. One such cytokine involved in osteoclast induction is RANKL. In 24 LCH lesions studied for RANKL expression, 17 were found to be positive. We found that this cytokine was not expressed by the endothelial cells and macrophages, as assessed by triple staining

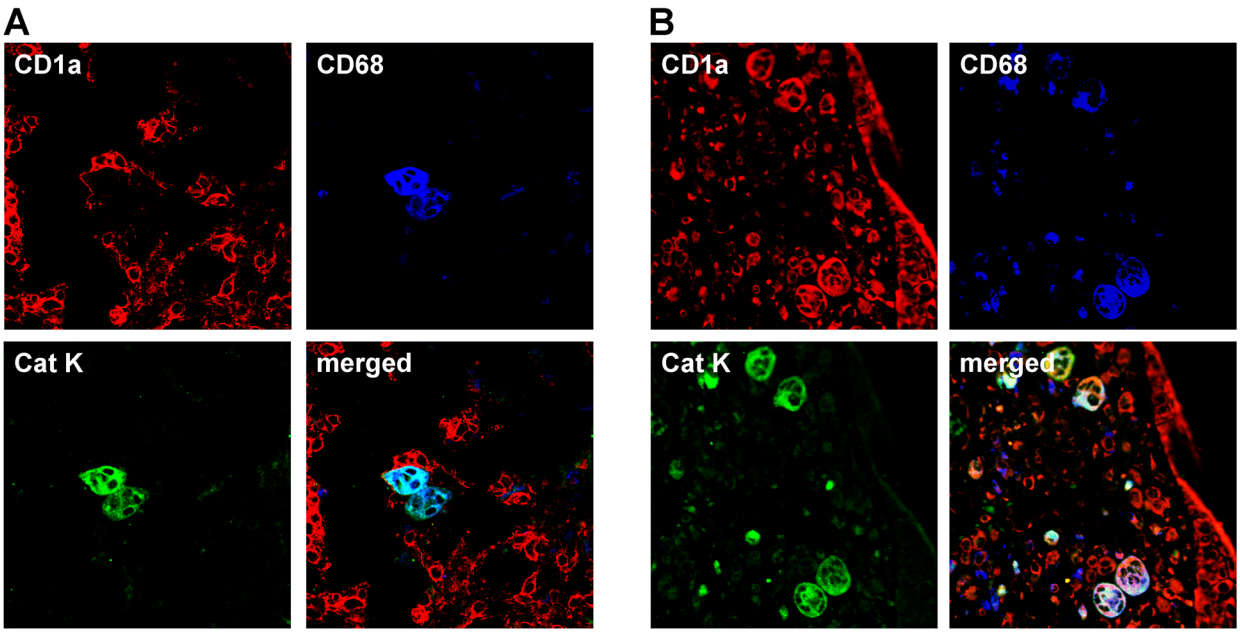


**Figure 2.** Phenotypic difference in osteoclast-like MGCs in bone versus nonbone lesions. Triple immunofluorescent staining for the monocyte lineage marker, CD68, DC marker, CD1a, and the enzyme CatK was performed in LCH bone (A), and skin (B). The osteoclasts in LCH skin lesions clearly expressed CD1a as well as CD68 and CatK (B). In contrast, LCH bone lesions never expressed CD1a (A). Original magnification: 260X.

combining RANKL with the CD31 and CD68 markers, respectively (unpublished data). Instead, triple staining for RANKL, CD1a to identify the LCH cells and the T cell marker CD3, revealed that the majority of CD1a+ LCH cells and T cells in close proximity to the LCH cells expressed RANKL (Figure 3 A). Thus, both the CD1a+ LCH cells and T cells contribute to osteoclastogenesis through up-regulated RANKL and, thus, provide a mechanism for the potentiation of osteoclast formation and bone resorption in LCH lesions.

One key feature of osteoclast differentiation is the interaction between RANKL and its receptor, RANK, commonly expressed by the osteoclast precursor cells. We looked at the presence of RANK receptor on CD68+ and CD1a+ cells by triple immunofluorescent staining. All the lesions that showed expression of RANKL were also positive for RANK, which was expressed by a high proportion of CD1a+ cells and to a lesser extent by CD68 cells as shown in Figure 3 B. The expression of RANK by CD1a+ cells as well as the presence of its ligand by activated T cells in LCH lesions is also important, as this interaction is known to induce a survival signal to DCs (27).

Furthermore, we looked at the expression of another cytokine known to be involved in osteoclast differentiation, M-CSF. M-CSF is normally produced by osteoblasts and/or stromal cells and is involved in the differentiation of osteoclasts from an early stage. We found it to be expressed by the MGCs and strikingly also by CD1a cells in 11 out of 15 LCH bone lesions. Interestingly, we found that this cytokine was even expressed by the CD1a+ cells in 1/3 skin (Figure 3 C) and 3/4

lymph node lesions that contained MGCs. The expression of M-CSF by the lesional CD1a+ cells seemed particularly relevant as there was clearly no expression of this cytokine by the normal LCs in both LCH skin lesions (Figure 3 C) and normal skin (not depicted). The presence of cytokines involved in osteoclast differentiation in LCH lesions provides an explanation for the presence of osteoclast-like cells in ostotic as well as nonostotic sites in LCH. An attractive hypothesis would be that excessive amounts of osteoclast-inducing cytokines, such as RANKL and M-CSF, induce osteoclast-like differentiation of inappropriate precursors (e.g., CD1a+ LCH cells). Strong support for this hypothesis was demonstrated in a very recent paper by Rivollier *et al.* (28). Using human monocyte-derived DCs generated *in vitro* they showed that immature DCs can transdifferentiate into functional osteoclasts in the presence of M-CSF and RANKL. Furthermore, they showed that the proinflammatory cytokines, TNFα and IL-1α, both of which are highly expressed in LCH lesions (26), promote cell fusion during DC transdifferentiation and lead to larger MGCs than M-CSF plus RANKL alone. Thus, the *in vitro* differentiation plasticity seen in cells of the mononuclear phagocyte system also appears to occur *in vivo* in LCH lesions.

*Rationale for the use of bisphosphonates in the treatment of LCH*

Due to the lack of fresh biopsy material, it was not possible to perform functional studies such as the use of dentine discs to determine the resorbing capacity of the MGCs in LCH lesions. However, the finding that the MGCs in LCH lesions are expressing various matrix-degrading enzymes supports the hypothesis of a destructive role for these cells in LCH lesions. Such a role would also help to explain the predominant symptom of bone pain suffered by patients with LCH bone lesions. The present report has provided support for the hypothesis that the excessive bony destruction found in LCH is likely mediated by osteoclast-like giant cells. Therefore, these cells are a potential target in LCH lesions. To date, only a few case reports, including one we authored, have indicated the use of bisphosphonates as a successful treatment of bone LCH (29). However, all of these case-reports lack the fundamental background for the rationale. Bisphosphonates appear to act, when administered at therapeutic doses, only in bone, which is probably due to their specific affinity to this tissue. This group of compounds is known to have an inhibitory effect in the number and activation of osteoclasts (30). Thus, this study has provided a rationale for the use of bisphosphonates in the treatment of LCH patients.

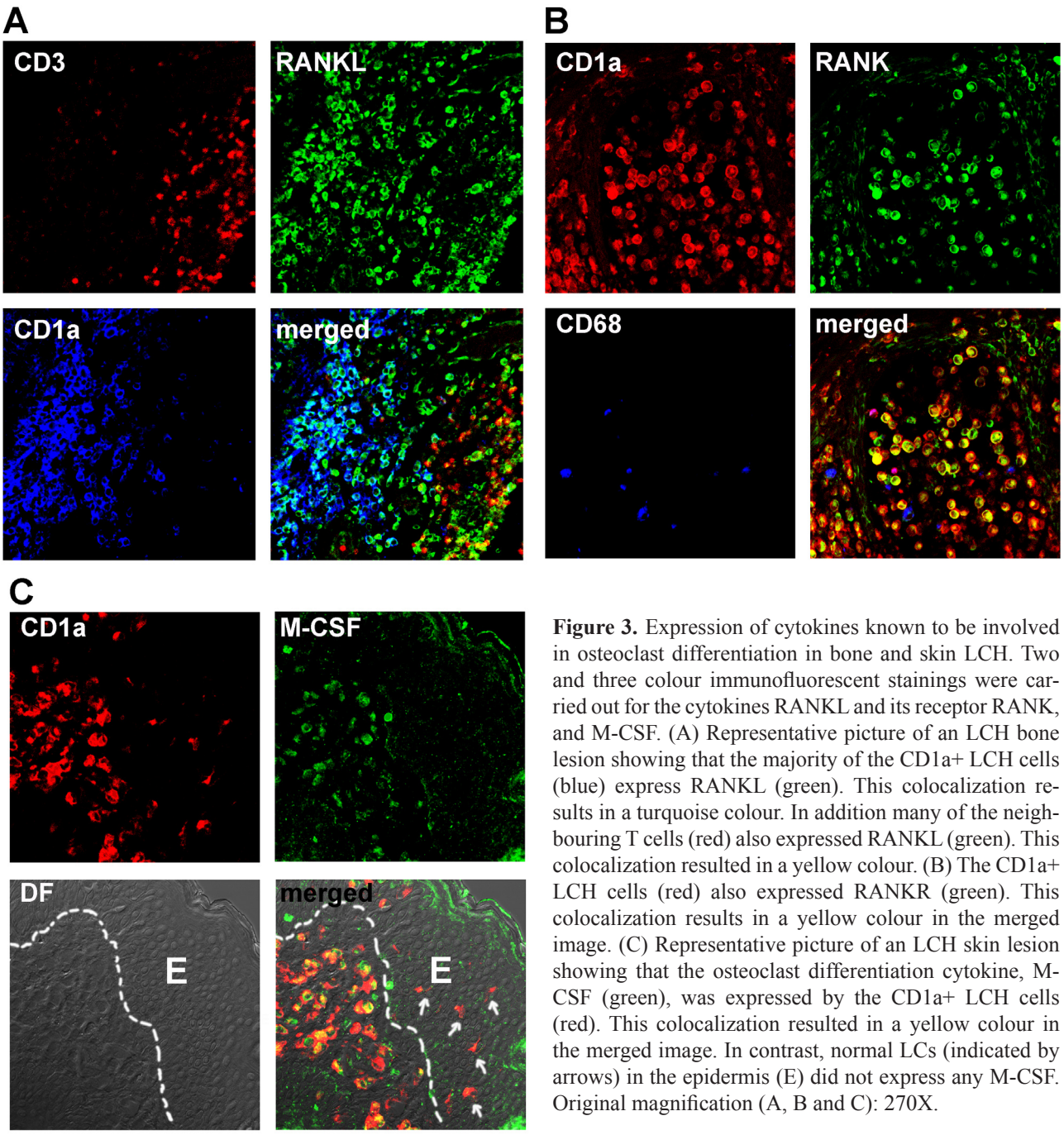


**Figure 3.** Expression of cytokines known to be involved in osteoclast differentiation in bone and skin LCH. Two and three colour immunofluorescent stainings were carried out for the cytokines RANKL and its receptor RANK, and M-CSF. (A) Representative picture of an LCH bone lesion showing that the majority of the CD1a+ LCH cells (blue) express RANKL (green). This colocalization results in a turquoise colour. In addition many of the neighbouring T cells (red) also expressed RANKL (green). This colocalization resulted in a yellow colour. (B) The CD1a+ LCH cells (red) also expressed RANKR (green). This colocalization results in a yellow colour in the merged image. (C) Representative picture of an LCH skin lesion showing that the osteoclast differentiation cytokine, M-CSF (green), was expressed by the CD1a+ LCH cells (red). This colocalization resulted in a yellow colour in the merged image. In contrast, normal LCs (indicated by arrows) in the epidermis (E) did not express any M-CSF. Original magnification (A, B and C): 270X.

**Acknowledgements**

We would like to thank Dr Ronald Jaffe for supplying some of the LCH skin lesions used in this work. Cristiana Costa is supported by Fundação para a Ciência e a Tecnologia, Portugal. This work was partly funded by The Histiocytosis Association of America.

**References**


1. Egeler, R. M. and G. J. D'Angio. 1995. Langerhans cell histiocytosis. J Pediatr. 127(1):1-11.
2. Favara, B. E. and R. Jaffe. 1994. The histopathology of Langerhans cell histiocytosis. Br J Cancer Suppl. 23:S17-23.
3. Anderson, J.M. 2000. Multinucleated giant cells. Curr Opin Hematol. 7:40-47.
4. Byrd, T.F. 1998. Multinucleated giant cell formation induced by IFNg/IL-3 is associated with restriction of virulent Mycobacterium tuberculosis cell to cell invasion in human monocyte monolayers. Cell Immunol. 188:89-96.
5. McNally, A.K. and J.M. Anderson. 1995. Interleukin-4 induces foreign body giant cells from human monocytes/macrophages: differential lymphokine regulation of macrophage fusion leads to morphological variants of multinucleated giant cells. Am J Pathol. 147:1487-1499.
6. Teitlelbaum, S. L. 2000. Bone resorption by osteoclasts. Science. 289:1504-1508.
7. Murch, A.R., M.D. Grounds, C.A. Marshall and J.M. Papadimitriou. 1981. Direct evidence that inflammatory multinucleated cells form by fusion. J Pathol. 137:117-123.
8. Kreipe, H., H.J. Radzun, P. Rudolph, J. Barth, M. Hansmann, M.R. Parwaresch. 1988. Multinucleated giant cells generated *in vitro*. Terminally differentiated macrophages with down-regulated c-fms expression. Am J Pathol. 130:232-238.
9. DeFife, K. M., C. R. Jenney, A. K. McNally, E. Colton, and J. M. Anderson. 1997. Interleukin-13 induces human monocyte/macrophage fusion and macrophage mannose receptor expression. J Immunol. 158:3385-3390.
10. Kudo, O., Y. Fujikawa, I. Itonaga, A. Sabokbar, T. Torisu, and N.A. Athanasou. 2002. Proinflammatory cytokine (TNFalpha/IL-1alpha) induction of human osteoclast formation. J Pathol. 198:220-227.
11. Tsurukai, T., N. Udagawa, K. Matsuzaki, N. Takahashi, and T. Suda. 2000. Roles of macrophage-colony stimulating factor and osteoclast differentiation factor in osteoclastogenesis. J Bone Miner Metab. 18:177-184.
12. Paquette, R. L., N.C. Hsu, S.M. Kiertscher, A.N. Park, L. Tran, M.D. Roth, and J.A. Glaspy. 1998. Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potent antigen-presenting cells. J Leukoc Biol. 64:358-367.
13. Duperrier, K., A. Eljaafari, C. Dezutter-Dambuyant, C. Bardin, C. Jacquet, K. Yoneda, D. Schmitt, L. Gebuhrer, and D. Rigal. 2000. Distinct subsets of dendritic cells resembling dermal DCs can be generated *in vitro* from monocytes, in the presence of different serum supplements. J Immunol Methods. 238:119-131.
14. Ferlazzo, G., J. Klein, X. Paliard, W. Z. Wei and A. Galy. 2000. Dendritic cells generated from CD34+ progenitor cells with flt3 ligand, c-kit ligand, GM-CSF, IL-4, and TNF-alpha are functional antigen-presenting cells resembling mature monocyte-derived dendritic cells. J Immunother. 23(1):48-58.
15. Servet-Delpart, C., S. Arnaud, P. Jurdic, S. Nataf, M.F. Grasset, C. Soulas, C. Domenget, O. Destaing, A. Rivollier, M. Perret, C. Dumontel, D. Hanau, G.L. Gilmore, M.F. Belin, C. Rabourdin-Combe, and G. Mouchiroud. 2002. Flt3+ macrophage precursors commit sequentially to osteoclasts, dendritic cells and microglia. BMC Immunol. 3:15-25.
16. Annels, N. E., C.E.T. da Costa, F.A. Prins, A. Willemze, P.C. Hogendoorn, and R.M. Egeler. 2003. Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. J Exp Med. 197:1385-1390.
17. Halleen, J.M., S. Raisanen, J. J. Salo, S. V. Reddy, G. D. Roodman, T. A. Hentunen, P. P. Lehenkari, H. Kaija, P. Vihko and H. K. Vaananen. 1999. Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. J Biol Chem. 274:22907-22910.

18. Bossard, M. J., T. A. Tomaszek, S. K. Thompson, B. Y. Amegadzie, C.R. Hanning, C. Jones, J. T. Kurdyla, D. E. McNulty, F. H. Drake, M. Gowen and M. A. Levy. 1996. Proteolytic activity of human osteoclast cathepsin K. Expression, purification, activation, and substrate identification. J Biol Chem. 271:12517-12524.
19. Okada, Y., K. Naka, K. Kawamura, T. Matsumoto, I. Nakanishi, N. Fujimoto, H. Sato and M. Seiki. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase B) in osteoclasts: implications for bone resorption. Lab Invest. 72:311-322.
20. Shankar, G., I. Davison, M. H. Helfrich, W. T. Mason and M.A. Horton. 1993. Integrin receptor-mediated mobilisation of intranuclear calcium in rat osteoclasts. J Cell Sci. 105:61-68.
21. Ruco, L. P., A. Stoppacciaro, D. Vitolo, S. Uccini, and C.D. Baroni. 1993. Expression of adhesion molecules in Langerhans' cell histiocytosis. Histopathology. 23:29-37.
22. Fais, S., V.L. Burgio, M. Sivestri, M.R. Capobianchi, A. Pacchiarotti and F. Pallone. 1994. Multinucleated giant cells generation induced by interferon-gamma. Changes in the expression and distribution of the intercellular adhesion molecule-1 during macrophages fusion and multinucleated giant cell formation. Lab Invest. 71:737-744.
23. Gerdes, J., L. Li, C. Schlueter, M. Duchrow, C. Wohlenberg, C. Gerlach, I. Stahmer, S. Kloth, E. Brandt and H.D. Flad. 1991. Immunobiochemical and molecular biologic characterisation of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol. 138:867-873.
24. Schouten, B., R.M Egeler, P.J. Leenen, A.H. Taminiau, L.J. van den Broek and P.C. Hogendoorn. 2002. Expression of cell cycle-related gene products in Langerhans cell histiocytosis. J Pediatr Hematol Oncol. 24:727-732.
25. Bank, M.I., P. Rengtved, H. Carstensen and B.L. Petersen. 2003. Langerhans cell histiocytosis: an evaluation of histopathological parameters, demonstration of proliferation by Ki67 and mitotic bodies. APMIS. 111:300-308.
26. Egeler, R.M., B.E. Favara, M. van Meurs, J.D. Laman and E. Claassen. 1999. Differential in situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. Blood. 94:4195-4201.
27. Cremer, I., M.C. Dieu-Nosjean, S. Marechal, C. Dezutter-Dambuyant, S. Goddard, D. Adams, N. Winter, C. Menetrier-Caux, C. Sautes-Fridman, W.H. Fridman, and C.G. Mueller. 2002. Long-lived immature dendritic cells mediated by TRANCE-RANK interaction. Blood. 100:3646-3655.
28. Rivollier, A., M. Mazzorana, J. Tebib, M. Piperno, T. Aitsiselmi, C. Rabourdin-Combe, P. Jurdic, and C. Servet-Delprat. 2004. Immature dendritic cell transdifferentiation into osteoclasts: a novel pathway sustained by the rheumatoid arthritis microenvironment. Blood. 10.1182/blood-2004-01-0041.
29. Farran, R. P., E. Zaretski, and R.M. Egeler. 2001. Treatment of Langerhans cell histiocytosis with pamidronate. J Pediatr Hematol Oncol. 23:54-56.
30. Green, J. R. and P. Clezardin. 2002. Mechanisms of biphosphonate effects on osteoclasts, tumor cell growth and metastasis. Am J Clin Oncol. 25(6 Suppl 1):S3-S9.

# 6

# Differences in telomerase expression by the CD1a+ cells in Langerhans cell histiocytosis reflect the diverse clinical presentation of the disease

Cristiana ET da Costa
R Maarten Egeler
Manja Hoogeboom
Karoly Szuhai
Ramses G Forsyth
Marieke Niesters
Ronald R de Krijger
Abdellatif Tazi
Pancras CW Hogendoorn
Nicola E Annels

*The Journal of Pathology*

2007; 212: 188-197

**Abstract**

Langerhans cell histiocytosis (LCH) is a disease characterised by an uncontrolled clonal proliferation of Langerhans cells, whose aetiology is still unclear. The clonal nature of LCH could support the hypothesis that it is a neoplastic disease with unlimited growth potential. One requirement for unlimited proliferation is the maintenance of telomere length. In a group of 70 patients we set out to investigate whether a telomere maintenance mechanism is indeed active in LCH cells. This work showed that LCH cells from all restricted skin LCH lesions (6/6) expressed telomerase as assessed by human telomere reverse transcriptase (hTERT) immunohistochemistry, whereas LCH cells from the majority of the bone lesions analysed did not express hTERT (26/34). Interestingly, in contrast to the solitary bone lesions, LCH cells from lesions of multisystem patients always expressed telomerase (11/11), regardless of the lesional site. *In situ* telomeric repeat amplification protocol (TRAP) assays performed on different lesional sites showed that this telomerase was active. In addition, the telomere length of LCH cells from a hTERT-positive skin multisystem lesion was long and homogeneous when compared to that in the LCH cells from hTERT-negative bone single system LCH lesions, which was heterogeneous in length. No evidence for an alternative lengthening of telomeres mechanism was found in hTERT-negative lesions. The difference in telomerase expression and telomere length at the different lesional sites and in biopsies from patients with solitary versus multisystem disease appears to reflect the diverse clinical presentation and course of this disease. The results from this study have important implications for understanding the nature of this disease.

**Introduction**

The aetiology of Langerhans cell histiocytosis (LCH), a disease mainly occurring during childhood and characterized by an uncontrolled clonal proliferation of Langerhans cells (LCs) in several sites of the body, is unknown. While some believe that it occurs due to an external trigger, others argue that it is likely caused by an intrinsic defect and is neoplastic (1, 2). Arguments supporting a reactive origin include the granulomatous character of LCH lesions rather than the cellular homogeneity of a malignant neoplasm, frequent cases of spontaneous clinical regression and the failure to establish a cell line from LCH cells *in vitro* (3). In contrast, observations of several cases of familial clustering, together with studies showing evidence for cytogenetic aberrations confirm that there indeed exists a component of genetic instability in LCH cells (4-7). Furthermore, the clonal nature of LCH could support the hypothesis that this disease is neoplastic (8, 9).

The massive accumulation of LCs that characterizes LCH lesions may result not only from the ab-

normal local proliferation of these cells but also from deregulation of apoptosis. As well as their local proliferative activity, as evidenced by their Ki-67 positivity, LCH cells have been shown to express elevated levels of the anti-apoptotic proteins bcl-2 and survivin (10-12). Furthermore, only a small number of apoptotic cells have been evidenced in LCH lesions by terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) (13), suggesting that, despite the uncontrolled proliferative capacity of LCH cells, they are able to survive. One requirement for such unlimited proliferation is the maintenance of telomere length (14, 15). Telomeres are DNA sequence repeats complexed with specific binding proteins that are located at the ends of every normal human chromosome. They play an important role in chromosome structural integrity and protection against the activation of DNA damage checkpoints. They also counter the loss of terminal DNA segments that occurs when linear DNA is replicated. In normal human cells, telomeres shorten with each cell division and become dysfunctional, leading to chromosomal instability and ultimately cell death (16,17). Therefore, normal human committed cells have a limited proliferative potential. In contrast, pathological telomere elongation is found in a large majority of cancers, either via telomerase enzymatic activity or by an alternative telomerase-independent pathway thought to involve homologous recombination, known as alternative lengthening of telomeres (ALT) (18-20). To date, evidence for ALT activity has only been found in a subset of *in vitro* immortalized and tumour-derived cell lines, as well as in spontaneous human tumours (21).

The presence of a possible telomere maintenance mechanism in the pathogenic LCs of LCH lesions has not been investigated to date. However, it is clear that the proliferation and survival of LCH cells lies at the heart of the development and persistence of LCH lesions. Therefore, determining how telomeres are regulated in LCH cells has important implications for the nature of this disease.

## Methods

*LCH tissue samples and controls*

Fifty-one paraffin embedded biopsies and 28 frozen biopsies from 70 children with single system (SS) (n = 59) or multisystem (MS) (n = 11) disease were studied, including 39 bone, 15 skin, eight lymph node (LN), nine lung LCH cases and eight cases involving other lesional sites. All received a definite diagnosis of LCH based on morphology and CD1a and S100 immunoreactivity. Dermatopathic lymphadenopathy (DL) (n = 3), was used for comparison. The osteosarcoma cell line (U2OS) was used as a positive control and Ewing's sarcoma was used as a negative control for the ALT mechanism (22, 23). Giant cell tumour (GCT) and normal testis were used as positive controls for the expression and activity of telomerase, respectively (24, 25). The expression of telomerase by multinucleated giant

cells (MGCs) was also studied in Paget's disease of bone (n = 3), sarcoidosis (n = 2) and tuberculosis (n = 3). The experiments were approved by the ethics committee of Leiden University Medical Center, and were in accordance with national ethical guidelines (*Code of Proper Secondary Use of Human Tissue in The Netherlands*, Dutch Federation of Medical Scientific Societies).

*Immunohistochemistry for telomerase*

Paraffin-embedded and frozen sections (4 μm thick) were cut and stained as described previously (26), using a mouse monoclonal anti-hTERT antibody (Novocastra, Newcatle upon Tyne, UK) (27). The primary antibody was detected enzymatically for brightfield microscopy and by immunofluorescence. For enzymatic detection, a rabbit-anti-mouse biotinylated antibody and streptavidin-biotin complex coupled with horse radish peroxidase system (DAKO, Denmark) were used. The colour was developed with 3,3'-diaminobenzidine, the slides were mounted with Pertex and analysed by brightfield microscopy. Double immunofluorescent staining was performed using mouse monoclonals anti-CD1a (Neomarkers, Fremont, CA, USA) in the case of LCH tissue, or anti-vitronectin receptor (Novocastra) in the case of GCT, and anti-hTERT antibodies, followed by detection with goat anti-mouse Alexa Fluor 488 and 594 (Invitrogen, Carlsbad, CA, USA) and mounting the slides in anti-fading medium. As a negative control, the primary antibodies were replaced by phosphate-buffered saline with 1% bovine serum albumin. For each lesion studied, at least 100 CD1a+ cells were scored for the typical nuclear and/or nucleolar staining of hTERT positivity (27). A complete absence of CD1a+ cells expressing hTERT was considered negative and a positive case scored according to whether it contained 1-30%, 30-70% or 70-100% of hTERT-positive (hTERT+) CD1a+ cells.

*Detection of ALT-associated promyelocytic leukaemia bodies (APBs)*

ALT-APBs were detected in sorted LCH cells from single cell suspensions obtained from paraffin-embedded biopsies (28). Staining was performed using a mouse anti-human Langerin monoclonal antibody (Novocastra) and the secondary antibody goat anti mouse-488 (Invitrogen). Flow cytometric analysis and sorting were performed using a BD FACSAria (BD Biosciences, San Jose, CA, USA). Controls included isotype-negative control mouse IgG2b from DAKO (Glostrup, Denmark).

Langerin/488+ sorted cells were spotted on slides, dried and swollen for 15 min with a borate solution (0.1 M $Na_2B_4O_7$). Detection of APBs was performed by fluorescent *in situ* hybridization (FISH) as described (22). The slides were hybridized with a Cy3-labelled telomere-specific peptide nucleic acid (PNA) probe (DAKO), followed by labelling with a goat anti-PML polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA) and detected with an Alexa Fluor 488 labelled secondary antibody. An APB was considered to be present if the telomeric DNA was colocalized with a PML in the nucleus.

An LCH biopsy was considered positive for APBs if present in larger or equal to 20% of the sorted Langerin+ cells. The slides were visualized using a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany) and analysed with Leica QFISH software (Leica Imaging System, Cambridge, UK).

*In situ TRAP assay*

The telomerase PCR ELISA detection kit from Roche (Basel, Switzerland) was used and adapted for *in situ* detection of telomerase activity, as described by Youssef *et al.* (29). This assay enables specific *in situ* detection of PCR-amplified telomerase-mediated elongation products on frozen sections.

*Telomere length determination in sorted CD1a+ cells from LCH and DL frozen biopsies*

Frozen LCH and DL biopsy sections, 50 μm thick, were fixed for 1 hr at 4°C with 4% paraformaldehyde. Following mechanical disaggregation, the single cell suspensions were stained with a mouse monoclonal CD1a-APC antibody (BD Pharmingen, San Diego, CA, USA) for 1hr at 4°C and CD1a+ cells were sorted as above. Sorted cells from the CD1a+ and CD1a-negative ($CD1a^-$) fractions were spotted on glass slides, dried and double stained fluorescently with the mouse anti-CD1a and anti-Langerin monoclonal antibodies, followed by counterstaining with 4'-6-Diamidino-2-phenylindole (DAPI) to confirm the purity of the sorting.

The telomere length of the CD1a+ sorted cells was determined using the TeloTAGGG Telomere Length Assay from Roche (Basel, Switzerland), according to the manufacturer's instructions.

## Results

*Expression of telomerase by CD1a+ cells in skin but not bone LCH lesions*

To investigate whether LCH cells display a telomere maintenance mechanism, the expression of telomerase was studied in LCH lesions from various tissue sites. The staining pattern of the anti-hTERT antibody was confirmed by a control tissue, GCT, where the MGCs express hTERT, the catalytic subunit of telomerase (Figure 1 A). In all the LCH skin lesions studied (n = 15), double immunofluorescent staining of CD1a and hTERT revealed hTERT positivity, not only by the keratinocytes in the epidermis but also by CD1a+ LCH cells in the dermis of LCH skin lesions (Table 1, Figure 1 C). In contrast, LCs in normal control skin were negative for hTERT (Figure 1 D) and only the keratinocytes expressed hTERT (30). The same staining was also performed in SS LN and bone LCH lesions. However, in contrast to SS skin LCH lesions, only 3/5 SS LN lesions and 8/34 SS bone LCH lesions contained CD1a+ LCH cells expressing hTERT (Figures 1 E and F, respectively). Double immun-

ofluorescence for CD1a and hTERT used to study three cases of the reactive disease, DL, showed the LCs to be hTERT-positive (hTERT+; data not shown).

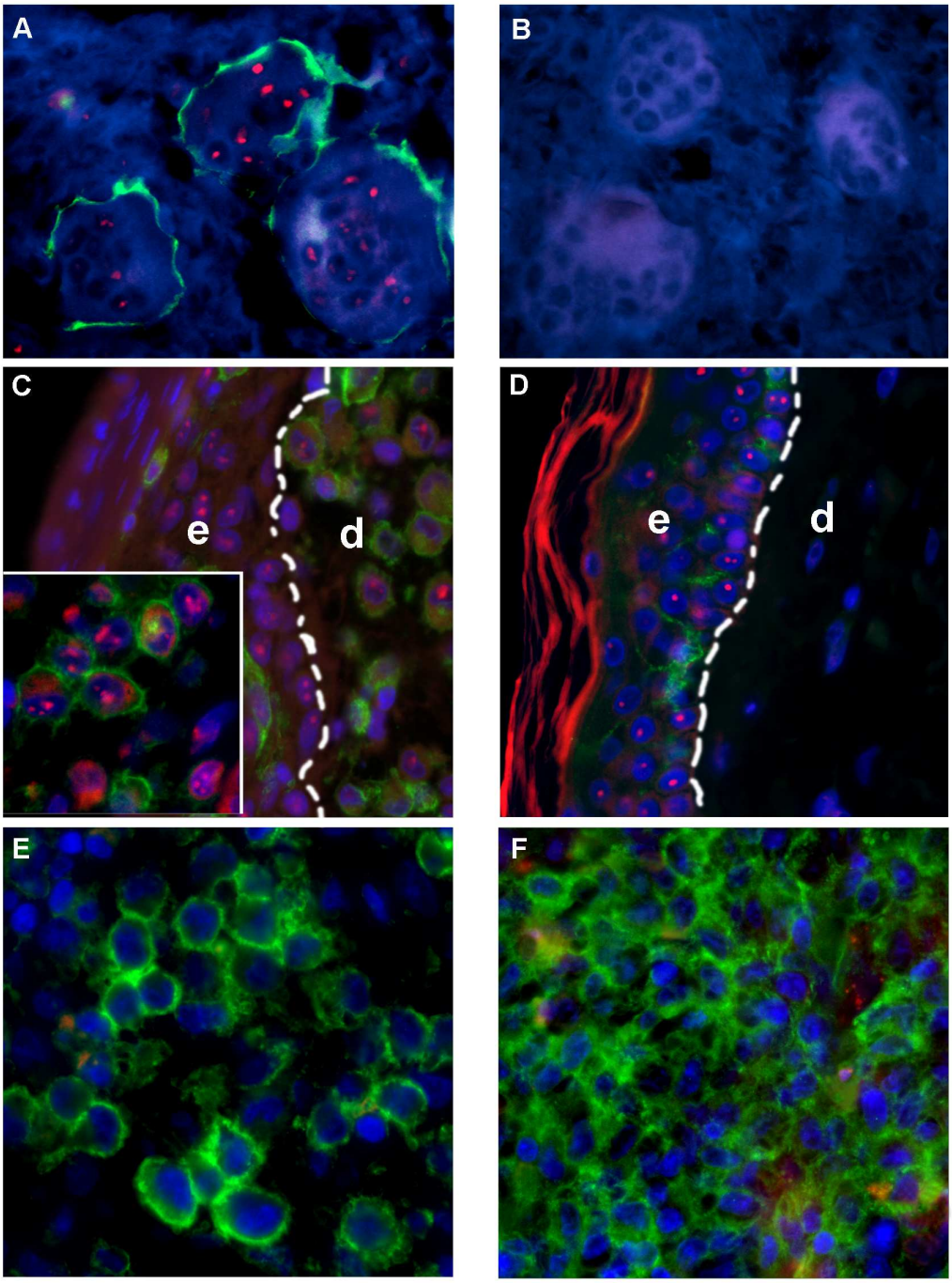


**Figure 1.** Expression of hTERT by CD1a+ LCH cells is consistently observed in skin LCH lesions. (A) hTERT staining was confirmed on control tissue known to be positive for telomerase, giant cell tumour (GCT). Double immunofluorescent staining was performed using the vitronectin receptor (green) to identify the multinucleated giant cells in GCT, in combination with hTERT (red) and DAPI (blue). (B) The omission of the primary antibodies in GCT confirmed the specificity of this staining. (C) Double immunofluorescence staining was performed using CD1a as a LCH cell marker (green) in combination with hTERT (red). DAPI was used to distinguish the cell nuclei (blue). Besides the keratinocytes in the epidermis (e), hTERT was also expressed by CD1a+ LCH cells in the dermis (d) of skin LCH lesions (see inset picture), in contrast to (D) normal skin, where the LCs were negative for hTERT. (E, F) Representative examples of LN and bone LCH lesions in which the CD1a+ cells were negative for hTERT in the majority of cases. The broken white line depicts the epidermal-dermal junction in the skin (magnifications: A, B, 40x; C, D, E, F, 100x).

**Table 1.** Detection of telomere maintenance mechanisms in LCH lesions.

| Type of lesion | hTERT+ CD1a cells[§] | | | | hTERT+ MGCs[*] | | | | Telomerase activity[†] | ALT[‡] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1-30% | 30-70% | 70-100% | | 1-30% | 30-70% | 70-100% | | |
| SS bone | 8/34: | 2 | 5 | 1 | 5/11: | 2 | 1 | 2 | 1/5 | 0/5 |
| MS bone | 4/4: | 1 | 2 | 1 | 2/2: | 1 | 1 | 0 | - | - |
| SS skin | 6/6: | 3 | 0 | 3 | 1/1: | 1 | 0 | 0 | 1/1 | - |
| MS skin | 9/9: | 3 | 1 | 5 | - | | | | 1/1 | 0/1 |
| SS LN | 3/5: | 3 | 0 | 0 | 1/1: | 1 | 0 | 0 | 1/1 | 0/2 |
| MS LN | 3/3: | 0 | 3 | 0 | - | | | | - | 0/1 |
| SS lung | 7/9: | 2 | 3 | 2 | - | | | | 1/3 | - |
| SS other lesional site | 2/5: | 2 | 0 | 0 | - | | | | 1/3 | 0/3 |
| MS other lesional site | 3/3: | 1 | 1 | 1 | - | | | | - | - |

[§]Number of lesions out of the total studied which contain CD1a+ cells expressing hTERT. In each of the cases of an hTERT+ lesion, the percentage of CD1a+ cells expressing hTERT was scored into three groups: 1-30%, 30-70% or 70-100%.
[*]Number of lesions out of the total studied which contain MGCs expressing hTERT. In each of the cases of an hTERT+ lesion, the percentage of MGCs expressing hTERT was scored into three groups: 1-30%, 30-70% or 70-100%.
[†]Number of lesions out of the total studied which contain CD1a+ cells displaying telomerase activity.
[‡]Number of lesions out of the total studied which contain CD1a+ cells displaying an alternative lengthening of telomere (ALT) mechanism.
SS = single system; MS = multisystem; (-) = no data.

*Multinucleated giant cells in skin LCH lesions also express hTERT*

Another cell type intrinsic to LCH lesions is the MGC. The expression of hTERT by these cells was also investigated by single enzymatic staining. In addition to the CD1a+ LCH cells displaying hTERT positivity, the MGCs also expressed hTERT in the skin (Figure 2 A), LN and a few cases of bone LCH lesions that contained MGCs (Figure 2 B). This was observed in nine cases of LCH, of which five were LCH lesions that contained hTERT+ LCH cells. These findings were compared to other diseases that contain activated MGCs, such as sarcoidosis and Paget's disease. In these diseases MGCs were also positive for hTERT (Figures 2 C and D, respectively).

*Telomerase detection in bone LCH lesions from multisystem patients*

As well as lesions from SS disease patients, the CD1a+ LCH cells of different lesional sites from 11 patients with MS LCH were also studied for their expression of telomerase. In 11 out of 11 MS patients, hTERT was expressed by the LCH cells regardless of the lesional site. Even the LCH cells from bone lesional sites showed hTERT positivity, in contrast to most SS bone LCH lesions. Figures 3 A, B and C show a representative example of a MS LCH patient with skin, bone and gut involvement, respectively. In all these sites LCH cells were hTERT+.

*In situ detection of telomerase activity in skin LCH lesions*

Although hTERT is a major component of telomerase, its expression does not strictly correlate with telomerase activity. Therefore, the activity of telomerase in hTERT+ LCH biopsies was assessed using an *in situ* telomeric repeat amplification protocol (TRAP) assay which allows direct visualiza-

tion of telomerase activity on tissue sections. Testis, which contains many germ cells known to have telomerase activity, was used as positive control (Figures 4 A and B). To check the specificity of the technique, negative controls which included the omission of the reaction mixture were routinely used (Figures 4 A, C and E). Telomerase activity was observed in cells present in the dermis of two hTERT+ skin LCH lesions (Figures 4 D and Table 1). In contrast, SS bone LCH lesions which were negative for hTERT (hTERT$^-$) did not show any evidence for telomerase activity (Figure 4 F) From the five SS bone cases analysed, only one showed telomerase activity, which corresponded to a lesion containing CD1a+ hTERT+ cells (Table 1).

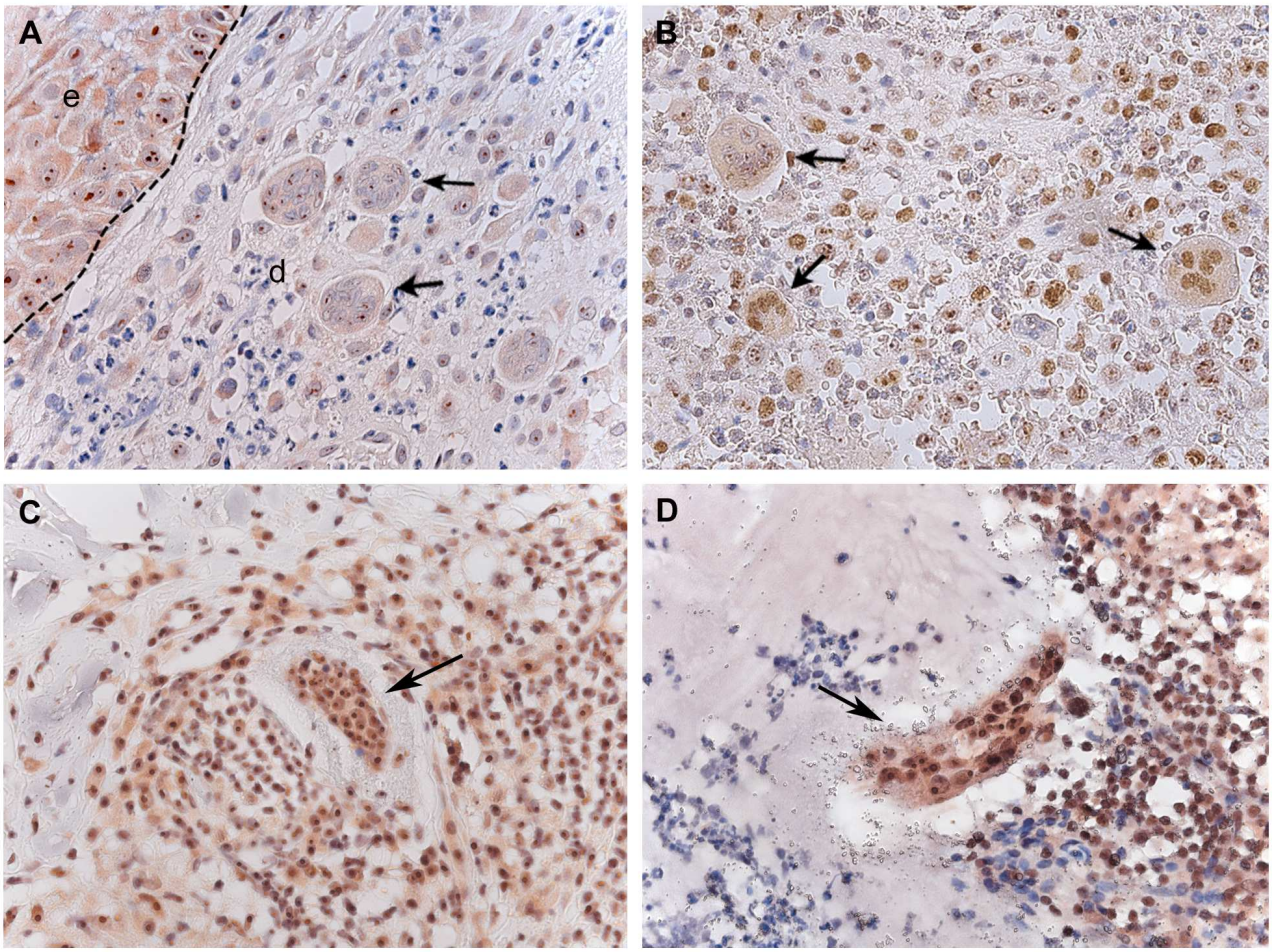


**Figure 2.** Expression of hTERT by MGCs in LCH lesions compared to other diseases that typically contain these cells. Immunohistochemistry was performed with an anti-hTERT monoclonal antibody and DAB detection. hTERT was detected in the osteoclast-like MGCs of skin (A) and a few bone LCH lesions (B) (arrows). The expression of hTERT in sarcoidosis (C) and Paget's disease of bone (D) also showed positivity of the MGCs (arrows) (magnifications: A, B, C, D, 40x).

*Lack of an ALT mechanism in hTERT-negative lesions*

To determine whether the hTERT$^-$ LCH cells in bone and LN lesions were displaying an ALT mechanism, the presence of APBs in these cells was determined. This was performed by FACS-sorting the LCH cells using

the LC-marker langerin (Figure 5 A, right panel) and carrying out FISH on the sorted LCH cells, using a PNA probe in combination with fluorescence immunolabelling for PML. The osteosarcoma cell line U2OS was used as a positive control for ALT positivity (Figure 5 B, left panel). hTERT$^-$ LCH cells from SS bone LCH lesions did not show any co-localization of the PNA probe and PML protein (Figure 5 B, right panel and Table 1), demonstrating the lack of an ALT mechanism. In addition, the skin and LN LCH lesions where LCH cells were hTERT+ (15 out of 15 and 6 out of 8, respectively) did not show any co-localization of PML/PNA (data not shown).

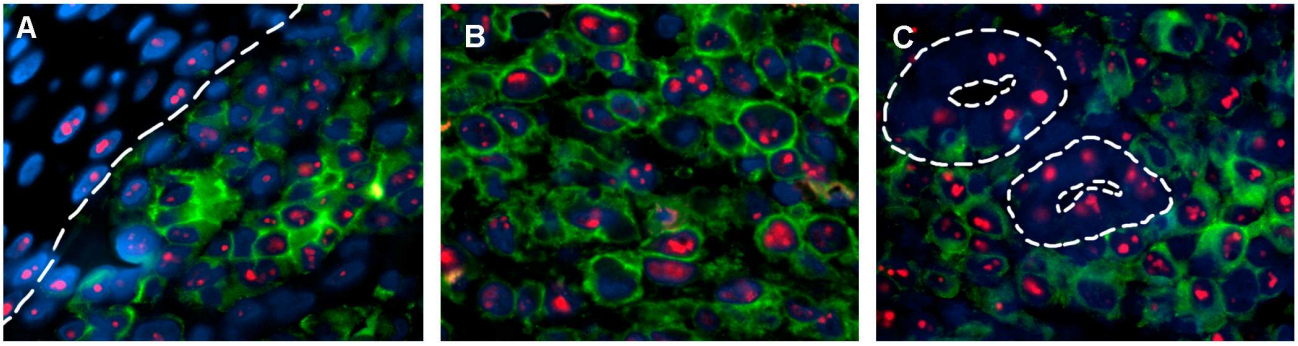


**Figure 3.** hTERT expression by CD1a+ LCH cells of a patient with multisystem disease, affecting skin, bone and gut. Double immunofluorescence staining using CD1a (green) in combination with hTERT-specific antibodies (red) was performed in skin (A), bone (B), and gut lesions (C) of a representative multisystem LCH patient. DAPI was used to distinguish cell nuclei. The broken white lines depicts the epidermal-dermal junction in the skin (A) and the crypts of the gut (C) (magnifications: A, B, C: 63x).

*Determination of telomere length in sorted CD1a+ LCH cells*

To evaluate the telomere length of LCH cells, the telomere repeat fragment (TRF) southern blot method was performed with DNA from sorted LCH cells of four LCH biopsies. The specificity of the CD1a staining (Figure 6 A, right panel) was confirmed using an IgG1-APC control (Figure 6 A, left panel). The purity of the sorting was confirmed by spotting the CD1a+ and CD1a$^-$ sorted fractions on glass slides and restaining them with CD1a and Langerin antibodies. Only the CD1a+ fraction stained for these LC-specific markers (Figure 6 B). Due to the great difficulty in obtaining frozen LCH biopsies, as well as isolating enough LCH cells from tissues to obtain sufficient amounts of DNA from these sorted cells, we were only able to include one hTERT+ biopsy (skin MS LCH) and three hTERT$^-$ biopsies (all SS bone LCH) in this experiment. Nevertheless, a difference between the telomere length of hTERT+ and hTERT$^-$ LCH cells was observed. Whereas hTERT+ LCH cells from the MS biopsy showed a very homogeneous long telomere length (mean TRF 9.9 Kbp), hTERT$^-$ cells from the three SS biopsies displayed a shorter (mean TRFs 5.9, 6.7 and 5.7 Kbp) and much more heterogeneous telomere length (Figure 6 C). The same analysis performed on sorted CD1a+ cells from two hTERT+ DL biopsies, showed the LCs from this reactive condition to display a very heterogeneous telomere length (Figure 6 C).

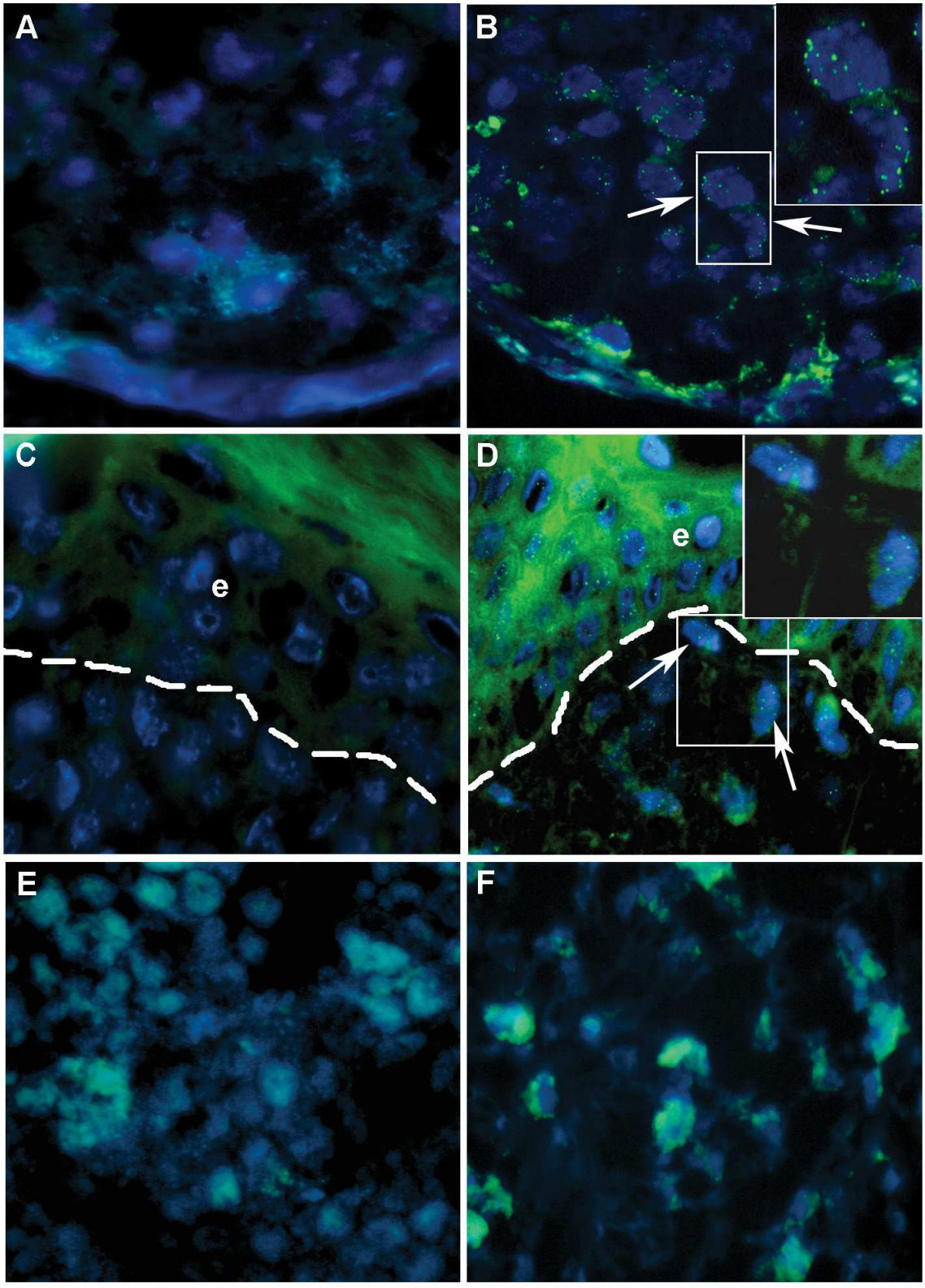


**Figure 4.** *In situ* detection of telomerase activity in hTERT+ LCH lesions. The activity of telomerase in positive control tissue, testis, and hTERT+ and negative LCH biopsies (B, D and F, respectively) was assessed using an *in situ* telomeric repeat amplification protocol (TRAP) assay. The omission of the reaction mixture confirmed the specificity of the TRAP detection (A, C and E). Only the hTERT+ and not the hTERT- LCH biopsies showed cells positive for TRAP, as demonstrated by representative pictures of (D) a hTERT+ LCH MS skin lesion and (F) a hTERT- LCH SS bone lesion. The broken white line depicts the epidermal-dermal junction in the skin and the arrows and inset pictures demonstrate the TRAP positivity of the cells (green spots) (magnifications: A, B, C, D, E, F, 63x).

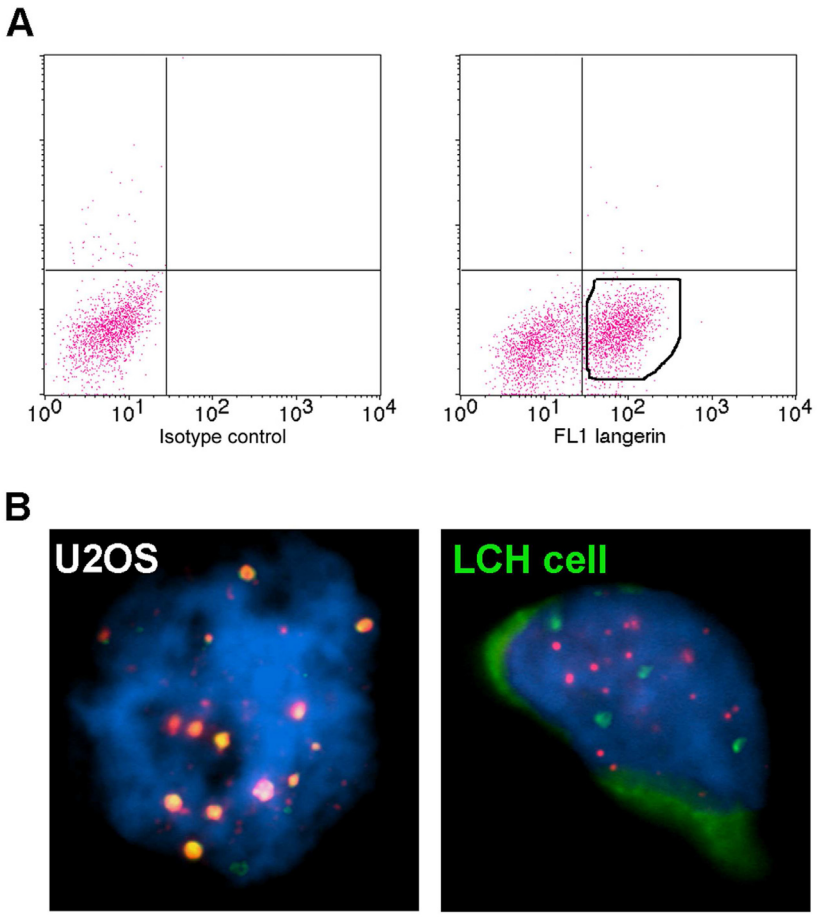


**Figure 5.** Absence of an ALT mechanism in lesions with hTERT negative LCH cells determined by lack of co-localization of PML with telomeres. Langerin+ cells were FACS-sorted from hTERT- and hTERT+ LCH lesions (A, right panel, gated cells). An isotype control was used as the negative control (A, left panel). These cells were spotted onto a slide and dried. FISH using a telomere probe (red) was performed in combination with fluorescence immunolabelling for the protein PML (green dots). Confirmation of ALT positivity was first detected in osteosarcoma cell lines which are known to be positive for this mechanism. (B, left panel) Co-localization of the telomeric DNA and PML (yellow), confirming the presence of an ALT mechanism in the osteosarcoma cell line U2OS. In contrast, absence of an ALT mechanism was observed by the lack of co-localization of PML with the PNA probe in LCH cells, identified by their Langerin–positivity (green), from a bone lesion of a single system patient (B, right panel).

## Discussion

LCH is a rare disease whose aetiology remains unknown. The clonal, proliferative nature of LCH cells, together with the higher incidence of secondary neoplasms, familial clustering and reports of loss of heterozygosity all contribute to the hypothesis that LCH is a neoplastic disease (4, 5, 8, 9). As a characteristic of neoplastic cells is their ability to survive through the maintenance of their telomere length, we hypothesized that LCH cells are able to activate a telomere maintenance mechanism, resulting in stabilization of telomere length and immortalization.

This study showed that the lesional CD1a+ cells express telomerase in all SS skin LCH lesions, in

contrast to SS bone lesions, in which only ca. 25% of the lesions analysed contained LCH cells that expressed telomerase. In addition, the lesional CD1a+ cells from multisystem patients always express telomerase, regardless of the lesional site. The correlation between hTERT expression and telomerase

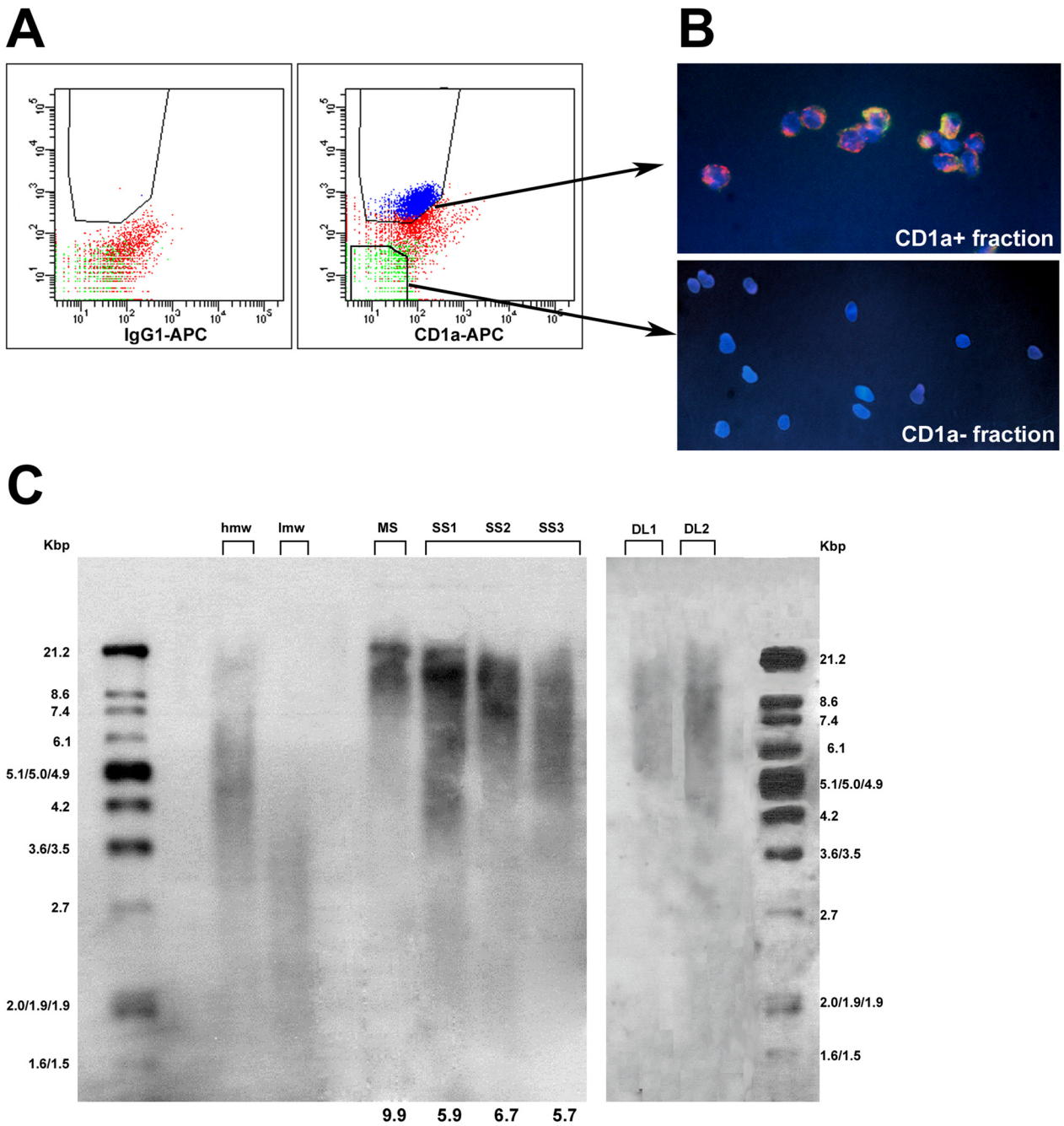


**Figure 6.** Long, homogeneous telomere length of a hTERT+ LCH lesion in contrast to a heterogeneous telomere length of hTERT- LCH lesions. CD1a+ LCH cells were FACS-sorted from frozen biopsies (A, right panel) and the specificity of the staining was confirmed by using an IgG1-APC control (A, left panel). (B) The purity of the CD1a+ and CD1a- sorted fractions was performed by restaining the cells with Langerin and CD1a. (C) Southern blotting of the TRFs was carried out, using DNA from CD1a+ cells from the affected skin of a multisystem (MS) LCH patient, three single system (SS) LCH bone lesions and two DL biopsies. The controls included high (hmw) and low (lmw) molecular weight DNA supplied with the kit. The CD1a+ cells from the MS patient displayed a long homogeneous telomere length, in contrast to the more heterogeneous length displayed by CD1a+ cells from the three SS bone LCH lesions and two DL biopsies (C). The telomere length (Kbp) is depicted at the bottom of each lane.

activity, as shown by the TRAP assay, suggests that hTERT expression is indeed a reliable marker of telomerase activity. Furthermore, we determined the telomere length of LCH cells from an hTERT+ MS skin lesion, compared to three SS bone LCH lesions that were hTERT$^-$. Although the number of biopsies studied was small, our results showed a difference in telomere length between the hTERT+ lesion and the three hTERT$^-$ lesions. Whereas the telomere length of LCH cells in the MS skin lesion was homogeneous and long, the telomere length of LCH cells in the three hTERT$^-$ SS LCH lesions was heterogeneous. This difference in telomere length and telomerase expression in the different lesional sites and forms of the disease may reflect the broad clinical spectrum of LCH, which ranges from a lethal leukaemia-like disorder, in which multiple organs are involved, to a curable solitary lytic lesion of bone. Indeed, in our patient cohort, telomerase was consistently expressed by LCH cells in MS disease and appeared to result in elongated telomeres. However, this correlation should be further investigated, as the hTERT positivity of LCs in the reactive condition, DL, did not appear to correlate with increased telomere length. In contrast, as the loss of telomerase activity has been correlated with cell senescence (31), the finding that the majority of SS bone lesions were negative for any telomere maintenance mechanism suggests that the LCH cells in these lesions may indeed have a more limited proliferative capacity and life-span. This could explain the fact that many patients with SS disease affecting the bone require minimal treatment or the lesions resolve spontaneously and hardly ever recur.

The lack of telomerase expression by LCH cells in the majority of SS bone lesions compared to skin lesions, which were always positive for this enzyme, was quite striking. One possible explanation for this difference could be due to the lesional environment, as different cytokines have been shown to have a profound impact on the regulation of telomerase (32). Although telomerase expression is mainly associated with cancers and immortalized cell lines, and is therefore thought to be involved in malignant transformation and cellular immortality (33), telomerase activity can also be detected at low levels in normal bone marrow and peripheral blood lymphocytes and at higher levels in activated T and B cells (34, 35). To date, there are only very limited data on the telomere length and telomerase activity in myeloid-lineage cells. In our study, the pathological LCs in skin LCH lesions expressed telomerase, in contrast to normal LCs from unaffected skin, which were negative for telomerase. Importantly, the LCs from the reactive condition DL were positive for telomerase, as they were in the majority of lung LCH lesions which is thought to be a more reactive disease, associated with cigarette smoking (36). Furthermore, although the MGCs in some LCH lesions were telomerase-positive, this was also the case for the MGCs found in the non-neoplastic conditions GCT, Paget's disease, sarcoidosis and tuberculosis. Thus, the expression of telomerase does not necessarily distinguish malignant proliferations from reactive states. The detection of telomerase positivity in LCH lesions, where there is a clear immunological response occurring, must therefore be interpreted with caution.

Telomerase activation may instead serve to protect cellular, proliferative capacity and to prevent apoptosis, hence potentiating LC survival in LCH lesions.

This study shows for the first time a clear difference between SS skin and bone LCH lesions and SS versus MS disease, based on the expression of telomerase. This difference in telomerase expression appears to be reflective of the diverse clinical presentation and course of the disease. However, the measurement of telomere length of LCH cells in a larger cohort of LCH patients is needed to validate our findings and will help to increase our understanding of the nature of the various clinical forms of this disease.

**Acknowledgements**

We would like to thank Dr. Nick Henriquez for the help with the southern blotting performed during this study for measurement of the telomere length of LCH cells. Our special thanks to the Cooperative Human Tissue Network (CHTN) in Columbus (OH, USA) for supplying many of the biopsies used in this study. Cristiana Costa is supported by Fundação para a Ciência e a Tecnologia, Portugal. Financial support from the Histiocytosis Association of America as well as the Dutch Langerhans Cell Histiocytosis Patient Group are very much appreciated.

**References**

(1) Egeler RM, D'Angio GJ. Langerhans cell histiocytosis. J Pediatr 1995; 127(1):1-11.

(2) Laman JD, Leenen PJ, Annels NE, Hogendoorn PC, Egeler RM. Langerhans-cell histiocytosis 'in sight into DC biology'. Trends Immunol 2003; 24(4):190-196.

(3) Nezelof C, Basset F, Rousseau-Merck MF, Barbey S. [Dynamic studies of Langerhans cell histiocy tosis cells. Their contribution to the current concept of the disease. Report of a series of 38 cases]. Bull Acad Natl Med 1999; 183(4):815-822.

(4) Arico M, Nichols K, Whitlock JA, Arceci R, Haupt R, Mittler U *et al*. Familial clustering of Langer hans cell histiocytosis. Br J Haematol 1999; 107(4):883-888.

(5) Murakami I, Gogusev J, Fournet JC, Glorion C, Jaubert F. Detection of molecular cytogenetic aber rations in langerhans cell histiocytosis of bone. Hum Pathol 2002; 33(5):555-560.

(6) Betts DR, Leibundgut KE, Feldges A, Pluss HJ, Niggli FK. Cytogenetic abnormalities in Langerhans cell histiocytosis. Br J Cancer 1998; 77(4):552-555.

(7) Ornvold K, Carstensen H, Larsen JK, Christensen IJ, Ralfkiaer E. Flow cytometric DNA analysis of lesions from 18 children with langerhans cell histiocytosis (histiocytosis x). Am J Pathol 1990; 136(6):1301-1307.

(8) Willman CL, Busque L, Griffith BB, Favara BE, McClain KL, Duncan MH *et al*. Langerhans'-cell histiocytosis (histiocytosis X)--a clonal proliferative disease. N Engl J Med 1994; 331(3):154-160.

(9) Yu RC, Chu C, Buluwela L, Chu AC. Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis. Lancet 1994; 343(8900):767-768.

(10) Bank MI, Rengtved P, Carstensen H, Petersen BL. Langerhans cell histiocytosis: an evaluation of histopathological parameters, demonstration of proliferation by Ki-67 and mitotic bodies. APMIS

2003; 111(2):300-308.
(11) Bank MI, Gudbrand C, Rengtved P, Carstensen H, Fadeel B, Henter JI *et al*. Immunohistochemical detection of the apoptosis-related proteins FADD, FLICE, and FLIP in Langerhans cell histiocytosis. J Pediatr Hematol Oncol 2005;27(6):301-306.
(12) Schouten B, Egeler RM, Leenen PJ, Taminiau AH, van den Broek LJ, Hogendoorn PC. Expression of cell cycle-related gene products in Langerhans cell histiocytosis. J Pediatr Hematol Oncol 2002; 24(9):727-732.
(13) Petersen BL, Rengtved P, Bank MI, Carstensen H. High expression of markers of apoptosis in Lang erhans cell histiocytosis. Histopathology 2003; 42(2):186-193.
(14) Oulton R, Harrington L. Telomeres, telomerase, and cancer: life on the edge of genomic stability. Curr Opin Oncol 2000; 12(1):74-81.
(15) Reddel RR. The role of senescence and immortalization in carcinogenesis. Carcinogenesis 2000; 21(3):477-484.
(16) Blackburn EH. Structure and function of telomeres. Nature 1991; 350(6319):569-573.
(17) Zakian VA. Telomeres: beginning to understand the end. Science 1995; 270(5242):1601-1607.
(18) Reddel RR. Alternative lengthening of telomeres, telomerase, and cancer. Cancer Lett 2003; 194(2):155-162.
(19) Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med 1997; 3(11):1271-1274.
(20) Bryan TM, Cech TR. Telomerase and the maintenance of chromosome ends. Curr Opin Cell Biol 1999;11(3):318-324.
(21) Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mamma lian cells. Oncogene 2002; 21(4):598-610.
(22) Henson JD, Hannay JA, McCarthy SW, Royds JA, Yeager TR, Robinson RA *et al*. A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res 2005; 11(1):217-225.
(23) Ulaner GA, Hoffman AR, Otero J, Huang HY, Zhao Z, Mazumdar M *et al*. Divergent patterns of telomere maintenance mechanisms among human sarcomas: sharply contrasting prevalence of the alternative lengthening of telomeres mechanism in Ewing's sarcomas and osteosarcomas. Genes Chromosomes Cancer 2004;41(2):155-162.
(24) Schwartz HS, Juliao SF, Sciadini MF, Miller LK, Butler MG. Telomerase activity and oncogenesis in giant cell tumor of bone. Cancer 1995; 75(5):1094-1099.
(25) Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL *et al*. Specific association of hu man telomerase activity with immortal cells and cancer. Science 1994; 266(5193):2011-2015.
(26) Annels NE, da Costa CE, Prins FA, Willemze A, Hogendoorn PC, Egeler RM. Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. J Exp Med 2003; 197(10):1385-1390.
(27) Lantuejoul S, Soria JC, Moro-Sibilot D, Morat L, Veyrenc S, Lorimier P *et al*. Differential expres sion of telomerase reverse transcriptase (hTERT) in lung tumours. Br J Cancer 2004; 90(6):1222-1229.
(28) Corver WE, Ter Haar NT, Dreef EJ, Miranda NF, Prins FA, Jordanova ES *et al*. High-resolution multi-parameter DNA flow cytometry enables detection of tumour and stromal cell subpopulations in paraffin-embedded tissues. J Pathol 2005; 206(2):233-241.
(29) Youssef N, Paradis V, Ferlicot S, Bedossa P. *In situ* detection of telomerase enzymatic activity in hu man hepatocellular carcinogenesis. J Pathol 2001; 194(4):459-465.
(30) Yasumoto S, Kunimura C, Kikuchi K, Tahara H, Ohji H, Yamamoto H *et al*. Telomerase activity in normal human epithelial cells. Oncogene 1996; 13(2):433-439.
(31) Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. Dev Genet 1996; 18(2):173-179.

(32) Engelhardt M, Kumar R, Albanell J, Pettengell R, Han W, Moore MA. Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. Blood 1997; 90(1):182-193.
(33) Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer 1997; 33(5):787-791.
(34) Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci U S A 1995; 92(20):9082-9086.
(35) Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA *et al*. Activation of telomer ase in human lymphocytes and hematopoietic progenitor cells. J Immunol 1995; 155(8):3711-3715.
(36) Tazi A, Moreau J, Bergeron A, Dominique S, Hance AJ, Soler P. Evidence that Langerhans cells in adult pulmonary Langerhans cell histiocytosis are mature dendritic cells: importance of the cytokine microenvironment. J Immunol 1999; 163(6):3511-3515.

# 7

# No genomic aberrations in Langerhans cell histiocytosis as assessed by diverse molecular technologies

Cristiana ET da Costa
Karoly Szuhai
Ronald van Eijk
Manja Hoogeboom
Raphael Sciot
Fredrik Mertens
Helga Björgvinsdóttir
Maria Debiec-Rychter
Ronald R de Krijger
Pancras CW Hogendoorn
R Maarten Egeler
Nicola E Annels

*Submitted for publication*

**Abstract**

The aetiology of Langerhans cell histiocytosis (LCH), a disease characterized by uncontrolled proliferation of Langerhans cells, is unknown. While some believe that LCH is reactive, others support a neoplastic origin. We tested the hypothesis that LCH is neoplastic by investigating potential consistent chromosomal aberrations in LCH cells. We used multiparameter DNA flow cytometry to analyse the DNA ploidy of LCH cells in 20 cases, performed karyotype analysis in 31 cases, array-based comparative genomic hybridization (arrayCGH) and single nucleotide polymorphism (SNP) arrays with DNA from flow-sorted CD1a-positive and CD1a-negative cells in 19 cases. Ploidy analysis revealed diploid DNA content in all cases. The karyotype of all patients analysed was normal, excluding presence of balanced translocations. ArrayCGH and SNP arrays did not show genome abnormalities. Despite positive immunohistochemical staining for p53 protein, sequencing exon 5 to 8 of p53 gene showed no alterations in 7 studied cases. This study strongly suggests that gross chromosomal abnormalities do not cause the onset of LCH as shown by a multi-targeted molecular approach. Although we cannot exclude cryptic point mutations in as yet unidentified genes, this large study of 72 LCH cases, shows that LCH may be the result of restricted oligoclonal stimulation rather than an unlimited neoplastic proliferation.

**Introduction**

Langerhans cell histiocytosis (LCH), a disease characterised by the abnormal accumulation of CD1a+ Langerhans cells (LCs), is most common in the first or second decade of life (1). The clinical presentation of LCH can range from a single system (SS) disorder, in which patients require minimal treatment or the lesions resolve spontaneously, to a disseminated form with or without organ failure. The disseminated form with organ failure is usually associated with a worse prognosis and leads, in 20% of the patients who do not respond to treatment, to a fatal outcome (2). As for the disseminated form of LCH without organ failure, although less critical, this often results in serious residual disabilities (3). The treatment of these severe, multisystem (MS) forms of LCH remains empirical at best, with the use of corticosteroids in combination with systemic chemotherapy being the most common therapeutical approach.

Despite much effort in the past years, the fundamental question whether LCH is a reactive or neoplastic disease has not yet been answered. The presence of immunologically active cells in LCH lesions and increased cytokine levels has led researchers to suggest that an exaggerated response of Langerhans cells to an antigen or reaction of LCH cells to abnormal signals from other immune

cells cause this disease (4). However, the finding of proliferation of LCH cells together with evidence for monoclonal expansion of these cells has renewed the arguments that LCH may indeed be a neoplasm (5-8). Strong evidence that LCH cells are indeed neoplastic would be the identification of clonal genetic abnormalities in these cells. However, to date there are only a few studies, mostly consisting of case-reports or few patients, providing support for the argument that LCH may be a neoplastic disorder. One feature of neoplastic disease might be the presence of DNA-aneuploidy. Only a few studies investigating the DNA content of LCH lesions have so far been performed with conflicting results (9-11). Whereas two studies showed a normal DNA content of the lesional LCH cells, one study reported a small number of aneuploid cells in LCH lesions. In addition, a few studies performed chromosome analysis in LCH. Betts *et al*. reported five cases of confirmed LCH with cytogenetic abnormalities (12). From one of the cases they detected a t(7;12)(q11.2;p13) in a small percentage of cells. In this and three other cases non-clonal acquired abnormalities were observed and in a fifth case, a constitutional paracentric inversion of chromosome 13q was observed. In another study, Murakami *et al*. evaluated 7 bone lesions using comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analysis and found losses on chromosomes 1p, 5, 6, 9, 16, 17 and 22q and gains on chromosomes 2q, 4q and 12 (ref. 13). In addition, LOH was found on 1p region in 3 of 7 cases and on chromosome 7 in 4 cases. Allelic loss was also detected on chromosome 9 in 2 of 7 cases and on 22q in 1 of 7 cases. However, all these reported abnormalities were non recurrent. More recently, a study showed that mutational events at tumor suppressor genes resulted in immunogenetic heterogeneity in SS and MS disease (14), suggesting that the lesional cells of LCH acquire additional mutations as the disease progresses from being localized to more extensive. Finally, further evidence for a genetic component in LCH came from a single study who reported a high number of twins and siblings affected by LCH (15). Taken together, these data suggest that there may be a component of genetic instability in LCH, as observed in some types of neoplasms and myelodysplastic disorders.

In this study, we performed a comprehensive genetic analysis of LCH biopsies using state-of-the-art molecular cytogenetic techniques. These included multiparameter DNA flow cytometry for analysis of the DNA ploidy, conventional karyotyping, arrayCGH and high density SNP microarrays. ArrayCGH is an established high resolution method that studies the whole genome for chromosomal gains and losses (16). A limitation of arrayCGH is the lack of genotype information, as it does not provide information about regions of LOH without copy number alteration. Thus, SNP arrays offer the possibility to analyse LOH and generate accurate copy number simultaneously in a high-resolution genome-wide manner, thus making it possible to distinguish between LOH regions with underlying hemizygous deletions and those with copy neutral events (17). This genetic analysis was carried out on LCH cells sorted from LCH biopsies, in order to enhance the detection level of potential subtle genome abnormalities masked by the abundant presence of reactive normal inflammatory cells. Us-

ing all these genome screening techniques, no evidence for genomic abnormalities was found in the LCH cells.

## Materials and Methods

*Patient material*

LCH biopsies were obtained from the Leiden University Medical Center (n= 4), Utrecht Medical Center (n= 1), Erasmus Medical Center (n= 11) and Academic Medical Center Amsterdam (n= 2; the Netherlands), the University Hospital Gent (n= 4) and Leuven (n= 22; Belgium), the Lund University Hospital (n= 9; Sweden), the Royal Orthopaedic Hospital in Birmingham (n= 1) and Children's Cancer and Leukaemia Group in Leicester (n= 9; UK) and the Cooperative Human Tissue Network (n= 9; Ohio, USA). Samples from 72 children with SS (n= 41), polyostotic (n= 15) and MS LCH (n= 16) were used in this study, including bone, skin and lymph node (LN; Table 2). All received a definite diagnosis of LCH, based on morphology and CD1a and S-100 immunoreactivity. The experiments were approved by the ethics committee of Leiden University Medical Center, and were in accordance with national ethical guidelines (Code of Proper Secondary Use of Human Tissue in The Netherlands, Dutch Federation of Medical Scientific Societies).

*Karyotype analysis*

G-banded metaphase spreads were obtained from short term cultures from thirty one LCH samples, according to standard protocols at Lund and Leuven centers. Karyotypes were evaluated according to the International System of Human Cytogenetic Nomenclature (ISCN) (18,19).

*FACS staining of the DNA content of the Lang+ and Lang- cell populations*

Dissociation and staining of LCH cells and the remaining cell fraction from paraffin-embedded LCH biopsies were performed as described previously (20,21). After staining the cells with Langerin antibody (Novocastra, Newcastle, UK) and Alexa Fluor 488 (Invitrogen, Carlsbad, CA) or omission of primary antibody as negative control, cells were washed with phosphate buffer and 1% albumin (PBA)/Tween and then incubated with 500ml PBA/Tween containing 10 mM PI (Calbiochem, San Diego, CA) and 0.1% DNase-free RNase (Sigma, St. Louis, MO). The cells were left at room temperature for 30 min to activate the RNase and stored overnight at 4°C. The samples were analysed the day after by flow cytometry (FACS Calibur, BD Biosciences, San Jose, California).

*Isolation of CD1a+ and CD1a- cells from frozen biopsies*

Frozen LCH 50 mm biopsy sections were fixed for 1 hr at 4°C with 4% paraformaldehyde. Following mechanical disaggregation, single cell suspensions were stained with a mouse monoclonal CD1a-APC antibody (BD PharMingen, San Diego, California) for 1hr at 4°C and CD1a+ cells were sorted with BD FACSAria (BD Biosciences, San Jose, California). Sorted cells from the CD1a-positive (CD1a+) and CD1a-negative(CD1a-) fractions were spotted on glass slides, dried and double stained fluorescently with the mouse anti-CD1a and anti-Langerin monoclonal antibodies, followed by DAPI counterstaining to confirm the purity of the sorting.

*ArrayCGH and FISH confirmation*

Genomic DNA of CD1a+ and CD1a- sorted cells was isolated with the Blood and Cell Culture DNA Mini Kit (Qiagen Benelux b.v., Venlo, The Netherlands) and samples were labelled using a random prime reaction incorporating Cy3 or Cy5 dye labelled nucleotides for test and reference samples, respectively. Hybridization was done on array slides containing ~3500 BACs, produced in house at Leiden University Medical Center (22).

Hybridization and post-hybridization washing steps were performed on a HS400 hybridization station and analysis was done as described previously (23).

*Agilent 244k Oligo-array*

For further high-resolution analysis of gains and losses, oligo arrayCGH was performed using the Agilent Human Genome CGH Microarray kit 244K (Agilent, Amstelveen, The Netherlands) according to the manufacturer's instructions following protocol 4.0. Data analysis was performed with the CGH Analytics 3.4 software platform.

*Illumina HumanHap300 BeadChips arrays*

The samples and HumanHap300 BeadChips were processed according to the Infinium II assay system manual, Rev. A (Illumina, Inc.) at the Leiden Genome Technology Center (Leiden, The Netherlands) and Service XS (Leiden, The Netherlands). HumanHap300 BeadChips were scanned using four different Illumina BeadArray Readers, using the FastScan Whole-Genome Genotyping scan setting, BeadScan 3.2. The intensity from the HumanHap300 Chips was extracted using both the original and enhanced methods for each sample. The intensity files were imported into Illumina BeadStudio 3.1.0.0, the HumanHap300 (v 1.0) cluster file was applied to the extracted intensity files and genotypes were called. The analysis was performed by standard settings with Illumina Genome Viewer 3.1.4 software. Using the paired sample editor option, paired sample copy number and LOH differences were calculated. For LOH and default value, 0.46 Mb was used.

*P53 mutation analysis*

PCR was performed on P53 exons 5 to 8 in 10 µL reactions using 10 ng DNA, 1 x Amplitaq Buffer II, 3 mM MgCl and 0.375 U AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA) and 2 pmol M13 tailed primers. The primer sequences are displayed in Table 1 (ref. 24). The PCR was carried out at an initial denaturation step of 10 min 95 oC subsequently followed by 40 cycles of 5 sec 95 oC , 10 sec 60 oC and 15 sec 72 oC and a final elongation step of 10 minutes at 72 oC. PCR products were purified using a filter system (Millipore Montage, Millipore, MA, USA). DNA was eluted in 25 µL sterile water and DNA concentration was measured spectrophotometrically (Nano-drop). 5-10 ng DNA was sequenced with 6 pmol of M13 forward (tgtaaaacgacggccagt) or reversed (caggaaacagctatgacc) primer on an ABI 3700 DNA Analyzer using Big Dye Terminator Chemistry (Applied Biosystems). Sequences were analyzed with Mutation Surveyor TM DNA variant analysis software (version 2.61 Softgenetics, PA, USA).

Table 1. PCR primer sequences for p53 exons 5 to 8.*

| *Fragment Name* | *Forward* | *Reversed* |
|---|---|---|
| fpTP53_x5 | TGTAAAACGACGGCCAGTTTTCAACTCTGTCTCCTTCCTCTT | CAGGAAACAGCTATGACCAGCCCTGTCGTCTCTCCAG |
| fpTP53_x6 | GACGTTGTAAAACGACGGCCAGTAGGCCTCTGATTCCTCACTG | CAGGAAACAGCTATGACCATGAGAGACCCCAGTTGCAAACC |
| fpTP53_x7 | GACGTTGTAAAACGACGGCCAGTCTTGGGCCTGTGTTATCTCC | CAGGAAACAGCTATGACCATGAGTGTGCAGGGTGGCAAG |
| fpTP53_x8 | GACGTTGTAAAACGACGGCCAGTTGCTTCTCTTTTCCTATCCTGAG | CAGGAAACAGCTATGACCATGAGCTTCTTGTCCTGCTTGC |

* Based on Tennis *et al.* (2006)[24]

*P53 and langerin stainings*

Both p53 and Langerin stainings were carried out enzymatically, according to standard procedures. Mouse monoclonal antibodies to p53 (clone DO-7) and langerin (clone 12D6) were obtained from Novocastra (Newcastle, UK).

## Results

*Ploidy analysis reveals a normal DNA content by sorted LCH cells*

A multiparameter DNA flow cytometric method was used to study the DNA content of Langerin-positive (Lang+) and Langerin-negative (Lang-) cell populations dissociated from 20 LCH biopsies (Figure 1 B right and left panels). These biopsies contained more than 50% of pathologic LCH cells out of the total number of cells in the lesions (Figure 1 A). This method involved staining single cell suspensions prepared from the paraffin biopsies with one of the LC markers, Langerin, in combina-

tion with propidium iodide (PI), which stains the DNA content of cells. In 100% of the SS and MS bone, LN and skin lesions analysed (20 out of 20), the Lang+ LCH cells were diploid, as were the Lang- cells, which served as the internal DNA ploidy reference.

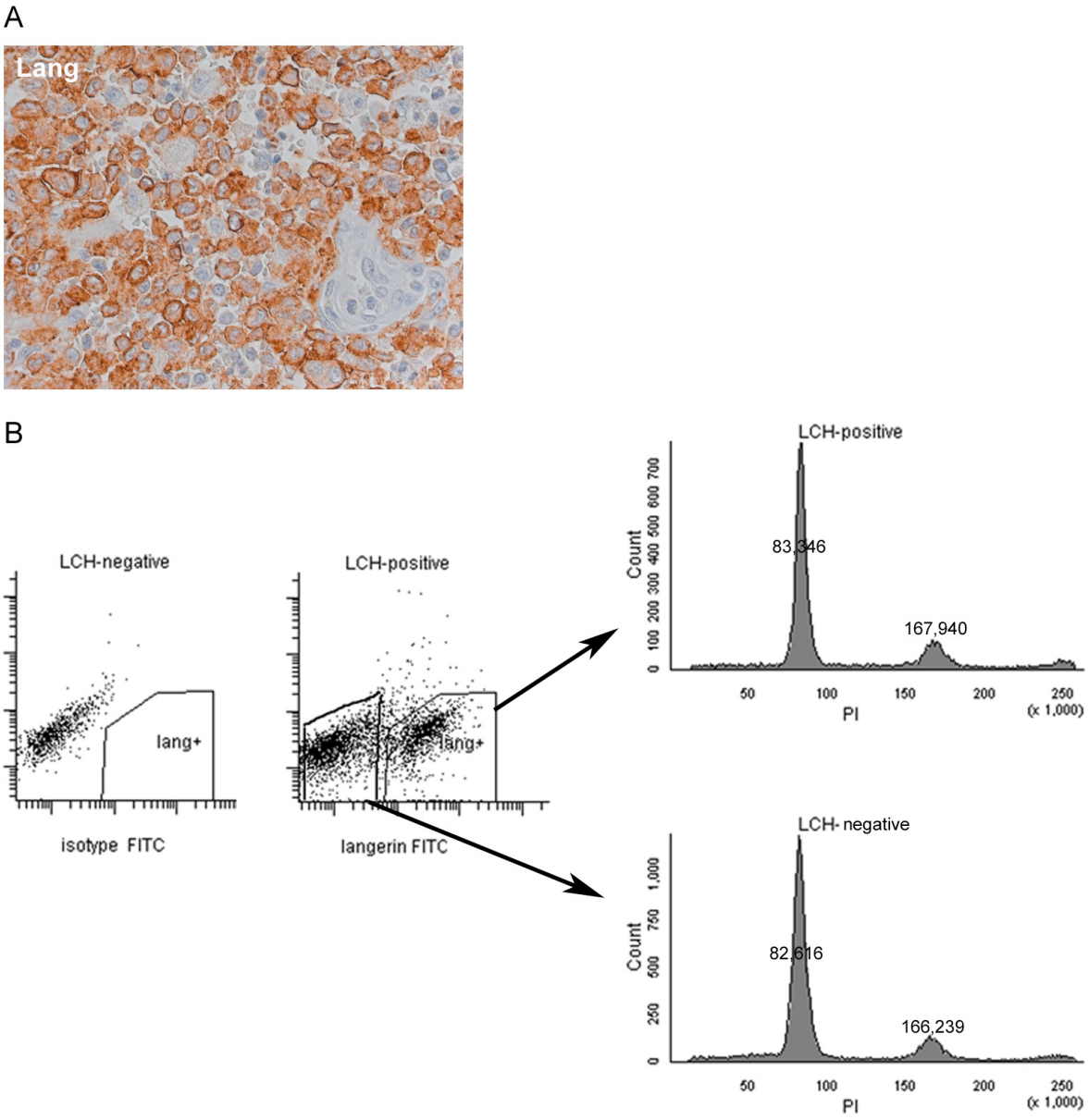


**Figure 1.** Sorted Lang+ LCH cells display a normal DNA content. Example of an LCH lesion used for FACS-sorting the LCH cells, typically full with CD1a+ round-shaped pathologic Langerhans cells (A). Representative example of disaggregated LCH cells from a paraffin biopsy stained with Langerin antibody and analysed by FACS (B, right panel). The specificity of the staining was confirmed using an isotype control (B, left panel). Analysis of the ploidy of both the Lang+-sorted fraction (B, upper panel) and Lang--sorted fraction (B, lower panel) was carried out by staining the cell suspension with propidium iodide.

*Karyotype data on LCH biopsies/BM cells*

In order to detect possible structural or numerical chromosomal abnormalities in LCH biopsies or bone marrow (BM) cells from LCH patients cultured in suspension, a karyotype analysis was per-

formed in 31 LCH patients. The analysis on these LCH patients, which included 7 patients with MS involvement, 7 patients with polyostotic disease and 17 patients with SS involvement or monostotic disease, revealed that the karyotype was, at the resolution of conventional banding, normal, i.e., 46,XX or 46,XY. Thus, no balanced genomic rearrangements were detected in LCH.

*BAC array analysis of DNA from LCH sorted cells*

As ploidy analysis does not reveal all possible chromosomal abnormalities that could be present in LCH cells, the use of high resolution arrayCGH was applied to the DNA of CD1a+ and CD1a- cells FACS-sorted from four LCH biopsies (3 monostotic lesions, 1 polyostotic lesion; patients 52 to 55, Table 2). A ~3500 BAC arrayCGH was performed in order to detect genomic copy number variation in both CD1a+ and CD1a- cell fractions. In all 4 of the LCH biopsies analysed, except known polymorphic region specific changes, no BAC clones showed altered copy number in either the CD1a+ cell fraction or the CD1a- cell fraction.

*Higher resolution oligo arrayCGH confirms the absence of any genetic alterations in LCH biopsies*

In order to detect possible alterations below the resolution of a BAC arrayCGH approach, a 244K Agilent arrayCGH was performed on CD1a+ and CD1a- flow-sorted cells from one LCH biopsy (patient 55, Table 2). The advantage of this array is having approximately 80x higher resolution than the BAC arrayCGH. In keeping with the previous findings, even at this high resolution, no DNA copy number alterations in the CD1a+ or CD1a- cell fractions were observed.

*SNP-LOH analysis*

From 16 patients with SS LCH (n=10), polyostotic LCH (n=2), and MS LCH (n=4), 30 DNA samples isolated from the CD1a+ and CD1a- cell fractions were analysed for allelic imbalance and copy number alterations using the Illumina's HumanHap300 BeadChips. In 2 patients only the CD1a+ cell fraction was analysed. Both copy number alteration and allele frequency readout were used in this test. The overall pattern of hybridisation of the 16 LCH samples showed that there was no copy number alteration in any of the cases. In several samples, small (less than 1Mb) non-recurrent, homozygosity regions were detected. Allele frequency read-out showed no alterations between the flow sorted normal and lesional samples indicating that these alterations were constitutional in all 16 cases. In one case however (patient 56, Table 2), multiple extended (more than 1Mb containing at least 75 SNPs), LOH pattern was observed involving 15 regions of 10 chromosomes, in a total of 80.194 Mb (Table 3). Intriguingly, the same homozygosity pattern was observed in the sorted normal cell fraction (CD1a- cells) proving that these alterations were not LC-related but constitutional (Figures 2 A and B).

Table 2. Clinical information on all the LCH cases used in this study, including the corresponding applications.

| Patient | Gender | Age (yr) | Form/lesional site | Treatment | Relapse | Outcome | Application |
|---|---|---|---|---|---|---|---|
| 1 | F | 8 | MO | Curettage | - | AML after 2 yr | K |
| 2 | M | 11 | MO | Curettage | - | NED | K |
| 3 | M | 5 | PO | Curettage | - | NED | K |
| 4 | M | 56 | MO | Curettage | - | NED | K |
| 5 | M | 22 | MO | Intralesional steroids | - | NED | K |
| 6 | M | 27 | MO | Curettage | - | NED | K |
| 7 | F | 19 | PO | Curettage | - | NED | K |
| 8 | F | 45 | MO | Curettage | - | NED | K |
| 9 | M | 5 | MO | Curettage | - | NED | K |
| 10 | M | 10 | MO | Curettage | - | NED | K |
| 11 | F | 2 | MO | Intralesional steroids | - | NED | K |
| 12 | M | 4 | PO | CHT | - | SD | K |
| 13 | M | 5 | PO | Intralesional steroids | - | NED | K |
| 14 | M | 31 | MS- | Curettage + systemic steroids | - | SD | K |
| 15 | M | 27 | MO | Curettage | - | NED | K |
| 16 | F | 10 | PO | Curettage + intralesional steroids | - | NED | K |
| 17 | M | 10 | MO | Intralesional steroids | - | NED | K |
| 18 | F | 13 | MS | Curettage | - | SD | K |
| 19 | M | 14 | MO | Intralesional steroids | - | NED | K |
| 20 | M | 47 | MO | Curettage | - | NED | K |
| 21 | F | 15 | MO | Curettage | - | NED | K |
| 22 | F | 32 | MS | RDT | - | NED | K |
| 23 | M | 0,5 | MS- | CHT | - | CR-1 | K |
| 24 | M | 10 | MS+ | Curettage of bone lesion | - | CR-1 | K |
| 25 | F | 2,5 | MS- | CHT | - | CR-1 | K |
| 26 | F | 1 | MO | Biopsy | - | CR-1 | K |
| 27 | F | 6,5 | PO | CHT | + (multiple skeletal sites) | CR-2 | K |
| 28 | M | 9,5 | MO | Curettage of bone lesion | + | CR-2 | K |
| 29 | M | 2 | PO | CHT | - | CR-1 | K |
| 30 | F | 12,5 | SS brain | Biopsy | - | CR-1 | K |
| 31 | M | 1,5 | MS+ | CHT | + (MR) | SD | K |
| 32 | F | NA | PO | NA | + (1x, MS) | CR (?) | P |
| 33 | F | 10 | SS LN | Excision biopsy | - | CR | P |
| 34 | F | 6 | MO | NA | + (1x) | CR | P |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 35 | F | 5 | PO | CHT | + (2x, bone) | CR | P |
| 36 | NA | NA | MO | NA | NA | MR | P |
| 37 | M | 3 | PO | CHT | + (at least 2x) | MR | P |
| 38 | F | 8 | MO | Biopsy/brace only | NA | CR | P |
| 39 | F | 2 | MS+ | CHT | - | DOD (01) | P |
| 40 | M | 1 | MO | Biopsy only | - | CR | P |
| 41 | M | 7 | MO | Curettage | - | CR | P |
| 42 | NA | 75 | MS | NA | NA | DOD | P |
| 43 | M | 9 mo | SS LNs | NA | NA | NA | P |
| 44 | M | 2 | SS brain | NA | NA | NA | P |
| 45 | NA | NA | SS skin | NA | NA | NA | P |
| 46 | F | 7 | SS soft tissue | CHT | - | CR | P |
| 47 | M | 7 mo | MS- | CHT | - | NDF | P |
| 48 | M | 1 | MS- | CHT | - | NDF | P |
| 49 | M | 2 | $MS^-$ | Intralesional steroids | + (2x ) | NDF | P |
| 50 | F | 15 | MO | NA | NA | NA | P |
| 51 | M | 11 | MO | Intralesional steroids | - | CR | P |
| 52 | NA | NA | MO | NA | NA | NA | C, M |
| 53 | F | 4 | MO | NA | NA | NA | C |
| 54 | M | 13 | PO | RDT | + (MR) | Alive | C |
| 55 | M | 8 | MO | NA | NA | Alive | C, S, M |
| 56 | M | 7 | $MS^-$ | NA | NA | NA | S, M |
| 57 | M | 8 | MO | NA | NA | NA | S, M |
| 58 | F | 7 | SS LN | CHT | NA | NA | S, M |
| 59 | M | 1 | PO | NA | NA | NA | M |
| 60 | M | 1 | PO | NA | NA | NA | M |
| 61 | M | 3 | MO | Curettage | + (2x) | CR | S |
| 62 | M | 2 | MS | Intralesional steroids | - | Alive | S |
| 63 | NA | NA | MS | NA | NA | Alive | S |
| 64 | F | 9 | MO | NA | NA | NA | S |
| 65 | NA | NA | MO | CHT | - | CR | S |
| 66 | NA | NA | SS | Curettage | - | CR | S |
| 67 | NA | NA | PO | CHT | + | Alive, NED | S |
| 68 | NA | NA | SS | Biopsy + curettage | - | CR | S |
| 69 | NA | NA | MS | CHT | - | Alive, NED | S |
| 70 | NA | NA | SS | CHT | - | CR | S |
| 71 | M | NA | MO | CHT | - | CR | S |
| 72 | NA | NA | PO | CHT | - | NED | S |

Legend: MS= multisystem; MS-= multisystem disease without organ dysfunction; MS+= multisystem disease with organ dysfunction; SS= single system; MO= monostotic; PO= poliostotic; (-)= no relapse; (+)= relapse; NED= no evidence of disease; CR-1= first complete remission; CR-2= second complete remission; CR= complete remission; MR= multiple relapses; DOD= death of disease; NDF= never disease free; SD= stable disease; PD= progressive disease; CHT= chemotherapy; RDT= Radiotherapy; AML= acute myeloid leukaemia; K= karyotype analysis; P= Ploidy analysis; C= arrayCGH; S= SNPs; M= p53 mutation analysis; NA= not available.

Table 3. Overview of detected homozygous regions of case 56 using Homozygosity Detector 1.0.3 (Illumina).

| *Chromosome* | *Start* | *End* | *Size* | *Chi square* |
|---|---|---|---|---|
| 2p | 62.611 | 64.279 | 1.668 | 56.01 |
| 3p | 33.623 | 23.652 | 1.029 | 60.91 |
| 3p | 79.160 | 80.509 | 1.348 | 51.045 |
| 4q | 64.795 | 66.157 | 1.362 | 69.389 |
| 4q | 151.074 | 152.485 | 1.411 | 51.602 |
| 6p | 27.823 | 32.298 | 4.474 | 456.570 |
| 6q | 65.886 | 91.905 | 26.019 | 1518.12 |
| 6q | 94.065 | 95.173 | 1.108 | 63.113 |
| 7q | 116.627 | 120.055 | 3.428 | 139.280 |
| 10q | 54.242 | 61.513 | 7.270 | 424.085 |
| 12q | 89.243 | 90.287 | 1.043 | 48.636 |
| 14q | 21.409 | 42.258 | 20.848 | 1346.530 |
| 17q | 57.812 | 64.943 | 7.131 | 386.185 |
| 20p | 22.724 | 23.733 | 1.008 | 70.034 |
| 20q | 36.804 | 37.851 | 1.047 | 49.268 |

*P53 mutation analysis of CD1a+ and CD1a- cell fractions from LCH biopsies*

The finding of lack of genetic alterations using the techniques described above led us to readdress previous reports of high expression of p53 protein by LCH cells, a feature often associated with genetic alterations. Thus, DNA sequencing on sorted CD1a+ and CD1a- cell populations from 7 LCH biopsies (patients 52 and 55 to 60, Table 2) was performed on P53 exons 5 to 8. The complete coding sequence from these exons was analyzed including at least 5 bp up- and downstream of the intron exon boundaries. No insertions, deletions or other protein changing mutations were observed.

*P53 staining of LCH biopsie*s

In order to confirm that p53 protein is indeed expressed by the LCH cells in LCH lesions, we performed p53 staining analysis in LCH lesions. Despite the lack of mutations in exons 5 to 8 of the p53 gene, p53 protein is highly expressed by these cells in LCH lesions (Figure 3).

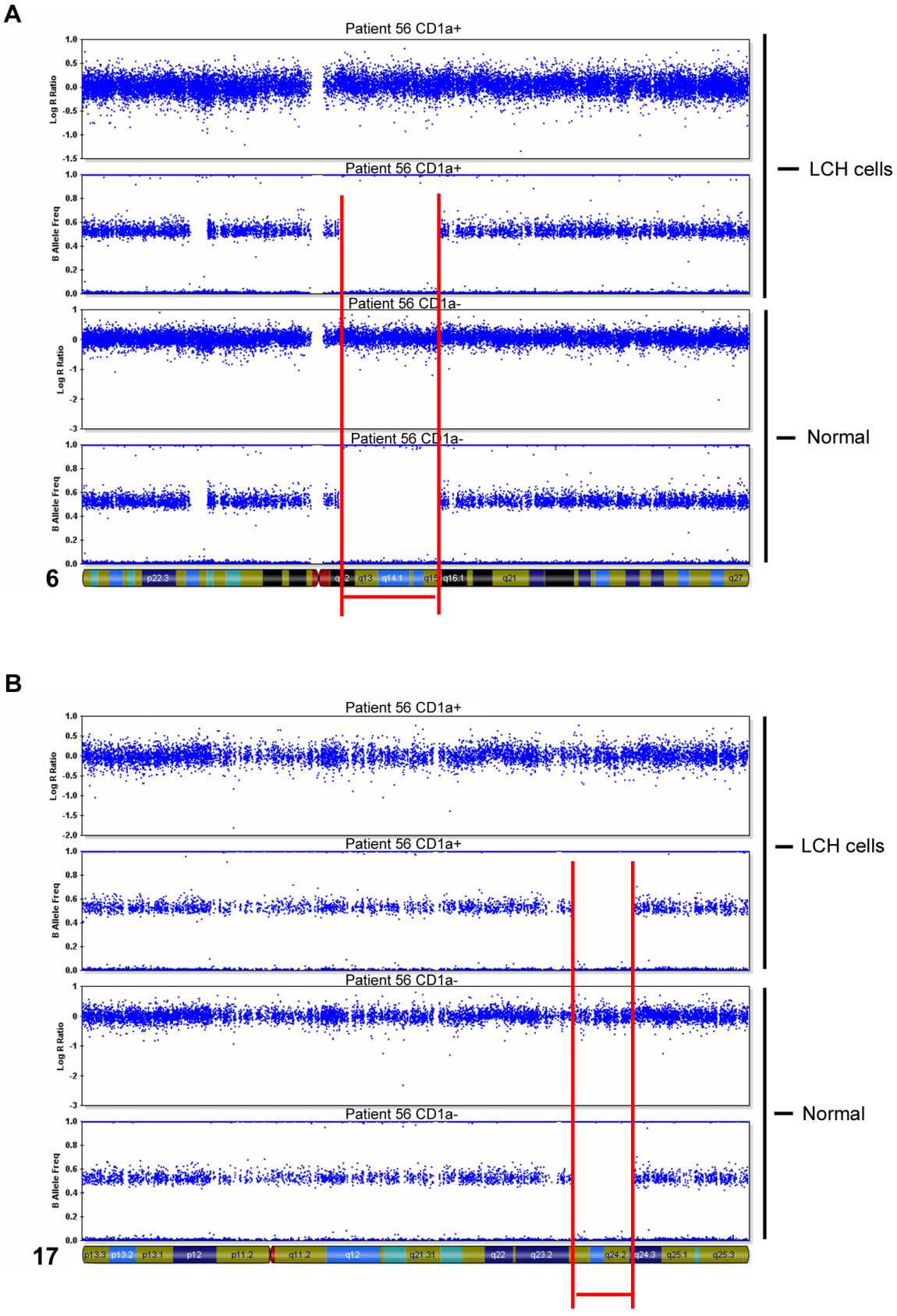


**Figure 2.** Lack of copy number and allele frequency alterations between normal and LCH cells. In a single case several homozygosity regions were detected both in the CD1a+ and CD1a- sorted cells from a multisystem LCH lesion (patient 56 in Table 1). Examples of homozygosities on chromosomes 6p and q (A) and 17q (B). Homozygosity regions indicated by the lack of A/B allele frequencies (bottom panels, between bars). Log R ratio (copy number alteration) was normal. Centromere indicated by red colour on the ideogram.

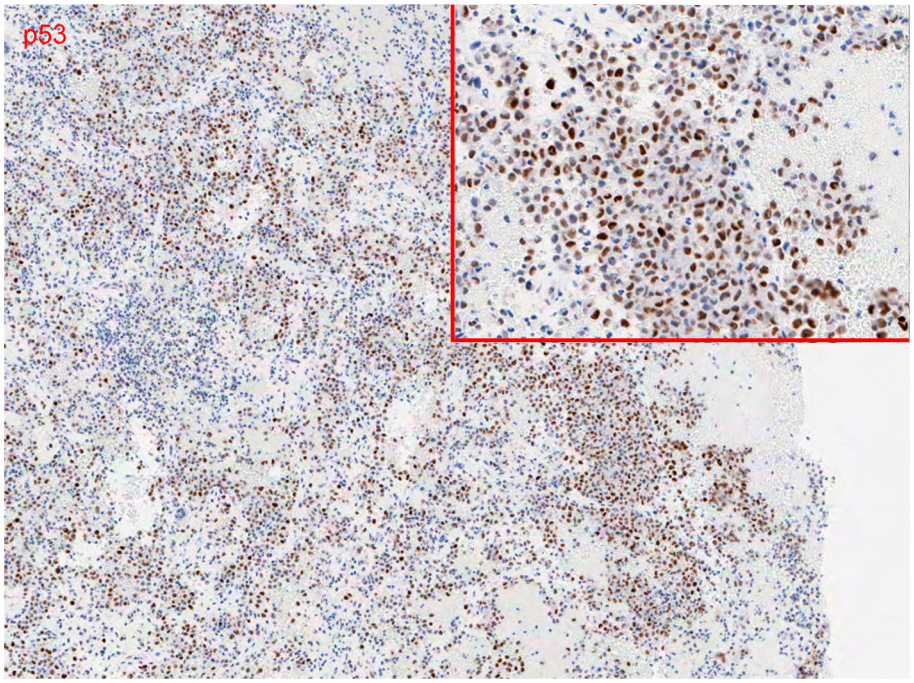


**Figure 3.** Expression of p53 protein in LCH lesions. Representative LCH lesion from patient 55 stained for the p53 protein (dark brown) in LCH cells and negative staining in non-LCH cells indicated by the nuclear haematoxylin counterstaining. The insert picture shows a strong nuclear staining pattern for the p53 protein.

## Discussion

The origin of LCH is still a matter of debate. To date, several studies have attempted to show that the aetiology of LCH could be due to cytogenetic abnormalities. However, these past studies were limited not only by the small number of LCH biopsies studied but also by the fact that the characterisation of genetic aberrations in such lesions is impaired by contaminating normal cells. In contrast, in the current study, the CD1a+ LCH cells were first isolated by flow cytometric sorting which allowed analysis of not only the DNA content of these cells but also subsequent high-resolution methods such as arrayCGH and SNP array to study potential genome-wide alterations. The results from all these techniques showed no genomic abnormalities by the CD1a+ pathologic LCH cells from patients with SS and MS involvement. In addition to the DNA content of the LCH cells being normal, genome-wide copy number and genotype analysis of the sorted CD1a+ cells from 19 LCH biopsies by arrayCGH and SNP array showed no genetic alterations. From one of the LCH patients studied, several (15 regions of 10 different chromosomes) homozygous regions more than 1 Mb in size were detected by SNP array involving approximately 80 Mb in total from the genome. However, this was observed in the DNA from both the CD1a+ and CD1a- fractions showing that these alterations were not disease-specific but rather the profile of the patient's constitutional DNA. Such genomic regions of homozygosity have been observed in children with parental consanguinity (25) but we were unable to confirm this for this patient.

The finding of normal diploid LCH cells from 20 LCH lesions in this study was in keeping with three previous studies that, although not gating for LCH cells, in general found a normal DNA content from the lesional cells of LCH biopsies by flow cytometry9-11. Furthermore, by analysing the karyotype data we showed that LCH cells have a normal karyotype in all patients studied. This is in contrast to the finding of Betts *et al*. who described the existence of an abnormal clone of cells with an unbalanced translocation in 1 out of 5 LCH lesions analysed12. However, the literature available on LCH case-reports is in general biased towards reporting results with positive findings, often from small studies with few cases with alterations. In addition, the described alterations are not recurrent.

A striking feature in LCH, that could be associated with genetic alterations, is the reported high expression of the p53 protein in LCH lesions. The p53 protein is normally expressed at low levels under unperturbed conditions. However, the p53 pathway is activated by cellular stress that alters the normal cell-cycle progression and can induce mutations of the genome leading to the transformation of a normal cell into a cancerous cell. In light of our finding of no genetic alterations by LCH cells we wanted to confirm that the over-expression of p53 was not due to mutations in the p53 gene, but to a physiological response that causes a blockade in the cell-cycle and thus accumulation of the p53 protein. By screening for mutations in exons 5 to 8 of the p53 gene and sequencing no mutations could be detected in pathologic CD1a+ LCH cells confirming the results from a previous study which also reported lack of mutations in the p53 gene in LCH lesions (26). This discrepancy between p53 expression and absence of genetic mutation in its gene has been reported before in EBV infections, where EBV-induced p53 expression in the course of immunoblast transformation was associated with normal cell cycle progression, instead of with apoptosis (27). Thus, a possibility is that LCH could be caused by environmental factors (i.e. infection, more specifically viral infection) and the normal genome or lack of alterations in a large panel of LCH biopsies would be in line with this.

In a previous study we observed differential expression of telomerase in different lesional sites and forms of LCH20. Although, according to a recent report there is no evidence for telomere lengthening in LCH in any stages of the disease (28). Regardless of telomerase function, like is the case for p53, there seems to be no genetic basis for the observed telomerase upregulation. However, the expression of telomerase is not exclusively related to the neoplastic process and different signals for activation of telomerase are known to exist such as immune stimulation (29). Furthermore, epigenetic mechanisms are known to be responsible for the diverse expression status of telomerase that is manifested in a tissue and cell-type-dependent manner (30).

Whereas previous reports have always focused on a specific molecular technique and were done in the lesion as a whole, this study used conventional karyotype analysis, arrayCGH, genome-wide SNP-array, and p53 mutation analysis on lesional CD1a+ sorted cells. All of these different approaches for studying a possible genetic basis for LCH did not reveal any evidence of genomic abnormalities,

even in patients with MS involvement. However, we cannot exclude the possibility that other genetic defects could still underlie this disease. These alterations include cryptic (below the resolution of conventional karyotyping) balanced genomic rearrangements and point mutations. Due to the lack of genomic abnormalities in our LCH patient cohort it remains intriguing that a single study reported familial cases, such as twin pairs and siblings affected with LCH. To date there have been no reports analysing these familial cases at the genetic level and we were unable to obtain any DNA from these reported cases. It is tempting to speculate here that epigenetic events may play an important role in LCH, which may explain the phenomenon of spontaneous regression seen in some cases of LCH as well as the familial cases, as epigenetic imprinting is a heritable factor.

In conclusion, although a comprehensive panel of genetic techniques was used in this study none has revealed genomic abnormalities in the SS and MS LCH. Obviously we cannot exclude the possibility that cryptic mutations may still underlie the cause of LCH. Nevertheless, these and other findings from literature suggest that LCH results from an immune dysregulation. Indeed, a recent study implicates IL-17 dysregulation in LCH (31). These findings appear to be in keeping with other diseases such as systemic lupus erythematosus and rheumatoid arthritis, which involve immune dysregulation and where no genetic abnormality is implicated. It remains to be investigated whether epigenetic factors or e.g. immune abnormalities can trigger LCH.

**Acknowledgements**

We would like to thank the Children's Cancer and Leukaemia Group (CCLG) Tissue Bank (Leicester, UK) for access to specimens. The Bank is funded by Cancer Research UK and the Leukaemia Research Fund. We would also like to thank the Cooperative Human Tissue Network (CHTN) in Columbus (OH, USA) for providing many of the biopsies used in this study. We are also grateful to Dr. D. Sie-Go from the Utrecht Medical Center and Dr. J. de Kraker from the Amsterdam Medical Center (the Netherlands) for contributing 1 and 2 biopsies used in this study, respectively. Samples were also supplied by partners in Leiden, Lund, Gent, Leuven and Birmingham, which are part of EuroBoNet, a European Commission-granted Network of Excellence for studying the pathology and genetics of bone tumors (LSHC-CT-2006-018814). This study was partially supported by The Histiocytosis Association of America and by KIKA funds (the Netherlands). Cristiana Costa has a grant from Fundação para a Ciência e a Tecnologia, Portugal, which is co-funded by POCI 2010 and FSE.

**References**

1. Egeler RM, D'Angio GJ. Langerhans cell histiocytosis. J.Pediatr. 1995;127:1-11.

2. Minkov M, Grois N, Heitger A, Potschger U, Westermeier T, Gadner H. Treatment of multisystem Langerhans cell histiocytosis. Results of the DAL-HX 83 and DAL-HX 90 studies. DAL-HX Study Group. Klin Padiatr. 2000;212:139-144.
3. Haupt R, Nanduri V, Calevo MG, *et al*. Permanent consequences in Langerhans cell histiocytosis pa tients: a pilot study from the Histiocyte Society-Late Effects Study Group. Pediatr Blood Cancer. 2004;42:438-444.
4. Nezelof C, Basset F. An hypothesis Langerhans cell histiocytosis: the failure of the immune system to switch from an innate to an adaptive mode. Pediatr Blood Cancer. 2004;42:398-400.
5. Schouten B, Egeler RM, Leenen PJ, Taminiau AH, van den Broek LJ, Hogendoorn PC. Expression of cell cycle-related gene products in Langerhans cell histiocytosis. J Pediatr Hematol Oncol. 2002;24:727-732.
6. Bank MI, Rengtved P, Carstensen H, Petersen BL. Langerhans cell histiocytosis: an evaluation of his topathological parameters, demonstration of proliferation by Ki-67 and mitotic bodies. APMIS. 2003;111:300-308.
7. Willman CL, Busque L, Griffith BB, *et al*. Langerhans'-cell histiocytosis (histiocytosis X)--a clonal proliferative disease. N Engl J Med. 1994;331:154-160.
8. Yu RC, Chu C, Buluwela L, Chu AC. Clonal proliferation of Langerhans cells in Langerhans cell his tiocytosis. Lancet. 1994;343:767-768.
9. Ornvold K, Carstensen H, Larsen JK, Christensen IJ, Ralfkiaer E. Flow cytometric DNA analysis of lesions from 18 children with langerhans cell histiocytosis (histiocytosis x). Am J Pathol. 1990;136:1301-1307.
10. McLelland J, Newton J, Malone M, Camplejohn RS, Chu AC. A flow cytometric study of Langer hans cell histiocytosis. Br J Dermatol. 1989;120:485-491.
11. Rabkin MS, Wittwer CT, Kjeldsberg CR, Piepkorn MW. Flow-cytometric DNA content of histiocyto sis X (Langerhans cell histiocytosis). Am J Pathol. 1988;131:283-289.
12. Betts DR, Leibundgut KE, Feldges A, Pluss HJ, Niggli FK. Cytogenetic abnormalities in Langerhans cell histiocytosis. Br J Cancer. 1998;77:552-555.
13. Murakami I, Gogusev J, Fournet JC, Glorion C, Jaubert F. Detection of molecular cytogenetic aberra tions in langerhans cell histiocytosis of bone. Hum Pathol. 2002;33:555-560.
14. Chikwava KR, Hunt JL, Mantha GS, Murphy JE, Jaffe R. Analysis of loss of heterozygosity in sin gle-system and multisystem Langerhans' cell histiocytosis. Pediatr Dev Pathol. 2007;10:18-24.
15. Arico M, Nichols K, Whitlock JA, *et al*. Familial clustering of Langerhans cell histiocytosis. Br J Haematol. 1999;107:883-888.
16. Oostlander AE, Meijer GA, Ylstra B. Microarray-based comparative genomic hybridization and its applications in human genetics. Clin Genet. 2004;66:488-495.
17. Huang J, Wei W, Zhang J, *et al*. Whole genome DNA copy number changes identified by high den sity oligonucleotide arrays. Hum Genomics. 2004;1:287-299.
18. Mandahl N, Heim S, Arheden K, Rydholm A, Willen H, Mitelman F. Three major cytogenetic sub groups can be identified among chromosomally abnormal solitary lipomas. Hum Genet. 1988;79:203-208.
19. Holtzman NA, Merz JF. ISCN 2005 - An International System for Human Cytogenetic Nomenclature (2005) Introduction. Community Genetics. 2005;8:201-202.
20. Da Costa CE, Egeler RM, Hoogeboom M, *et al*. Differences in telomerase expression by the CD1a+ cells in Langerhans cell histiocytosis reflect the diverse clinical presentation of the disease. J Pathol. 2007;212:188-197.
21. Corver WE, Ter Haar NT, Dreef EJ, *et al*. High-resolution multi-parameter DNA flow cytometry ena bles detection of tumour and stromal cell subpopulations in paraffin-embedded tissues. J Pathol. 2005;206:233-241.
22. Knijnenburg J, Szuhai K, Giltay J, *et al*. Insights from genomic microarrays into structural chromo some rearrangements. Am J Med Genet A. 2005;132:36-40.

23. Knijnenburg J, van der Burg M, Tanke HJ, Szuhai K. Optimized amplification and fluorescent labe ling of small cell samples for genomic array-CGH. Cytometry. 2007;71A(8):585-591.
24. Tennis M, Krishnan S, Bonner M, *et al*. p53 Mutation analysis in breast tumors by a DNA microar ray method. Cancer Epidemiol Biomarkers Prev. 2006;15:80-85.
25. Woods CG, Cox J, Springell K, *et al*. Quantification of homozygosity in consanguineous individuals with autosomal recessive disease. Am J Hum Genet. 2006;78(5):889-896.
26. Weintraub M, Bhatia KG, Chandra RS, Magrath IT, Ladisch S. p53 expression in Langerhans cell histiocytosis. J Pediatr Hematol Oncol. 1998;20:12-17.
27. Pokrovskaja K, Okan I, Kashuba E, Lowbeer M, Klein G, Szekely L. Epstein-Barr virus infection and mitogen stimulation of normal B cells induces wild-type p53 without subsequent growth arrest or apoptosis. J Gen Virol. 1999;80( Pt 4):987-995.
28. Bechan GI, Meeker AK, de Marzo AM *et al*. Telomere length shortening in Langerhans cell histiocy tosis. Br J Haematol. 2007;140(4):420-428.
29. Hiyama K, Hirai Y, Kyoizumi S, *et al*. Activation of telomerase in human lymphocytes and hemat opoietic progenitor cells. J Immunol. 1995;155:3711-3715.
30. Liu L, Lai S, Andrews LG, Tollefsbol TO. Genetic and epigenetic modulation of telomerase activity in development and disease. Gene. 2004;340:1-10.
31. Coury F, Annels N, Rivollier A, *et al*. Langerhans cell histiocytosis reveals a new IL-17A-dependent pathway of dendritic cell fusion. Nat Med. 2008;14:81-87.

# 8

# General Discussion

# General discussion

*Is LCH a reactive or neoplastic disease?*

Since its first description as histiocytosis X in 1953, the etiology of LCH remains to be clarified. LCH is commonly labelled as an orphan disorder, as its incidence in children is 2-5 per million per year peaking at 1-4 years (1, 2). LCH may be considered as a heterogeneous disorder, in which the clinical outcome depends on the presenting disease entity. Many patients die, especially younger patients presenting with the disseminated form of LCH (3). This form of LCH appears to reflect a more malignant phenotype. On the other hand, patients suffering from localized LCH often show a sudden recovery (4), which is more in keeping with a reactive type of disorder. In addition, LCH biology is complex: the lesions are composed of pathologic Langerhans cells (LCH cells) which, in the majority of the cases, appear in high numbers as a cluster, surrounded by T lymphocytes, eosinophils and other cell types (5). So, the question arises whether these lesions represent a typical granuloma, resulting from a frustrated reaction to an external trigger (e.g., a pathogen) or are the lesions a consequence of a genetic defect of the LCH cells, which in turn trigger the large immunological response observed locally? Arguments supporting or disproving the various theories behind the pathogenesis will be discussed in the next sections.

**1. Maturation block of LCH cells.**

A definite finding in LCH is that the Langerhans cells (LCs) in LCH lesions have an aberrant behaviour. Whatever the initial trigger is, either a genetic defect or a pathogen, these cells are unable to become fully activated. This is, for example, illustrated by their inability to switch the expression of the chemokine receptor CCR6, typically expressed by immature DCs, to the mature DC chemokine receptor, CCR7 (chapter 3). In fact, LCH cells are hardly ever found in the lymph nodes that drain the lesional sites, which suggest that these cells do not migrate. In contrast to this finding, Fleming *et al*. reported that LCH cells expressed CCR6 and CCR7 simultaneously in all cases analysed (6). However, their technique involved using single stainings for each of the markers (CD1a, CCR6 and CCR7), whereas we used triple immunofluorescence stainings in our study. In addition, upon swapping biopsies for confirmation using our pattern of stainings, we found the CD1a cells in all of their cases to be CCR7 negative. Under normal circumstances, upon contact with antigens or in the context of inflammation, LCs will take up and process the antigen and migrate to the draining lymph nodes at an accelerated rate, where they can present the antigen to the immune system for an appropriate response. LCs migrate specifically into T cell areas of draining lymph nodes where they secrete

chemokines that permit the attraction of naïve T cells and induce the proliferation and differentiation of antigen-specific T cells (7). These processes are accompanied by a downregulation of CCR6 and upregulation of CCR7 (8, 9). This normal function of LCs is a complex cascade of events involving antigen recognition, uptake, degradation, migration, cellular activation, contact, signalling, and differentiation. For unknown reasons LCH cells in contrast to normal LCs do not loose their expression of CCR6 and do not up-regulate CCR7 after antigen uptake despite the presence of inflammatory cytokines in LCH lesions, such as TNF-α (10-12). The expression of CCR6 is thus likely responsible for the retention of LCH cells in their peripheral tissue sites, subsequent accumulation and likely even survival (figure 1). In addition, CCR6 was also found on the surface of CD4+ T cells in LCH lesions, which implies that CCL20 is also responsible for the attraction of T cells to the lesions (figure 1). Finally, chemokines such as CCL5 and CXCL11 were present in LCH lesions (chapter 3) and are thus likely responsible for the recruitment of other inflammatory cells present in the lesions (figure 1). Interestingly, in pulmonary LCH we have found a differential expression of CCR6 in the lesions. However, a similar finding of lack of CCR7 expression in these lesions was observed as well (chapter 4). This is intriguing as it has been reported that LCH cells in pulmonary LCH lesions display a more mature phenotype (13), which is thus not in keeping with the absence of CCR7 expression. Although important for the ability of the host to control infections, it has been reported that chemokines are implicated in the pathogenesis of many human diseases. These include asthma, atherosclerosis, rheumatoid arthritis or multiple sclerosis, where inflammatory cells are recruited into tissue sites by the chemokine/chemokine receptor interaction, causing an inflammatory infiltrate, which results in tissue damage (14-16). Therefore, it has been suggested that chemokines and their receptors could be used as therapeutic targets for controlling pathologic inflammations. Such an approach may also be applied to LCH, where targeting the chemokine/chemokine receptor interaction may help break up the lesion.

**2. Is LCH reflecting an intrinsic tumorigenic process?**

What is then inducing the formation of such an aberrant LC? For a long time the question has remained whether LCH could be the result of a specific genetic defect of LCH cells. In fact, a number of studies have shown evidence that LCH cells are monoclonal in all forms of LCH except for pulmonary LCH, where LCH cells are polyclonal and occasional dominant clones may emerge (17-19). Thus, could LCH be a neoplastic disorder, arising from somatic mutations, which affect cell biology leading to the clonal expansion of LCs or their precursors in the bone marrow and other organs? An indication that there are abnormalities in cells is the disruption of a limited number of cellular regulatory pathways. In LCH there is an up-regulation of the anti-apoptotic protein bcl-2 (20, 21). There is also a relatively high expression of the proliferation marker Ki-67 (21-23). However, it is

important to note that the clonal proliferation of rare progenitor cells resident in or attracted to lesions in response to cytokines may produce a "nonneoplastic" clonal proliferation of histiocytes. "Clonality", therefore, does not necessarily indicate a malignant process: clonal cells have been detected in several disorders that are not malignant (24, 25). In addition, there are no studies in LCH showing that LCH cells obtained from different sites in the case of multisystem (MS) LCH are derived from a single clone. However, the evidence for survival and proliferation of LCH cells led us to investigate the possible presence of telomerase in these cells (chapter 6). Telomerase activation has been shown to be an almost universal property of malignant tumors, indicating immortality better than either the alterations of telomere length or the actual presence of telomerase RNA (26-28). In addition, although telomerase activity is detectable in germline cells as well, it is shown to be absent in most normal somatic tissues where its reappearance is associated with the development of malignancy (26, 29). Finally, the expression of this enzyme has been reported to correlate with cell proliferation in many different types of cells (30, 31). We found consistent presence of telomerase in all single system (SS) skin LCH lesions as well as all lesional sites from MS cases. In these cases, we also found evidence for functional activity of this enzyme. However, in the majority of SS bone lesions LCH cells did not show any expression of telomerase. This difference of telomerase expression in different forms of LCH may reflect the diverse clinical presentation observed in LCH as mentioned earlier in this chapter. In fact, many of the patients with SS bone LCH require minimal treatment or the lesions resolve spontaneously (4). In contrast, in cases of MS disease with organ failure mortality may be as high as 20% irrespective of treatment (3). As for cases of MS LCH without organ failure, the disease runs a fluctuating course and may eventually "burn out", often leaving serious residual disabilities. Again, this observation supports the view that LCH may be sub-divided into different categories. On the other hand, a recent study from Bechan *et al*. (32) showed, by using immunofluorescence in combination with fluorescence in situ hybridisation, that LCH cells display a significant telomere shortening in all stages of disease involvement. This was compared to LCs from reactive lymph nodes or unaffected skin, which displayed longer telomeres. This discrepancy may reflect the differences in sensitivity of the techniques used to assess the telomere length of the cells. Interestingly, a report from Ping *et al*. (33) showed evidence that telomerase activity is largely increased during the differentiation and maturation process of DCs. Thus, it is possible as observed from our results that SS bone LCH is either likely a reactive disease or the reflection of a disease where LCs are more immature, since LCH cells are telomerase negative and the SS skin and MS LCH likely represent a malignant disease or an indication that the LCs here are more mature. This is in keeping with the findings from a study where they looked at the phenotype of LCH cells in SS bone, skin and lymph node LCH. In this study, LCH cells from bone lesions had both immature phenotype and function, whereas LCH cells from cutaneous LCH had a more mature phenotype (34).

**3. No evidence for genetic defects in LCH cells.**

Could LCH be the result of a series of genetic and cellular events that may govern the formation of most types of human cancers? In addition to clonality and survival, other arguments strengthening this hypothesis are the reported familial cases as well as the higher than expected incidence of malignancies in LCH patients (35-37). It is known that increased mutability is essential for the development of many types of human cancers (38). Such increased mutability is acquired when the genes and proteins that ordinarily protect the genome by detecting and repairing damage in chromosomal DNA are inactivated. In addition, the cellular mechanisms (particularly apoptosis) that usually eliminate cells with damaged DNA are often compromised in tumor cells; the result is the survival of a mutant cell and the possible outgrowth of a large population of its similarly mutated descendants (39). Thus, in order to provide strong evidence that LCH could be a malignant disease the finding of consistent genetic abnormalities was crucial. We carried out a comprehensive study using state-of-the-art molecular techniques to investigate whether LCH cells were pathological due to genomic abnormalities (chapter 7). Thus far, a few reports have showed with limited molecular techniques that LCH cells contain genetic losses and gains (40-42), but these findings have never been studied in depth and they present inconsistencies. However, we found neither copy number nor copy neutral alterations both in LCH lesions from patients with single system involvement and multisystem disease. In addition, the karyotype of 31 single system and multisystem LCH patients was normal. Of course we cannot exclude the possibility that LCH could still be the result of a point mutation. Neither can we overlook that, despite the lack of consistent evidence for genetic alterations, LCH cells display several phenotypic changes that appear to distinguish them from normal counterparts. Furthermore, there is an unexpectedly high association between LCH and the occurrence of malignancy in patients (37). One group consists of patients in whom LCH appears concomitantly with the malignancy and resolves with effective treatment of the malignancy. These patients provide support for the concept that LCH may be the consequence of a reactive process in response to a foreign or abnormal antigen. A second group of patients are those who developed a malignancy, frequently a leukaemia, after treatment for LCH. These may be therapy-associated malignancies. A third group consists of patients who developed LCH long after the diagnosis of a malignancy, suggesting a possible biologic or genetic link between the two processes. In fact, a case report from a patient who developed LCH after T cell acute lymphoblastic leukaemia showed that LCH cells and the precursor T-lymphoblastic leukaemia/lymphoma cells had identical rearrangements of the gene for T cell receptor g, confirming a clonal relation between the two diseases (43). This case resembles another case where, in a patient who developed histiocytic sarcoma while on maintenance chemotherapy for acute lymphoblastic leukaemia, the original leukaemia blasts and the subsequent histiocytic-sarcoma cells showed identical gene rearrangements, confirming a clonal origin (44). Thus, it would still be of interest to look at the genetics

of LCH lesions associated to malignancies, such as leukaemias, lymphomas and Hodgkin's disease, specifically using the array platforms CGH and SNPs carried out by us in chapter 7. The aim would be to investigate whether LCH cells in these cases share common mutations with the malignancy to which the LCH lesions are associated. However, this high incidence of malignancies in LCH patients may be the consequence of impaired immunity secondary to the nature of LCH (LC dysfunction) or to the immunosuppressive/anti-inflammatory treatment. Thus, this is not a strong argument for a malignant origin of LCH itself. Furthermore, the fact that no permanent *in vitro* LC cell line has been successfully established, neither have xenografts been successfully transplanted to nude or SCID mice or monkeys, supplies more support for LCH being non-neoplastic.

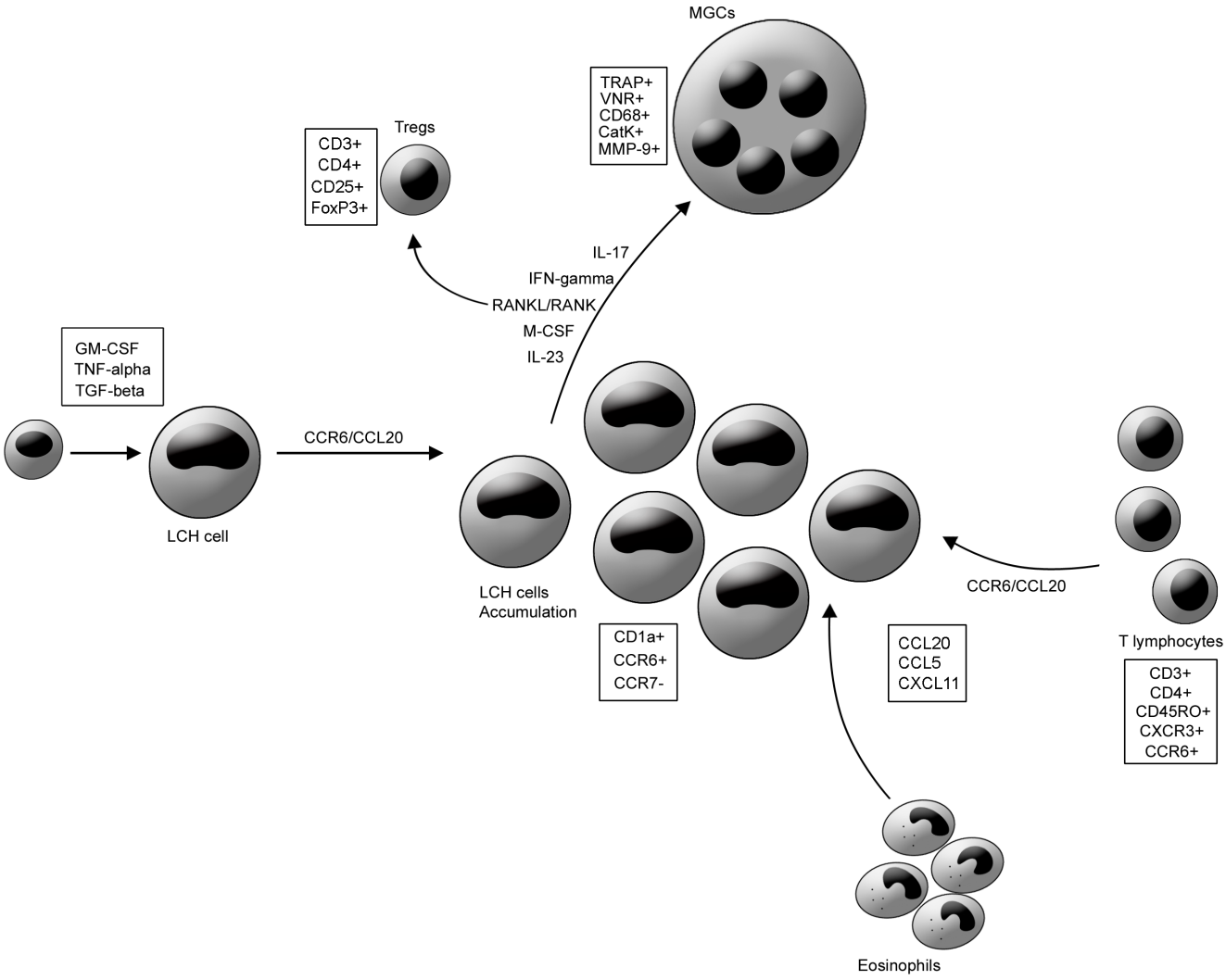


**Figure 1.** Overview of the immunological events occurring in Langerhans cell histiocytosis lesions, based on the findings described in this thesis.

**4. No evidence for infections triggering the onset of LCH.**

A question that we pose is whether LCH could reflect a misbalance of pro-inflammatory and anti-inflammatory signals upon exposure to an external trigger leading to chronic inflammation. A number of studies have actually investigated whether LCH could be triggered by an abnormal response to a pathogen, especially to viruses, causing the enhanced immunological reaction typical of the lesions which are composed of many immune cell types (45-47). Thus, a specific virus could be responsible for the activation of histiocytes but also for the impairment of immune regulation of subsequent histiocytic proliferation. However, despite much postulation of viral aetiology, this has never been substantiated. No positive results were obtained when probes were used against viral DNAs, such as human T cell viruses type I, II, and III (human immunodeficiency virus), adenovirus, cytomegalovirus, Epstein-Barr virus, parvovirus, herpes simplex virus, and human herpesvirus type 6 (45, 46). In contrast to these findings, Glotzbecker *et al*. (48) found immunohistochemical evidence for HHV-6 in 71% of LCH biopsies. However, there was no serologic indication that showed that a recent infection had occurred. In addition, the prevalence of HHV-6 in the tissue of LCH patients is the same as that found in the tissue of healthy individuals. Although no clear viral trigger has been detected to date, this does not exclude the involvement of other microbial agents in disease development. Indeed, the phenotype of the LCs present in LCH lesions resembles that of normal activated dendritic cells (DCs) that have encountered bacterial products (49). Following this line of thought, we ourselves have looked at the expression of toll-like receptors (TLRs) 1 through 9 by the LCH cells, with the goal of finding a consistent pattern of upregulation that could indicate the recognition of a microbial product. However, the results from this study showed that LCH cells display a pattern of TLR expression similar to that of normal LCs (data not published). Thus, it is unlikely that a specific bacterial or viral protein recognized by TLRs play a role in the triggering process of LCH.

Despite the lack of evidence for a role of microorganisms in the aetiology of LCH, there are several characteristics of LCH lesions that are suggestive of a reactive condition. These include the granulomatous character of these lesions, resembling diseases such as tuberculosis, sarcoidosis or Crohn's disease (50-52). Another feature typical of a reactive condition is the occurrence of spontaneous clinical regressions of most bone lesions, the consistently favourable outcome of the restricted forms and the frequent recovery in disseminated forms of LCH.

**5. LCH: an immune dysregulation.**

The fact that the cells present in the different LCH lesions are typical of an innate immune response strengthens the theory that LCH may be caused by an immune dysregulation. The absence of plasma cells, the relative scarceness of T cells in LCH lesions and the limited involvement of lymph node draining sites of LCH where no LCH cells are found, represent unusual features for a condition

characterised by chronicity. This apparently self-sustaining cellular response may indicate either the local persistence of some initiating agent(s) or, possibly, an inability of the innate immune response to switch to a more effective adaptive immune response. One possible cause for such a block could be an inhibitory action of some of the molecules released in the "cytokine storm" that accompanies LCH. Tobacco, so frequently associated with pulmonary LCH, might act by this mechanism. Cigarette smoke contains tobacco glycoprotein, which is an immunostimulant that induces lymphocyte differentiation and lymphokine production (53). Interestingly, lymphocytes obtained from patients with pulmonary LCH respond abnormally to tobacco glycoprotein (54). This abnormal response by the lymphocytes in LCH together with the fact that cigarette smoke also causes an increased number of LCs in the bronchoalveolar lavage fluid of smokers may explain the LC accumulation in pulmonary LCH as a reaction to cigarette smoke (55, 56).

The pathogenesis of granulomas in general is known to be influenced by the milieu in which the cells are found. The lesional microenvironment in LCH is characterised by the presence of many pro-inflammatory cytokines, as already mentioned. In a recent report by Coury *et al*. (57), it is described that LCH cells express the pro-inflammatory cytokine IL-17. In this study, it is shown that IL-17 has a direct effect on monocytes resulting in multinucleated giant cells (MGCs) formation. IFN-γ, when added together with IL-17, also has a role in this process as it was shown that it leads to an increase in the MGCs size. Previous studies have shown that IL-17 is normally expressed by T cells, has a role in the development and maintenance of several auto-immune inflammatory conditions, and is implicated in the defence against bacterial infections (58-60). However, as already mentioned, thus far there is no evidence for infections triggering the onset of LCH. Another role of IL-17 described in literature is its involvement in osteoclastogenesis by inducing the secretion of RANKL by osteoblasts (61, 62). In fact, RANKL is present in LCH lesions (chapter 5; figure 1). These findings fit very nicely with the observed IL-23 expression in LCH lesions (data not published; figure 1), which is also known to induce RANKL expression (63). Together with M-CSF (also expressed in these lesions, chapter 5), expression of RANKL may help explain the presence of osteoclast-like MGCs in LCH lesions. Thus, LCH cells, besides directly participating in the granulomatous response typical of LCH lesions by abnormally synthesizing IL-17, may also have a direct effect on monocytes or LCH cells themselves resulting in their fusion and generating the MGCs typically observed in LCH lesions (figure 1). IFN-γ, which is also present in LCH lesions, potentiates this IL-17-dependent pathway for DC fusion by increasing the size of MGCs and leading them to express tissue-destructive enzymes (57). In fact, the observed presence of matrix-degrading enzymes in the osteoclast-like MGCs of LCH lesions suggests a destructive role of the tissue by these cells (figure 1). Therefore, the cytokine production in LCH is clearly an important element in the progression of the disease. In addition, besides the involvement in osteoclastogenesis, RANKL is known to induce regulatory T cells (Tregs) via activation

of DCs (64). Interestingly, Senechal *et al*. (65) recently suggested that LCs in LCH lesions are also abnormal as they seem to induce the accumulation of Tregs within the granuloma. We have performed double immunofluorescent staining for Treg markers and shown that indeed these cells are present in high numbers in LCH lesions (data not published; figure 1). However, despite the presence of Tregs in LCH lesions, the inflammatory process is not controlled and is instead amplified. Since Tregs usually control T cells, it is likely that inflammation in LCH is not driven by T cells but rather by LCH cells. Moreover, instead of proliferation being the driving force behind the development of LCH, it is the accumulation of the pathological LCs that leads to the abnormal immune response seen in LCH (figure 1). The accumulation of LCs in LCH and the release of abundant inflammatory molecules can be in fact regarded as a reactive and compensatory phenomenon aimed at overcoming the patient's inability of the adaptive immune response to downregulate innate immunity.

**6. Future directions.**

Based on the assumption that LCH may be the result of an immune dysregulation by exclusion of both genetic defects and microbial triggers, it is of importance to further delineate the pathogenesis of LCH by investigating the immunological mechanisms behind this disease. Thus, it is essential to study the expression of chemokines, since LCH cells were already proven to have a dysregulated pattern of expression level of chemokine receptors which does not allow them to migrate out of the lesional sites to the lymph nodes. It is then possible that additional chemokine receptors are also involved in keeping LCH cells in the different lesional sites. In fact, a study has showed that the chemokine CXCL14/BRAK controls the epidermal recruitment of monocyte dendritic precursors, which enables their in situ differentiation into functional LC-like cells under steady-state conditions (66). Likewise, CCL2/MCP-1 has also been shown to recruit DCs and LCs to the skin (67). It is possible that an aberrant expression of chemokines such as CXCL14 and/or CCL2 is causing LC-committed monocytes to be abnormally retained in other sites than skin. Along this line of thought, it is of interest to compare the chemokine ligand and receptor expression pattern analysis in pulmonary LCH, as this is a prototype of a reactive form of LCH. There are now research-friendly chemokine array platforms available that allow the analysis of chemokine receptor/ligand gene expression profiles and are suited to use for this purpose.

As mentioned earlier in this chapter, it is still essential to look at the genetics of LCH lesions associated to malignancies, such as leukaemias, lymphomas and Hodgkin's disease, particularly to LCH lesions developed after the onset of a malignancy. To date our results from LCH lesions without clinical history of having an associated malignancy have revealed no genomic abnormalities in the DNA of sorted lesional LCH cells. Thus, it would be interesting to investigate whether LCH cells in cases associated with malignancies share common mutations with the malignancy to which the LCH

lesions are associated.

Another area of interest is to compare the cell subsets present in the blood as well as in the lesional tissue site of LCH patients. There is evidence that the peripheral blood of treated LCH patients contains decreased levels of (CD4+ CD25 high) Tregs, NK cells and monocytes compared to healthy controls and is deficient in both plasmacytoid and conventional/myeloid DC populations (personal communication, Christine Delprat). Thus, it is crucial to investigate the presence of these cells both in the blood and in the lesional tissue sites of LCH patients as well. If the decreased levels or deficiency of these cells in the blood of LCH patients are confirmed, this may be a consequence of cell recruitments due to the aberrant chemoattractant expression to LCH lesions, which exhaust the blood compartment. This may reveal that an impairment of haematopoiesis or recruitment of the cells to the LCH lesions is occurring.

The work produced for this thesis along with other published studies strongly suggests that LCH could be the result of an immune dysregulation, where a prolonged inflammatory signalling co-exists with defects in anti-inflammatory mechanisms, which lead to the chronic inflammation and benefit the accumulation of LCH cells. It is thus likely that future research in LCH pathogenesis will take some of its leads from other inflammatory disease research.

**References**


1. Egeler,R.M. and D'Angio,G.J. 1995. Langerhans cell histiocytosis. J.Pediatr. 127:1-11.
2. Bhatia,S., Nesbit,M.E., Jr., Egeler,R.M., Buckley,J.D., Mertens,A., and Robison,L.L. 1997. Epi demiologic study of Langerhans cell histiocytosis in children. J.Pediatr. 130:774-784.
3. Minkov,M., Grois,N., Heitger,A., Potschger,U., Westermeier,T., and Gadner,H. 2000. Treatment of multisystem Langerhans cell histiocytosis. Results of the DAL-HX 83 and DAL-HX 90 studies. DAL-HX Study Group. Klin.Padiatr.212:139-144.
4. Titgemeyer,C., Grois,N., Minkov,M., Flucher-Wolfram,B., Gatterer-Menz,I., and Gadner,H. 2001. Pattern and course of single-system disease in Langerhans cell histiocytosis data from the DAL-HX 83- and 90-study. Med.Pediatr.Oncol. 37:108-114.
5. Schmitz,L. and Favara,B.E. 1998. Nosology and pathology of Langerhans cell histiocytosis. Hema tol.Oncol.Clin.North Am. 12:221-246.
6. Fleming,M.D., Pinkus,J.L., Fournier,M.V., Alexander,S.W., Tam,C., Loda,M., Sallan,S.E., Nichols,K.E., Carpentieri,D. F., Pinkus,G.S. *et al*. 2003. Coincident expression of the chemokine re ceptors CCR6 and CCR7 by pathologic Langerhans cells in Langerhans cell histiocytosis. Blood 101:2473-2475.
7. Ohl,L., Mohaupt,M., Czeloth,N., Hintzen,G., Kiafard,Z., Zwirner,J., Blankenstein,T., Henning,G., and Forster,R. 2004. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. Immunity. 21:279-288.
8. Caux,C., Ait-Yahia,S., Chemin,K., de Bouteiller,O., Dieu-Nosjean,M.C., Homey,B., Massacrier,C., Vanbervliet,B., Zlotnik,A., and Vicari,A. 2000. Dendritic cell biology and regulation of dendritic cell trafficking by chemokines. Springer Semin.Immunopathol. 22:345-369.
9. Kimber,I., Cumberbatch,M., Dearman,R.J., Bhushan,M., and Griffiths,C.E. 2000. Cytokines and

chemokines in the initiation and regulation of epidermal Langerhans cell mobilization. Br.J.Dermatol. 142:401-412.
10. Andersson,B.U., Tani,E., Andersson,U., and Henter,J.I. 2004. Tumor necrosis factor, interleukin 11, and leukemia inhibitory factor produced by Langerhans cells in Langerhans cell histiocytosis. J.Pediatr.Hematol.Oncol. 26:706-711.
11. Egeler,R.M., Favara,B.E., van Meurs,M., Laman,J.D., and Claassen,E. 1999. Differential In situ cy tokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expres sion of cytokines relevant to disease and treatment. Blood 94:4195-4201.
12. de Graaf,J.H., Tamminga,R.Y., Dam-Meiring,A., Kamps,W.A., and Timens,W. 1996. The presence of cytokines in Langerhans' cell histiocytosis. J.Pathol. 180:400-406.
13. Tazi,A., Moreau,J., Bergeron,A., Dominique,S., Hance,A.J., and Soler,P. 1999. Evidence that Langer hans cells in adult pulmonary Langerhans cell histiocytosis are mature dendritic cells: importance of the cytokine microenvironment. J.Immunol. 163:3511-3515.
14. Gerard,C. and Rollins,B.J. 2001. Chemokines and disease. Nat.Immunol. 2:108-115.
15. Charo,I.F. and Ransohoff,R.M. 2006. The many roles of chemokines and chemokine receptors in in flammation. N.Engl. J.Med. 354:610-621.
16. Luster,A.D. 1998. Chemokines--chemotactic cytokines that mediate inflammation. N.Engl.J.Med. 338:436-445.
17. Willman,C.L., Busque,L., Griffith,B.B., Favara,B.E., McClain,K.L., Duncan,M.H., and Gilliland,D. G. 1994. Langerhans'-cell histiocytosis (histiocytosis X)--a clonal proliferative disease. N.Engl. J.Med. 331:154-160.
18. Yu,R.C., Chu,C., Buluwela,L., and Chu,A.C. 1994. Clonal proliferation of Langerhans cells in Lang erhans cell histiocytosis. Lancet 343:767-768.
19. Yousem,S.A., Colby,T.V., Chen,Y.Y., Chen,W.G., and Weiss,L.M. 2001. Pulmonary Langerhans' cell histiocytosis: molecular analysis of clonality. Am.J.Surg.Pathol. 25:630-636.
20. Savell,V.H., Jr., Sherman,T., Scheuermann,R.H., Siddiqui,A.M., and Margraf,L.R. 1998. Bcl-2 ex pression in Langerhans' cell histiocytosis. Pediatr.Dev.Pathol. 1:210-215.
21. Amir,G. and Weintraub,M. 2007. Association of cell cycle-related gene products and NF-kappaB with clinical parameters in Langerhans cell histiocytosis. Pediatr.Blood Cancer.
22. Bank,M.I., Rengtved,P., Carstensen,H., and Petersen,B.L. 2003. Langerhans cell histiocytosis: an evaluation of histopathological parameters, demonstration of proliferation by Ki-67 and mitotic bod ies. APMIS 111:300-308.
23. Schouten,B., Egeler,R.M., Leenen,P.J., Taminiau,A.H., van den Broek,L.J., and Hogendoorn,P.C. 2002. Expression of cell cycle-related gene products in Langerhans cell histiocytosis. J.Pediatr.Hema tol.Oncol. 24:727-732.
24. Weiss,L.M., Wood,G.S., Trela,M., Warnke,R.A., and Sklar,J. 1986. Clonal T-cell populations in lym phomatoid papulosis. Evidence of a lymphoproliferative origin for a clinically benign disease. N.Engl.J.Med. 315:475-479.
25. Kurahashi,H., Hara,J., Yumura-Yagi,K., Murayama,N., Inoue,M., Ishihara,S., Tawa,A., Okada,S., and Kawa-Ha,K. 1991. Monoclonal nature of transient abnormal myelopoiesis in Down's syndrome. Blood 77:1161-1163.
26. Kim,N.W., Piatyszek,M.A., Prowse,K.R., Harley,C.B., West,M.D., Ho,P.L., Coviello,G.M., Wright,W.E., Weinrich,S.L., and Shay,J.W. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.
27. Hiyama,E., Gollahon,L., Kataoka,T., Kuroi,K., Yokoyama,T., Gazdar,A.F., Hiyama,K., Piatyszek,M. A., and Shay,J.W. 1996. Telomerase activity in human breast tumors. J.Natl.Cancer Inst. 88:116-122.
28. Avilion,A.A., Piatyszek,M.A., Gupta,J., Shay,J.W., Bacchetti,S., and Greider,C.W. 1996. Human tel omerase RNA and telomerase activity in immortal cell lines and tumor tissues. Cancer Res. 56:645-650.
29. Counter,C.M., Hirte,H.W., Bacchetti,S., and Harley,C.B. 1994. Telomerase activity in human ovarian

carcinoma. Proc.Natl.Acad.Sci.U.S.A 91:2900-2904.
30. Smith,D.L., Soria,J.C., Morat,L., Yang,Q., Sabatier,L., Liu,D.D., Nemr,R.A., Rashid,A., and Vauthey,J.N. 2004. Human telomerase reverse transcriptase (hTERT) and Ki-67 are better predictors of survival than established clinical indicators in patients undergoing curative hepatic resection for colorectal metastases. Ann.Surg.Oncol. 11:45-51.
31. Ikeda,S., Shibata,T., Eishi,Y., Takizawa,T., and Koike,M. 2003. Correlation between the expression of telomerase reverse transcriptase and proliferative activity in breast cancer cells using an immuno cytochemical restaining method. Pathol.Int. 53:762-768.
32. Bechan,G.I., Meeker,A.K., De Marzo,A.M., Racke,F., Jaffe,R., Sugar,E., and Arceci,R.J. 2008. Tel omere length shortening in Langerhans cell histiocytosis. Br. J. Haematol. 140(4):420-428.
33. Ping,L., Asai,A., Okada,A., Isobe,K., and Nakajima,H. 2003. Dramatic increase of telomerase activ ity during dendritic cell differentiation and maturation. J.Leukoc.Biol. 74:270-276.
34. Geissmann,F., Lepelletier,Y., Fraitag,S., Valladeau,J., Bodemer,C., Debre,M., Leborgne,M., Saeland,S., and Brousse,N. 2001. Differentiation of Langerhans cells in Langerhans cell histiocyto sis. Blood 97:1241-1248.
35. Arico,M., Nichols,K., Whitlock,J.A., Arceci,R., Haupt,R., Mittler,U., Kuhne,T., Lombardi,A., Ishii,E., Egeler,R.M. *et al*. 1999. Familial clustering of Langerhans cell histiocytosis. Br.J.Haematol. 107:883-888.
36. Egeler,R.M., Neglia,J.P., Arico,M., Favara,B.E., Heitger,A., Nesbit,M.E., and Nicholson,H.S. 1998. The relation of Langerhans cell histiocytosis to acute leukemia, lymphomas, and other solid tumors. The LCH-Malignancy Study Group of the Histiocyte Society. Hematol.Oncol.Clin.North Am. 12:369-378.
37. Egeler,R.M., Neglia,J.P., Puccetti,D.M., Brennan,C.A., and Nesbit,M.E. 1993. Association of Langer hans cell histiocytosis with malignant neoplasms. Cancer 71:865-873.
38. Lengauer,C., Kinzler,K.W., and Vogelstein,B. 1998. Genetic instabilities in human cancers. Nature 396:643-649.
39. Hahn,W.C. and Weinberg,R.A. 2002. Rules for making human tumor cells. N.Engl.J.Med. 347:1593-1603.
40. Betts,D.R., Leibundgut,K.E., Feldges,A., Pluss,H.J., and Niggli,F.K. 1998. Cytogenetic abnormalit ies in Langerhans cell histiocytosis. Br.J.Cancer 77:552-555.
41. Murakami,I., Gogusev,J., Fournet,J.C., Glorion,C., and Jaubert,F. 2002. Detection of molecular cy togenetic aberrations in langerhans cell histiocytosis of bone. Hum.Pathol. 33:555-560.
42. Chikwava,K.R., Hunt,J.L., Mantha,G.S., Murphy,J.E., and Jaffe,R. 2007. Analysis of loss of hetero zygosity in single-system and multisystem Langerhans' cell histiocytosis. Pediatr.Dev.Pathol. 10:18-24.
43. Feldman,A.L., Berthold,F., Arceci,R.J., Abramowsky,C., Shehata,B.M., Mann,K.P., Lauer,S.J., Pritchard,J., Raffeld,M., and Jaffe,E.S. 2005. Clonal relationship between precursor T-lymphoblastic leukaemia/lymphoma and Langerhans-cell histiocytosis. Lancet Oncol. 6:435-437.
44. Feldman,A.L., Minniti,C., Santi,M., Downing,J.R., Raffeld,M., and Jaffe,E.S. 2004. Histiocytic sar coma after acute lymphoblastic leukaemia: a common clonal origin. Lancet Oncol. 5:248-250.
45. McClain,K., Jin,H., Gresik,V., and Favara,B. 1994. Langerhans cell histiocytosis: lack of a viral etiol ogy. Am.J.Hematol. 47:16-20.
46. Mierau,G.W., Wills,E.J., and Steele,P.O. 1994. Ultrastructural studies in Langerhans cell histiocyto sis: a search for evidence of viral etiology. Pediatr.Pathol. 14:895-904.
47. Leahy,M.A., Krejci,S.M., Friednash,M., Stockert,S.S., Wilson,H., Huff,J.C., Weston,W.L., and Brice,S.L. 1993. Human herpesvirus 6 is present in lesions of Langerhans cell histiocytosis. J.Invest Dermatol. 101:642-645.
48. Glotzbecker,M.P., Carpentieri,D.F., and Dormans,J.P. 2004. Langerhans cell histiocytosis: a primary viral infection of bone? Human herpes virus 6 latent protein detected in lymphocytes from tissue of children. J.Pediatr.Orthop. 24:123-129.

49. Ricciardi-Castagnoli,P. and Granucci,F. 2002. Opinion: Interpretation of the complexity of innate im mune responses by functional genomics. Nat.Rev.Immunol. 2:881-889.
50. Perez,R.L., Rivera-Marrero,C.A., and Roman,J. 2003. Pulmonary granulomatous inflammation: From sarcoidosis to tuberculosis. Semin.Respir.Infect. 18:23-32.
51. Ulrichs,T. and Kaufmann,S.H. 2006. New insights into the function of granulomas in human tubercu losis. J.Pathol. 208:261-269.
52. Zumla,A. and James,D.G. 1996. Granulomatous infections: etiology and classification. Clin.Infect. Dis. 23:146-158.
53. Francus,T., Klein,R.F., Staiano-Coico,L., Becker,C.G., and Siskind,G.W. 1988. Effects of tobacco glycoprotein (TGP) on the immune system. II. TGP stimulates the proliferation of human T cells and the differentiation of human B cells into Ig secreting cells. J.Immunol. 140:1823-1829.
54. Youkeles,L.H., Grizzanti,J.N., Liao,Z., Chang,C.J., and Rosenstreich,D.L. 1995. Decreased tobacco-glycoprotein-induced lymphocyte proliferation *in vitro* in pulmonary eosinophilic granuloma. Am.J.Respir.Crit Care Med. 151:145-150.
55. Soler,P., Moreau,A., Basset,F., and Hance,A.J. 1989. Cigarette smoking-induced changes in the number and differentiated state of pulmonary dendritic cells/Langerhans cells. Am.Rev.Respir.Dis. 139:1112-1117.
56. Casolaro,M.A., Bernaudin,J.F., Saltini,C., Ferrans,V.J., and Crystal,R.G. 1988. Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in associa tion with cigarette smoking. Am.Rev.Respir.Dis. 137:406-411.
57. Coury,F., Annels,N., Rivollier,A., Olsson,S., Santoro,A., Speziani,C., Azocar,O., Flacher,M., Djebali,S., Tebib,J. *et al*. 2008. Langerhans cell histiocytosis reveals a new IL-17A-dependent path way of dendritic cell fusion. Nat.Med. 14:81- 87.
58. Lockhart,E., Green,A.M., and Flynn,J.L. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J.Immunol. 177:4662-4669.
59. Happel,K.I., Dubin,P.J., Zheng,M., Ghilardi,N., Lockhart,C., Quinton,L.J., Odden,A.R., Shellito,J.E., Bagby,G.J., Nelson,S. *et al*. 2005. Divergent roles of IL-23 and IL-12 in host defense against Kleb siella pneumoniae. J.Exp.Med. 202:761-769.
60. Vaknin-Dembinsky,A., Balashov,K., and Weiner,H.L. 2006. IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. J.Immunol. 176:7768-7774.
61. Kotake,S., Udagawa,N., Takahashi,N., Matsuzaki,K., Itoh,K., Ishiyama,S., Saito,S., Inoue,K., Kamatani,N., Gillespie,M.T. *et al*. 1999. IL-17 in synovial fluids from patients with rheumatoid ar thritis is a potent stimulator of osteoclastogenesis. J.Clin.Invest 103:1345-1352.
62. Sato,K., Suematsu,A., Okamoto,K., Yamaguchi,A., Morishita,Y., Kadono,Y., Tanaka,S., Kodama,T., Akira,S., Iwakura,Y. *et al*. 2006. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J.Exp.Med. 203:2673-2682.
63. Yago,T., Nanke,Y., Kawamoto,M., Furuya,T., Kobashigawa,T., Kamatani,N., and Kotake,S. 2007. IL-23 induces human osteoclastogenesis via IL-17 *in vitro*, and anti-IL-23 antibody attenuates col lagen-induced arthritis in rats. Arthritis Res. Ther. 9:R96.
64. Loser,K., Mehling,A., Loeser,S., Apelt,J., Kuhn,A., Grabbe,S., Schwarz,T., Penninger,J.M., and Beissert,S. 2006. Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells. Nat.Med. 12:1372-1379.
65. Senechal,B., Elain,G., Jeziorski,E., Grondin,V., Patey-Mariaud,d.S., Jaubert,F., Beldjord,K., Lellouch,A., Glorion,C., Zerah,M. *et al*. 2007. Expansion of regulatory T cells in patients with langer hans cell histiocytosis. PLoS.Med. 4:e253.
66. Schaerli,P., Willimann,K., Ebert,L.M., Walz,A., and Moser,B. 2005. Cutaneous CXCL14 targets blood precursors to epidermal niches for Langerhans cell differentiation. Immunity. 23:331-342.
67. Nakamura,K., Williams,I.R., and Kupper,T.S. 1995. Keratinocyte-derived monocyte chemoattractant

protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin. J.Invest Dermatol. 105:635-643.

# Summary
# Samenvatting
# Sumário

## Summary

The main goal of this thesis was to gain insight into the immunological and genetic aspects of LCH. We set out to address several previously unanswered questions in LCH, namely, why are the lesional CD1a+ LCs in LCH aberrantly present in many body sites; what is the origin – whether via recruitment or in situ formation – of the many cells characteristic of the lesion composition and finally, the ultimate question of what is the cause behind this disease, a reactive trigger or a neoplastic defect.

LCH is characterised by an abnormal accumulation and proliferation of lesional CD1a+ LCH cells in many sites in the body. The unusual retention of these lesional LCs in tissue sites other than skin prompted us to investigate, in **chapter 3**, the expression of chemokine ligands and receptors that play an important role in the migration and functioning of DCs in general. The finding of expression of CCR6 and the absence of CCR7 on the lesional CD1a+ LCH cells confirms that these cells are indeed in an immature state, as CCR6 is typically expressed by immature DCs and, in contrast, CCR7 is indicative of DC maturation, guiding DCs to lymphoid organs by responding to CCR7 agonists. The lack of CCR7 by the lesional CD1a+ cells prevents them from leaving their peripheral tissue sites and results in their accumulation. It is also demonstrated that the lesional CD1a+ cells are the likely source of up-regulated CCL20/MIP-3a, ligand for CCR6, as well as of other inflammatory chemokines, such as CCL5/RANTES and CXCL11/I-TAC. This finding may explain the recruitment of other inflammatory cell types characteristic of LCH lesions. The results of this study showed that CD1a+ lesional cells are consistently positive for CCR6 and its corresponding ligand CCL20 and the different lesional sites express the same profile of inflammatory chemokines studied. In **chapter 4** we set out to analyse the expression of CCR6 and CCR7 in pulmonary LCH lesions. We found a differential expression of CCR6 by the LCH cells, ranging from 100% CCR6+ CD1a LCH cells, to 50% CCR6+ CD1a LCH cells, to no CCR6+ CD1a LCH cells. Interestingly, no CCR7 was present in the CCR6- CD1a LCH cells, despite previous reports describing the expression of typical cell activation markers by these cells in pulmonary LCH lesions.

**Chapter 5** focuses on understanding the role of multinucleated giant cells (MGCs) in LCH lesions. Due to the characteristic osteolysis of bone lesions and fibrosis in other LCH lesional sites, these cells were investigated for the presence of characteristic osteoclast markers. The finding that the MGCs in LCH lesions express osteoclastic phenotypic markers, such as TRAP, VNR and CD68, as well as osteoclast-secreting enzymes, such as Cathepsin K (CatK) and MMP-9 confirms that these cells are indeed osteoclast-like MGCs. The presence of these osteoclast-like giant cells in LCH bone lesions is not that unusual as this is the normal tissue site for osteoclasts which, through their resorbing activity, help to maintain the normal homeostasis of the bone. However, even in the ostotic lesions, these osteoclast-like cells are present in relatively high numbers than in normal bone and appeared to be

"floating" within the cellular infiltrate, whereas normally close contact with bone would be expected. In contrast, the finding of osteoclast-like cells in non-ostotic LCH sites raises the question of their origin. Thus, by looking at the expression of specific cellular markers, such as CD68 for macrophages and CD1a for LCs, we found that in all bone lesions MGCs seem to be normal, as the CatK+ osteoclast-like cells co-expressed the macrophage marker CD68 and not CD1a. However in a number of skin and lymph node lesions that contained osteoclast-like cells, these MGCs expressed both CD68 and CD1a. Thus, it is likely that the lesional environment in these unusual sites induces the local formation of the osteoclast-like MGCs derived from a different origin, such as the CD1a+ cells. Further evidence to support this came with the finding that RANKL, a cytokine involved in osteoclast induction was found in the majority of LCH lesions, expressed by the lesional CD1a+ cells and T cells. The interaction of RANKL with its receptor, RANK, is a key feature of osteoclast differentiation and thus we looked for the presence of the receptor as well. RANK was present on CD68+ and CD1a+ cells, and the lesions that showed expression of RANKL were also positive for this receptor. M-CSF, another cytokine involved in osteoclast differentiation normally produced by osteoblasts and stromal cells, was expressed by MGCs and strikingly also by the CD1a cells in most LCH lesions. This study provided support for the hypothesis that the excessive bony destruction found in LCH is likely mediated by osteoclast-like giant cells. These cells are thus a potential target in LCH lesions, with the use of bisphosphonates being the most adequate therapeutic approach.

In **chapter 6**, based on the proliferation and non-apoptotic character of LCH cells, the potential presence of a telomere maintenance mechanism in these cells was investigated. If such a telomere maintenance mechanism were to be found active in LCH cells this would provide stronger evidence that LCH could be a neoplasm, as telomerase expression, one of the most common telomere maintenance mechanisms, is strongly associated to cancers. Thus, this study showed that the lesional CD1a+ cells express telomerase in all SS skin LCH lesions, in contrast to SS bone lesions, in which the majority of the lesions contained LCH cells negative for telomerase. Furthermore, lesional CD1a+ cells from MS patients always expressed telomerase, regardless of the tissue site. More evidence for telomerase activity in LCH came from the use of the TRAP assay, where evidence for telomerase activity was found in lesions reported to contain LCH cells that expressed telomerase. In addition, the telomere length of a MS LCH lesion was long and homogeneous, in contrast to three other single system LCH lesions which displayed a much shorter telomere length. This difference of telomere length and telomerase expression in the different lesional sites and forms of the disease may reflect the broad clinical spectrum of LCH. This study showed for the first time that there is a clear difference between SS skin and bone LCH lesions and SS versus MS disease, based on the expression of telomerase.

To date it has remained unknown whether the abnormal behaviour displayed by LCH cells was due to defects at the genetic level. Thus, in **chapter 7** a comprehensive study using several state-of-the-art

molecular techniques was performed to address this. LCH cells were isolated from LCH paraffin and frozen biopsies using methodology developed by us and others. The20
evidence against a genetic basis for LCH.

Finally in **chapter 8** the findings of this thesis and their implications for future research are discussed.

## Samenvatting

Het hoofddoel van dit proefschrift was om inzicht te krijgen in de immunologische en genetische aspecten van LCH. We zijn begonnen met het behandelen van een aantal voorgaand onbeantwoorde vragen betreffende LCH, zoals “waarom zijn de aangedane CD1a+ LCs in LCH abnormaal aanwezig in vele lichaamsdelen”, “wat is de oorzaak – hetzij via aantrekking of in situ formatie – van de vele karakteristieke cellen van de laesio/lesion samenstelling” en tenslotte de uiteindelijke vraag “wat is de oorzaak achter deze ziekte: een reactieve ontlading of een neoplastisch defect”.
LCH wordt gekarakteriseerd door een abnormale ophoping en groei van aangedane CD1a+LCH cellen op vele plekken in het lichaam. De ongewone aanwezigheid van deze aangedane LCs in weefsels anders dan de huid, zette ons er toe om, in **hoofdstuk 3**, de expressie te onderzoeken van chemokine ligands en receptoren die een belangrijke rol spelen in de migratie en het functioneren van DCs in het algemeen. De vondst van de expressie van CCR6, en de afwezigheid van CCR7 bij de aangedane CD1a+LCH cellen bevestigen dat deze cellen inderdaad in een onvolwassen stadium zijn, zoals dit bij CCR6 typisch tot uiting komt in onvolwassen DCs en, in tegenstelling, dat CCR7 indicatief is voor rijping van DC, die DCs leiden naar lymphoide organen door te reageren naar CCR7 agonisten. Het tekort aan CCR7 in de aangedane CD1a+ cellen voorkomt dat ze de periferale weefsel locaties verlaten wat resulteert in ophoping. Het is ook bewezen dat de aangedane CD1a+ cellen waarschijnlijk de bron zijn van op-gereguleerde CCL20/MIP-3a, ligand voor CCR6, en tevens voor andere geïriteerde chemokines, zoals CCL5/RANTES en CXCL11/I-TAC. Deze vondst zou de aantrekking van andere geïrriteerde celtypen, karakteristiek voor LCH lesions, kunnen verklaren. De resultaten van deze studie laten zien dat aangedane CD1a+ cellen blijvend positief zijn voor CCR6 en haar corresponderende ligand CCL20, en de verschillende aangedane locaties laten hetzelfde profiel zien voor de bestudeerde geïrriteerde chemokines. In **hoofdstuk 4** wordt de expressie van CCR6 en CCR7 in LCH lesions in de longen geanalyseerd. We vonden een verschil in de expressie van CCR6 in de LCH cellen, variërend van 100% CCR6+CD1a LCH cellen, tot 50% CCR6+CD1a LCH cellen, en geen CCR6+CD1a LCH cellen. Belangwekkend genoeg was er geen CCR7 aanwezig in de CCR6-CD1a LCH cellen, ondanks eerdere rapporten waarin de expressie van typische celactiviteitmarkers bij deze cellen in long LCH lesions worden beschreven.
**Hoofdstuk 5** focussed meer op het begrijpen van de rol van multinucleated reuze cellen (MGCs) in LCH lesions. Door de karakteristieke osteolyse van bot lesions en fibrose in andere door LCH aangedane locaties, zijn deze cellen onderzocht op de aanwezigheid van karakteristieke osteoclast markers. De bevinding dat de MGCs in LCH lesions osteoclastische fenotypische markers tonen, zoals TRAP, VNR en CD68, als ook osteoclast-uitscheidende enzymen, zoals Cathepsin K (CatK) en MMP-9 bevestigt dat deze cellen inderdaad osteoclast-gelijkende MGCs zijn. De aanwezigheid van deze os-

teoclast-gelijkende reuze cellen in LCH bot lesions in niet zo ongewoon omdat dit de normale weefsel locatie is voor osteoclasts welke, door de resorptie activiteit, helpt de normale homeostase van het bot te behouden. Echter, zelfs in de ostotic lesions zijn de osteoclast-gelijkende cellen in relatief grotere hoeveelheden aanwezig dan in normaal bot, en lijken ze te "zweven" in het cellulaire infiltraat, waar normaal dicht contact met het bot verwacht wordt. Aan de andere kant doet de vondst van osteoclast-gelijkende cellen in niet-ostotische LCH locaties de vraag naar hun origine rijzen. Dus door naar de expressie van cellulaire markers te kijken, zoals CD68 voor macrofagen en CD1a voor LCs, vonden we dat in alle bot lesions de MGCs normaal waren, doordat de CatK+ osteoclast-gelijkende cellen co-expressie toonden voor de macrofaag marker CD68 en niet CD1a. Echter in een aantal huid- en lymfeknoop lesions die osteoclast-gelijkende cellen bevatten, toonden MGCs zowel expressie in CD68 als in CD1a. Het is dus waarschijnlijk dat de aangedane omgeving in deze ongewone locaties de lokale formatie van de osteoclast-gelijkende MGCs van een andere origine is afgeleid, zoals de CD1a+ cellen. Meer bewijs hier voor kwam met de bevinding dat RANKL, een cytokine dat betrokken is bij osteoclast inductie, in de meeste LCH lesions werd gevonden, uitgedrukt in de aangedane CD1a+ cellen en T cellen. De interactie van RANKL met haar receptor, RANK, is een sleutel kenmerk voor osteoclast differentiatie en hierdoor keken we tevens naar de aanwezigheid van de receptor. RANK was aanwezig op CD68+ en CD1a+ cellen, en de lesions die expressie toonden voor RANKL waren ook positief voor deze receptor. M-CSF, een andere cytokine betrokken in osteoclast differentiatie dat normaal geproduceerd wordt door osteoblast en stroma cellen, werd uitgedrukt door MGCs en verrassend ook door de CD1a cellen in de meeste LCH lesions. Deze studie ondersteund de hypothese dat de buitengewone botvernietiging gevonden in LCH waarschijnlijk gemedieerd wordt door osteoclast-gelijkende reuze cellen. Deze cellen zijn dus een mogelijke doel in LCH lesions, waarbij het gebruik van bifosfonaten de meest adequate therapeutische aanpak is.

In **hoofdstuk 6**, gebaseerd op de groei en het niet-apoptische karakter van LCH cellen, wordt de potentiële aanwezigheid van een telomeer onderhoudsmechanisme in deze cellen onderzocht. Als zo'n telomeer onderhoudsmechanisme gevonden zou worden die actief is in LCH cellen, zou dit een sterker bewijs vormen dat LCH een neoplasme zou zijn, doordat telomerase uitdrukking, een van de meest voorkomende telomeer onderhoudsmechanisme, sterk geassocieerd is aan kanker. Dus, deze studie toont aan dat de aangedane CD1a+ cellen telomerase uitdrukken in alle SS huid LCH lesions, in tegenstelling to SS bot lesions, waar de meerderheid van de lesions bevattende LCH cellen negatief zijn voor telomerase. Bovendien drukken aangedane CD1a+ cellen van MS patienten altijd telomerase uit, ongeacht de weefsel locatie. Meer bewijs voor telomerase activiteit in LCH kwam uit het gebruik van de TRAP analyse, waar bewijs voor telomerase activiteit werd gevonden in lesions die LCH cellen bevat die telomerase uitdrukten. Daarbij was de telomeerlengte van een MS LCH lesion lang en homogeen, in tegenstelling tot drie andere enkelvoudige systemen LCH lesions die een veel

kortere telomeerlengte gaven. Dit verschil in telomeerlengte en telomerase uitdrukking in de verschillende aangedane locaties en vormen van de ziekte zou het breed klinische spectrum van LCH kunnen weerspiegelen. Deze studie toont voor de eerste keer aan dat er een duidelijk verschil is tussen SS huid en bot LCH lesions en SS versus MS aandoening, gebaseerd op de uitdrukking van telomerase.
Tot op heden was het onduidelijk of het abnormale gedrag uitgedrukt door LCH cellen door defecten kwam op genetisch niveau. Daarom is er in **hoofdstuk 7** een brede studie uitgevoerd om dit nader te onderzoeken, door het gebruik van verscheidene hoogstaande moleculaire technieken. Uit LCH paraffine en bevroren biopsies zijn LCH cellen geïsoleerd door methodes te gebruiken die door ons en anderen zijn ontwikkeld. Het DNA werd geëxtraheerd en gebruikt voor arrayCGH, SNPs en p53 mutatie en ploidy analyse. In alle geanalyseerde lesions vertoonden LCH cellen noch kopie nummer noch kopie neutrale veranderingen. Aanvullend op deze technieken liet analyse van karyotype van deze cellen nogmaals normaliteit zien. Dus, buiten de mogelijkheid van punt mutaties waarvan we niet in staat waren ze te bestuderen, geven deze resultaten een sterk bewijs tegen een genetische basis voor LCH.
Tenslotte worden in **hoofdstuk 8** de bevindingen van dit proefschrift en haar implicaties voor toekomstig onderzoek bediscussieerd.

## Sumário

O principal objectivo desta tese é aprofundar o conhecimento sobre os aspectos imunológicos e genéticos da histiocitose das células de Langerhans (LCH). Assim, procuramos abordar várias questões que permanecem em aberto na LCH, nomeadamente a razão pela qual as células de Langerhans CD1a+ lesionais (células de LCH) se encontram anormalmente em diversos territórios do corpo; qual é a origem – se por via de recrutamento ou por formação *in situ* – dos vários tipos de células características da composição lesional de LCH; e finalmente, a grande questão sobre a origem desta doença, se é devida a um estímulo reactivo ou a um defeito neoplásico.

A LCH é caracterizada por acumulação e proliferação anormais de células de LCH CD1a+ em várias zonas do corpo. A presença invulgar destas células lesionais de Langerhans em tecidos não cutâneos levaram-nos a investigar, no **capítulo 3**, a expressão de ligandos e receptores de quimocinas que desempenham um papel importante na migração e função das células dendríticas em geral. A descoberta da expressão de CCR6 e da ausência de CCR7 na superfície das células de LCH confirma que estas células estão num estado imaturo, já que CCR6 é tipicamente expresso por células dendríticas imaturas. Por outro lado, CCR7, que é indicativo de maturação das células dendríticas, não foi encontrado à superfície de nenhuma destas células. CCR7 dirige as células dendríticas para os orgãos linfáticos por resposta a agonistas de CCR7. A ausência de expressão de CCR7 pelas células de LCH impede-as de abandonarem os tecidos periféricos, o que resulta na sua acumulação. Também é demonstrado neste capítulo que as células de LCH são a provável fonte de sobre-expressão da quimocina CCL20/MIP-3a, ligando de CCR6, assim como de outras quimocinas inflamatórias, como CCL5/RANTES e CXCL11/I-TAC. Esta descoberta ajuda a explicar o recrutamento de outros tipos celulares inflamatórios tipicamente observados nas lesões de LCH. Os resultados deste estudo demonstram que as células de LCH expressam consistentemente CCR6, apresentando à sua superfície o seu ligando, CCL20, assim como o facto de os diferentes locais lesionais expressarem o mesmo perfil de quimocinas inflamatórias. No **capítulo 4**, analisamos a expressão de CCR6 e CCR7 em lesões de LCH pulmonares. Os resultados mostram um padrão diferencial de expressão de CCR6 pelas células de LCH, variando desde 100% de células de LCH positivas para CCR6, a 50% de células positivas, e, finalmente, a 0% de células LCH CCR6+. Curiosamente, CCR7 também estava ausente das células de LCH negativas para CCR6, apesar de estudos anteriores descreverem a expressão de marcadores celulares típicos de activação nestas células presentes em lesões pulmonares de LCH.

O **capítulo 5** investiga o papel das células gigantes multinucleadas (MGCs) nas lesões de LCH. Devido à osteólise típica das lesões ósseas e à fibrose observada noutros locais lesionais, a presença de marcadores específicos de osteoclastos nas MGCs foi investigada. A descoberta de que as MGCs nas lesões de LCH expressam marcadores fenotípicos de osteoclastos, como TRAP, VNR e CD68,

bem como enzimas produzidas por osteoclastos, como catepsina K (CatK) e MMP-9, confirma que estas células são, de facto, MGCs tipo-osteoclasto. A presença destas células gigantes tipo-osteoclasto em lesões ósseas de LCH não é de todo invulgar já que este é o tecido onde se encontram os osteoclastos que, através da sua actividade de digestão, ajudam a manter a homeostase do osso. No entanto, mesmo nas lesões ósseas, estas células estão presentes em números relativamente mais elevados do que no osso saudável e aparentam "flutuar" por entre o infiltrado celular, não estando em contacto próximo com o osso. Em contraste, a descoberta de células tipo osteoclasto em locais de LCH não ostóticos levanta a questão da sua origem. Assim, ao analisar a expressão de marcadores celulares específicos, como CD68 para macrófagos e CD1a para células de Langerhans, verificamos que em todas as lesões ósseas as MGCs aparentam ser normais, já que as células tipo-osteoclasto positivas para CatK expressam o marcador de macrófago CD68 e não expressam CD1a. No entanto, em algumas lesões cutâneas ou dos nódulos linfáticos de LCH que contêm células tipo-osteoclasto, estas MGCs expressam CD68 e CD1a. Assim, é provável que o ambiente lesional nestes locais invulgares induza a formação local de MGCs tipo-osteoclasto originadas a partir de um percursor diferente, como as células positivas para CD1a. Evidência posterior que suporta estes resultados vem da descoberta de que RANKL, uma citocina envolvida na diferenciação de osteoclastos, foi encontrada na maioria das lesões de LCH, sendo expressa pelas células de LCH e pelas células T. A interacção de RANKL com o seu receptor, RANK, é uma característica-chave da diferenciação de osteoclastos e, por isso, também analisamos a expressão do receptor. RANK está de facto presente nas células positivas para CD68 e para CD1a, e as lesões que contêm células que expressam RANKL também são positivas para o seu receptor. M-CSF, outra citocina envolvida na diferenciação de osteoclastos e normalmente produzida por osteoblastos e células estromais, é expressa pelas MGCs e, curiosamente, também pelas células de LCH na maioria das lesões de LCH. Este estudo fortalece a hipótese de que a destruição óssea excessiva encontrada em LCH é provavelmente mediada pelas MGCs tipo-osteoclasto. Estas células são, por isso, um alvo potencial em lesões de LCH, e o uso de bifosfonatos torna-se a abordagem terapêutica preferencial.

No **capítulo 6**, com base na proliferação e no carácter anti-apoptótico das células de LCH, a provável presença nestas células de um mecanismo de manutenção de telómeros foi investigada. No caso de tal mecanismo de manutenção de telómeros estar activo nas células de LCH, a hipótese de que LCH possa ser um neoplasma é fortalecida, já que a expressão de telomerase, um dos mecanismos mais vulgares de manutenção de telómeros, está fortemente associada a cancros. Este estudo demonstra que as células de LCH expressam telomerase em todas as lesões cutâneas de LCH solitárias, em oposição às lesões ósseas solitárias, nas quais a maioria das lesões contêm células de LCH negativas para telomerase. Adicionalmente, todas as células de LCH de lesões de pacientes com LCH multisistémica expressam telomerase, qualquer que seja o tecido analisado. Evidência crescente para

actividade da telomerase em LCH provém dos resultados obtidos com o ensaio de TRAP, em que telomerase activa foi encontrada em lesões que contêm células de LCH com expressão de telomerase. Para além disso, o comprimento dos telómeros nas células de LCH de uma lesão multisistémica é longo e homogéneo, em contraste com três outras lesões solitárias de LCH que contêm células de LCH com comprimentos de telómeros mais curtos. Estas diferenças no comprimento dos telómeros e na expressão de telomerase nos diferentes tecidos lesionais e formas de LCH pode reflectir o largo espectro clínico de LCH. Assim, este estudo demonstra pela primeira vez que existe uma diferença evidente entre as lesões de LCH solitárias cutâneas e ósseas e entre a LCH solitária e multisistémica, baseada na expressão de telomerase.

Até à data é desconhecido se o comportamento anormal das células de LCH é devido a defeitos do foro genético. Assim, no **capítulo 7**, foi desenvolvido um estudo extensivo usando várias técnicas moleculares inovadoras para tentar esclarecer esta questão. As células de LCH foram isoladas de biópsias de LCH embebidas em parafina ou congeladas, utilizando metodologia desenvolvida pelo nosso e por outros grupos. O ADN foi isolado destas células e usado em técnicas de arrayCGH, SNPs, sequenciação do gene que codifica a proteína p53, e analisado para detectar a ploidia. Em todas as lesões analisadas, as células de LCH não apresentaram nem alterações no número nem alterações neutras de cópias de material genético. Complementando estas técnicas, a análise do cariótipo destas células revelou-se normal. Assim, exceptuando a possibilidade de ocorrência de mutações pontuais que fomos incapazes de estudar, estes resultados fornecem forte evidência de que a origem de LCH não tem uma base genética.

Finalmente, no **capítulo 8**, as descobertas descritas nesta tese e as suas implicações para investigação futuras são discutidas.

## Curriculum vitae

The author of this thesis, Cristiana Elizabete Teixeira da Costa, was born on October 14th 1979 in Braga, Portugal. In 1997 she received the high school diploma in área científico-natural from Escola Secundária Alberto Sampaio in Braga, Portugal, equivalent to the Dutch VWO. From 1997 until 2001 she studied Applied Biology at the Faculty of Sciences in University of Minho, Braga, Portugal. In her last year of the Degree she came to Holland to enrol in a training period entitled "Detection of cross-presented (tumor)-antigen by different in vitro assays", carried out at the Tumor Immunology group in the Department of Immunohematology and Blood Bank at the Leiden University Medical Center, under supervision of Dr. Renée Toes and Dr. Annemieke Th. den Boer. After her graduation in 2001, she returned to Holland in the beginning of 2002 to pursue another training period entitled "The role of chemokines in the pathophysiology of Langerhans cell histiocytosis", at the Immunology Laboratory (head: Dr. Maarten van Tol), in the Department of Paediatrics, at the Leiden University Medical Center, under supervision of Prof. Dr. R. Maarten Egeler, Prof. Dr. Pancras Hogendoorn and Dr. Nicola E. Annels. She continued in this project as a PhD student which she started officially in 2003. In 2008 she started working as a project coordinator, and 5 months later she was promoted to project leader at OctoPlus BV in Leiden, The Netherlands.

## List of publications

*Full papers*

- Annels NE, **da Costa CET**, Prins FA, Willemze A, Hogendoorn PCW and Egeler RM. Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. Journal of Experimental Medicine. 197: 1-7, May 2003

- **Costa CET**, Annels NE, Faaij CM, Forsyth RG, Hogendoorn PCW and Egeler RM. Presence of osteoclast-like multinucleated giant cells in bone and nonostotic lesions of Langerhans cell histiocytosis. Journal of Experimental Medicine. 201(5): 687-693, Mar 2005

- **Da Costa CET**, Egeler RM, Hoogeboom M, Szuhai K, Forsyth RG, Niesters M, de Krijger R, Tazi A, Hogendoorn PCW and Annels NE. Differences in telomerase expression by the CD1a+ cells in Langerhans cell histiocytosis reflects the diverse clinical presentation of the disease. J Pathol. 212: 188-197, Jun 2007

- **Da Costa CET**, Szuhai K, van Eijk R, Hoogeboom M, Sciot R, Mertens F, Bjorgvinsdóttir, H, Debiec-Rychter M, de Krijger RR, Hogendoorn PCW, Egeler RM and Annels NE. No genomic aberrations in Langerhans cell histiocytosis as assessed by diverse molecular technologies. Submitted

- **Da Costa CET**, Tazi A, Matsumoto Y, Annels NE and Egeler RM. Differential expression of CCR6 in Langerhans cell histiocytosis. Submitted

*Book Chapters*

- The immunological basis of Langerhans cell histiocytosis. **Costa CET**, Annels NE and Egeler RM. In : Weitzman S and Egeler RM, Editors. Histiocytic disorders of children and adults. Cambridge press. 2005; 66-82

*Peer-reviewed abstracts*

- **Costa C**, Egeler M, Hoogeboom M, Forsyth R, Szuhai K, Niesters M, de Krijger R, Tazi A, Hogendoorn P and Annels N. Differences in telomerase expression by the CD1a+ cells in Langerhans cell histiocytosis reflects the diverse clinical presentation of the disease. Clinical Immunology. 123: S50, Jun 2007

- **Costa CET**, Annels NE, Faaij CM, Hogendoorn PCW and Egeler RM. The role of multinucleated giant cells in Langerhans cell histiocytosis. Pediatric Blood and Cancer. 43(2): 199-199, Aug 2004

## Oral Presentations

**Costa *et al***. *Amsterdam-Leiden Institute for Immunology Meeting*, Zandvoort, The Netherlands, April 2003.

**Costa *et al.*** Presence of multinucleated giant cells in Langerhans cell histiocytosis. 1*9th Annual Meeting of the Histiocyte Society*, Philadelphia, USA, September 2003.

**Costa *et al***. Do LCH cells display a telomere maintenance mechanism? *20th Annual Meeting of the Histiocyte Society, Stockholm*, Sweden, September 2004.
Awarded with the Nezelof prize, as the best scientific work present in the congress

**Costa *et al***. The immunological basis of Langerhans cell histiocytosis – a biological update. *37th International Congress of Paediatric Oncology*, Vancouver, Canada, September 2005.

**Costa *et al***. Investigation of potential chromosomal abnormalities in the lesional cells of Langerhans cell histiocytosis. *The Nikolas Symposium XVI*, Athens, Greece, May 2006.

**Costa** *et al*. Consistent genetic alterations in the lesional cells of Langerhans cell histiocytosis. *22nd Annual Meeting of the Histiocyte Society*, Buenos Aires, Argentina, October 2006.