In its rare occurrence, Langerhans cell histiocytosis (LCH) is a dangerous but intriguing deviation of mononuclear phagocytes, especially dendritic cells (DCs). Clinically, the disease ranges from self-resolving or well manageable to severe and even fatal. LCH lesions in skin, bone, and other sites contain high numbers of cells with phenotypic features resembling LCs admixed with macrophages, T cells, eosinophils, and multinucleated giant cells. Here we review current progress in the LCH field based on two central questions: (i) are LCH cells intrinsically aberrant, and (ii) how does the lesion drive pathogenesis? We argue that LCH cells may originate from different sources, including epidermal LCs, tissue Langerin+ DCs, or mononuclear phagocyte precursors. Current and prospective in vitro and in vivo models are discussed. Finally, we discuss recent insights into plasticity of T-helper cell subsets in light of the lesion microenvironment. LCH continues to provide urgent clinical questions thereby inspiring innovative DC lineage research.

Keywords: dendritic cells, monocytes/macrophages, cell proliferation

Outline of the review

Langerhans cell histiocytosis (LCH) is a relatively rare disease and hence has limited notoriety among immunologists and hematologists at large. Yet, it provides a unique and unfortunate experiment of nature offering mechanistic insight into differentiation and function of cells of the macrophage–dendritic cell (DCs) lineage. The structure of this review has been designed to be comprehensive, while still allowing readers with distinct types of expertise rapid access to parts most relevant to their interest. After a brief introduction to the disease, clinical presentation and treatment are discussed. Next, the main body of the review is organized along two major questions, namely whether LCH cells are intrinsically aberrant, and how the lesion potentially drives LCH pathogenesis. Figures and tables were designed to cover and summarize the complete topic matter, capturing the essence of the review. Conclusions and perspectives were intentionally phrased to provoke discussion and stimulate future experimentation and collaboration.
Overview of the disease

The term ‘histiocytoses’ identifies a group of disorders that have in common the proliferation of cells of the mononuclear phagocyte system, including DCs (1). The individual entities are diagnosed on the basis of symptoms, signs, and laboratory information that, when taken together, fulfill internationally accepted criteria for the diagnosis of that particular disorder. Histiocytes (formally connective tissue macrophages, but here representing tissue macrophages in general) and DCs constitute two of the major types of non-lymphoid mononuclear cells and are involved in immune and non-immune inflammatory responses. Each of the histiocytoses is characterized by localized or generalized reactive or neoplastic accumulation of cells similar, if not identical, to one of these cell types. In case of LCH, the accumulating cells have many features in common with the epidermal LCs (2).

Major advances have been made in defining the clinical and pathologic criteria needed for diagnosis and treatment. Standardization of nomenclature and speaking one histiocytic language has made it possible to accumulate and record coherent data and has promoted international studies on the natural evolution of LCH and its response to treatments (3, 4).

Clinical presentation

LCH can present at any age from the neonatal period until old age (5, 6). In a population of 15 million people, LCH is diagnosed ±30 times per year. These figures probably still underestimate the problem, as many patients with localized LCH are likely to go undiagnosed. Our own experience indicates that there are as many cases in adults as in children, with the notice that in a country like the Netherlands the number of children versus adults is approximately 1:4, making the incidence in children 4 times higher. Therefore, LCH is usually considered a pediatric disease.

In its clinical presentation, LCH is a very diverse disease, ranging from a spontaneously regressing single lesion to a life-threatening extensive multi-system disorder with rapid progression and death, similar to acute myeloid leukemia. Besides common systemic symptoms such as fever, weight loss and fatigue, the symptoms depend on the involvement of specific organs (Table 1). The severity of the disease tends to be age-related, with extensive multi-system LCH with or without organ failure seen mostly in the very young. Multifocal restricted single-system LCH is often diagnosed in children between 2 and 5 years, while half of the unifocal bone disease occurs in children over 5 years (7). Pulmonary LCH is most often seen in the third or fourth decade of life but can also be part of multi-system LCH in the young child.

About 66% of children present with restricted disease, usually single-system disease in bone (8). Single-system disease is further subdivided as unifocal or multifocal. Extensive LCH should be divided into low-risk multi-system and high-risk multi-system LCH, respectively, without and with organ dysfunction.

Besides a clinical and thus therapeutic classification as stated in Table 1, the central nervous system involvement and endocrinopathies are striking and can develop before, during, or years after single-system bone involvement or before, during, or after extensive multi-system disease. The most common endocrinopathy in LCH is diabetes insipidus indicating posterior pituitary dysfunction. Anterior pituitary involvement might follow, resulting in growth retardation because of growth hormone deficiency. Approximately 5% of patients

| Table 1: Clinical features of Langerhans cell histiocytosis (2, 16, 129, 130) |
|-------------------|---------------------------------------------------------------|
| **Restricted (single-system) LCH** | The restricted, single system form of LCH occurs particularly in skin, bone or lymph nodes, with or without diabetes insipidus (polyuria and polydipsia due to pituitary involvement), but without other sites |
|                   | * The lesions of skin (scaly, erythematous, seborrhea-like brown to red papules), bone (monostotic and/or polyostotic lesions; painful swelling; irregularly margined lytic lesions of bone), or lymph node (enlarged) are biopsy-proven. This form of the disease is mostly diagnosed between the age of 5-15 years |
|                   | * Multifocal, restricted single system LCH is often diagnosed in children between 2 and 5 years, while half of the unifocal bone disease occurs in children over 5 years |
|                   | * The single system skin disease is often seen in infants |
|                   | * Localized lung involvement is most commonly diagnosed during the third decade (can however occur at any age as part of extensive LCH). In case of restricted disease, there is a strong, but not absolute link with smoking |
| **Extensive (multi-system) LCH** | Multi-organ LCH manifests itself with visceral organ involvement (lung, liver, spleen, gastrointestinal tract) or involvement of the hematopoietic system (pancytopenia), with or without bone lesions, diabetes insipidus, adjacent lymph node involvement, and/or skin rash. In the worst case scenario the lung (tachypnea, large bullae, spontaneous pneumothorax), liver (hepatomegaly, ascites), or hematopoietic system show signs of organ dysfunction. Gastroenterologic involvement (malabsorption, diarrhea, protein-losing enteropathy) can also be life-threatening. The incidence is highest in infants (children younger than 2 years of age) |

LCH, Langerhans cell histiocytosis
develop central nervous system involvement or central nervous system impairment with symptoms like progressive ataxia, dysarthria, nystagmus, hyperflexia, dysdiadochokinesia, dysphagia, and blurred vision.

Although the prognosis for survival in single-system disease and for the low-risk multi-system LCH without organ dysfunction is good, repeated reactivations may be associated with significant long-term complications. In contrast, the extensive multi-system LCH with organ dysfunction has an extremely variable course with still a high mortality, especially if the hematopoietic system is involved (9).

Every organ can be affected as a solitary symptom, but careful assessment is needed to ensure that they are not part of more extensive disease. Although the diagnosis of LCH might be considered based solely on clinical findings, a pathological confirmation, showing the accumulation of CD1a+ LCH cells, is required. To decide which organs are involved and to what extent, a standardized evaluation for every patient is performed. Besides an extensive laboratory investigation radiographic evaluation has to be carried out (10).

Treatment options

Therapeutic decisions vary for single-system LCH compared with multi-system LCH with organ dysfunction (Table 2).

Table 2. Treatment of Langerhans cell histiocytosis*

<table>
<thead>
<tr>
<th>Restricted (single-system) LCH</th>
<th>Extensive (multi-system) LCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>The restricted form of LCH is treated minimally, as obtaining the biopsy to prove the diagnosis might be enough to let the lesion resolve spontaneously.</td>
<td>Extensive LCH requires chemotherapy with corticosteroids, vinblastine and 6-mercaptopurin.</td>
</tr>
<tr>
<td>For skin involvement</td>
<td>Salvage therapy consists of cladribine (2-CdA) and cytosine-arabinoside (Ara-C).</td>
</tr>
<tr>
<td>Local application of corticosteroids is first option (secondly tacrolimus locally).</td>
<td>In specific cases, hematopoietic stem cell transplantation, lung transplantation or liver transplantation is indicated.</td>
</tr>
<tr>
<td>The next step is corticosteroids centrally (orally or intravenously).</td>
<td>Extensive LCH with organ dysfunction has an extremely variable course with still a high mortality, especially if the hematopoietic system is involved (9).</td>
</tr>
<tr>
<td>In severe cases mild chemotherapy might be needed (adding vinblastine to the corticosteroids).</td>
<td>For bone involvement, biopsy, curettage, intralesional injection of corticosteroids (11), non-steroidal anti-inflammatory drugs, bisphosphonates, anti-neoplastic chemotherapy, low-dose radiation therapy, and various forms of immunotherapy (12). Treatment of multi-system LCH has dual aims: to improve survival and to prevent late diabetes insipidus and other late complications due of the disease or therapy. Patients with extensive disease but without involvement of the risk organs liver, spleen, and hematopoietic system have an excellent survival rate with minimal therapy. For patients with multi-system LCH with organ dysfunction, results of the large randomized cooperative group trials suggest that early therapy with relatively nontoxic chemotherapy improves survival and may reduce the incidence of late complications. These same studies demonstrated that around 80% of young children with multi-system LCH survive long term (9, 13–15). For patients who responded to initial therapy, survival was very good. The very high-risk group of non-responders (around 30%) should be moved early to a salvage protocol. In this setting, toxicity and late effects become secondary considerations.</td>
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<tr>
<td>For bone involvement</td>
<td>Early results of a prospective Histiocyte Society study utilizing 2-CdA suggest that 2-CdA is more effective in low-risk chronically relapsing patients and that less than a third of refractory high-risk patients responded (12). A number of successful hematopoietic progenitor cell transplantations as well as lung and liver transplantations have been reported in refractory LCH patients upon specific indication (16). Organ transplantation is the only proven effective therapy for end-stage lung and liver disease, and the results appear to be durable.</td>
</tr>
<tr>
<td>Together with a biopsy, curettage is often sufficient, especially in mono-ostotic cases.</td>
<td>Some sporadic patients have been treated with agents like cyclosporine-A (17), thalidomide (18), and interferon-alpha (IFN-α) (19), all drugs with significant activity in low-risk LCH. These results, however, are based on a few patients, and non-published discussions through the grape-vine indicate no long-lasting results. The same is true for blocking tumor necrosis factor (TNF)-α. Etanercept, a soluble TNF receptor/Fc fusion protein, was added to the therapy of a child with non-responsive multi-system LCH, with good effect and no toxicity. Prolonged therapy was necessary (20). Others, however, have not repeated this positive result.</td>
</tr>
<tr>
<td>Local injection of corticosteroids releases pain often within 48 h.</td>
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<td>With poly-ostotic LCH (≥3 lesions) systemic chemotherapy is required (corticosteroids and vinblastine).</td>
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<tr>
<td>Single-system pulmonary LCH (in adults)</td>
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<td>Cessation of smoking is indicated, often supplied with systemic corticosteroid treatment.</td>
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<tr>
<td>Chemotherapy might be required, but some of the drugs often used in children, like vinblastine give severe side-effects in adults.</td>
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</table>

LCH, Langerhans cell histiocytosis.

*References (9, 11, 12, 16).
Other forms of therapy have led to the experimental testing of anti-CD1a antibody for diagnostic immunolocalization and therapy of LCH (21). These clinical trials provide encouraging evidence that it may be possible to target LCH cells in patients and development of human CD1a antibody is currently pursued (G. Bechan, D. Lee, and R.J. Arceci, unpublished observations).

The sequelae and late effects of LCH are dependent on the disease itself and on the intensity of treatment and exist in high incidence, ranging from 33% to 50%. Besides sequelae including intellectual problems, neurologic symptoms, endocrine abnormalities such as diabetes insipidus and growth failure, and orthopedic disabilities, ‘second’ malignancies have been recognized in LCH (22, 23). The risk/benefit ratio in the use of radio- or chemotherapy in LCH, and the manner of their use, need to be weighed carefully. Patients with systemic, extensive LCH, in whom the mortality rate can reach 50%, should not be denied treatment for fear of a <5% incidence of therapy-related second malignancies; the basic disease without effective therapy poses a much greater risk (22).

### Diagnosis of LCH

The diagnosis of LCH is based on hematologic and histologic criteria established by the International Histiocyte Society in 1987 (2). The LCH cell, the hallmark of the lesion is characterized by the expression of CD1a and CD207/Langerin, regardless of the site of the lesion (skin, lymph nodes, bone, or lung) (Table 3). These proteins can easily be detected by

<table>
<thead>
<tr>
<th>Marker expression by LCH cells compared with their normal human counterparts*†</th>
<th>Bone LCH</th>
<th>Skin-LCH</th>
<th>Epidermal LCs</th>
<th>Dermal CD1a+ DCs</th>
<th>Dermal CD14+ DCs</th>
<th>MGC</th>
<th>Monocytes</th>
<th>Dermal macrophages</th>
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</thead>
<tbody>
<tr>
<td><strong>General markers</strong></td>
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<tr>
<td>CD45</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ (dim)</td>
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<tr>
<td>MHC class II</td>
<td>Intracellular</td>
<td>Intracellular</td>
<td>Intracellular</td>
<td>Extracellular</td>
<td>Extracellular</td>
<td>?</td>
<td>Extracellular</td>
<td>Extracellular</td>
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<tr>
<td><strong>DC markers</strong></td>
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<tr>
<td>CD1a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Langerin/CD207</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>DC-SIGN/CD209</td>
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<td>-</td>
<td>+/?</td>
<td>+?</td>
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<tr>
<td>CD83</td>
<td>-/+</td>
<td>-/+</td>
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<td>?</td>
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<td>E-cadherin</td>
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<td>+</td>
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<td>?</td>
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<td><strong>Mφ markers</strong></td>
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<td>CD14</td>
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<td>CD68</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Factor XIIIa</td>
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<td>-</td>
<td>-</td>
<td>?</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

LCH, Langerhans histiocytosis cell; LCs, Langerhans cell; DCs, dendritic cell; Mφ, macrophage.
*Expression as determined by immunohistochemical or flow cytometric analysis; (−) no expression, (±) expression limited to a subset of cells, (+) expressed by the vast majority of cells, (?) not defined.
†Based on references: (25, 35, 138–141).

Fig. 1. Langerhans cell histiocytosis (LCH) cells differ morphologically from normal skin Langerhans cells. The images show immunoperoxidase-labeled paraffin sections of normal skin (A) and a typical LCH bone (B) lesion stained with CD1a antibodies. The majority of LCH cells is also CD207/Langerin positive (C), which is mostly intracellular in contrast to the surface expression on freshly isolated LCs. Note the rounded morphology of the LCH cells, in contrast to the dendritic, ramified appearance that characterizes normal epidermal LCs. Magnification 300X.
conventional (conv.) immunohistochemistry (Fig. 1). The identification of these cells is an absolute prerequisite for the diagnosis. Previously, presence of Birbeck granules was regarded as the ‘gold standard’ for diagnosis of LCH. Interestingly, formation of these granules is induced by expression of Langerin (24). It is reasonable, therefore, to replace the diagnostically obsolete electron microscopic technique by the more convenient immunohistochemical detection of Langerin expression. The diagnostic LC can be masked by the overwhelming admixture of eosinophils (Eo), T cells, and macrophages and osteoclast-like multinucleate giant cell (MGC) (25). By virtue of this complex cellular composition, particularly bone lesions have been called granulomatous, although usually a classic, walled-off granuloma structure is lacking.

Histologically the differential diagnosis includes osteomyelitis, (non-)Hodgkin lymphoma, and in case of skin involvement a variety of immune-mediated dermatites. Hematoxylin-eosin-stained LCH cells at all distinct tissue sites are rounded, lacking prominent dendritic extensions, and typically have a moderate amount of homogeneous, pink, granular cytoplasm, and distinct cell margins. The nucleus shows folding and indistinct nucleoli (25).

Thus, despite a heterogeneous appearance, LCH lesions invariably feature a population of CD1a⁺ CD207/Langerin⁺ histiocytes with a phenotype akin to that of epidermal LCs. Therefore, LCs have long been considered the origin of LCH cells. The recent identification, at least in the mouse reviewed in (26), of distinct non-epidermal populations of DCs with LC-like features has raised the intriguing possibility that other or maybe multiple cell types could give rise to the dysregulated LCH cells (Table 4).

Table 4. Possible origins of LCH cells

Recent studies in mice and humans provide evidence that other populations of DCs express markers that were previously thought to be unique to epidermal LCs, such as CD207/Langerin or Birbeck granules, the intracellular organelles that are formed upon ligand binding to surface Langerin (reviewed in 26). This notion has raised the question where LCH cells actually originate from. Currently, four putative origins of LCH cells can be hypothesized, assuming that the Langerin-expressing subsets of DCs that have been identified in the mouse also exist in human.

1. Epidermal Langerhans cells

The identification of Birbeck granules, which were the identifying feature of epidermal LCs, inside LCH cells caused a paradigm change and justified to change the name of the disease from Histiocytosis X into LCH (131). Many LCH lesions, however, are found at sites that are atypical for normal LCs, such as the bones. Although skin is often affected in the disease, LCH cells are even then primarily located in the dermis.

2. Dermal Langerin⁺ DCs

Recently, a distinct population of dermal Langerin⁺ cells has been identified in the mouse (reviewed in 26). This population is independent from the epidermal population of LCs and is, under steady state conditions, maintained through immigration of precursors from the blood, in contrast to the epidermal LCs, which are maintained locally.

3. Lymphoid tissue-resident Langerin⁺ DCs

In the mouse, a separate population of lymph node DCs has been identified that expresses Langerin as well as CD8α, in contrast to the dermal Langerin⁺ DC population (26). Also other markers are differentially expressed between the populations when they occur in the lymph node, arguing in favor of distinct subtypes, both derived from blood-borne precursors.

4. Mononuclear phagocyte precursors

The findings that human and mouse monocytes can be induced to acquire an LC-like phenotype suggests that the specific phenotype of LCH cells can be locally induced in common mononuclear phagocyte precursors or even more mature cells by environmental conditions. Stimulating factors such as TGF-β, Notch-L, or activin induce LC characteristics (132-134).

Etiology of LCH

There is a long standing dispute whether LCH should be regarded as a reactive or a neoplastic disorder (Table 5). Most forms of LCH appear to consist of clonal populations of LCH cells as shown by X-chromosome inactivation studies (27, 28). The exception to this is pulmonary LCH, where both clonal and non-clonal populations of LCH cells have been found (29). As this form of the disease is closely associated with cigarette smoking, this suggests that pulmonary LCH is a reactive disorder. In contrast, the clonality found in non-pulmonary LCH is used as an argument to regard the lesion as neoplastic.

This is further substantiated by the strong immunoreactivity of the LCH cells for p53 protein, combined with strong expression of the proliferation marker Ki-67 (30). Also the reports of familial clustering, either in siblings or with vertical transmission, have been used to argue for a neoplastic disease (31). Some cases have been reported with cytogenetic abnormalities like a t(7, 12) translocation (32). These isolated findings stimulated speculations on the neoplastic character while others argued that clonality per se is not an argument for neoplasia (33, 34). Restricted clonal proliferations in immune disorders and p53 immunoreactivity could also point to non-neoplasia-related active cell damage control.

Together, the considerations whether LCH is caused by a cell-intrinsic, tumorigenic process, or by a pathological response to environmental triggers have been central to our research efforts over the last years. Hence, we use the following leading questions as guide for the discussion in the subsequent parts of this review: (i) are LCH cells intrinsically aberrant, and (ii) how does the lesion drive LCH pathogenesis?

LCH, Langerhans cell histiocytosis; TGF, transforming growth factor; DCs, dendritic cells.
Are LCH cells intrinsically aberrant?

Phenotypic diversity of LCH cells

Besides the diagnostic marker CD1a, three additional markers are commonly used for co-staining lesional LCH cells, i.e. CD207/Langerin, CD14, and CD68 (35). CD207/Langerin antibodies stain a type II lectin receptor typically expressed by normal LC residing in the epidermis (24). This receptor binds a variety of specific glycans, for instance expressed by microbial ligands such as HIV-1, M. leprae, but also endogenous ligands exist such as extracellular matrix components (36, 37). CD14 and CD68 are routinely used to visualize cells derived from the monocyte–macrophage lineage and for DCs, expression of these markers is regarded as an indication of relative immaturity (35, 38). An initial confocal microscopy study described that antibodies against CD1a and Langerin labeled the same cells present in LCH lesions (35). Later studies confirmed this observation (39, 40). Two more recent studies reported, however, the existence of additional types of DCs in childhood LCH, i.e. CD1a+ CD207− cells [author reply in reference (41)] and CD1a−CD207+ cells (42) as is also represented in Fig. 2. The former DCs type seems to be

Table 5. LCH: a neoplastic or a reactive disease?

A crucial question in understanding LCH etiology and pathogenesis is whether this is a neoplastic or a reactive disease. To define both terms unequivocally: a neoplasm results from the clonal proliferation of genetically defective, hence intrinsically abnormal precursor cells. Conversely in a reactive disorder genetically normal cells proliferate and accumulate in response to an exogenous stimulus. Tumors arising from neoplastic cells can be classified as benign or malignant depending upon their pathological appearance and clinical behavior. In addition, neoplastic processes typically evoke inflammatory responses leading to the accumulation of bystander cells. Conversely, certain reactive immune-mediated diseases are characterized by the accumulation of activated white blood cells. Occasionally, these white blood cells form lesions that resemble neoplastic tumors. Thus, neoplastic and reactive disorders may share many clinical and pathological features, which can make it difficult to distinguish between the two. Arguments in favor of a neoplastic versus reactive origin of LCH are listed below.

<table>
<thead>
<tr>
<th>Neoplastic</th>
<th>Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonality of LCH cells in all studied cases of non-pulmonary LCH (27, 28)</td>
<td>Non-clonality of pulmonary LCH, related to smoking (29)</td>
</tr>
<tr>
<td>Recurrent genetic abnormalities, incl. deletion of chromosome segments in 7 pts (135)</td>
<td>No gross genetic abnormalities observed in 72 pts (50)</td>
</tr>
<tr>
<td>More extensive and higher-risk forms of LCH have evidence of more mutational events at tumor suppressor genes (136)</td>
<td>No mutations in genetic master switch p53 (50, 57)</td>
</tr>
<tr>
<td>Rare cases of familial clustering with high concordance between monozygotic twins (31)</td>
<td>Sporadic disease in vast majority of cases</td>
</tr>
<tr>
<td>Clinically aggressive behavior of some LCH forms</td>
<td>Indolent, clinically benign behavior of most LCH cases, sometimes involving</td>
</tr>
<tr>
<td></td>
<td>- Spontaneous remissions</td>
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<tr>
<td></td>
<td>- Flare up when patients develop a cold or other infectious process</td>
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<td></td>
<td>- Favorable response to antibiotic treatment</td>
</tr>
<tr>
<td>Immature LCs may accumulate in inflammatory processes, e.g. in lymph nodes that drain chronically inflamed skin (137)</td>
<td>Immature LCs may accumulate in inflammatory processes, e.g. in lymph nodes that drain chronically inflamed skin (137)</td>
</tr>
<tr>
<td>LCH cells cannot be maintained in vitro or in vivo in humanized mouse models</td>
<td>LCH cells cannot be maintained in vitro or in vivo in humanized mouse models</td>
</tr>
<tr>
<td>LCH cells are cytologically benign</td>
<td>LCH cells are cytologically benign</td>
</tr>
<tr>
<td>Granulomatous composition of apparently immune-activated cells</td>
<td>Granulomatous composition of apparently immune-activated cells</td>
</tr>
</tbody>
</table>

LCH, Langerhans cell histiocytosis.

Fig. 2. Langerhans cell histiocytosis (LCH) cells do not necessarily co-express CD207/Langerin and CD1a. The merged immunofluorescent picture of a skin-LCH biopsy displays CD207+ CD1a− LCH cells in green in a dermal lesion, CD207− CD1a+ LCH cells in red versus CD207 and CD1a co-expressing normal epidermal LCs in yellow; dashed line indicates the epidermal-dermal junction.

Egeler et al. DC dynamics in LCH

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overrepresented especially in bone lesions (C. Delprat, personal communication). Thus, LCH lesions may contain not only cells with a classical LCs phenotype but also other subtypes of DCs. This is an important issue that needs to be clarified. CD1a+ LCH cells may also co-express CD14 (35). Likewise, CD207+ CD14+ cells have been described in LCH lesions, particularly in bone and lymph node and to a lesser extent in skin-LCH lesions. This study also demonstrated similar staining patterns for CD68, CD1a, and CD207 in skin LCH lesions. However, normal epidermal LCs also express CD14 and CD68 to some extent (35).

In vitro studies have provided evidence that interleukin (IL)-17A may play a role in the induction of this immature macrophage-like DCs phenotype (43). Healthy monocyte-derived DCs cultured with recombinant IL-17A display similar phenotypic characteristics as expressed by DCs found in LCH lesions, i.e. CD14, CD68, CD1a, and CCR6. In contrast to DCs generated from healthy monocytes, DCs generated from LCH patients as well as lesional LCH cells both display the intrinsic capacity to express IL-17A, which may affect their phenotypic features in an autocrine fashion. In particular, the below-described MGC co-express CD1a and CD68 (44). Macrophages expressing CD68 but not CD1a are also present in LCH lesions (35, 44). Taken together, these phenotypic analyses indicate that LCH lesions comprise a spectrum of cells derived from the macrophage/DC lineage.

Despite this heterogeneity, LCH cells with strong expression of cell surface markers CD1a, S-100 and the ultrastructural presence of Birbeck granules are found in all cases. These markers are highly characteristic of LCs residing in the epidermis but are readily lost after activation (45). In addition, LCH cells, like epidermal LCs, express MHC class II molecules intracellularly and hardly ever express mature DCs (mDC) molecules such as CD83, CD86, or DC-LAMP (35). Although the resemblance to LCs is remarkable, LCH cells also differ from normal LCs, not only in morphology, as they lack dendritic extensions, but also in expressing abnormal levels of several phenotypic markers like CD54 (intercellular adhesion molecule-1) and CD58 (leukocyte function antigen-3) (46). In normal LCs, upregulation of CD54 and CD58 is seen on activated LCs migrating from the skin to the draining lymph node (47). Thus, LCs accumulating in LCH lesions share some characteristics with resting, immature LCs but additionally express some features of their activated counterparts.

Based on the inappropriate accumulation of LCs at one or more LCH-affected tissues, it could be hypothesized that these cells either fail to mature to the full extent, and therefore persist in the tissue of origin, or exploit other abnormal chemokine-mediated mechanisms to traffic to aberrant anatomic sites, such as bone. While CCR6 is typically expressed by immature DCs (iDCs), CCR7 is a chemokine receptor expressed by DCs that have undergone activation-induced maturation. This allows them to traffic to regional lymph nodes (48), where mDC interact with naive T cells. This physiologic process appears to be inhibited in LCH cells as we found that cells at the various lesional sites clearly express CCR6 but not CCR7 (49).

In accordance with an arrested maturation state of CD1a+ cells, LCH cells are hardly ever found in the lymph nodes that drain lesional sites. This underlines the concept that these cells are not able to migrate. As stated before, LCH can be localized, however, in lymph nodes as primary lesions. LCH cells lack the ability to activate T cells when tested ex vivo (35). Only when these cells are treated in vitro with CD40L, they strongly upregulate MHC class II expression at the cell membrane and acquire allo-stimulatory activity to a level similar to that of mDC. This indicates that LCH cells have the intrinsic ability to mature when stimulated appropriately. Yet, it remains puzzling why they do not do so in vivo, despite the presence of an array of maturation-stimulating mediators as well as CD40L-expressing T cells (see below).

The deviant chemokine-receptor profile of the LCH cells and their local interaction with T cells is currently a research endeavor in our laboratory. Furthermore, it remains to be studied whether other, more tissue-specific, chemokine receptors are expressed by LCH cells and whether the presence of the corresponding ligands specifically determines the different anatomic localization patterns of abnormal LCs.

LCH cells are frustrated in cell cycle regulation but probably not genetically affected

Several studies have provided evidence that LCH cells do not display abnormalities in their DNA content (50–53) or karyotype (50). One study has described the existence of an abnormal clone of LCH cells with an unbalanced translocation (32) albeit these alterations were found in a small fraction of LCH cells only. LCH cells do, however, display characteristic expression profiles of markers associated with cell proliferation, differentiation and survival (Table 6). Indeed, LCH cells display markedly upregulated levels of cell cycle-related proteins and oncogene-encoded products known to promote cell growth and survival (30). Between 3% and 25% of lesional cells may express the proliferation marker Ki-67 (30, 54), indicating that a substantial number of LCH cells is actively cycling. Paradoxically, only low num-
Anti-apoptotic over-expression of p53 in CD1a+ LCH cells is not the result of increased levels of the anti-apoptotic protein Bcl-2 (30, 58). By contrast, CD40-stimulated keratinocytes do not proceed toward cell cycle as shown by accumulation in the G2/M phase (62). This issue merits further research.

The evidence for survival and proliferation of LCH cells prompted us to investigate the presence of telomerase in these cells, given that telomerase activation is highly prevalent in malignancy. This is a better indicator of cellular immortality than alterations in telomere length or the actual presence of telomerase RNA (63). Telomerase activity is detectable in germline cells but absent in most normal somatic tissues. Notably, reappearance of telomerase activity in somatic tissue is associated with the development of malignancy. While all LCH cells in skin lesions express the critical enzyme telomerase, as assessed by human telomere reverse transcriptase (hTERT) immunohistochemistry, most LCH cells from restricted bone lesions do not express telomerase (64). In contrast, in case of multi-system involvement, the cells expressed the protein regardless of anatomic location. No alternative telomere lengthening mechanisms, such as mediated by homologous recombination, appear to be operative in hTERT-negative lesions. This may point to the heterogeneity of LCH: a telomerase-positive aggressive form and a telomerase-negative restricted form. In contrast to our results, a recent study from Bechan et al. (65), using quantitative immune fluorescence, showed that LCH cells display significant telomere shortening in all clinical phenotypes of LCH studied. This discrepancy may reflect the differences in sensitivity of the techniques used to assess the telomere length of the 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? It is known that increased mutability is essential for the development of many types of human cancers (66). 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. To provide evidence that LCH could be a malignant disease the finding of consistent genetic abnormalities was crucial.

In a large multi-institute study using a range of techniques, including conv. cytogenetics, array-based comparative genomic hybridization and single nucleotide polymorphism analysis on CD1a+-sorted cell populations no abnormalities could be substantiated. This largely rules out gross chromosomal abnormalities to be causative for the disease and suggests a restricted oligoclonal stimulation rather than an unlimited neoplastic proliferation (50). However, a cryptic point mutation or more probable a viral small insertion cannot be excluded.

Table 6. Expression of cell cycle–related and apoptosis pathway–related proteins by LCH cells a, b, c

<table>
<thead>
<tr>
<th>Indicators of cell cycle progression/proliferation</th>
<th>Bone lesions</th>
<th>Single-system skin lesions</th>
</tr>
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<tbody>
<tr>
<td>Ki-67</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>hTERT (telomerase)</td>
<td>±/+</td>
<td>+</td>
</tr>
<tr>
<td>MDM2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Rb</td>
<td>+</td>
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<table>
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<tr>
<th>Indicators of cell cycle arrest and/or apoptosis</th>
<th>Bone lesions</th>
<th>Single-system skin lesions</th>
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<tbody>
<tr>
<td>p53</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>p21</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p16</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>TGF-β receptor I and II</td>
<td>+</td>
<td>+</td>
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<tr>
<td>FADD (MORT1)</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>FLICE (caspase 8)§</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Caspase 3†</td>
<td>+</td>
<td>?</td>
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</table>

<table>
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<tr>
<th>Anti-apoptotic/survival markers</th>
<th>Bone lesions</th>
<th>Single-system skin lesions</th>
</tr>
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<tbody>
<tr>
<td>Bcl2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Survivin§</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>FLIP</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

LCH, Langerhans cell histiocytosis; TGF, transforming growth factor.
*aExpression is determined by immunohistochemical analysis; (−) no expression, (±) expression limited to a subset of cells, (+) expressed by the vast majority of cells, (?) not defined.

†Based on references (30, 50, 64).
‡Multisystem bone lesions express high levels of hTERT.
§B.L. Petersen, unpublished observations cited in ref. (142).

In a large multi-institute study using a range of techniques, including conv. cytogenetics, array-based comparative genomic hybridization and single nucleotide polymorphism analysis on CD1a+-sorted cell populations no abnormalities could be substantiated. This largely rules out gross chromosomal abnormalities to be causative for the disease and suggests a restricted oligoclonal stimulation rather than an unlimited neoplastic proliferation (50). However, a cryptic point mutation or more probable a viral small insertion cannot be excluded.
Involvement of the β-catenin signaling cascade in LCH

The dysregulated, proliferative nature of LCH cells inspired us to postulate that deviations in the E-cadherin – β-catenin – Wnt signaling cascade might be fundamental to the development of LCH (67). At the time, no experimental information at all was available concerning the putative expression and function of different β-catenin-cascade molecules in DCs. The primary cues serving as a rationale to hypothesize a causative role for the β-catenin cascade in LCH were (i) the role of E-cadherin as an adhesion molecule of epidermal LCs and keratinocytes (68); (ii) the absence of E-cadherin from LCH cells in the vast majority of lesions (69); (iii) the dual function of β-catenin as an E-cadherin-associated molecule connecting to the cytoskeleton and a transcriptional co-activator (70); and (iv) the fundamental role of β-catenin dysregulation in epithelial carcinogenesis (71). In the hypothesis, we assumed that stimulation of proliferation and survival by beta-catenin activation was similarly regulated in LCs as in epithelial cells by stimulating target genes such as c-Myc and cyclin D1. Thus, a predicted result would be that LCH cells have aberrantly elevated levels of transcriptionally active β-catenin.

Phosphorylation of β-catenin at different sites determines its role in adhesion and transcription (72, 73). Thus, GSK-3β-mediated phosphorylation at N-terminal serine/threonine residues leads to ubiquitination and proteasomal degradation (Fig. 3). Antibodies binding specifically the N-terminal non-phospho sites therefore show the presence of transcriptionally active beta-catenin (74, 75). This form can be found either at the membrane, in the cytoplasm or in the nucleus, and, obviously, only in the latter location it can be functional by binding to the nuclear Tcf/Lef transcription factor family members and activating transcription of target genes (73).

Using antibodies against total and transcriptionally active β-catenin we assessed their presence immunohistochemically in LCH biopsies obtained from patients with single-system and multi-system disease (G.I. Bechan, F. von Nederveen, B. den Broeder, R. de Kryger, A.M. Cleton-Jansen, P.C.W Hodendoorn, R.M. Egeler, P.J.M Leenen, unpublished data). Total β-catenin could be readily shown in LCH cells in most lesions. To our surprise, however, little if any of the active form of β-catenin could be detected in the vast majority of LCH lesions. The disparity between the unequivocal presence of beta-catenin, but virtually complete absence of its transcriptionally active form still might be related to a dysregulation of the pathway in LCH cells. In experiments aimed to establish the role of β-catenin signaling in the in vitro maturation of human monocytes to iDCs, we observed that both total and active β-catenin were found at increasing levels in monocyte-derived DCs. Moreover, pharmacological inhibition of β-catenin transcriptional activity strongly diminished the acquisition of DC markers such as CD1a and DC-SIGN by these cells, indicating the functional involvement of the cascade in maturation of monocytes to iDCs. Therefore, increasing β-catenin signaling appears to be involved in maturation of monocytes to iDCs and absence of active β-catenin in LCH cells may be considered aberrant indeed.

Recent studies by others strongly support the functional participation of β-catenin signaling in DCs differentiation and maturation. Using mouse bone marrow-derived DCs, investigators from the Mellman laboratory (76) observed that mechanical disruption of E-cadherin-mediated adhesion between clusters of iDCs induced these cells to reach an end-stage mature phenotype in a β-catenin-dependent manner (76). Interestingly, these β-catenin-mDCs lacked high level pro-inflammatory cytokine production and were tolerogenic. In contrast, LPS-stimulated maturation induced the development of immunogenic DCs with high level production of...
pro-inflammatory cytokines. In addition to its role in the terminal maturation of DCs, β-catenin also appears to be involved in the induction of DC development from the earliest myeloid progenitor cells. Recently, the Gabrilovich laboratory (77) showed that β-catenin signaling skewed mouse myeloid progenitor cells or human CD34+ bone marrow progenitor cells to develop into conv. DCs but not plasmacytoid DCs. Our results on human monocytes extend the recent findings by Gabrilovich and Mellman and coworkers. These studies substantiate the important role of β-catenin signaling in the earliest and in the final phases of DC development, respectively. The monocytic cells, however, represent intermediate stages of development, which still have the capacity to differentiate into either DCs or macrophages. In contrast to the findings in mouse terminal DC maturation, we could not demonstrate unequivocal involvement of the β-catenin pathway in terminal maturation of human monocyte-derived DCs, although this might be explained by technical limitations. Taken together, these studies indicate that β-catenin signaling appears to be involved in essentially all stages of DC development, from the earliest lineage decision in progenitor cells, the subsequent lineage decision in intermediate stage monocytes to the terminal maturation of end-stage cells (Fig. 4).

In view of these recent insights, the finding that LCH cells seem to lack functional β-catenin signaling makes these cells stand out from normal DC development, although in a different manner than proposed in our initial hypothesis. The notion that β-catenin signaling in normal iDCs stimulates their final maturation (76) feeds the speculation that defective β-catenin signaling underlies the maturation arrest of LCH cells. Moreover, LCH cells are known to produce high levels of pro-inflammatory cytokines, while β-catenin-mDCs do not. Potentially, activation of β-catenin in LCH cells by stimulation of the Wnt pathway could overcome their maturation arrest and limit cytokine production, and might thus have therapeutic promise.

Deviations in LCH cells: a summary

The currently available phenotypic and molecular data indicate that LCH cells are morphologically aberrant, when compared with regular LCs, and have an immunophenotype of immature, partially activated cells and a pro-inflammatory cytokine profile. Despite the presence of numerous stimulating factors in their environment (see below), LCH cells fail to mature to the full extent. The cell cycle machinery of LCH cells is active, although their actual level of proliferation is generally low. There is no consistent underlying genomic defect demonstrable with the current techniques, suggesting that LCH might not represent the uncontrolled neoplastic proliferation as previously assumed. However, the generalizations above may eventually prove to be misleading, as biopsy analyzes typically show a high degree of variability between as well as within different cases, which might reflect either different stages of disease or even the existence of different pathogenic pathways.

In vitro LCH models

The value of in vitro and in vivo models to study aspects of disease pathogenesis requires no argumentation, and therefore many attempts have been made also to generate model systems for LCH. Short-term cultures of LCH cells isolated from lesions have been established successfully and these have enabled the further characterization of these cells in terms of functional and developmental capabilities (35, 78). However, to our knowledge no investigator has been able to propagate these cells in vitro for more than a few days, nor has successful maintenance of LCH cells in immune-deficient mice been claimed (79). The only reported attempt in this direction is the generation of the DOR-1 cell line established from a
pediatric bone lesion LCH (80). However, as the authors state, the cellular origin of this cell line is probably not an LCH cell, as DOR-1 cells lack the typical features of LCH cells, such as CD1a, Langerin, and HLA-DR expression. In accordance, also no Birbeck granules were observed in these cells. The consistent binding of CD34, CD10, and CD117 antibodies, together with their adherent pleomorphic morphology suggest that DOR-1 cells have a stromal nature.

In vivo LCH models

The inability to maintain and propagate human LCH cells in immune-deficient mice has strongly hampered progress in understanding the etiology and pathogenesis of LCH and thus rational therapy development. It might be argued that most attempts for in vivo modeling using human LCH cells have been made with less sophisticated mouse models than are currently available for xenotransplantation of human cells and therefore renewed attempts into that direction are certainly worthwhile considering.

An early in vivo mouse model of histiocytosis was described in the mid 1980s by the Ostertag laboratory (81, 82). In this model, infection of mice with a newly isolated recombinant virus of Harvey murine sarcoma virus and Friend mink cell focus-forming virus caused transformation and accumulation of histiocytes. This occurred initially in spleen and bone marrow and was followed by widespread dissemination in virtually all visceral organs. Transformed cells were found to represent relatively mature macrophages, as indicated by the cellular phenotype, enzyme profile and phagocytic capacity. The remarkable cell type specificity of transformation and the characteristics of the resulting disease justified naming the pathogenic retrovirus ‘malignant histiocytosis sarcoma virus’ (MHSV). Evaluation of the genetic parameters important for disease susceptibility indicated that the resistance phenotype was linked with high probability to loci on mouse chromosome 13 (83). Interestingly, this area is homologous to human chromosome 5q35, which has been associated with human malignant histiocytosis as well as other proliferative diseases involving myeloid or histiocytic aspects (84, 85).

Human malignant histiocytosis is a rare disease. Moreover, improved molecular diagnostics have indicated that many cases diagnosed earlier as malignant histiocytosis appeared to represent either anaplastic large cell lymphoma, involving malignant lymphocytes that show a histiocyte-like appearance, or malignancies of dendritic cell origin (86, 87). Thus, the reappraisal of malignant histiocytosis as a human disease as well as the recently increased awareness of the heterogeneity of the dendritic cell lineage inspired us to re-evaluate the identity of the cells transformed in the MHSV mouse model (88).

In accordance with the earlier descriptions, we observed that infection of susceptible BALB/c or DDD mice with MHSV caused a rapid expansion of myeloid cells as well as erythroblasts expressing the virally encoded v-Ha-ras p21 protein in the primary target organs bone marrow and spleen, already from day 11 of infection. Detailed immunophenotypic marker analysis indicated that the virally transformed cells represented distinct populations of myeloid cells, rather than a homogeneous population of mature macrophages. Interestingly, DCs expressing various markers also expressed by LCs, such as Langerin, CD11c, and CD13 appeared to be a predominant target of viral transformation (Fig. 5). Furthermore, discrete nodules of transformed myeloid precursor cells, characterized by expression of CD11b and the myeloid precursor marker ER-MP58, and CD169/sialoadhesin-positive macrophages were also observed in spleen sections of animals euthanized 14 days after infection. Upon disease progression these

**Fig. 5.** Distinct myeloid populations are affected in a virus-induced mouse model of histiocytosis. DC-like cells (CD13^+; middle) and myelomonocytic cells (CD11b^+; right) expand in a mouse model of histiocytosis induced by the malignant histiocytosis sarcoma virus. Cells transformed by this virus express elevated levels of v-Ha-ras p21 protein (left). Virus-transformed DCs express an LC-like phenotype, including markers such as CD207/Langerin, DEC205, MHC class II. These serial spleen sections were obtained from a BALB/c mouse infected with virus 18 days earlier. In addition to these two populations, also marginal metallophilic macrophages, characterized by CD169 expression, are virally transformed (not shown).
distinct subpopulations of transformed cells expanded differentially in individual mice, leading to a heterogeneous picture of predominating myeloid tumor cells, varying between Lang- erin$^+$ DCs, CD169$^+$ macrophages, ER-MP58$^+$ precursor cells, and mixtures thereof in animals with end-stage disease (typically between 20 and 60 days after infection).

By in vitro propagation of cells isolated from bone marrows or spleens of diseased animals we were able to isolate a panel of MHSV-transformed cell lines that could be maintained without exogenous growth factor support. These cell lines showed distinct iDC-like morphology and phenotype. Reflective of the heterogeneous appearance of tumor cells in vivo, they were arrested in different stages of DC maturation. Despite this maturation arrest in vitro, the cells appeared to have a remarkable tissue-specific adaptation upon transfer into naive mice. In experiments where BMH1.1 cells, which have an iDC-like phenotype (CD11b$^+$, CD11c$^+$, and MHC class II$^+$), were transferred, the cells reproducibly acquired or lost expression of various markers depending on the tissue of homing. Apparently, these cells respond to micro-environmental signals that induce tissue-specific changes in their immunophenotype, making them switch between precursor cells, macrophages and Langerin-expressing DCs (Fig. 6). Therefore, these experiments strongly support the long-postulated paradigm of tissue-specific differentiation of mononuclear phagocytes (89). In addition, they argue in favor of a close relationship between Langerin-positive DCs and macrophages. It is important to note, however, that the Langerin-positive MHSV-transformed cells are probably unrelated to the epidermal LCs as their phenotype corresponds largely with the non-LC-derived population of Langerin-expressing DCs in secondary lymphoid organs (90), while in situ they have minimal expression of additional typical LCs markers such as CD11b or F4/80.

In a recent approach to generate a mouse model of histiocyte- tosis involving DCs, Acha-Orbea et al. (91) have generated transgenic mice that expresses SV40 large T antigen under control of the relatively DC-specific CD11c promoter. Different mouse lines with high and low expression levels of the transgene were generated. Related to this difference, these mice show early and late onset of disease, respectively, characterized by accumulation of DCs causing highly increased spleen and liver sizes and decreased hematocrit values. Interestingly, the affected DCs in this model are of the same subtype as the one transformed by MHSV, namely the Langerin/CD207$^+$ CD8$\alpha^+$ CD11b$^+$/CD205$^+$ non-LC-related lymphoid tissue DCs. Given the widespread dissemination of transformed DCs, these DC-specific SV40 large T-transgenic mice may serve as a model for multi-system histiocytic disease, hence the name Mushi mice.

What is the future of in vivo modeling in LCH research? The recent progress in cell type-specific modulation of gene expression, either or not inducible, in mice generates a wide range of possibilities to target LC-related cell types. Interestingly, the Langerin promoter has recently been shown to be a highly valuable tool for the generation of mouse models that enable LC-specific modulation (reviewed in 92). It can be envisaged that in similar models LC-specific expression is realized of oncogenes or pro-inflammatory cytokines such as IL-17A, or, alternatively, in which tumor suppressor genes are deleted. Furthermore, renewed attempts could be undertaken to propagate isolated human LCH cells in immunodeficient mice reconstituted with a human immune system, as major progress has been made over the last years in establishing such models (reviewed in 93). It should be realized, however, that both approaches for in vivo modeling assume that intrinsic deficits in LCH cells are prime to the disease etiology. As discussed above, this premise remains to be proven.

How does the lesion drive LCH pathogenesis?

Locally produced inflammatory mediators orchestrate leukocyte trafficking into the lesion

Although the CD1a$^+$ LCH cell appears to be the central player in LCH, MGC, macrophages, Eo, and T cells are all characteristic cell types, which may accompany LCH cells, particularly at ostotic locations. LCH lesions are characterized by a high level and diversity of locally produced cytokines also referred to as the lesional ‘cytokine storm’ (94, 95). The predominant source of these cytokines is the CD4$^+$ T-helper (T_h) cell; LCH cells and macrophages are additional cytokine producers (95). Cytokine release is one of the key effector functions of T_h cells.
requiring reciprocal interactions between T cells and antigen-presenting cells. As CD40 is also abundantly expressed (60), LCH lesions can be seen as inflammatory ‘niches’, which not only facilitate their own recruitment and retention, but also attract other types of inflammatory cells. T-cell-derived cytokines such as TNF-α, IFN-γ, and IL-5 (95), and LCH cell-derived chemokines such as MIP3α/CCL20, RANTES/CCL5, and I-TAC/CXCL11 (49) all participate in cellular trafficking to LCH lesions.

Tissue-destructive multinucleated giant cells

The origin and role of MGC remained unknown until recently. As a result of the fact that patients with LCH involving the bone often present with osteolytic lesions, work from our own laboratory addressed the question whether these MGC are indeed osteoclast-like cells which could cause tissue destruction as often seen at lesional sites (44). Analysis of MGC from three different lesional tissues, i.e. bone, skin, and lymph node, showed that the MGC expressed the characteristic osteoclast markers like tartrate-resistant acid phosphatase and vitronectin receptor as well as the enzymes Cathepsin-K and matrix metalloproteinase-9 (MMP9) (43, 44) (Fig. 7). While the vitronectin receptor supports cell adhesion to matrix proteins such as fibronectin (96), Cathepsin-K and MMP9 are proteases involved in bone degradation through catabolizing extracellular matrix proteins such as collagen (97). Interestingly, MMP9 is one of the key regulators affecting recruitment and differentiation of osteoclasts and osteoprogenitor cells (98). These osteoclast-like MGC were not just found in bone, the normal tissue site for osteoclasts to carry out their bone-resorbing activity, but also in skin and lymph node lesions. We also showed over-production of osteoclast-inducing cytokines such as receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) by both the CD1a+ LCH cells and T cells in these lesions. While in vitro studies have shown that monocyte fusion to MGC is driven by granulocyte-macrophage (GM)-CSF and IL-17A, DC fusion to MGC is mainly induced by

Fig. 7. Multinucleate giant cell (MGC) in Langerhans cell histiocytosis (LCH) lesions share functional and phenotypic features with normal osteoclasts. This triple color immunofluorescence staining shows MGC in bone LCH tissue by confocal laser scanning microscopy. In green: cathepsin K, an enzyme typically secreted by osteoclasts, in red: vitronectin receptor, which is commonly expressed by osteoclasts, and in blue: the macrophage marker CD68.
CCL20 and IL-17A. Both fusion processes are potentiated by IFN-γ, which significantly increases MGC size and nuclear content (43). Interestingly, the four molecular components necessary for MGC formation, i.e. GM-CSF (95), CCL20 (49), IFN-γ (95) and IL-17A (43), have all been detected, in situ, in LCH lesions. Thus, an attractive hypothesis would be that excessive amounts of osteoclast-inducing cytokines such as RANKL, locally produced by activated T cells, and M-CSF, released by LCH cells, induce either osteoclast-like differentiation of infiltrated blood-borne precursor cells such as monocytes or, alternatively, induce the fusion of mature tissue-residing macrophages (99) or DCs (43).

Macrophages

Lesional CD3⁺CD1a⁺CD207⁺ cells expressing high levels of CD68 represent classical tissue-residing macrophages (Table 3). Further confocal microscopy examination revealed the presence of IL-10 expressing macrophages, which are in close contact with LCH cells and T cells in bone and lymph node LCH lesions, but not in single-system skin lesions (35). In fact, macrophages are rarely found in skin-LCH patients with self-healing lesions. Exposure to IL-10 during in vitro differentiation is known to induce phenotypically aberrant immature monocyte-derived DC (100). This phenotype is hallmarkmed by preserved expression of CD14 and coincides with tolerogenic properties. LCH cells without stimulation are poor stimulators of T cells, but it is not known whether they are tolerogenic. After α vis expression to CD40L-expressing fibroblasts, they induce potent allogeneic T-cell proliferation. Nonetheless, exposure to IL-10 produced by bystander macrophages may affect the phenotypic characteristics of LCH cells.

Eosinophils

A third cell type which is attracted into LCH lesions, especially in bone, is the Eo. The presence of this seemingly innocent bystander cell is believed to result from the high levels of locally produced CCL5/RANTES, a crucial cytokine affecting Eo recruitment and effector function. This process may be further enhanced via eosinophilia-promoting IL-5 (101), which is produced by LCH lesion-infiltrating T cells (95). The role of Eo in LCH remains poorly explored.

Inflammation-promoting T cells

Limited information has thus far been reported on LCH lesion-infiltrating T cells. The majority of these cells express cell surface markers indicative of recent activation, such as CD40L (60), CD45RO (49), and RANKL (44). These T cells also express a broad range of T-cell receptor-encoding genes demonstrating their polyclonal nature (54); the antigen-specificity of these T cells, however, remains unclear. Lesional T cells seem to be attracted by two chemokines produced by LCH cells: MIP-3α/CCL20 and I-TAC/CXCL11 (49). In situ analyses support this hypothesis given the clear presence of T cells expressing either CXCR3, the receptor for CXCL11, or CCR6, the receptor which specifically binds CCL20. Together with CCR5, CXCR3 defines primarily inflammation-promoting IFN-γ-producing Th1 cells (102). CCR6 expressing T cells comprise other types of T_h cells such as the Th17 subset, discussed in more detail below (103). It needs to be clarified whether CCR6 and CXCR3 co-expressing, IL-17A- and IFN-γ-producing T cells are also present in LCH lesions. These T cells may represent converted Th17 cells as recently described (104). The exciting field of T_h subset biology is developing at very high pace, and to some extent is hampered by loose definitions of terms like subset, lineage commitment, and terminal differentiation, as insightfully discussed very recently (105). Current focus is on plasticity of T_h subsets and the genetic and epigenetic mechanisms underlying these functionalities. In conclusion, LCH lesions may provide the necessary signals for (i) CD40-CD40L driven local T-cell expansion; (ii) continuous new recruitment of different types of T cells; and (iii) T_h subset switches. The latter issue needs to be further investigated.

Inflammation-modulating T cells

Immunohistochemical analyzes have revealed the presence, albeit at variable numbers, of two types of T_h cells with inflammation-modulating capacity, i.e. CD25⁺FoxP3⁺ T cells (54) and CD3⁺ IL-17A⁺ T cells (43). While the former are thought to be so called ‘naturally occurring’ T-regulatory cells (nTreg), the latter belong to a recently described subset of T cells named Th17 cells, which develop via a lineage distinct from the Th1 and Th2 lineage (106). CCR6, expressed by lesional CD1a⁺ DCs and CD4⁺ T cells (49), plays a critical role in the recruitment of murine Tregs and Th17 to inflammatory tissues (107). Human CD4⁺CD25⁺ Tregs (108) and Th17 (109) also express CCR6. These observations suggest an important role for CCR6 in the recruitment of both nTreg and Th17 to LCH lesions. Interestingly, Coury et al. (43) have observed increased levels of IL-17A in serum samples of mostly multi-system LCH patients with active disease. However, this could not be confirmed by Allen et al. (41), who analyzed plasma mainly from patients with bone disease. The
main source of this cytokine is probably not the Th17 T cell but rather the LCH cell, as based on the abundant in situ presence of IL-17A+ CD1α+ DCs and IL-17A+ MGC (43). The discrepancy between this study and the study of Allen et al., who could not detect IL-17A mRNA in isolated LCH cells nor in CD3+ T cells derived from the same LCH lesions, might be explained by the notion that CD207+ cells rather than CD1α+ cells were used in the latter study. This issue needs to be clarified as more DCs subtypes may be present in LCH lesions.

CD3+ T cells derived from ostotic LCH lesions can suppress allogeneic T-cell proliferation when tested ex vivo in a co-culture assay (54). This study reported that approximately 20% of these T cells expressed FoxP3, a transcription factor generally considered as the ‘master gene’ for the development of Tregs (110). By preventing autoimmune T-cell responses at sites of inflammation, CD4+ CD25+ FoxP3+ nTregs play an important role in maintaining peripheral tissue integrity (111). Inflammation-controlling Tregs are either recruited from the circulation or arise locally from antigen-specific effector/memory type T cells, which also accumulate at sites of inflammation; the latter type of Tregs are called induced or adaptive Tregs (αTregs) (112). Interestingly, Bacillus Calmette-Guérin-vaccinated LCH patients display impaired delayed type hypersensitivity reactions to intradermally injected tuberculin when tested at the onset of disease (54). These observations suggest that LCH is associated with increased Tregs activity. These Tregs proliferate within LCH lesions as demonstrated by co-expression of FoxP3 and the proliferation marker Ki-67 in the nuclei of lesional CD25+ cells. This proliferation may be driven by RANK-expressing CD1α+ DCs (44), as RANK-RANKL signaling is of critical importance for local Tregs expansion at sites of inflammation as recently shown in mice displaying chronic colitis (113). Transforming growth factor (TGF)-β, a cytokine clearly present in LCH lesions, also supports the expansion of human Tregs (reviewed in 114). TGF-β released by activated Tregs could on the one hand act in an autocrine fashion on the accumulation of Tregs in LCH lesions and on the other hand block the activation of newly attracted CD4+ effector T cells. Together with MGC-released MMP9, Treg-derived TGF-β, designated as a major fibrinogenic cytokine, may also affect local extracellular matrix turnover (115), resulting in extensive tissue remodeling and fibrosis as generally observed in LCH lesions of bone, liver, and lungs. Along with TGF-β, dendritic cells are specialized cells for the differentiation of CD4+ FoxP3+ Tregs from blood-derived FoxP3+ precursor T cells (116). The observation that FoxP3+ T cells are found in close contact with LCH cells and FoxP3+ lymphocytes (54) suggests that, besides accumulation of nTregs, also αTregs may arise in LCH lesions. The latter is probably not driven by TGF-β, which, together with T-cell receptor triggering, induces stable FoxP3 expression in naive CD4+ FoxP3+ T cells but does not convert these cells into T cells with suppressive function (117).

IL-17A-producing Th17 were initially thought to play an important role in respectively host defense against pathogens (118, 119) and the induction of autoimmune inflammation (120). IL-17A exerts this pro-inflammatory effect by eliciting the production of neutrophil-attracting chemokines by IL-17 receptor-positive endothelial and epithelial cells (121). Recent studies in mice have shown that T-cell-derived IL-17A can also modulate intestinal inflammation induced by IFN-γ-producing Th1 effector T cells (122). These observations support the new concept that IL-17A exerts anti-inflammatory effects by suppression of the Th1 differentiation program. Likewise, differentiation into IL-17A-producing Th17 cells is negatively regulated by IFN-γ (118). The lineage-specific transcription factor required for the generation of Th17 cells is RORγt (109). However, not all RORγt+ T cells express IL-17A, given that a subset of these cells was found to co-expresses CD25, FoxP3, and IL-10; only the latter population displayed a suppressive function (123). While these two counteracting subsets may co-exist at different ratios, several recent in vitro studies have provided evidence that bona fide human CD4+ CD25+ FoxP3+ nTregs can acquire an IL-17 phenotype when activated in the presence of pro-inflammatory cytokines such as IL-1β (124–126). As LCH lesions display considerable heterogeneity in cellular composition and concomitant cytokine milieu (94), it remains to be studied whether or not Tregs expansion, Th17 differentiation, Th1–Treg conversion, Th17–Th1 conversion, and Tregs – Th17 conversion all occur in LCH (Fig. 8). If so, the next question would be which LCH or stromal cell-derived factors are driving these processes.

Concluding remarks and future directions

LCH continues to surprise and elude us, sorely regrettable from the clinical perspective while intriguing from the basic immunology standpoint. A prime example is the cellular origin of LCH cells, where it is now conceivable that the past focus on epidermal LCs may have been overly exclusive. Furthermore, the immunophenotype of LCH cells within and among lesions is more heterogeneous than thought before, while also a variety of mononuclear phagocytes other than LCH cells is typically present in LCH lesions. Based on these findings, we speculate that LCH is a disease of the mononuclear phagocyte system rather than a purely LC-related disease.
Newly recognized Langerin⁺ DC types as well as monocytes and macrophages are now under intense scrutiny as potential sources. It can also be envisioned, but without much firm experimental ground, that LCH cells in an individual lesion could have several origins, and conversely that different subtypes of LCH may occur dependent on the cell subtype giving rise to LCH cells. Could LCH range over a spectrum from immune-driven dysregulated accumulation of myeloid cells adopting an LCH phenotype induced by local conditions to accumulation of cells with clinical features of malignancy? This would translate into a great challenge to distinguish different disease types requiring distinct clinical approaches.

Altogether, the current evidence is in favor of a reactive origin of LCH and the absence of consistent genetic aberrancies brings back the possibility that LCH is an immune deviation that may develop on an unfortunate underlying background. But this issue is not conclusively solved and major questions remain. Are the putative driving agents microbial, otherwise environmental or maybe autoimmune in origin? What can be learned from the relation between smoking and pulmonary LCH, and its incidental reversibility upon smoking cessation? What can be learned from the more than expected rate of reactivations in LCH? What can be learned from the patients in whom spontaneous resolution occurs? Conversely, despite the extensive genetic studies performed until now, the analysis is by far not complete. How promising, for instance, is the Herculean task of searching for viral insertions and transposable elements using deep sequencing or other innovative molecular technology?

With respect to the contribution of the lesional bystander cells, many questions remain as well. A lesion cell type only recently receiving limelight exposure is the MGC, which displays many striking morphological, phenotypic, and functional characteristics with osteoclasts. It is amazing that these cells develop in skin and other lesions in the absence of mineralized bone, their natural substrate. Do these cells develop both from DCs and macrophages in LCH? Further quantitation of sources of IL-17A and IFN-γ, thought to drive and promote MGC differentiation, is critical. It also remains mysterious why, despite the presumably high IL-17A production, neutrophilic granulocytes are not recruited into the lesion in high numbers. Very recent work demonstrates that in vitro, primary myeloma cells stimulate DCs fusion and development into osteoclasts, a process requiring CD47 and thrombospondin-1...
Hence, therapy targeting this axis potentially could be beneficial in LCH (128).

The recent rapid advances in the field of T<sub>h</sub> subset biology offer new questions for LCH, and it will be a major challenge to dissect systematically questions about the recruitment of naïve or T-memory CD4<sup>+</sup> cells to LCH lesions. In addition, does the lesion act as a tertiary lymphoid organ where naïve T cells are locally activated? Are nTregs recruited, or do aTregs develop locally? Finally, what role does the lesion play in plasticity of T<sub>h</sub> subsets? We are pursuing some of these issues, including a further systematic analysis of chemokine-receptor pairs controlling recruitment of leukocytes into the lesion.

Advanced in vitro and in vivo model systems offer new opportunities for mechanistic dissection of LCH. Interestingly, not only lesional LCH cells show defects, but also patient peripheral blood monocytes display abnormalities when stimulated to develop into monocyte-derived DCs (43). This allows in vitro manipulation and underscores the possibility of an underlying generic problem which leads to LCH lesion formation upon specific local triggering.

Cell type-specific conditional mutagenesis now allows creation of mice lacking or expressing a single gene only in Langerin<sup>+</sup> cells, for instance the TGF-β receptor or β-catenin. Conversely, keratinocytes or fibroblasts can be modified to offer new questions for LCH, and it will be a major challenge to develop into monocyte-derived DCs (43). This allows in vitro manipulation and underscores the possibility of an underlying generic problem which leads to LCH lesion formation upon specific local triggering.

Cell type-specific conditional mutagenesis now allows creation of mice lacking or expressing a single gene only in Langerin<sup>+</sup> cells, for instance the TGF-β receptor or β-catenin. Conversely, keratinocytes or fibroblasts can be modified to assess their contribution to the LC environment. Perhaps combinations of transgenic modification with local exogenous or internal insults will allow partial recreation of the LCH skin lesion. Recent advances in deprogramming of fibroblasts and induced pluripotent stem cells may provide additional approaches comparing differentiation pathways and their molecular cues.

The lack of mechanistic insight into LCH hampers rational development of immunotherapy, and, ignoring important issues of safety for now, several existing and experimental drugs are potential candidates to be tested in clinical trials. These include antibodies aimed to inhibit cytokines such as TNF, IL-1, IL-17A, chemokines, leukocyte adhesion molecules such as VLA-4 (natalizumab), or antigen-presenting cell-T-cell interaction via CD40 (5D12). CD1a-directed depleting antibodies or immunotoxins are also putative therapeutics, as are pharmaceutical modulators of signaling pathways involving receptor tyrosine kinases or Wnt/β-catenin. And how much promise does local depletion of monocytes and macrophages hold, including the MGC, with classical methods such as clodronate-loaded liposomes or with advanced immunotherapy such as targeting CD47 (128)?

Intensified, multi-level interplay between LCH clinicians, researchers dedicated to LCH, and basic immunologists and hematologists, such as fostered by the Nikolas Symposia (8), will enhance the scientific and therapeutic gains from this disease.

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