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# Pathogenesis of Langerhans Cell Histiocytosis

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## Keywords

dendritic cells, *BRAF*

## Abstract

Langerhans cell histiocytosis (LCH) combines in one nosological category a group of diseases that have widely disparate clinical manifestations but are all characterized by accumulation of proliferating cells with surface markers and ultrastructural features similar to cutaneous Langerhans cells (LCs). Despite this unified nosology, important questions about LCH remain unanswered. First, despite having phenotypic features of LCs, LCH cell gene-expression patterns differ from those in LCs. Although this observation suggests that LCH may arise from an earlier precursor, it is not necessarily inconsistent with the hypothesis that LCs are the cell of origin for LCH. Second, LCH's prominent inflammatory component and occasional benign clinical course suggest that LCH may not be a neoplasm. However, the demonstration that LCH cells are clonal, along with the recent discovery of activating *BRAF* mutations in LCH cells, strongly suggests that LCH is a neoplastic disease. These new observations point the way to rationally targeted therapies.

# HISTORICAL CONSIDERATIONS

*To most physicians the rarer diseases of bone with their multiple polysyllabic and still more confusing eponymic terminologies constitute the most perplexing field in medicine, a field so esoteric that it is apt to be relegated far back into the realms of the subconscious.*

T.B. Mallory, 1942 (1)

## Recognizing the Disease

In 1893, Alfred Hand, Jr. (2), a resident physician at Children's Hospital in Philadelphia, described a child with exophthalmos, diabetes insipidus, skull lesions, and a cutaneous eruption "something like scabies," which he ascribed to tuberculosis. It was not until 1915 that Artur Schüller (3), working at the Allgemeines Krankenhaus in Vienna, reported another child and an adolescent with skull lesions and exophthalmos. The child had diabetes insipidus and the adolescent had adiposogenital dystrophy, which led Schüller to infer that both had a disease involving the hypophysis. However, the earliest suggestion that this collection of signs might be stereotypic was made by Henry A. Christian, the first physician-in-chief at Peter Bent Brigham Hospital in Boston. Christian conveniently rereported Schüller's cases in English and added a third case in a paper he prepared for a volume celebrating Sir William Osler's seventy-fifth birthday (4). Calling the presentations a "truly remarkable clinical picture," Christian enshrined the triad of exophthalmos, skull lesions, and diabetes insipidus, which came to be known as Christian or Schüller–Christian disease. He memorably wrote that the skull defects "can best be described by comparing them to the irregular holes in a bit of moth-eaten flannel or the appearance of a pasteboard box gnawed full of holes by mice." (Interestingly, both Schüller and Christian suggested that the bone changes in the skull were the consequence of a pituitary disorder, rather than the other way around.) Meanwhile, although Hand's 1893 case had been little noted, Hand long remembered it and reported two additional cases in 1921, citing similarities to Christian's and Schüller's cases (5). This later report led to the eponymous usage, especially in the European literature, of Hand–Schüller–Christian disease. Hand foreshadowed 90 years of controversy by suggesting that this disease could be either a neoplastic or chronic infectious process.

Meanwhile, in 1924, Erich Letterer (6) in Tübingen described an infant with a purpuric rash, hepatosplenomegaly, and anemia, in whom large, pale, reticuloendothelial cells invaded and defaced the structure of skin, liver, spleen, lymph nodes, and bone marrow. A few similar cases then followed in the literature; they were collected and added to by Sture A. Siwe (7) of the University of Lund in 1933. Siwe proposed that these cases were part of a defined clinicopathological entity of unclear etiology, but his concept did not gain traction until 1936, when Arthur F. Abt and Edward J. Denenholz (8) at Michael Reese Hospital in Chicago highlighted these cases, added another of their own, and proposed that the entity be named Letterer–Siwe disease. (Abt's contribution is sometimes honored by referring to the disease as Abt–Letterer–Siwe disease.) In addition to the rash, hepatosplenomegaly, and anemia, Abt and Denenholz noted frequent bone "tumors" that were often visible only by X-ray, and they described involvement of thymus, lung, and intestinal lymphoid tissue in addition to the organs mentioned by Letterer and Siwe. Microscopic pathology consisted of a generalized hyperplasia of macrophages in involved organs. Although Abt and Denenholz could not identify an etiology, they insisted that Letterer–Siwe disease not be characterized as a reticuloendothelial disease primarily because "a term which is as broad and inclusive as this is of questionable aid in understanding the process involved."

A third entity began receiving attention in 1940. Sadao Otani and John C. Ehrlich (9) at Mt. Sinai and Louis Lichtenstein and Henry L. Jaffe (10) at the Hospital for Joint Diseases, both in New York City, semi-independently reported a series of cases characterized by solitary bone lesions. (The latter physicians



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had consulted on at least one of the former two's cases.) Although the radiographic picture suggested a neoplasm, both sets of authors observed excellent clinical responses to curettage alone or curettage accompanied by radiotherapy. The lesions were characterized by infiltration of pale histiocytic cells with infolded nuclei. However, these cells were invariably accompanied by eosinophils and multinucleated giant cells, which led Otani & Ehrlich (9) to name the lesion solitary granuloma of bone. However, Lichtenstein & Jaffe (10) won the marketing battle by coining the term eosinophilic granuloma, although they conceded that the name they "proposed for this lesion is not altogether above criticism."

## Unification

These three syndromes are notable for their distinct and well-defined clinical presentations. However, by the 1940s, astute clinical scientists were beginning to sense common threads despite deeply ingrained assumptions that worked against unification. One of the most insidious assumptions appeared in a highly influential 1928 paper by Russell S. Rowland (11), who, solely on the basis of birefringent staining of histiocytes in some cases, inferred that Hand–Schüller–Christian disease was a cholesterol-storage disorder. For more than a decade, this disease's pathogenesis was thought to be analogous to what was then understood about Gaucher or Niemann–Pick disease; the only difference was the type of lipid that the histiocytes accumulated. This assumption created a problem for scientists who thought they saw similarities between Hand–Schüller–Christian and Letterer–Siwe diseases but were dissuaded from pursuing a unifying nosology because everyone knew that Letterer–Siwe disease was not a lipid-driven granulomatous disease. In fact, one of its names at the time was nonlipoid granulomatosis. Abt & Denenholz even entertained the notion that Letterer–Siwe disease might be similar to Hand–Schüller–Christian disease but dismissed the possibility because of the absence of lipid-containing cells in the former (8).

Arvid Wallgren (12) of Göteborg should be better recognized as a towering figure in this field because he marshaled substantial evidence from published case reports that Hand–Schüller–Christian disease is not a cholesterol-storage disorder. In particular, he cited several cases in which reticular cell proliferation was not associated with cholesterol, and he suggested that the appearance of foam cells is simply a late-stage phenomenon associated with tissue destruction. Furthermore, he cited cases in which the clinical boundaries between Hand–Schüller–Christian and Letterer–Siwe diseases seemed fluid: exophthalmos and diabetes insipidus accompanied by progressive anemia in some cases, Letterer–Siwe disease accompanied by lipid-laden foam cells in others. Wallgren stated clearly that cholesterol storage is not pathognomonic of Hand–Schüller–Christian disease and, more importantly, that the histologic picture of Hand–Schüller–Christian disease is generally similar to that of Letterer–Siwe disease.

**Histiocytes:** tissue-localized cells derived from the monocytic lineage (as distinguished from circulating monocytic cells)

Another impediment to unification was the assumption that the patterns of bone involvement are distinct among the three diseases: multiple calvarial lesions in Hand–Schüller–Christian disease, disseminated bone lesions in Letterer–Siwe disease, and single bone lesions in eosinophilic granuloma. The first person to challenge this assumption explicitly was Sidney Farber of Boston. At a meeting of the American Association of Pathologists and Bacteriologists in 1941, he presented 10 cases of eosinophilic granuloma (as defined by the criteria of Otani & Ehrlich or Lichtenstein & Jaffe) (13). In addition to noting that the radiographic appearance of these lesions was identical to that of Hand–Schüller–Christian bone lesions, he showed that the microscopic pathology was also identical not only to that of Hand–Schüller–Christian disease but also to that of Letterer–Siwe disease. Farber suggested that all three diseases are "variations in degree, stage of involvement, and localization of the same basic disease process." [Perhaps not surprisingly, Lichtenstein & Jaffe were recorded in the meeting proceedings as objecting



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Histiocytosis: general term denoting an accumulation of tissue macrophages

LC: Langerhans cell

Birbeck granules: pentalaminar membranous organelles found in the cytoplasm of LCs and the cells of LC histiocytosis

because they saw Farber present no criteria solidly linking Hand–Schüller–Christian and Letterer–Siwe diseases (13).] The following year, Farber published his cases with a detailed description of lesion evolution that could encompass the histopathological pictures of all three diseases (14). As for the disease-specific patterns of bone involvement, it is interesting to note in retrospect that both Schüller (3) and Christian (4) mentioned that the patients they described also had radiolucent lesions in their pelvises as well as their skulls. Appreciation for the potentially systemic nature of eosinophilic granuloma was solidified with the advent of safe open-lung biopsies, which demonstrated pulmonary lesions in some cases (15).

Over the next decade, researchers reached a consensus around the idea that Hand–Schüller–Christian disease, Letterer–Siwe disease, and eosinophilic granuloma were manifestations of the same fundamental disease process (see, e.g., Reference 1). But in 1953, Lichtenstein (16) scored another branding triumph by suggesting that all three diseases be grouped under the single heading of histiocytosis X. He rejected the then-popular alternative of reticuloendotheliosis because the cells in these diseases were clearly histiocytes (in the Aschoff sense) with phagocytic properties. Also, he liked the term histiocytosis because it connotes "an inflammatory proliferative reaction [that] is the one feature common to all of the various pathologic expressions of the disease." What kind of histiocytosis is it? Lichtenstein rejected the term idiopathic histiocytosis because it was too broad; after all, sarcoidosis is an idiopathic histiocytosis. Instead, he chose X because he thought it would "emphasize the necessity for an intensive search for the etiologic agent." Although it seems likely that the search for the etiology would have progressed just as vigorously without the mysterious X, the name found its way into general usage and remained unchallenged for nearly 35 years.

## Solving for X

The next wave of advances in understanding this disease came from progress in identifying the lineage of the histiocyte. The phagocytic capacity of the pathologic cell in histiocytosis X, whether for cholesterol or erythrocytes, suggested to most investigators that it was derived from a histiocyte, that is, a tissue macrophage. This view began to change in 1961, when an electron microscopic analysis of vitiliginous skin revealed the presence of pentalaminar, membranous granules in the high-level clear cells of the epidermis (17). These aureophilic cells had first been described by Langerhans as "nerves of human skin" (18) and have since been named Langerhans cells (LCs). The granules themselves were initially termed Langerhans granules but are more commonly known as Birbeck granules; they are named for the first author of the paper that described them (17).

By 1967, a handful of electron microscopy studies had been performed on histiocytosis X samples. Strikingly, they demonstrated the presence of Birbeck granules in most of the nonkeratinized cells in the epidermal samples (19, 20). Despite this significant clue, investigators resisted the idea that histiocytosis X cells were derived from LCs. In retrospect, this resistance is somewhat understandable because, prior to the groundbreaking demonstrations that LCs are antigen presenting cells, their function was unknown (21, 22). They had been variously suggested to be "exhausted" melanocytes, epidermal neural elements (on the basis of their dendritic morphology), or Schwann cells (17). Furthermore, prior to 1976 (23), the LC was thought not to be phagocytic, whereas the histiocytosis X cell was.

Resistance began to fade when Tarnowski & Hashimoto (24) first provided ultrastructural evidence for the phagocytic nature of pathologic LCs. They based their inference on the facts that Birbeck granules are present in dermal macrophages in Letterer–Siwe disease and that these cells are clearly phagocytic. Additional support came from the realization that Birbeck granules are not viral inclusion particles, as had originally been suggested (20), but rather are derived from cellular membranous structures that are internalized during endocytosis (24, 25).

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These results led, in part, to Nezelof et al.'s (26) grand synthesis in 1973. Reviewing all of the available data, these authors restated the clinical and pathological characteristics that would qualify a disease to be named histiocytosis X, then turned their attention to the question of the cell of origin. By this time, the phagocytic nature of LCs had become accepted, and Nezelof et al. stated that histiocytosis X is probably a "tumoral process in which the Langerhans cell would be the stem cell." The disease itself was proposed to be the result of the proliferation and dissemination of these pathologic LCs, although its etiology was still obscure.

During the subsequent decade, new technologies spawned observations that strengthened the connection between LCs and the cells of histiocytosis X. For example, the anti-T6 monoclonal antibody (27), which recognizes CD1a, was shown to stain LCs specifically (28, 29). The same antibody, as well as an anti-Ia antibody, stained Birbeck granule-positive histiocytosis X cells (30–32), demonstrating a strong phenotypic linkage between LCs and histiocytosis X cells. Of course, CD1a positivity is now considered a diagnostic marker for this disease.

Decades of unification and synthesis of formerly disparate diseases culminated in the 1987 recommendation by the Writing Group of the Histiocyte Society to rename this disorder Langerhans cell histiocytosis (LCH) (33). The Writing Group intended this designation to embrace histiocytosis X as well as all the other variant names, such as LC granulomatosis. The Writing Group's goal was to provide a broadly inclusive name while adhering to strict diagnostic criteria, and the rapid acceptance of LCH by the clinical and research community suggests that it succeeded. Notably, the Writing Group also offered a consensus on etiology: "There is no evidence that the disease is a malignant neoplastic process.... Some evidence suggests that the disorder is a manifestation of an immunological aberration" (33). (In contrast, the Writing Group readily suggested that the immunological deficiencies observed in hemophagocytic lymphohistiocytosis are secondary to the perforin mutations that cause the disease.) This viewpoint dominated the scientific approach to LCH for the next 10 years.

## CONTROVERSIES

Recognition of the close similarities between LCH cells and normal LCs, and an understanding that the distinct clinical entities defined in the first half of the twentieth century are manifestations of the same disease, has helped organize thinking about LCH. Nonetheless, at least two questions that are central to an understanding of LCH remain controversial. One is whether the LC is truly the precursor of the abnormal cell in LCH, and the other is whether LCH is a neoplasm.

### Origin of the Pathologic Cell in Langerhans Cell Histiocytosis

One of the most significant conceptual breakthroughs in the understanding of this disease was the discovery of a link between the pathologic cells of LCH and normal epidermal LCs on the basis of shared surface markers and ultrastructural features. The simplest pathogenetic inference that was drawn from this connection is that the normal epidermal LC is the precursor cell for LCH. Whether the disease arises in response to an external inflammatory stimulus or as the result of a cell-autonomous genetic event (see the section titled Neoplasm Versus Immune Disorder, below), the cellular target of these changes has been assumed to be the epidermal LC.

A 2010 study challenges this assumption (34). It compared global gene expression in CD207-positive cells from 12 LCH samples with CD207-positive LCs obtained from 13 samples of normal skin. CD207, also known as langerin, is a type II transmembrane protein that is expressed in LCH and normal LCs. It is unsurprising, and superficially consistent with their presumed common origin, that more than 95% of the >47,000 surveyed messenger RNAs were expressed at similar levels in the two cell types. Nonetheless, informatic analyses grouped the LCH samples into one cluster and

**CD1a:** a transmembrane protein structurally related to major histocompatibility group proteins and involved in lipid antigen presentation; expressed by human LCs

**LCH:** Langerhans cell histiocytosis

**CD207 (also known as langerin):** a C-type lectin involved in antigen presentation and expressed by a subset of DCs, including LCs

**DC:** dendritic cell

**Epithelial-mesenchymal transition (EMT):** a phenotypic alteration in which epithelial cells take on the motile and other characteristics of mesenchymal cells; thought to be a component of metastatic competency in cancer

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the normal LC samples into another; the correlation coefficient between them was only 0.2. Although this observation can be interpreted in different ways, the authors of the 2010 study suggested that it is consistent with a model in which the cell of origin for LCH is not a mature epidermal LC. Rather, because several genes encoding myeloid dendritic cell (DC) markers, including CD1d, CD33, and CD44, were overexpressed in CD207-positive cells from LCH patients compared with normal LCs, the authors suggested that this finding is evidence for a myeloid DC precursor of the pathologic cell in LCH. They pointed out that other DCs can also be CD207 positive (35, 36), and perhaps the precursor cell for LCH is also a precursor for this cell type. Perhaps the field has been misled by the CD207 positivity of LCs.

If LCH were to arise from a myeloid precursor rather than a mature LC, at what stage in myeloid DC maturation might this occur? Because the pathologic cell in LCH shares features with LCs that are nearly specific for this lineage, an understanding of LC ontogeny may help answer this question. Importantly, when LCs are placed broadly within the DC family, LCs are more closely related to classical DCs than to plasmacytoid DCs, given their shared expression of CD11d and major histocompatibility (MHC) class II antigens. However, careful studies of LC precursors in mouse models suggest that they arise from a lineage that is distinct from that of classical DCs. In particular, investigators have identified murine LC precursors that are CD207 and CD24 negative (37, 38). These cells begin to populate the epidermis during development of the stratum corneum and remain CD207 and MHC class II negative until approximately a week after birth (39–41). Merad and colleagues (42–45) identified the precursors of these CD207-negative cells as a population that derives from the monocyte lineage. Like other monocytoid cells, these CD207-negative cells are CD11b positive, F4/80 positive, colony-stimulating factor 1 receptor positive, and CX3CR1 positive, although, unlike most monocytes, they are Gr-1 negative.

Some lines of evidence point to a yolk sac origin for these early cells. For example, the CD207-negative LC precursors appear in limb buds at approximately the same time that yolk sac–derived macrophages begin to populate the skin. In addition, yolk sac–derived primitive macrophages have the same distinctive set of monocytoid surface markers as the early LC precursors, including the absence of Gr-1. The Merad group (46) suggests that during embryogenesis yolk sac–derived primitive macrophages migrate to the skin, where they differentiate into CD207-positive LCs. Thus, this lineage is distinct from that of classical myeloid DCs. If myeloid DC lineage specificity were the reason that the handful of genes that are expressed in myeloid DCs are also expressed in LCH cells, then the cellular target for the initiating event in LCH would have to be an even earlier precursor common to the LC and myeloid DC lineages—a very early precursor, indeed.

An alternative explanation for the expression of some myeloid DC precursor genes in LCH is that epidermal LCs are, in fact, the precursors of LCH cells but that they undergo reactivation of these early myeloid marker genes as a consequence of transformation-induced reprogramming of the LC transcriptional profile. This process used to be interpreted as dedifferentiation, but we now know that a similar transcriptional reprogramming forms the basis of epithelial-mesenchymal transition (EMT), which may be necessary for neoplastic cells to invade and spread (47). Despite the name EMT, the phenotypic changes associated with this transition are not restricted to epithelial cancers, and some of the alterations that are the most characteristic of EMT are also observed in LCH. In particular, the loss of E-cadherin is both a hallmark of EMT and a well-described change in LCH cells compared with resting LCs (48). Another marker of EMT is upregulation of osteopontin, which has also been described in LCH (34). Furthermore, the concept of EMT may be particularly relevant to LCH because of the evidence that tumor-associated inflammatory cells may be important inducers

and reinforcers of EMT (49, 50). Thus, the alterations in gene expression that may suggest a different cell of origin for LCH may be simply the consequence of an EMT-like change. Resolution of this question will require continued careful analyses of LC precursors in tractable settings, such as mouse models, coupled with ongoing references to human physiology and disease.

The idea that the epidermal LC is the cell of origin for LCH has also been questioned because LCs are a stable, localized cell population and are therefore unlikely to be responsible for disseminated forms of LCH (34). Again, there are different ways to interpret these facts, but an understanding of LC homeostasis can inform the discussion. As noted above, LCs are phenotypically related to classical DCs, and both classical DCs and LCs are derived from circulating hematopoietic precursor cells. The bone marrow source of classical DCs is convincingly demonstrated by the wholesale replacement of host DCs, including those residing in mucosal sites, by donor cells after hematopoietic stem cell transplantation (51–54). Similarly, donor bone marrow cells can replace a significant proportion of host LCs when transplanted into lethally irradiated recipients (55, 56), although there is a caveat: The replacement of host LCs is not complete. Approximately 20% of LCs retain host markers, which may be a consequence, in part, of the relative radioresistance of LCs compared with that of classical DCs. The lineage of the bone marrow–derived replacement cell is monocytoid, according to the finding that blood monocytes can replenish cutaneous LCs following UV irradiation (43); this process requires expression of both the chemokine receptor CCR2, for cells to enter the dermis, and CCR6, for the cells to reach their final destination in the epidermis.

However, these observations may have little relevance to patterns of steady-state LC homeostasis. For example, conditioning regimens for hematopoietic stem cell transplantation induce systemic hyperinflammatory states, which lead to LC activation and emigration from the epidermis. In contrast, in mouse models under steady-state, noninflammatory conditions, LC populations are extraordinarily stable; their half-lives in the epidermis are 53 to 78 days (57, 58), and the proliferating fraction is only 1% to 2% (59). In these settings, slow renewal of skin LCs is accomplished by local proliferation; bone marrow–derived precursors are not required. This renewal has been demonstrated directly through the use of parabiosis models in mice (45). Similarly, noninflammatory depletion of epidermal LCs achieved with transgenically targeted expression of diphtheria toxin suggests that proliferation of local precursors slowly leads to their replacement (60). Even in mild inflammatory states such as atopic dermatitis, in which the dermal-epidermal junction remains intact, replacement of LCs appears to occur primarily through local proliferation (39).

Thus, experimental and clinical evidence shows that epidermal LCs are a stable cell population that renews itself primarily through regulated proliferation of local precursor cells. However, this physiology does not necessarily preclude LCs' dissemination to other organs in the setting of LCH. An epidermal LC, activated either by an inflammatory stimulus or by an oncogenic event, may be capable of leaving the epidermis and migrating to distant organs. Even under normal conditions, activation of LCs induces their departure from the epidermis and migration to regional lymph nodes in a manner that may be dictated by patterns of chemokine receptor expression. Pathological activation in LCH may take advantage of elements of this preexisting program to produce characteristic patterns of organ involvement. Transformation may also be accompanied by EMT, as described above. The analogy to metastatic cancer is apparent. Thus, it is not necessary to posit a bone marrow–derived precursor to explain multiorgan involvement in LCH.

## Neoplasm Versus Immune Disorder

Uncertainty about so fundamental a question as whether or not LCH is a neoplasm is



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understandable. Unification of the various clinical presentations of LCH creates a single nosological category, but it simultaneously embraces a broad spectrum of clinical presentations: diseases with generally good outcomes, such as eosinophilic granuloma, along with diseases that behave as aggressive malignancies, such as some cases of infantile Letterer–Siwe disease. Furthermore, there are well-documented reports of spontaneous remissions even in multisystem LCH in infants (61). This propensity for favorable outcomes is of more than academic interest because, during the ascendancy of cytotoxic chemotherapy for cancer in the 1970s and 1980s, similar treatments were used for LCH. Even though those treatments were clinically effective in multisystem disease, recognition of the potential for spontaneous remissions and the possibility that LCH is not neoplastic appropriately tempered the tendency to administer overly aggressive treatments.

The granulomatous nature of LCH lesions and the generally benign morphology of pathologic LCs have suggested possible infectious, environmental, or autoimmune pathogeneses. Nonneoplastic etiologies do not preclude aggressive and occasionally fatal outcomes. For example, secondary hemophagocytic lymphohistiocytosis, which can also be fatal, arises after viral infection (62). However, despite decades of searching, no convincing evidence has emerged for an infectious cause of LCH. Herpesviruses such as Epstein–Barr virus, cytomegalovirus, and human herpesvirus 6 have been detected in clinical samples (cited in Reference 63), but they are unlikely to be pathogenetic because no differences in the overall prevalence or titers of antibodies against these viruses have been detected in comparisons between LCH patients and case-matched controls (63). This finding is consistent with earlier reports that were unable to document an association between herpesviruses and LCH (64).

A substantial literature has described immune dysregulation and abnormal cytokine expression in LCH patients and has been cited as support for the idea that the nature of LCH is predominantly immune or inflammatory. Although an initial crude examination of immune status in LCH patients revealed no gross abnormalities (65), later studies indicated a deficiency of circulating suppressor T lymphocytes (66). Considering the role that LCs play in shaping T cell responses, this observation raises the possibility that pathologic LCs may drive immune dysfunction. High levels of circulating cytokines such as granulocyte macrophage colony–stimulating factor (GM-CSF), macrophage colony–stimulating factor (M-CSF), and FLT3 ligand have been detected in LCH patients, although their cellular sources and their pathogenetic relevance have not always been well documented (67–71).

In this regard, a current controversy concerns the cytokine interleukin (IL)-17A. One group has reported high plasma levels of IL-17A in LCH and its expression by pathologic LCs (72). This observation is provocative because, in the same study, the authors demonstrated that IL-17A stimulates DC fusion in vitro, which may explain the frequent appearance of multinucleated giant cells in LCH lesions. However, a second group was unable to detect IL-17A messenger RNA or protein in a collection of LCH samples; these authors suggest that the original finding may have been the unfortunate consequence of a nonspecific antibody (73). Nonetheless, demonstrating the DC fusion activity of IL-17A in vitro did not depend on an IL-17A antibody and has not yet been challenged, so IL-17A secreted by some other cell type may still play a role in LCH pathobiology (74).

## Altered Transcriptional Programs in Langerhans Cell Histiocytosis

Several cell-surface markers that are characteristic of activated LCs, including CD2, CD11b, CD24, CD44, CD54, CD58, CD80, and CD86, have been identified on LCH cells (69, 75, 76). Also, similar to activated LCs, LCH cells express the $\gamma$ chain of the GM-CSF receptor (77), which may provide a mechanism whereby elevated circulating GM-CSF (70) stimulates their proliferation. Patterns of



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adhesion molecule expression are also consistent with an activated phenotype, namely the presence of β1 and β2 integrins, which resting LCs do not express (69, 75, 78), and the absence of E-cadherin, which is downregulated following LC activation (48, 79).

However, LCH cells also have some features of resting LCs. In particular, Birbeck granules, which are a sine qua non of pathologic LCs, are present in resting LCs and absent from activated cells (80). Furthermore, despite the expression of some of the activation surface markers described in the previous paragraph, LCH cells are poor stimulators of naïve T lymphocytes, which is a characteristic of resting LCs (81, 82). Similarly, although LCH cells show patterns of adhesion molecule expression that are characteristic of activated LCs, they also express VLA-5 and L-selectin, which are expressed by resting cells (83).

Analysis of chemokine receptor expression is also consistent with a mixed resting/activated phenotype in pathologic LCs. Resting LCs express CCR6, which is thought to anchor these cells in the epidermis through chemoattraction to CCL20, a CCR6 ligand that is secreted by epidermal keratinocytes. Following activation, LCs downregulate CCR6 and replace it with CCR7, which directs LCs to regional lymph nodes, the source of CCR7 ligands CCL19 and CCL21 (84–86). Some investigators have reported coincident expression of CCR6 and CCR7 by pathologic LCs in all the cases they examined (87). Another group has identified only CCR6 expression (88). Although the basis for this discrepancy has not been identified, both groups agree that LCH cells express CCR6, which is characteristic of resting LCs.

Taken together, these phenotyping studies paint a picture of disordered maturation in pathologic LCs. The mixture of markers that are characteristic of both resting and activated cells may be consistent with (*a*) maturation arrest after stimulation of resting cells; (*b*) partial reversion to a resting phenotype by an activated cell; or (*c*) the transcriptional program associated with EMT, as described above. The findings could also be consistent with a cell-autonomous abnormality that disrupts the LC activation program, an influence of the milieu (such as abnormal cytokine expression) that results in the same phenotype, or both as hypothesized in the EMT model (49). Notably, however, this phenotype is preserved after cells are explanted in vitro and requires further manipulation to alter it. For example, as mentioned above, pathologic LCs are not potent stimulators of resting T cells despite their expression of CD80 and CD86 (81, 82). CD40 ligand treatment in vitro can make pathologic LCs potent antigen presenting cells (89). These observations argue that the defect in pathologic LCs is stable and, to some extent, cell autonomous.

## LANGERHANS CELL HISTIOCYTOSIS AS A NEOPLASTIC DISEASE

Determining whether LCH is a neoplastic disease has therapeutic implications. If LCH were a reactive inflammatory disease, then proliferating LCH cells would be intrinsically normal and definitive treatment could focus on removing the inciting inflammatory stimulus. In contrast, documentation of recurrent genomic abnormalities in LCH cells may suggest that LCH is susceptible to molecularly targeted antineoplastic therapies, which would place the disease within the contemporary paradigm that has revolutionized cancer treatment. Resolution of this distinction will dictate the direction of developmental therapeutics in LCH.

### Molecular Evidence for Neoplasia

Careful molecular analyses performed over several years have been consistent with both of the models described above, but the finding that pathologic LCs in nonpulmonary LCH are clonal has lent the strongest support for the idea that LCH is a neoplastic disease (90, 91). However, although clonality may be a necessary attribute of neoplasia, it is not sufficient. For example, some clinically aggressive immune disorders may be characterized

by oligoclonal expansion of nonneoplastic T lymphocytes (92). To establish a neoplastic pathogenesis for LCH, investigators must identify recurrent genomic abnormalities.

**CGH:** comparative genomic hybridization

**FFPE:** formalin-fixed, paraffin-embedded

The search for molecular genetic alterations in LCH has been severely impeded by the rarity of the disease. Abnormalities identified in a small collection of samples have often not been confirmed in later studies of a larger number of samples. For example, cytogenetic alterations were reported in an examination of five LCH cases (93), but none recurred within the sample set and none were observed in a later study of 31 samples (94). In fact, the later study documented diploid genomes in all cases, which, if anything, casts additional doubt on the neoplastic origin of LCH. Copy number analysis has been attempted using array comparative genomic hybridization (array CGH) and polymerase chain reaction (PCR). One study that examined seven LCH bone samples with array CGH reported numerous copy number alterations throughout the genome and recurrent loss of heterozygosity at several loci (95). Similarly, a PCR-based study of 24 samples found a higher degree of fractional allelic loss in samples from multisystem and high-risk patients compared with those from single-system and low-risk patients (96). Again, however, a larger study using PCR and high-density single-nucleotide polymorphism arrays detected no significant copy number alterations (94).

Until recently, the only reliably recurrent molecular abnormality in LCH was overexpression of p53 (97), which is most commonly detected by immunohistochemistry. The molecular basis for its overexpression is not known. No mutations in TP53 or MDM2 were found in the 10 cases in which overexpression was originally described, nor were they found in a later survey of a larger number of cases (94), although that survey did confirm p53 overexpression in most cases. To date, only one TP53 mutation has been reported in LCH (98). In some settings, overexpression of p53 may reflect the persistence of an abnormal non- or hypofunctioning allele; in such cases, p53 may be a primary contributor to a hyperproliferative, transformed phenotype through its inability to induce apoptosis. However, p53 overexpression may, instead, be a normal secondary response to a proliferative stimulus. Furthermore, p53 overexpression by itself cannot be cited as evidence of the neoplastic nature of LCH because there are examples of benign proliferative disorders that are characterized by high levels of p53 expression (99–102).

Some of the most important mechanistic insights into cancer pathobiology have come from global, nonbiased assessments of small genomic abnormalities, such as point mutations and short insertions or deletions. Until recently, the relevant technologies have been insufficiently robust to provide reliable measurements through the use of DNA extracted from archived formalin-fixed, paraffin-embedded (FFPE) samples. This problem has placed LCH investigators at a disadvantage because the rarity of the disease is surpassed only by the rarity of fresh frozen samples that are sufficiently large for such analyses. Fortunately, an appreciation of the wealth of genomic information in archived clinical pathology specimens has driven an explosion in technological advances that permit reliable genomic analyses of these specimens. The study of LCH has benefited from this technological evolution.

Next-generation DNA sequencing platforms, especially those that perform targeted or whole-exome sequencing, will almost certainly be used to analyze DNA extracted from FFPE samples in the very near future. Meanwhile, however, an example of a relatively nonbiased technology that has already demonstrated its utility in the analysis of archived specimens is the mass spectrometry genotyping platform developed by the Sequenom Corporation (103). By use of genomic DNA as a template, short amplicons are generated by PCR around the polymorphic loci of interest, and the distinct products of polymorphic templates are identified by their characteristic molecular masses with MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry. Because this technology can separate

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and identify amplicons with very small differences in mass, the process can be multiplexed to permit the analysis of several loci in a single run. A version of this platform customized for oncology, OncoMap, can test a sample for the presence of more than 1,000 distinct alleles from 140 cancer-related genes in a single run (104).

The inherent difficulty of analyzing DNA from FFPE samples is that the DNA is fragmented. Although this drawback can make direct sequencing approaches difficult, it presents a less daunting challenge to the mass spectrometric approach because the amplicons it generates, generally 200–400 base pairs in length, are smaller than the median size of the DNA fragments from FFPE material (105). In a direct comparison between OncoMap's performance on fresh frozen samples and on FFPE samples of pediatric low-grade astrocytomas, the sensitivities and specificities of the test were nearly identical in both tissue types (106).

## *BRAF* Mutations

Recently, a version of OncoMap that tests 983 alleles from 115 cancer-related genes was used to analyze 61 archived LCH cases (98). The most striking finding was the presence of the oncogenic BRAF V600E mutation in 57% of the cases. The mutation was validated using pyrosequencing as an orthogonal technology in these samples and in a fresh frozen sample. The presence of mutated *BRAF* was not associated with disease stage, but it did correlate negatively with age. Other point mutations detected in this analysis included TP53 R175H, a known cancer-related mutation in p53; KRAS G13D, a transforming allele of K-Ras; and MET E168D, a possible transforming allele of c-Met, although that allele may be a nonpathogenic polymorphism. None of the other mutations assayed by this version of OncoMap were detected, but recall that OncoMap is not truly unbiased and other mutations not included in this testing panel may be present in LCH.

This study also employed several controls to demonstrate the specificity of BRAF V600E for LCH. For example, the mutation was not detected in dermatopathic lymphadenopathy, a disease characterized by proliferation of normal LCs, which suggests that BRAF V600E is specific for pathologic LCs and is not present in normal proliferating LCs. The mutation also was not detected in non-LC histiocytoses such as juvenile xanthogranulomatosis and Rosai–Dorfman disease (sinus histiocytosis with massive lymphadenopathy).

If BRAF V600E were pathogenetic in LCH, then this allele should be present specifically in the pathologic LCs, as opposed to other cell types in the samples. Identification of the cell that harbors this mutation in mixed cellularity samples such as LCH is challenging because of the absence of a validated antibody that specifically recognizes the mutant BRAF protein. Instead, the authors of this study inferred the specific presence of BRAF V600E in pathologic LCs by the severalfold-higher abundance of the mutant allele in CD1a-positive versus CD1a-negative cells harvested by laser-capture microdissection, as analyzed by pyrosequencing. Similarly, the relative abundance of the mutant allele compared with that of the wild-type allele, quantified either by OncoMap mass spectrometry or by pyrosequencing, was proportional to the number of pathologic LCs in all of the samples that contained the mutant allele. Interestingly, the proportion of mutated alleles was approximately half the proportion of pathologic LCs. This finding may suggest that the mutant allele is present as a single copy, which would be consistent with the pathogenic behavior of a dominant oncogene.

B-Raf is an important component of the signaling cascade that ordinarily begins when a growth factor or other ligand engages its receptor tyrosine kinase. The signal is passed via sequential phosphorylation steps to Ras, then Raf, then MEK and ERK kinases, which ultimately leads to modulation of gene expression (107). Investigators obtained evidence for the activation of this pathway in LCH by demonstrating that MEK and ERK are phosphorylated in

**OncoMap:** a mass spectrometry–based allelotyping system focused on cancer-related variants

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pathologic LCs. The fact that phospho-MEK and phospho-ERK were observed by immunofluorescence only in LCs and not in other cell types in LCH provides further evidence for the cell type specificity of mutant BRAF V600E.

Unexpectedly, however, phospho-MEK and phospho-ERK were observed in the LCs of all LCH samples, regardless of their *BRAF* genotype (98). This signaling pathway can also be activated by duplication of a wild-type *BRAF* locus, which has been observed in a significant number of pediatric low-grade astrocytomas (108, 109). However, *BRAF* duplication was not observed by fluorescence in situ hybridization analysis in 43 LCH cases (98). Thus, the molecular basis for RAS-RAF-MEK pathway activation is unknown in the ∼40% of LCH cases that do not harbor an activating *BRAF* mutation. One possibility is overexpression of a receptor tyrosine kinase in LCH cells. Amplification of a gene that might be responsible for such overexpression cannot be detected by OncoMap but could be revealed by next-generation sequencing approaches. Another possible explanation for pathway activation is high ambient levels of a growth factor or cytokine whose cognate receptor is expressed by pathologic LCs. Several candidates, including GM-CSF and M-CSF, are cited above (70, 71, 77).

## Is Langerhans Cell Histiocytosis a Neoplasm?

The demonstration of BRAF V600E in more than half of the archived LCH samples examined to date provides the recurrent genomic abnormality that is required, in addition to clonality, to assign LCH a neoplastic origin. This review is very careful to use the word neoplastic rather than malignant. The most clinically benign forms of LCH may be mechanistically analogous to nevi, which also contain BRAF V600E and are, of course, neoplastic. In fact, the benign nature of most nevi is now thought to be an in vivo example of oncogene-induced senescence in which the presence of an activating oncogenic mutation, such as BRAF V600E in melanocytes, can direct several rounds of cell division that are superseded by a senescence response within the same cell, presumably as an organism-level protection against malignancy (110). Perhaps, in some LCH cases, the appearance of BRAF V600E in LCs also leads to proliferation and the appearance of clinical LCH, but as in nevi, senescence supervenes, which causes the spontaneous remissions that have been well documented in the literature.

More aggressive LCH cases may also be explained by analogy to other diseases such as melanoma. Although some melanocytes that carry BRAF V600E produce nevi, others become true melanomas. Careful molecular analyses have demonstrated that melanoma cells contain additional genomic abnormalities, such as loss of *PTEN* or *CDKN2*, that are presumed to be required for the progression to malignancy (111). Although the OncoMap analysis that identified BRAF V600E did not find inactivating mutations of these genes and detected a p53 mutation in only one case, this technology is limited by its inability to detect novel mutations or deletions. Thus, next-generation sequencing approaches that can reveal novel mutations as well as copy number alterations (and, in the case of whole-genome sequencing, translocations) may identify additional abnormalities that could cause the development of aggressive forms of LCH. These abnormalities could bypass the senescence response in BRAF V600E–positive cases or could engage novel, non-BRAF pathways in BRAF V600E–negative cases.

The discovery of *BRAF* mutations in LCH has refocused attention on some areas of controversy. The first controversy is a debate about the source of pathologic LCs in LCH lesions. If LCH is a neoplasm, then the presumption is that lesions expand by local proliferation of neoplastic LCs. In contrast, if LCH is an oligoclonal reactive inflammatory disease, then LCs might be recruited to lesions from circulating or bone marrow pools. Although this distinction is hardly absolute (inflammatory cells can proliferate locally), the controversy was stoked by a 1998 paper with the provocative title "Langerhans Cells in Langerhans Cell

Granulomatosis Are Not Actively Proliferating Cells" (112). This study examined 25 cases from various anatomic sites for markers of cell proliferation. The mitotic index for LCs was 0.1% versus 0.3% for a small collection of non-small-cell lung cancer (NSCLC) cases; Ki67 staining was present in only 5% of LCs versus 18–33% of NSCLC cells; and assessment of DNA content by Feulgen staining showed only 5% to 6% of LCs with an S-$G_2$-M DNA content versus 26% of NSCLC cells. Although the authors observed comparable proportions of PCNA-positive cells in LCH and NSCLC samples, they suggested that the inconsistently high degree of staining in LCH was a consequence of PCNA's ability to be expressed in nondividing cells. Their overall conclusion was that local proliferation does not contribute to the mass of pathologic LCs observed in LCH lesions.

Although these investigators clearly overstated their case, inasmuch as they did document some degree of local proliferation, the growth fraction within LCH lesions was quite low in this study. However, other data indicate that pathologic LCs actively proliferate. One study demonstrated Ki67 staining in 3% to 24% of pathologic LCs in 26 cases and found that Ki67 positivity correlated closely with PCNA positivity (113). Another study examined 61 sections from 43 patients and found mitotic cells in 56% of the sections, all of which were CD1a positive (114). All but one case had Ki67-positive pathologic LCs; 34 of the 43 cases had greater than 10% Ki67-positive LCs, and 11 of the 43 cases had greater than 50% Ki67-positive cells. The discrepancy in Ki67 staining between these two reports and the first report could be due to differences in sample selection, sample preparation, antibody source, or staining protocols. However, the weight of evidence from the latter two studies, among others (115), suggests that significant local proliferation of pathologic LCs occurs in LCH. Of course, this finding does not preclude a contribution from circulating cells, which may have arisen from proliferating pools at other sites such as bone marrow.

The second controversy concerns pulmonary LCH, which has long been thought to be distinct from the nonpulmonary forms. Because it arises in adult smokers and the pathologic LCs are nonclonal, pulmonary LCH has been classified as a reactive inflammatory disease. Therefore, the finding that 40% of pulmonary cases examined in the original OncoMap study contained BRAF V600E was surprising (98). Two possible explanations for this finding have been suggested. First, the nonclonality of pulmonary LCH may be an overstatement. A careful analysis of clonality in pulmonary LCH demonstrated that 29% of the cases contained a clonal population of pathologic LCs (116). This proportion is not very different from the proportion of pulmonary cases with BRAF V600E; perhaps a subtype of pulmonary LCH has the same molecular attributes of clonality and oncogenic *BRAF* mutation as nonpulmonary LCH. Second, pulmonary LCH may arise in smokers who are rendered susceptible to the disease by their inability to repair the T1799A transversion that underlies the activating *BRAF* mutation. Their susceptibility may result in a "field defect" throughout the lung that could produce multiple independent clones of transformed LCs. The result may be numerous clonal sites of LCH that, in the aggregate, appear to be polyclonal. A similar mechanism may give rise to some nevi that contain BRAF V600E but are also polyclonal (117).

## CONCLUSIONS

The discovery of frequently recurring oncogenic *BRAF* mutations in LCH, along with rare *KRAS* and *TP53* mutations, supports the classification of this disease as a neoplasm. Although this concept is useful in many ways, it leaves several questions unanswered. For example, is the expression of BRAF V600E in LCs necessary or sufficient for the development of LCH? In all cancers in which BRAF V600E has been detected, additional genomic abnormalities are present. For example, expression of BRAF V600E by itself in melanocytes

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generally produces benign nevi through a process of oncogene-induced senescence (110). Several additional mutations or deletions are required for the development of melanoma. Similarly, expression of BRAF V600E by itself may produce only self-limited forms of LCH; additional abnormalities may be required, in addition to BRAF V600E, for the development of more aggressive LCH. Distinct suites of genetic changes may even be associated with the different clinical subtypes of LCH. Although this model fits well within the currently understood models of cancer progression, another unresolved question is whether the cellular target for these genetic changes is the mature LC itself or a precursor cell.

Some of these questions will be addressed through the application of more powerful and truly unbiased genomic technologies to clinical samples of LCH. As more tissues from the various clinical types of LCH are more fully analyzed, various characteristic abnormalities may emerge. Determining whether any of these changes is truly pathogenic will ultimately require the demonstration of a clinical response to therapies targeted against them. In the interim, evidence for the causal nature of a genetic change in LCH could be obtained through testing in mouse models. Unfortunately, no faithful murine models of LCH currently exist. Investigators have attempted to create models by using molecular tools that may not be relevant to LCH (for example, one group employed SV40 large T antigen to target the retinoblastoma pathway, which seems not to be affected in LCH) in cells that may also not be relevant (such as all CD11c-positive cells) (118). However, we can envision mouse models in which disease-associated genetic abnormalities such as BRAF V600E can be targeted to mature LCs or, to answer questions about cellular provenance, to various candidate precursor cells.

Fortunately, the clinical development of BRAF inhibitors has advanced so rapidly that it may be possible to document mutant BRAF's importance in LCH directly in a clinical trial. Despite the presence of other genomic abnormalities, in addition to BRAF V600E, in metastatic melanoma, targeted inhibition of this mutant protein alone produces profound clinical responses and prolonged survival (119). Furthermore, the presence of BRAF V600E in patients' tumors is a powerful predictor of response. This clinical precedent may be sufficient justification for a direct test of BRAF V600E inhibition in LCH. The current generation of inhibitors has a very favorable toxicity profile but can induce squamous cell carcinomas of the skin, probably through a mechanism-based effect (120, 121). This problem is considered to be easily manageable in adults who have life-threatening metastatic melanoma, but clinical trials in children with LCH should be carefully planned in order to optimize the risk-to-benefit ratio of BRAF V600E inhibition.

In focusing on LCH as a neoplasm, we should not lose sight of its prominent inflammatory component. This feature was a major contributor to the century-long controversy about the fundamental nature of LCH. The granulomas, the immune dysfunction, and the cytokine "storm" contribute to LCH's clinical presentation and point to additional targets for therapeutic intervention. Nonetheless, the driver of this disease appears to be the pathologic LC, which we now know is itself likely to be driven by oncogenic mutations. This situation may be similar to that of Hodgkin lymphoma, which is characterized by intense inflammatory or fibroblastic reactions accompanying rare neoplastic and causal Reed–Sternberg cells.

Our understanding of LCH has been greatly enhanced by the application of advanced genomic technologies to the analysis of primary human material. The demonstrations of clonality and *BRAF* mutations should be only the beginning. The next phase of LCH research will include an even broader application of next-generation technologies, which will reveal additional abnormalities. Eventually, we should be able to understand, at the molecular level, the basis for the multiple and distinct clinical entities that are classified as LCH.

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## SUMMARY POINTS

1. Evidence continues to support the rationale for unifying the disparate clinical entities Hand–Schüller–Christian disease, Letterer–Siwe disease, eosinophilic granuloma, and histiocytosis X under the single designation of LCH.
2. There is insufficient evidence to conclude that LCH cells either are or are not derived from LCs, as opposed to a common precursor.
3. On the basis of LCH cell clonality and the presence of activating somatic *BRAF* mutations in most patient samples, LCH is best understood as a neoplastic disease.

## DISCLOSURE STATEMENT

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