Langerhans cell histiocytosis: A reactive or neoplastic disease?

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General Discussion

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Is LCH a reactive or neoplastic disease?

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

1. Maturation block of LCH cells.

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

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

2. Is LCH reflecting an intrinsic tumorigenic process?

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

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

3. No evidence for genetic defects in LCH cells.

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

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

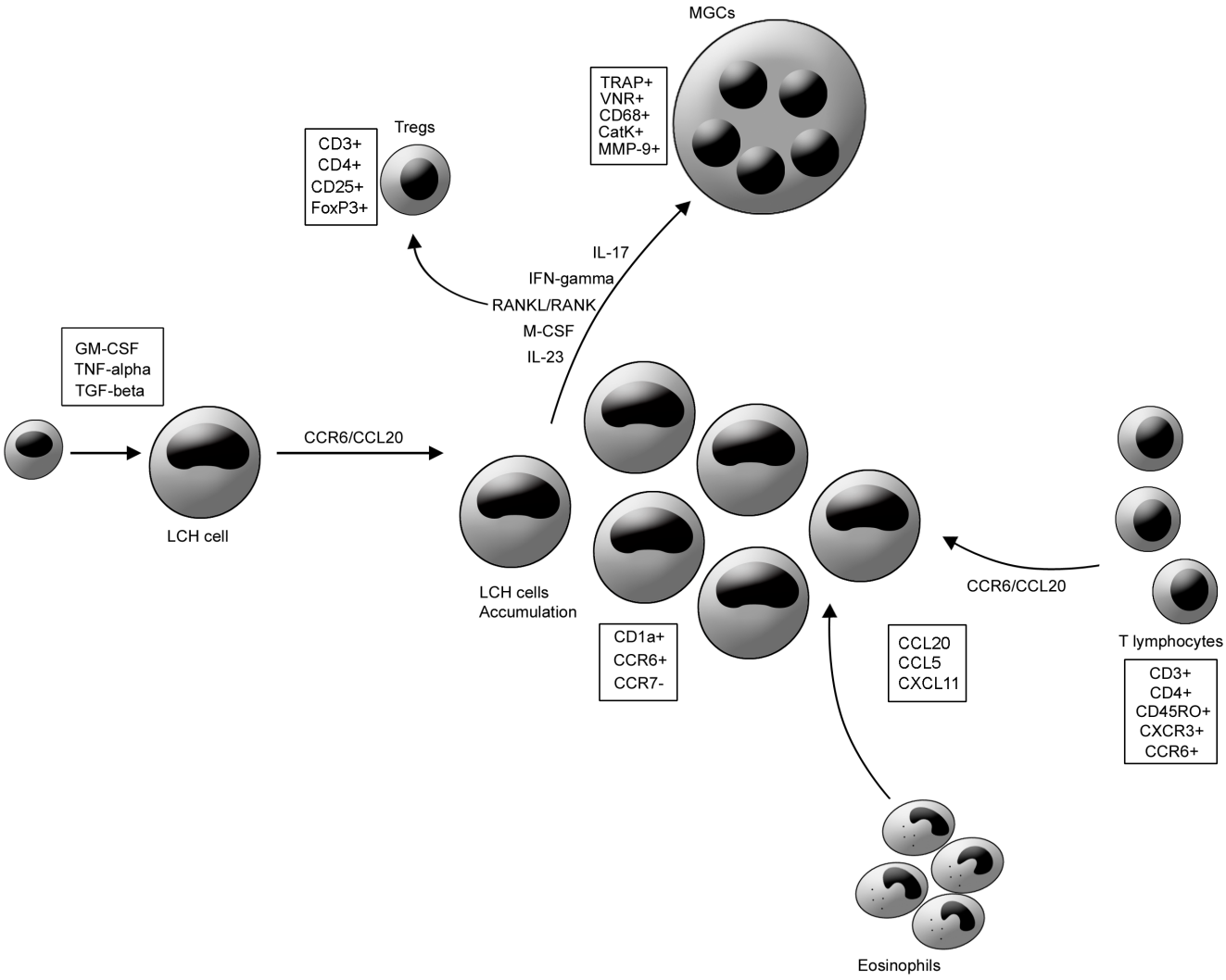

Figure 1. Overview of the immunological events occurring in Langerhans cell histiocytosis lesions, based on the findings described in this thesis.

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

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

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

5. LCH: an immune dysregulation.

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

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

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

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

6. Future directions.

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

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

lesions are associated.

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

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

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