Brief Definitive Report

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

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Abstract

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

Key words: dendritic cell • migration • retention • inflammatory signalling • differentiation

Introduction

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

Due to the integral role that migration plays in the normal function and distribution of LCs as well as the other lesional cells, it seems possible that dysregulation of chemokine production and/or chemokine receptor expression plays a role in LCH. Chemokines have already emerged as major regulators of DC migration (4–8). DC subsets express a distinct pattern of functional chemokine receptors at different stages of their maturation. Immature DCs express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5, CCR6, and CXCR1 which enable the recruitment of immature DCs to sites of inflammation where cognate ligands are produced. Maturation of DCs is associ-

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ated with the coordinated down-regulation of receptors for inflammatory chemokines and the up-regulation of receptors for constitutive chemokines such as CXCR4 and CCR7. This results in the responsiveness of these cells to lymphoid chemokines causing the migration of mature DCs to draining lymph nodes where they are effective at activating naive and central memory T cells (9, 10).

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

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

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

Materials and Methods

Tissue. Paraffin blocks of tissues from 13 patients with LCH were identified by pathologists at Leiden University Medical Center, which acts as a reference center for bone tumors. In all cases the diagnosis was reviewed and confirmed by immunohistochemistry for S100 and CD1a. All biopsies showed the presence of characteristic lesions containing histiocytes, macrophages, lymphocytes, and eosinophil granulocytes. Nine of the specimens were from bone in cases of ostotic LCH, two were from skin biopsies in cases of isolated skin disease and two were from excisional lymph node biopsies from patients with solitary lymph node involvement.

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

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

Primary antibodies were diluted in 1% BSA in PBS and incubated overnight at room temperature in a humidity chamber. The bound primary antibodies were detected using several approaches. Single staining with antibodies specific for chemokines was detected enzymatically using either MouseEnvision or a rabbit anti–goat-HRP antibody followed by VECTOR NovaRed detection. Double and triple staining with primary anti-chemokine receptors in combination with cell-specific markers was detected fluorescently using the relevant secondary goat anti–mouse or goat anti–rabbit isotype-specific Alexa Fluor 488, Alexa Fluor 647, or Alexa Fluor 546 antibodies. To test the specificity of immunostaining, primary antibodies were omitted or replaced by an isotype-matched control antibody. Under these conditions no

Table I. *Technical Details of Antibodies Used in Immunohistochemical Study*

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

positive cells were identified. In addition, sections of suitable tissues were used as positive controls (Table I).

Immunogold Labeling. To carry out double staining of $CD1a^+$ cells and chemokines, immunofluorescent staining of CCL20/MIP-3α was combined with immunogold labeling of $CD1a^+$ cells. As both primary antibodies were mouse IgG1, the anti–human CCL20/MIP-3α was applied after direct labeling with Alexa Fluor 488 using a monoclonal antibody labeling kit (A-20181; Molecular Probes). The first primary antibody, CD1a, was diluted in 0.1% cationic BSA (Aurion) in PBS, and the incubation was performed overnight at room temperature in a humidity chamber. Prior to immunogold labeling, an incubation step with 5% BSA (diluted in PBS) for 30 min was introduced to block nonspecific labeling. The secondary immunoreagent, goat anti–mouse IgG coupled to ultra small colloidal gold particles (Aurion) was diluted 1:50 in 0.1% cationic BSA in PBS and the conditions of incubation were 2 h at room temperature. After rinsing several times with PBS followed by several washes in MilliQ water silver enhancement was performed for 20 min at room temperature. Slides were then washed again with MilliQ water followed by several rinses in PBS. The second directly labeled fluorescent antibody, CCL20/MIP-3α, was diluted 1:25 in PBS and incubated overnight on the sections at room temperature. The sections were mounted using Mowiol and then analyzed by confocal microscopy using a Carl Zeiss MicroImaging, Inc. LSM 510 confocal fluorescence microscope in fluorescence and brightfield mode.

Results

Accumulation of CCR6-expressing $CD1a^+$ Cells in LCH Lesions. To investigate whether LCs in LCH lesions are in an arrested state of activation and/or differentiation, the expression of particular chemokine receptors known to be characteristic of different stages of DC maturation, namely CCR6 (marker of immature DCs) and CCR7 (marker of mature DCs) were studied. For this analysis double immunofluorescent staining of CD1a and CCR6 as well as CD1a and CCR7 was performed. In all the LCH tissues studied double staining of CCR6 and CD1a on the same cells was consistently found irrespectively of the site of the lesion (Fig. 1 A). In contrast, expression of CCR7 was not observed on the $CD1a^+$ cells in these lesions (Fig. 1 B).

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

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

Expression of Other Inflammatory Chemokines by Lesional $CD1a^+$ Cells. To determine whether the recruitment of various inflammatory cell types characteristic of LCH le-

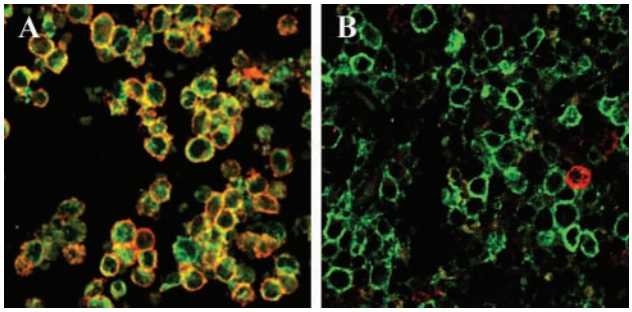

Figure 1. Expression of CCR6 but not CCR7 by LCH $CD1a^+$ cells. Immunofluorescence staining of a representative LCH bone lesion using antibodies specific for CD1a (green), CCR6 (red), and CCR7 (red). Double immunofluorescent staining shows that all the $CD1a^+$ cells are positive for CCR6, which appear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

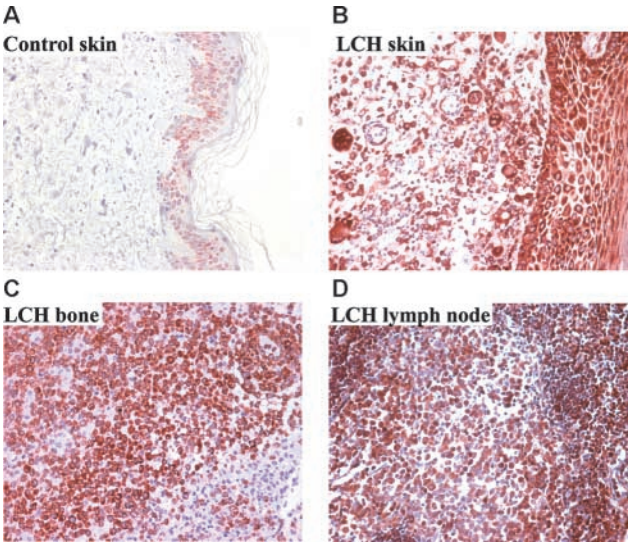

Figure 2. High expression level of CCL20/MIP-3α in LCH lesions. Immunohistochemistry was performed with an anti-hCCL20/MIP-3α monoclonal antibody and NovaRed detection. CCL20/MIP-3α was weakly expressed by epidermal keratinocytes in normal skin (A) in contrast to LCH skin lesions where CCL20/MIP-3α expression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

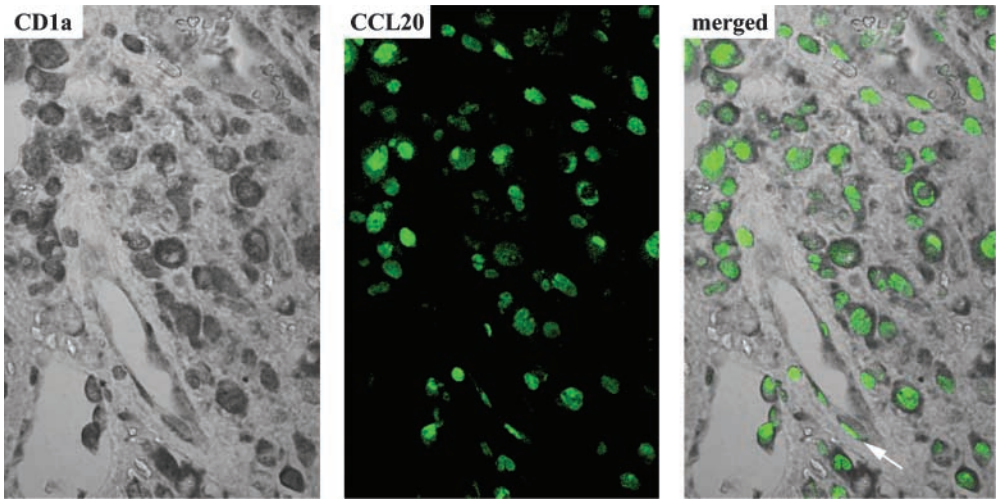

Figure 3. Expression of CCL20/MIP-3α by CD1a$^+$ cells in LCH lesions. Immunohistochemistry was performed using antibodies specific for CD1a and CCL20/MIP-3α. The CD1a was detected by an immunogold/silver method (black) and the CCL20/MIP-3α by immunofluorescence (green). The merged image shows the same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

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

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

Discussion

LCH is a disease characterized by the abnormal accumulation and retention of cells with a LC-like phenotype at various tissue sites. LCH cells do not acquire typical dendritic-like processes and their phenotype reflects only partial maturation when compared with the normal DC life

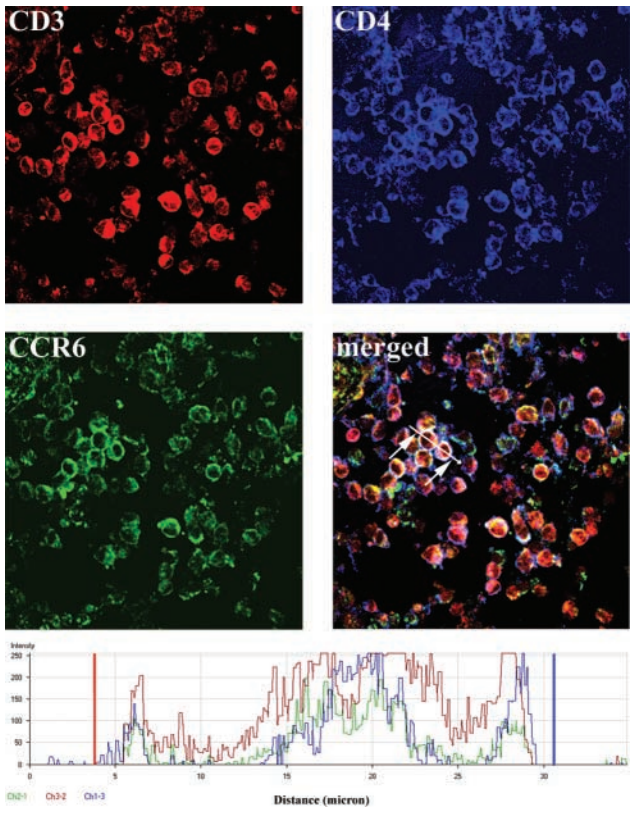

Figure 4. Lesional CD4$^+$ T cells express CCR6. Triple immunofluorescent staining on a representative LCH bone lesion for CD3 (red), CD4 (blue), and CCR6 (green). The intensity profile measured between the arrows demonstrates on two representative cells the three different fluorescent labels. Original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

cycle. Due to the integral role that migration plays in the normal functioning of DCs at their distinct stages of maturation we hypothesized that inappropriate expression and/or function of chemokine receptors on the lesional $CD1a^+$ cells may help explain the pathophysiology of this disease. As the presence of $CD1a^+$ cells uniquely define these lesions it can be speculated that the accumulation of other lesional cells is secondary to that of the presence of aberrant $CD1a^+$ cells.

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

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

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

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

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