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

Introduction

Langerhans Cell Histiocytosis (LCH), a rare disorder often presenting during childhood, is uniquely characterized by a clonal proliferation of CD1a+ dendritic Langerhans cells (LCs) (1). Signs and symptoms of LCH can be explained by the existence of the granulomatous lesions, not only present in skin or lymph node, where LCs normally reside, but also in many other sites like bone marrow, lung and liver (2). Other inflammatory cells may also accumulate within the lesions, such as eosinophils, T cells and macrophages. Particularly the described lesional “cytokine storm” with LCH cells and T cells as major producers are accountable for the more systemic symptoms like fever, failure to thrive, as well as for the well-known sequellae like osteolysis and fibrosis leading to organ dysfunction (3). Despite the rarity of this disease, with an annual incidence in the pediatric age range estimated at 2-5 per 106/ year, studies on LCH should help contribute to our understanding of human in vivo dendritic cell (DC) biology.

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

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

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

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

Materials and Methods

Tissue

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

**Reagents**

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

**Immunohistochemistry**

Paraffin sections were cut at 4 μm and placed onto aminopropyltriethoxysilane coated slides. The sections were dried overnight at 37°C, dewaxed, and rehydrated. Endogenous peroxidase was blocked using methanol/0.3% H2O2 for 20 minutes. The sections were then subjected to heat mediated antigen retrieval in a microwave using either citrate buffer (10 mM, pH 6.0) or EDTA buffer (1 mM, pH 8.0).

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

**Immunogold Labelling**

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

Table 1. Technical details of antibodies used in immunohistochemical study

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Results

Accumulation of CCR6-expressing CD1α+ cells in LCH lesions
To investigate whether LCs in LCH lesions are in an arrested state of activation and/or differentiation,
the expression of particular chemokine receptors known to be characteristic of different stages of DC
maturation, namely CCR6 (marker of immature DC) and CCR7 (marker of mature DC) were studied.
For this analysis double immunofluorescent staining of CD1a and CCR6 as well as CD1a and CCR7
was performed. In all the LCH tissues studied double staining of CCR6 and CD1a on the same cells was consistently found irrespectively of the site of the lesion (Figure 1 A). In contrast, expression of CCR7 was not observed on the CD1a+ cells in these lesions (Figure 1 B).

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

Due to the expression of CCR6 by CD1a cells in LCH lesions the presence of its cognate ligand, CCL20/MIP-3α, in the affected tissues was investigated. First, single enzymatic staining for CCL20/MIP-3α on normal control skin was carried out. As previously reported, the epidermis showed weak CCL20/MIP-3α expression by keratinocytes (Figure 2 A). However, the same staining procedure on LCH skin lesions revealed an increased level of CCL20/MIP-3α immunoreactivity, not only in the epidermis but also in the dermal region (Figure 2 B). This marked expression of CCL20/ MIP-3α staining was also consistently found in LCH bone and lymph node lesions (Figures 2 C and D). The pattern of CCL20/MIP-3α staining displayed in LCH lesions appeared to closely match the distribution of the lesional CD1a+ cells. In order to evaluate CCL20/MIP-3α expression by the lesional CD1a+ cells, a double staining was performed using immunogold labelling followed by silver enhancement to detect the CD1a+ cells in combination with immunofluorescent staining for CCL20/MIP-3α. As shown in Figure 3 this immunostaining of LCH lesions consistently showed expression of CCL20/MIP-3α by CD1a+ cells. Most of the CCL20/MIP-3α up-regulation could thus be attributed to the CD1a+ cells themselves.

![Figure 1](image-url) 

**Figure 1.** Expression of CCR6 but not CCR7 by LCH CD1a+ cells. Immunofluorescence staining of a representative LCH bone lesion using antibodies specific for CD1a (green), CCR6 (red) and CCR7 (red). Double immunofluorescent staining shows that all the CD1a+ cells are positive for CCR6, which appear yellow in the merged image (A). In contrast CCR7 is negative on the CD1a+ cells (B). Original magnification 400x.
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 250x.

Expression of other inflammatory chemokines by lesional CD1a+ cells

To determine whether the recruitment of various inflammatory cell types characteristic of LCH lesions could be explained by the production of chemokines by the CD1a+ cells, we investigated the expression of particular chemokines associated with the infiltration of other lesional cells. Besides CCL20/MIP-3α, prominent expression of the inflammatory chemokines CCL5/RANTES and CXCL11/I-TAC was found in all lesions studied. Similar to the CCL20/MIP-3α staining the pattern of CCL5/RANTES and CXCL11/I-TAC expression appeared to closely match the distribution of the lesional CD1a+ cells (unpublished data). As eosinophils are an important infiltrating population in LCH lesions, the expression of CCL5/RANTES seems relevant as this chemokine is known to be a potent activator of not only eosinophil chemotaxis but also eosinophil effector function. CXCL11/I-TAC on the other hand is a well-known chemotactic agent for IL-2 activated memory T cells expressing CXCR3.
Figure 3. Expression of CCL20/MIP-3α by CD1a+ cells in LCH lesions. Immunohistochemistry was performed using antibodies specific for CD1a and CCL20/MIP-3α. The CD1a was detected by an immunogold/silver method (black) and the CCL20/MIP-3α by immunofluorescence (green). The merged image shows the same cells positive for CD1a and CCL20/MIP-3α. Note: the arrow points to endothelial cells expressing CCL20/MIP-3α. Original magnification 400x.

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

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

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

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

Although it is now clear that lesional CD1a+ cells in vivo remain in an immature state, it has been shown that in vitro CD1a+ LCH cells could differentiate toward mature DCs in response to CD40 triggering (16). This raises the question then why in vivo are these CD1a+ cells not responding to inflammatory maturation signals, such as TNF-α which are abundantly expressed in LCH lesions? In the present study it was also shown that CD1a+ cells are the probable source of up-regulated CCL20/MIP-3α production in all LCH lesions studied. It is now known that CCL20/MIP-3α expression is under the direct control of TNF-α signalling (20, 21). Thus, these appear to be conflicting observations which will require in vitro experiments to elucidate whether the failure to up-regulate CCR7 is due to a signalling defect by inflammatory cytokines or due to conflicting cytokine signals e.g., IL-10 as suggested by Geissman et al. (16). Evidence showing signalling defects would be more supportive of an aberrant/malignant phenotype of the CD1a+ cells underlying the disease which has been suggested by groups who have shown clonality and proliferation to be present in LCH lesions (22-24). Alternatively, conflicting signalling would be more supportive of a reactive disease.
Although the etiology of LCH is not clear, certainly the CD1a+ cells are capable of maintaining and progressing the disease. In the present study it was shown that lesional CD1a+ cells express not only CCL20/MIP-3α but other inflammatory chemokines such as CCL5/RANTES and CXCL11/I-TAC. These chemokines are the likely factors responsible for the recruitment of other inflammatory cell types characteristic of LCH lesions. Although the presence of T cells in all LCH lesions is a striking feature, the mechanism by which these T cells are recruited has not so far been addressed. Most of the T cells surround the lesions in the reactive ‘rim’, however a few are present within most active lesions (25). To date there has been little information on the in situ characterisation of these T cells. The present study has now clearly shown that the T cells in LCH lesions mainly comprise CD4+ CD45RO+ T cells with very few CD8+ T cells. Furthermore, most of the lesional T cells express CXCR3 which is known to be closely related to cell-mediated immunity (Th1-type immune response). This memory/activated phenotype found on the lesional T cells fits with previous findings of CD154 on these cells (26). Immunohistochemical analysis consistently detected expression of the CCL20/MIP-3α receptor, CCR6, on the infiltrating T cells in LCH lesions. This finding along with the fact that it has already been shown that CCL20/MIP-3α specifically attracts the memory subset of T cells in vitro (27), strongly implicates CCL20/MIP-3α as an important chemoattractant responsible for T cell recruitment in LCH lesions. Thus the lesional CD1a+ cells, the probable source of CCL20/MIP-3α in the LCH lesions, are not only causing their own recruitment and retention but that of other inflammatory cells as well.

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

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References


