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Increased Blood Myeloid Dendritic Cells and Dendritic Cell-Poietins in Langerhans Cell Histiocytosis

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Langerhans cell histiocytosis (LCH), previously known as histiocytosis X, is a reactive proliferative disease of unknown pathogenesis. Current therapies are based on nonspecific immunosuppression. Because multiple APCs, including Langerhans cells and macrophages, are involved in the lesion formation, we surmised that LCH is a disease of myeloid blood precursors. We found that lin− HLA-DR+CD11c+ precursors of dendritic cells, able to give rise to either Langerhans cells or macrophages, are significantly (p = 0.004) increased in the blood of LCH patients. The analysis of serum cytokines in 24 patients demonstrated significantly elevated levels of hemopoietic cytokines such as fms-like tyrosine kinase ligand (FLT3-L, a dendritic cell-mobilizing factor, ~2-fold) and M-CSF (~4-fold). Higher levels of these cytokines correlated with patients having more extensive disease. Serum levels of FLT3-L and M-CSF were highest in high risk patients with extensive skin and/or multisystem involvement. Finally, patients with bone lesions had relatively higher levels of M-CSF and of stem cell factor. Thus, early hemopoietic cytokines such as FLT3-L, stem cell factor, and M-CSF maybe relevant in LCH pathogenesis and might be considered as novel therapeutic targets. The Journal of Immunology, 2005, 174: 3067–3071.

Materials and Methods

Patient characteristics

Peripheral blood specimens and clinical information from 24 patients with LCH (23 pediatric and 1 adult patient) were obtained after receiving written consent on a protocol approved by the local committee for use of human subjects in research (IRB no. H-6927). All LCH patients were treated at the Texas Children’s Cancer Center and evaluated by K. McClain. The status of their disease and time of specimen relative to treatments are given in Table I. This pilot study was done using archival, retrospective samples. Serum samples from 21 patients and PBMC samples from 10 patients were available for analysis. In some of the patients, blood could be collected at different time points in the course of disease (Table I). Controls were either adult HVs (n = 19) or children visiting the clinic for the reasons other than autoimmunity or infectious disease (n = 15; IRB no. 0199017).

Quantitation of blood DC precursors by flow cytometry

PBMCs were isolated by Ficoll and frozen in 10% DMSO. Thawed PBMCs were analyzed in batches by flow cytometry using the following fluorochrome-conjugated anti-human Abs: LINEAGE-FITC mixture (containing CD3, CD14, CD16, CD19, CD20, and CD56), CD123-PE, HLA-DR-PerCP, and CD11c-APC. Samples were acquired on a FACScan Calibur and analyzed with CellQuest software (BD Biosciences). mDC and pDC subsets were defined by simultaneous lack of lineage markers, HLA-DR, and CD11c.
**Table I. Patient characteristics and serum cytokine levels**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time from Orig. Dx</th>
<th>Dx Type</th>
<th>Dx Status</th>
<th>ON/OFF Rx</th>
<th>FLT3-L</th>
<th>M-CSF</th>
<th>SCF</th>
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<tbody>
<tr>
<td>1</td>
<td>1 yr</td>
<td>Pitu.</td>
<td>Stable</td>
<td>OFF</td>
<td>84.5</td>
<td>1356</td>
<td>626</td>
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<tr>
<td>2</td>
<td>1.5 yr</td>
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<td>R</td>
<td>OFF</td>
<td>207</td>
<td>1965</td>
<td>2000</td>
</tr>
<tr>
<td>3</td>
<td>7 yr</td>
<td>B/LN</td>
<td>R</td>
<td>ON</td>
<td>99.5</td>
<td>1108</td>
<td>1047</td>
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<td>4</td>
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<td>R</td>
<td>ON</td>
<td>125</td>
<td>523</td>
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<td>5</td>
<td>3 yr</td>
<td>MSD</td>
<td>P</td>
<td>OFF</td>
<td>85</td>
<td>1071</td>
<td>498</td>
</tr>
<tr>
<td>6</td>
<td>7 yr</td>
<td>MFB</td>
<td>R</td>
<td>OFF</td>
<td>308</td>
<td>368</td>
<td>831</td>
</tr>
<tr>
<td>7</td>
<td>0.75 yr</td>
<td>MSD</td>
<td>P</td>
<td>OFF</td>
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<td>4544</td>
<td>473</td>
</tr>
<tr>
<td>8A</td>
<td>1.25 yr</td>
<td>MSD</td>
<td>R</td>
<td>OFF</td>
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<td>1722</td>
<td>646</td>
</tr>
<tr>
<td>8B</td>
<td>1.5 yr</td>
<td>MSD</td>
<td>P</td>
<td>ON</td>
<td>32</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>8C</td>
<td>2 yr</td>
<td>MSD</td>
<td>R</td>
<td>ON</td>
<td>25</td>
<td>1462</td>
<td>332</td>
</tr>
<tr>
<td>9A</td>
<td>6 yr</td>
<td>MFB</td>
<td>R</td>
<td>ON</td>
<td>64</td>
<td>803</td>
<td>239</td>
</tr>
<tr>
<td>9B</td>
<td>7.5 yr</td>
<td>B</td>
<td>R</td>
<td>ON</td>
<td>130</td>
<td>258</td>
<td>247</td>
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<tr>
<td>9C</td>
<td>7.75 yr</td>
<td>B/LN</td>
<td>R</td>
<td>ON</td>
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<tr>
<td>9D</td>
<td>7.9 yr</td>
<td>B</td>
<td>R</td>
<td>ON</td>
<td>404</td>
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<td>284</td>
</tr>
<tr>
<td>10</td>
<td>7 yr</td>
<td>R</td>
<td>R</td>
<td>ON</td>
<td>20</td>
<td>382</td>
<td>287</td>
</tr>
<tr>
<td>11A</td>
<td>0.75 yr</td>
<td>MSD</td>
<td>P</td>
<td>ON</td>
<td>16</td>
<td>2071</td>
<td>249</td>
</tr>
<tr>
<td>11B</td>
<td>1.2 yr</td>
<td>MSD</td>
<td>Pitu.</td>
<td>R</td>
<td>28</td>
<td>839</td>
<td>366</td>
</tr>
<tr>
<td>12</td>
<td>2.5 yr</td>
<td>B</td>
<td>R</td>
<td>OFF</td>
<td>42</td>
<td>864</td>
<td>2000</td>
</tr>
<tr>
<td>13</td>
<td>7 yr</td>
<td>R</td>
<td>R</td>
<td>OFF</td>
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<td>950</td>
<td>552</td>
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<tr>
<td>14A</td>
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<td>DI</td>
<td>R</td>
<td>OFF</td>
<td>91</td>
<td>1681</td>
<td>261</td>
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<tr>
<td>14B</td>
<td>10.3 yr</td>
<td>B</td>
<td>R</td>
<td>R</td>
<td>56</td>
<td>614</td>
<td>379</td>
</tr>
<tr>
<td>15</td>
<td>2 mo</td>
<td>MFB</td>
<td>N</td>
<td>ON</td>
<td>37</td>
<td>416</td>
<td>327</td>
</tr>
<tr>
<td>16</td>
<td>2 yr</td>
<td>B</td>
<td>R</td>
<td>OFF</td>
<td>59</td>
<td>586</td>
<td>299</td>
</tr>
<tr>
<td>17A</td>
<td>1.2 yr</td>
<td>MSD</td>
<td>P</td>
<td>ON</td>
<td>147</td>
<td>1385</td>
<td>757</td>
</tr>
<tr>
<td>17B</td>
<td>2 yr</td>
<td>MSD</td>
<td>P</td>
<td>ON</td>
<td>121</td>
<td>742</td>
<td>693</td>
</tr>
<tr>
<td>17C</td>
<td>2.2 yr</td>
<td>MSD</td>
<td>O</td>
<td>ON</td>
<td>128</td>
<td>128</td>
<td>118</td>
</tr>
<tr>
<td>18</td>
<td>1 yr</td>
<td>MSD</td>
<td>R</td>
<td>ON</td>
<td>150</td>
<td>3036</td>
<td>421</td>
</tr>
<tr>
<td>19</td>
<td>3 mo</td>
<td>B</td>
<td>N</td>
<td>OFF</td>
<td>94</td>
<td>333</td>
<td>218</td>
</tr>
<tr>
<td>20</td>
<td>6 yr</td>
<td>B/SK</td>
<td>R</td>
<td>OFF</td>
<td>93</td>
<td>826</td>
<td>688</td>
</tr>
<tr>
<td>21A</td>
<td>2.75 yr</td>
<td>B/SK</td>
<td>R</td>
<td>OFF</td>
<td>197</td>
<td>774</td>
<td>508</td>
</tr>
<tr>
<td>21B</td>
<td>3.75 yr</td>
<td>B/SK</td>
<td>H</td>
<td>ON</td>
<td>135</td>
<td>491</td>
<td>330</td>
</tr>
</tbody>
</table>

* D, diagnosis disease type and status at diagnosis, treatment status at the time of serum analysis. Disease type: B, bone; Pitu, pituitary; SK, skin; MFB, multifocal bone; DI, diabetes insipidus; MSD, multisystem disease. Disease status: Rx, treatment; N, new; P, persistent; R, recurrent; O, no evidence of disease; H, healing. Chemotherapy: (Chemo), M, methotrexate; AR, arabinoside i.v.; Vc, vincristine i.v.; AR = cytarabine i.v.; CSA, cyclosporine oral; M, methotrexate i.v.; VP, VP-16 (etoposide) i.v.; TH, thalidomide oral. A, B, and C: consecutive serum samples from the same patient.

*flt3-l, m-csf, and stem cell factor (SCF)*

Quantification was conducted with ELISA using respective immunoassay kits (R&D Systems). Standards and undiluted serum samples were run in duplicate wells for each immunoassay. Serum samples were pipetted into the wells of the microplate (precoated with specific mAbs). After washing, HRP-linked specific polyclonal Abs were added to the wells. After another wash, a substrate solution consisting of both hydrogen peroxide and tetramethylbenzidine was added to the wells. This reaction was then stopped with sulfuric acid. The OD was then measured at 450 nm with a microplate reader (with the correction wavelength set at 540 nm). A four-parameter logistic standard curve was generated from the standards, and mean concentrations were then calculated in picograms per milliliter.

**Results**

**LCH patients show increased blood mDCs**

Given the predominant LC and MΦ phenotype in LCH lesions, we surmised that the major alterations in LCH may be at the level of circulating myeloid DC precursors. To this end, we have analyzed the frequency of DC subsets in the blood in a cohort of seven LCH patients. pDCs and mDCs were identified in whole blood as lin-CD123 or CD11c, respectively (Fig. 1a). We found increased frequency of lin- HLA-DR+CD11c+ cells expressing either CD123 or CD11c, respectively (Fig. 1a). A two-parameter logitistic standard curve was generated from the standards, and mean concentrations were then calculated in picograms per milliliter.

**LCH patients show increased serum levels of FLT3-L**

We have previously shown that FLT3 ligand (FLT3-L) mobilizes both DC subsets upon repeated administration (15). Thus, increased serum levels of FLT3-L could explain increased numbers of circulating DCs. Indeed, LCH patients showed significantly elevated (p < 0.05) serum levels of FLT3-L as compared with control cohorts, either pediatric or adult HVs (Fig. 2 and Table I). The median serum concentration of FLT3-L in samples from LCH patients was 91 pg/ml (range, 1–404; n = 31). Adult and pediatric controls showed a median of 0.23–2; n = 10), pediatric controls showed a median of 0.56% (range, 0.23–2; n = 10), and the median of adult controls was 0.33% (range, 0.21–0.44; n = 8). These results suggested that LCH sera may contain a factor(s) that mobilizes DC and/or their precursors.

**LCH patients show increased serum levels of M-CSF**

Because LCH lesions are also characterized by the infiltration of MΦ and eosinophils, we have analyzed serum levels of M-CSF and SCF, two cytokines involved in the mobilization and differentiation of these cells. As shown in Fig. 3 and Table I, LCH patients displayed significantly higher levels of serum M-CSF (p < 0.0001) than did healthy adult controls with median serum concentration of 803 pg/ml (range, 20–4544; n = 31) and 50 pg/ml (range: 0–153; n = 15), respectively. When compared with the median of pediatric and/or adult controls, 11 samples from LCH patients showed >2-fold increased serum concentration of FLT3-L. Thus, increased FLT3-L may mobilize blood mDCs leading to increased influx of these cells into LCH lesions.
concentration was not significantly affected (data not shown). Altogether, these results suggest that early hemopoietic cytokines, such as FLT3-L and M-CSF, which are involved in DC/MΦ differentiation, may play a role in LCH etiopathogenesis.

**Serum levels of FLT3-L, M-CSF, and SCF correlate with disease activity**

We next analyzed whether serum cytokines would correlate with clinical status and disease activity. Indeed, in the cohort of patients in whom serial specimens could be analyzed (patients 8, 9, 11, 14, 17, and 21), a decline in levels of all three cytokines was noted when the LCH responded to therapy (Table I). Moreover, the appearance of new lesions was accompanied by elevations of each cytokine (patients 8 and 9). When patients were categorized according to the type of disease (single or multiple bone lesions, bone and skin or lymph node, or multisystem LCH), several patterns of cytokine expression were evident (Fig. 4). Thus, M-CSF and SCF levels were lower in patients with bone disease only, whether in single or multiple lesions. However, M-CSF levels were markedly increased in patients who, in addition to bone disease, also showed skin or lymph node involvement (Fig. 4). These patients also showed markedly increased levels of FLT3-L. Patients with multisystem LCH were the most ill, having large livers...
and spleens in addition to bone and skin lesions. These patients had highest M-CSF levels, most particularly when they were near death (Fig. 4, patients 7, 8, 11, and 18). SCF levels were not clearly associated with disease status in the multisystem disease group.

Discussion
LCH includes a wide range of clinical presentations from a single system involvement such as the skin or bone lesions to multifocal disease involving liver, lungs, bone marrow, and the CNS. Currently, the disease is widely accepted to be a reactive process rather than a malignancy, although there is a clonal proliferation of the LC in lesions from children (22). Although the etiology of LCH is unknown, immunologic alterations at either cellular level (dysregulation of LCs) (23) or molecular level (TNF family molecules) (24) are being considered. The clinical course of LCH is unpredictable, varying from rapid progression and death to repeated recurrence and recrudescence with chronic sequelae or to spontaneous regression and resolution (1). Those patients with limited organ systems involved and no dysfunction of those systems have a very good prognosis and need minimal treatment. Patients with multisystem disease appear to benefit from nonspecific immunosuppression with cytotoxic drugs and/or steroids, either alone or in combination (26), yet mortality is ~20% for all patients and is especially high in children with involvement of the liver, of whom up to 50% die despite treatment. Our results demonstrate significantly elevated levels of DC-poietins, FLT3-L, and M-CSF, most particularly in patients having more extensive disease.

Similarly to FLT3-L and SCF, M-CSF is an early hematopoietic growth factor and shares structural homology with both SCF (27) and FLT3-L (17). M-CSF is a multipotent cytokine that controls macrophage differentiation and function (28), promotes angiogenesis (29), and is essential for osteoclast function (30). Until recently, M-CSF was not considered a DC-poietin and M-CSF-deficient (op/op) mice demonstrate normal LC differentiation (31). However, there is emerging evidence that M-CSF may play a role in LC differentiation upon contact with cutaneous fibroblasts in synergy with TGF-β1 (32, 33). Further, recent studies by Rivollier et al. show that M-CSF together with RANK-L allows in vitro trans-differentiation of immature DCs into osteoclasts (34). Thus, elevated M-CSF could explain osteolytic lesions in LCH patients via promotion of trans-differentiation of immature mDCs mobilized by FLT3-L into osteoclasts. Such model is consistent with the increased expression of M-CSF gene observed in bronchoalveolar lavage cells of a patient with pulmonary LCH (35).

Thus, DC-poietins maybe relevant in LCH pathogenesis and might be considered as novel therapeutic targets. The next question to address is why LCH patients display high serum levels of these two cytokines. Is that related to alterations at genomic level (for example, mutations in gene promoter), or at protein level (for example, alterations in regulatory feedback)? Our results warrant further investigations that may unravel LCH pathogenesis.

Disclosures
The authors have no financial conflict of interest.

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histiocytosis: serum-free conditions without granulocyte-macrophage colony-stimulating factor-deficient mice ho-
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