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**Supplementary Material**

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Cell-Specific Gene Expression in Langerhans Cell Histiocytosis Lesions Reveals a Distinct Profile Compared with Epidermal Langerhans Cells

Carl E. Allen,*† Liunan Li,* Tricia L. Peters,‡ Hon-chiu Eastwood Leung,*†§ Alexander Yu,* Tsz-Kwong Man,*† Sivashankarappa Gurusiddappa,* Michelle T. Phillips,* M. John Hicks,* Amos Gaikwad,* Miriam Merad,§ and Kenneth L. McClain*†

Langerhans cell histiocytosis (LCH) is a rare disease characterized by heterogeneous lesions containing CD207+ Langerhans cells (LCs) and lymphocytes that can arise in almost any tissue and cause significant morbidity and mortality. After decades of research, the cause of LCH remains speculative. A prevailing model suggests that LCH arises from malignant transformation and metastasis of epidermal LCs. In this study, CD207+ cells and CD3+ T cells were isolated from LCH lesions to determine cell-specific gene expression. Compared with control epidermal CD207+ cells, the LCH CD207+ cells yielded 2113 differentially expressed genes (false discovery rate < 0.01). Surprisingly, the expression of many genes previously associated with LCH, including cell-cycle regulators, proinflammatory cytokines, and chemokines, were not significantly different from control LCs in our study. However, several novel genes whose products activate and recruit T cells to sites of inflammation, including SPP1 (osteopontin), were highly overexpressed in LCH CD207+ cells. Furthermore, several genes associated with immature myeloid dendritic cells were overexpressed in LCH CD207+ cells. Compared with the peripheral CD3+ cells from LCH patients, the LCH lesion CD3+ cells yielded only 162 differentially regulated genes (false discovery rate < 0.01), and the expression profile of the LCH lesion CD3+ cells was consistent with an activated regulatory T cell phenotype with increased expression of FOXP3, CTLA4, and SPP1. Results from this study support a model of LCH pathogenesis in which lesions do not arise from epidermal LCs but from accumulation of bone marrow-derived immature myeloid dendritic cells that recruit activated lymphocytes. The Journal of Immunology, 2010, 184: 4557–4567.

Langerhans cells histiocytosis (LCH) is a potentially fatal disease characterized by invasive lesions infiltrated with multiple cell types, including CD1a+/CD207+ cells presumed to be pathologic Langerhans cells (LCs). The incidence of LCH is approximately five cases per million children and 1/10,000 live births per year (1). Approximately 30% as many adults are afflicted, although this incidence is likely underestimated (2). LCH includes a spectrum of clinical presentations from single-system involvement in skin or bone to diffuse multisystem involvement of liver, lungs, bone marrow, CNS, and other organ systems (reviewed in Refs. 3 and 4). Patients with limited organ system involvement have a very good prognosis, and patients with multisystem disease have survival rates ~80% (5). However, survival is poor in patients with high-risk multisystem disease who fail to respond to induction therapy (6). Chemotherapy for LCH is based on a lymphoma model of general immune suppression and cytotoxicity to rapidly proliferating cells (reviewed in Ref. 7).

The etiology of LCH is not known. Scientific debate has focused on LCH resulting from malignant transformation or from functional proliferation of epidermal LCs in response to external stimuli (8–12). Regardless of whether clonal proliferation is the initiating factor in LCH, the CD207+ cells from these lesions require multiple interactions with other cell types, including T cells, eosinophils, and macrophages (13). LCs will not grow in isolation in vitro or as xenografts in immune-deficient mice. The mainstay of research on LCH tissues has been immunohistochemical analysis of biopsy samples. Although this approach has been useful, it is also limited by various difficulties, such as testing multiple Abs simultaneously, variable sensitivity of Abs, inability to quantitatively interpret results, and lack of control tissues. RNA and protein studies from whole-biopsy samples are also difficult to interpret because of the heterogeneous composition of LCH lesions. To overcome some of the experimental challenges in studying LCH, we devised a robust procedure to study cell-specific gene-expression profiling in the cells that most likely contribute to pathology in LCH patients: LCs (CD207+) and T cells (CD3+).

Materials and Methods

Subjects

LCH diagnosis was established by the presence of CD1a+ or CD207+ histiocytes in clinical biopsy specimens. Samples from the 15 individuals with LCH in this study included patients with relapsed disease and high-
risk multisystem disease (Supplemental Table 1A). Control epidermal LCs were isolated from discarded skin, primarily elective circumspections, from patients <18 y of age. Control tonsil CD3 cells were isolated from discarded samples from elective tonsillectomy in patients <18 y of age. Studies were performed according to protocols approved by the Institutional Review Board of Baylor College of Medicine.

Isolation of LCs and T cells

**LCH samples.** Fifteen fresh LCH biopsy samples were collected. They were transported in RPMI 1640 media (Invitrogen, Carlsbad, CA) and processed within 24 h. All samples were processed into single-cell suspensions by passing through a 70-μm-mesh filter. Cells were washed twice with RPMI 1640 supplemented with 10% FBS and then incubated with conjugated Abs, CD207-PE (Beckman Coulter, Fullerton, CA) and CD3-FTTC (BD Biosciences, San Jose, CA) for 30 min on ice. Cells were washed again and resuspended in RPMI 1640/FBS with 2 μg/ml propidium iodide (PI; Molecular Probes, Eugene, OR). Cells were then separated by flow cytometry by gating on the PI− population and Ab-specific fluorescence. Cells were sorted with a MoFlo Sorter (Beckman Coulter) directly into PicoPure RNA Extraction Buffer (Molecular Devices, Sunnyvale, CA) (Supplemental Table IB). Select samples were reanalyzed by flow cytometry for purity (Fig. 1).

**Control skin LC samples.** Control LCs were isolated from 12 skin samples that were transported in RPMI 1640 media and processed within 24 h. Tissue was incubated in RPMI 1640 with 5 U/ml dispase II (Roche, Indianapolis, IN); incubation times were 8 and 16 h for the 12 and 8 control LCs respectively. The epidermal layer was further treated with 0.25% trypsin-EDTA (Invitrogen) for 15 min at 37˚C and then LCs were isolated with CD207-PE-conjugated Ab (Beckman Coulter), as described above. Patient details from the individual skin samples were not available. However, review of elective circumspections performed at Texas Children’s Hospital in an operating room shows a range of ages from 1 mo to 18 y. Fifteen fresh LCH biopsy samples were collected. They were transported in RPMI 1640 media (Invitrogen, Carlsbad, CA) and processed within 24 h. Tissue was incubated in RPMI 1640 with 5 U/ml dispase II (Roche, Indianapolis, IN); incubation times were 8 and 16 h for the 12 and 8 control LCs respectively. The epidermal layer was further treated with 0.25% trypsin-EDTA (Invitrogen) for 15 min at 37˚C and then LCs were isolated with CD207-PE-conjugated Ab (Beckman Coulter), as described above. Patient details from the individual skin samples were not available. However, review of elective circumspections performed at Texas Children’s Hospital in an operating room shows a range of ages from 1 mo to 18 y.

**Peripheral T cells.** Peripheral T cells were isolated from seven patients with active LCH prior to chemotherapy (Supplemental Table I). Peripherial blood was collected and stored in EDTA tubes and processed within 24 h. PBMCs were isolated after centrifuging blood over a Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient at 450 g for 30 min. PBMCs were washed twice in RPMI 1640 and then T cells were isolated with CD3-FTTC (BD Biosciences)-conjugated Abs, as described above.

**Tonsil T cells.** T cells were isolated from 20 tonsil samples from children who underwent elective tonsillectomy. Tonsils were processed in an identical fashion to the LCH lesions, and CD3+ cells were isolated by flow cytometry by gating on the PI− population and Ab-specific fluorescence. Cells were sorted with a MoFlo Sorter (Beckman Coulter) directly into PicoPure RNA Extraction Buffer (Molecular Devices, Sunnyvale, CA) (Supplemental Table IB). Select samples were reanalyzed by flow cytometry for purity (Fig. 1).

RNA purification and CDNA amplification

Total RNA was processed and cDNA was amplified for 13 LCH CD207 samples, 12 control CD207 samples, 12 LCH lesion CD3 samples, 7 LCH peripheral CD3 samples, and 4 pooled tonsil CD3 samples (5 individual RNA samples/tonsil pool). Total RNA was processed from the sorted cells, according to the PicoPure RNA Isolation Kit protocol (Molecular Devices). RNA concentration and quality were verified using the 6000 Pico Chip according to the PicoPure RNA Isolation Kit protocol (Molecular Devices). Total RNA was processed from the sorted cells, according to the PicoPure RNA Isolation Kit protocol (Molecular Devices). RNA concentration and quality were verified using the 6000 Pico Chip according to the PicoPure RNA Isolation Kit protocol (Molecular Devices).

Quantitative real-time PCR

Real-time PCR reactions were performed with TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA), which include a mix of two unlabeled PCR primers (900 nM final concentration) and 1 FAM dye-labeled TaqMan MGB Probe (250 nM final concentration). Single-stranded cDNA was generated by RNA amplification, as described above, in experiments independent from the amplifications used to generate cDNA probe for microarray studies. For the CD207+ analysis, one cDNA pool was made from equal contributions from the 13 LCH CD207+ samples and another from equal contributions from the 12 control CD207+ samples. For the CD3+ analysis, one cDNA pool was made from equal contributions from the seven peripheral LCH CD3+. For the CD3+ analysis, one cDNA pool was made from equal contributions from the seven peripheral LCH CD3+ samples and another from equal contributions from the seven matched LCH lesion CD3+ samples. Each reaction included 20 ng cDNA. TaqMan Fast Universal PCR Master Mix (Applied Biosystems) was used in 25-μl reactions in 96-well plates on a qT5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Assays were plated in triplicate in two independent experiments. Thermal cycling conditions were set at 2 min at 50˚C and 10 min at 95˚C and then 40 cycles of 95˚C and 60˚C for 1 min. Relative mRNA levels were determined using standard ΔΔ Ct calculations, with expression levels of experimental samples normalized to GAPDH (18). TaqMan probe sets (Applied Biosystems) included AFF3, Hs00289335_s1; CCL5, Hs01475757_m1; CCR1, Hs00174298_m1; CD36, Hs01567888_g1; CD74, Hs02069961_m1; CD80, Hs00170423_m1; CD83, Hs01546812_m1; HOXB7, Hs00270131_m1; HSPA1A, Hs00359147_s1; IL-17A, Hs00174383_m1; IL-2, Hs00174114_m1; IL-8, Hs00174103_m1; MMP1, Hs00899660_g1; MMP9, Hs00234579_m1; NR1P1, Hs00821628_m1; PEP, Hs00751717_s1; S100A7, Hs00162488_m1; S100A8, Hs00374263_m1; SMYD3, Hs00225208_m1; SPP1, Hs00959010_m1; TACSTD1, Hs01058908_m1; TNFRSF9, Hs00155512_m1; and VNN1, Hs01546812_m1.

Immunohistochemistry

Fresh LCH biopsy tissue, skin samples, and tonsil samples were snap-frozen in embedding medium (Tissue-Tek OCT Compound; Sakura Finetech USA, Torrance, CA) for tissue specimen and stored at −80˚C within 8 h from the time of collection. Tissue was cut at a thickness of 4–8 μm from frozen blocks and adhered to microscope slides. Slides were dried at −80˚C until use. After removal from acetone, slides were air-dried for 30 min, and tissue was subsequently fixed by incubating slides in ice-cold acetone for 15 min. After being air-dried for 10 min, the tissue was rehydrated by incubation in PBS for 10 min. Immunohistochemical staining was performed using the Dako Envision + Dual Link Kit (Dako, Carpenteria, CA). Endogenous peroxidase was blocked using Dako Block Buffer for 15 min. Primary Abs were used in 1:50 dilution. Peroxidase reaction was detected using DAB Substrate Chromagen System. Tissue was then stained with hematoxylin, counterstained, and stored at room temperature. Abs included CD11b and CD11c were diluted 1:10. All other Abs were diluted 1:50. Mouse and rabbit Abs were detected using the previously diluted HRP-linked secondary Ab included in the kit. Goat Abs were detected using anti-goat/HRP Ab (DakoCytomation) diluted 1:100. Slides were washed three times for 5 min each in Dako Wash Buffer between incubations. HRP activity was detected using the DAB + Substrate Chromagen System. Tissue was then stained with hematoxylin, counterstained, and stored at room temperature. Abs used included CD207: mouse monoclonal – ab49730 (Abcam, Cambridge, MA); CD3: mouse monoclonal – ab6999 (Abcam); HLA-DR+DP+DQ: mouse monoclonal – sc59251 (Santa Cruz Biotechnology, Santa Cruz, CA); CTLA4: mouse monoclonal – sc1271 (Santa Cruz Biotechnology); Osteopontin: mouse monoclonal – sc65028 (Santa Cruz Biotechnology); CD207: mouse monoclonal – ab51037 (Abcam); Neutrophil1: mouse monoclonal – sc5307 (Santa Cruz Biotechnology); Vanin1: goat polyclonal – sc16776 (Santa Cruz Biotechnology); CEACAM6: rabbit polyclonal – ab56234.
(Abcam); MMP1: rabbit monoclonal – ab52631 (Abcam); MMP9: goat polyclonal – sc6840 (Santa Cruz Biotechnology); CD13: goat polyclonal – sc6995 (Santa Cruz Biotechnology); CD33: rabbit monoclonal – ab59940 (Abcam); CD1d: mouse monoclonal – ab11076 (Abcam); ICAM1: mouse monoclonal – ab20 (Abcam); CD11b: mouse monoclonal – MAB1699 (R&D Systems, Minneapolis, MN); and CD11c: mouse monoclonal – MAB1777 (R&D Systems). Images were magnified using the Olympus BX51 microscope, ×40 objective. Images were captured using the Olympus DP71 digital camera with Olympus DP Controller and DP Manager software.

**Results**

**Robustness of the gene-expression profiling procedures**

Validity of the data derived from these gene-expression profiling experiments is predicated on fidelity of the methods used to isolate cells and generate cDNA probe. Cells were sorted using fluorescent markers conjugated to CD3 and CD207 Abs using standard methods (Fig. 1). Cell purity was evaluated in several samples by sorting cells into RPMI 1640, then reanalyzing with the MoFlo

![Scatter plot of isolated cells.](http://www.jimmunol.org/)
Sorter. Purity exceeded 95% in all CD3+ and CD207+ samples tested (Fig. 1). The NuGen WT-Ovation Pico System was used to amplify RNA to generate cDNA probe for the gene-expression profiling studies. The yield of cDNA consistently ranged between 4 and 6 μg. Previous studies validated this experimental strategy (19, 20). In this series of experiments, gene-expression datasets generated from technical replicates of amplified probe from 5 ng input RNA had a correlation >90% (data not shown).

Cell-specific gene expression from LCH biopsy specimens was evaluated by comparing hybridization signals from amplified cDNA on Affymetrix gene chips (U133A Plus 2.0) (Tables I, II). Three sets of comparisons were performed: 13 LCH CD207+ cDNA on Affymetrix gene chips (U133A Plus 2.0) (Tables I, II).

Validity of the data derived from this experimental strategy is evidenced by cell-specific grouping of the datasets in dendrograms constructed with unsupervised clustering (Supplemental Fig. 1).

Cell-specific gene-expression array analyses

CD207+ cells. Analysis of global gene expression in LCH CD207 cells showed a consistent and distinct profile from control skin CD207 cells, with overall correlation <0.2. Although the LCH CD207 datasets were grouped with unsupervised clustering, the overall intergroup correlation was >30%. By comparison, the control skin CD207 cells showed less overall variability, with an intergroup correlation >50% (Supplemental Fig. 1) (excluding sample #1, which appears to be an outlier).

Expression of 2,113 genes (of >47,000) was significantly different between the LCH CD207 cells and the control CD207 cells using stringent statistical criteria (false discovery rate < 0.01). Overall, 747 probes (520 genes) showed increased expression and 2153 probes (1593 genes) showed decreased expression in the LCH CD207 cells (Supplemental Data 1). Several genes involved in cell-cycle regulation, inflammation, and LC development have been evaluated in LCH lesions and implicated in models of LCH pathogenesis. The most widely accepted current model of LCH can be described as the “Activated-Immature” Model, in which immature epidermal CD207+ LCs undergo malignant transformation or pathologic immune stimulation, and subsequently take on some properties of an activated LC, including down-regulation of anchoring molecules (E-cadherin) and increased expression of proinflammatory cytokines (TNF-α and IL-1β), chemokine receptors (CCR6 and CCR7), T cell costimulatory molecules (CD40, CD80, and CD86), and markers of cell proliferation (Ki67 and PCNA) (reviewed in Refs. 8, 10, and 21). Furthermore, several studies described the LCH lesion microenvironment as a “cytokine storm”, with expression of several proinflammatory cytokines (10, 21–24).

A comprehensive review of PubMed for genes and proteins associated with LCH pathogenesis was performed, and results were compared with cell-specific gene expression in this study (Fig. 2, Supplemental Table IIA). The results from our study supported some previous observations, including significantly decreased expression of E-cadherin and relatively increased expression of MMP9 (22, 25–28). However, the lack of differential gene expression in many molecules previously associated with LCH was surprising. In some cases, such as TNF-α and IL-1β, genes were expressed at very high levels in control skin CD207+ cells and LCH lesion CD207+ cells. For others, such as IL-2 and IL-17A, we failed to find significant levels of expression in control epidermal or LCH lesion CD207+ cells. Additionally, some genes, such as CCL20, were not found to be significant, despite high levels of expression in some LCH CD207+ cells due to highly variable expression. Specific results of differential expression of genes reportedly associated with LCH in these experiments are detailed below.

Further review of this dataset identified several genes of interest not previously associated with increased expression in LCH, including genes involved in the regulation of cell cycle (CDK2A, AF4/FMR2 [member 3], SMYD3, and HOXB7), apoptosis (BAX, BCL2L1, and CFLAR), signal transduction (DUSP4, JAK3, PRKCA, TLR2, TLR4, SOCS3, and JAG2), tumor invasion and

Table I. Comparison of real-time PCR and microarray results: LCH CD207 versus control skin CD207

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Real-Time PCR</th>
<th>Array</th>
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</thead>
<tbody>
<tr>
<td>SPP1</td>
<td>162.6</td>
<td>22.5–37.0</td>
</tr>
<tr>
<td>CEACAM6</td>
<td>76.7</td>
<td>10.1</td>
</tr>
<tr>
<td>CD2K2NA</td>
<td>73.9</td>
<td>2.1–4.9</td>
</tr>
<tr>
<td>JAK3</td>
<td>46.1</td>
<td>1.9–7.1</td>
</tr>
<tr>
<td>VNN1</td>
<td>44.0</td>
<td>5.2–7.7</td>
</tr>
<tr>
<td>SMYD3</td>
<td>34.8</td>
<td>9.3–12.4</td>
</tr>
<tr>
<td>AFF3</td>
<td>24.7</td>
<td>3.1–11.3</td>
</tr>
<tr>
<td>HOXB7</td>
<td>20.7</td>
<td>6.6–6.8</td>
</tr>
<tr>
<td>DUSP4</td>
<td>15.7</td>
<td>3.4–10.3</td>
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<td>MMP9</td>
<td>15.5</td>
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</tr>
<tr>
<td>MMP1</td>
<td>11.5</td>
<td>7.2</td>
</tr>
<tr>
<td>CCR1</td>
<td>8.6</td>
<td>4.5–5.7</td>
</tr>
<tr>
<td>CCL5</td>
<td>8.6</td>
<td>4.6</td>
</tr>
<tr>
<td>NR1P1</td>
<td>4.8</td>
<td>4.5–9.5</td>
</tr>
<tr>
<td>TNFRSF9</td>
<td>4.6</td>
<td>5.8–6.4</td>
</tr>
<tr>
<td>DCAL1</td>
<td>3.2</td>
<td>5.1–6.6</td>
</tr>
<tr>
<td>CD36</td>
<td>−28.1</td>
<td>(1.4–14.4)</td>
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<tr>
<td>S100A8</td>
<td>−20.4</td>
<td>(22.5–26.2)</td>
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<tr>
<td>EpCAM</td>
<td>−20.4</td>
<td>38.1</td>
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<tr>
<td>CDH1</td>
<td>−269.6</td>
<td>(52.2–123.6)</td>
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<tr>
<td>PERP</td>
<td>−1,172</td>
<td>(3.9–227.5)</td>
</tr>
<tr>
<td>S100A7</td>
<td>−14,201</td>
<td>(1.7–74)</td>
</tr>
<tr>
<td>IL-2</td>
<td>NA</td>
<td>1.0</td>
</tr>
<tr>
<td>IL-17</td>
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<td>1.1</td>
</tr>
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Table II. Comparison of real-time PCR and microarray results: LCH lesion CD3 versus LCH peripheral CD3

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Real-Time PCR</th>
<th>Array</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP1</td>
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<td>IL-8</td>
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<td>21.6–45.7</td>
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<tr>
<td>MMP9</td>
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<td>43.6</td>
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<tr>
<td>HSPA</td>
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</tr>
<tr>
<td>FOXP3</td>
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<td>6.7</td>
</tr>
<tr>
<td>HLADDR</td>
<td>18.9</td>
<td>11.6–22.0</td>
</tr>
<tr>
<td>CTLA4</td>
<td>11.4</td>
<td>7.5–13.4</td>
</tr>
<tr>
<td>DUSP4</td>
<td>10.3</td>
<td>6.7–54.6</td>
</tr>
<tr>
<td>CD74</td>
<td>5.7</td>
<td>5.2–6.2</td>
</tr>
</tbody>
</table>

NA, no PCR product was amplified in the real-time PCR reaction, suggesting the absence of target template for IL-2 and IL-17.
metastasis/tissue invasion (CEACAM6, MMP1, and TGFBI), myeloid cell maturation (CD11D, CD13, CD14, CD33, ITGA2B, ITGAX, ITGAM, and CD300F), and lymphocyte trafficking (SPP1, VNN1, NR1P1, CCR1). A large number of the genes with decreased or no expression in the LCH CD207 cells are involved in cell–cell adhesion, including TACSTD1 (64). PERP, an apoptosis effector, is one of the genes with the most significantly decreased expression in LCH CD207 cells (65, 66). TIMP2, a metalloproteinase inhibitor, also has reduced expression in LCH CD207 cells (Fig. 2, Supplemental Table IIA).
CD3+ cells. Analysis of global gene expression in the LCH lesion CD3 cells also showed a consistent and distinct profile from the peripheral LCH CD3 cells and the tonsil control CD3 cells. Therefore, although lymphocytes from peripheral blood may factor into the variability of the gene-expression profiles, these results suggest that the CD3+ cells isolated from the LCH lesions primarily represent tumor-infiltrating lymphocytes. Differential T cell populations within the LCH lesions may also contribute to the variability between the LCH lesion CD3 datasets (correlation >20%) compared with the peripheral LCH CD3 datasets (correlation >70%) or the control tonsil CD3 datasets (correlation >70%) (Supplemental Fig. 1).

**FIGURE 3.** LCH-associated genes: CD3+.  
A, LCH CD3+ (LCH lesion CD3 versus LCH peripheral CD3) gene heatmap expression profile results of CD3+ cells isolated from LCH lesions (LCH tumor CD3) (right columns) and CD3+ cells isolated from peripheral blood of patients with active LCH (LCH peripheral CD3) (left columns). Genes associated with lymphocyte migration and activation had increased expression in tumor-infiltrating lymphocytes. This suggests that lymphocytes may play an active role in tumor formation and progression in LCH. Increased expression of FOXP3 and CTLA-4 specifically suggests overrepresentation of regulatory CD3+CD4+ T cells, which are thought to arise from Ag-driven activation. Osteopontin (SPP1) showed the highest relative expression in LCH CD207+ cells and LCH lesion CD3+ cells.  
B, CD3+ (LCH lesion CD3 versus tonsil CD3) gene heatmap expression profile results of CD3+ cells isolated from LCH lesions (LCH tumor CD3) (right columns) and control CD3+ cells isolated from tonsils (left columns). These results suggest that, although LCH lesion T cells may be activated, they have significantly less expression of IL-17 and IL-21, associated with acute inflammation, compared with control tonsil T cells. Genes/proteins previously associated with LCH, LCH CD3+ arrays: CCL20/MIP3α (32), CCL5/RANTES (32), CCR6 (32), CD40LG (41, 44, 58), CXCR3 (10), FOXP3 (56), GMCSF/CSF2 (10, 52, 53), HLA-DR/DQ (10, 41, 68), IFN-γ (10, 23, 24), IL-10 (10, 41, 56), IL-17A (22), IL-1β (10, 23, 24), IL-2 (10, 23, 24), IL-22 (22), IL2Ra/CD25 (56, 58), IL-3 (10, 24), IL-4 (10, 23, 24), IL-5 (10, 24), IL-6 (10), TGF-β (10, 23, 24), TNFRSF11B (58, 59), TNFSF11/RANKL (22, 58), and TNFa (10, 24, 25, 63).
Using stringent statistical criteria, 162 probe sets (129 genes) showed significant differences in hybridization between cDNA derived from CD3 cells isolated from LCH lesions and peripheral CD3 cells isolated from peripheral blood from the same patient at the time of biopsy. Overall, 117 probe sets (84 genes) had increased expression and 45 probe sets (45 genes) showed decreased expression in LCH lesion CD3 cells (Supplemental Data 2). Differentially expressed genes in this group represent several markers of T cell activation, including MHC class II genes, CD58, CD74, and HSP70. Interestingly, FOXP3, a gene specific to regulatory T cells (Tregs), was overexpressed in the LCH lesion CD3 cells, supporting a similar observation reported by other investigators (56). CTLA4, an activation marker associated with Tregs and inhibition of inflammation, was also overexpressed. LCH lesion-infiltrating T cells also significantly overexpressed several genes involved in leukocyte chemotaxis, including SPP1, IL-8, CCL3, CCL16, CCR1, CCR5, vitamin D receptor, and plasminogen activator/urokinase receptor. SPP1 had the highest relative expression in the group 1 (LCH lesion CD207 versus skin CD207) and group 2 (LCH lesion CD3 versus LCH peripheral blood CD3) datasets. DUSP4, a negative regulator of MAPK, was also highly overexpressed by CD207 cells and CD3 cells from the LCH lesions (67) (Fig. 3A, Supplemental Table IIB).

Comparing the 12 LCH lesion CD3 samples and the 4 control tonsil CD3 pooled samples, only 81 probe sets (70 genes) were significantly differentially expressed, all of which had decreased relative expression (Supplemental Data 3). Genes associated with an activated Th1 (or Th17) response, including IL-17, IL-21, and CD40L, were significantly underexpressed in LCH lesion T cells compared with control tonsil T cells (Fig. 3B).

Validation

We performed real-time PCR on independently amplified cDNA samples to validate the microarray data. Although there were some variations in the amplitude of differences in relative gene expression determined by chip hybridization and real-time PCR in the group 1 (LCH lesion CD207+ cells versus control CD207+ samples) and group 2 (LCH lesion CD3+ cells versus LCH peripheral CD3+ cells) samples, the trends were consistent. Quantitative differences between the two methodologies were likely due to signal compression with the chips at very high levels of expression and baseline fluctuation of noise at very low levels of expression (Tables I, II).

Immunohistochemistry performed on a sample of LCH-associated proteins also substantiated the gene-expression profiling data identified by the array experiments. Immunohistochemistry was performed on four LCH biopsy samples as well as normal skin and tonsil biopsy samples used as controls for Ab staining. The results were reproducible and are representative of all LCH biopsy samples tested. Protein products of all candidate genes identified by these experiments as overexpressed in LCH lesions (CD207+ or CD3+ cells) were validated by immunohistochemistry (Fig. 4). The results from this study call into question a current model of LCH pathogenesis in which the epidermal Langerhans cell is the...
cell of origin. The cell-specific gene expression data support a model in which CD207+ cells in LCH lesions arise directly from circulating myeloid DCs rather than from transformed or activated LCs (Fig. 5).

Discussion

The concept that LCH arises from epidermal LCs was first proposed by the landmark observation by Nezelof et al. (11) that the histiocytes in LCH lesions contain Birbeck granules. Subsequently, cell-surface expression of langerin (CD207), a protein associated with Birbeck granules in Ag processing in LCs, was identified as pathognomonic of normal LCs and pathological LCs in LCH (69, 70). Therefore, models of LCH have developed around the concept of aberrant activation or malignant transformation of the resident epidermal LC, as described above in the “Activated-Immature” Model (Fig. 5) reviewed in Refs. 8, 10, and 21. However, results from our cell-specific gene expression analysis prompted us to question the origin of the LCH CD207+ cell. First, global expression patterns between control epidermal LCs and LCH CD207+ cells showed low overall correlation (Supplemental Fig. 1). Furthermore, expression levels of specific genes central to the “Activated-Immature” Model were not significantly different between the two groups in our experiments.

This study is the first to establish the gene-expression profile of CD207+ cells isolated from patient LCH lesions, rather than from in vitro models derived from monocytes or monocyte-derived dendritic cells (DCs). LCH-associated genes and proteins were identified through a comprehensive literature review, and relative LCH lesion CD207+ and CD3+ expression in this study are detailed in Figs. 2 and 3 and Supplemental Table II. Differences between the gene-expression results from this study and some previously published observations may be due to the experimental limitations of immunohistochemistry, including heterogeneity of LCH lesions, variable specificity of Abs, and lack of reliable control tissues. It is also possible that tissue processing altered gene expression relative to cells that are fixed or frozen in tissue blocks.

This study was designed to identify gene-expression patterns common to all LCH lesions. Datasets from all LCH CD207+ samples clustered in a group distinct from control epidermal LCs, with no distinction between the profiles from high- and low-risk samples. Additional samples will be required to power a study to determine whether there are significant cell-specific differences in gene transcription in biopsies from different patient groups. In addition to testing previous concepts of LCH and associated markers, the experimental approach of cell-specific gene expression revealed some previously unrecognized patterns of gene expression. LCH CD207+ cells had increased expression of early myeloid markers compared with control epidermal LCs. Epidermal LCs are derived from myeloid precursors that take up residence in the skin in embryonic life but may be replenished by peripheral monocytes in inflamed skin (reviewed in Ref. 71) (Fig. 5A). Therefore, it has been difficult to understand how LCs, which are normally restricted to the epidermis, could give rise to such a multifocal disorder. We recently identified in mice a population of interstitial CD207+ (langerin+) DCs in most tissues, including the lung, liver, and lymph nodes. In contrast to LCs that are confined to the epidermis and derive from skin-resident hematopoietic precursors, interstitial CD207+ DCs are derived from blood-borne precursor cells and are present in most of the tissues affected by LCH (71, 72). Therefore,
langerin expression is inducible and not exclusive to epidermal LCs. These observations, together with the results of the LCH gene-expression studies presented in this article, suggest that LCH may not derive from abnormal LCs but rather from abnormal circulating DC precursors. The observation that plasma M-CSF levels and myeloid DC precursors (Lin^H^HLA-DR^CD11c^) are overabundant in patients with active LCH also supports bone marrow-derived precursors, rather than epidermal LCs, as the source of pathologic CD207+ cells in LCH.

We propose a model for LCH pathogenesis in which bone marrow-derived myeloid DC precursors, rather than epidermal LCs, migrate to sites of LCH lesions and differentiate into CD207+ cells (Fig. 5C). In the “Misguided Myeloid DC Precursor” model, blood-derived myeloid DCs are recruited to sites of disease where they accumulate and recruit activated T cells. Together, these cells produce mediators of inflammation and tissue destruction. These experiments do not answer the ongoing question of whether LCH arises from intrinsic defects in a transformed clonal population of precursor cells or whether it is caused by a functional response to a pathologic stimulus. Lack of differential expression of markers of proliferation supports a model in which CD207+ LCH cells arise from the accumulation of pathologic cells, which is also supported by other recent studies (56).

The role of T cells in LCH is not well understood. There is no evidence of a functional interaction between T cells and CD207+ cells in vivo in LCH (41). The results from this study showed highly variable ratios of CD207/CD3 cells in LCH lesions (Supplemental Table IB). The CD3 cells are unlikely to arise primarily from contamination of lesions at the time of biopsy because they have unique gene-expression profiles compared with peripheral blood (Supplemental Fig. 1). Several genes with significant gene expression in LCH CD207+ cells play roles in lymphocyte activation, recruitment, and DC interaction, including osteopontin, neuropilin-1, and vanin-1. Osteopontin, expressed by SPP1, also known as early T lymphocyte activation 1, had the highest differential expression in the LCH CD207+ versus control LC and infiltrating LCH CD3+ versus peripheral LCH CD3+ datasets. It is a secreted phosphoprotein with putative functions, including T cell activation, Treg differentiation, homing of macrophages and T cells, cell survival, and tumor progression (reviewed in Ref. 73). Osteopontin-deficient mice have deficient type 1 immunity to bacterial and viral infections and are unable to form granulomas (74). Osteopontin overexpression has been found in a variety of cancers, including breast cancer, lung cancer, colorectal cancer, stomach cancer, ovarian cancer, and melanoma (75). One of the major osteopontin ligands, CD44, is also significantly overexpressed in LCH CD207 cells compared with control LCs. Neuropilin-1, expressed by NRP1, is a membrane-bound coreceptor to a tyrosine kinase receptor both vascular endothelial growth factor and semaphorin molecules. Neuropilin-1 is involved in axonal guidance of nerves. In the immune system, it is important in establishing the T cell–DC synapse, as well as migration and adhesion of thymocytes (76, 77).

Tregs are thought to develop as an Ag-specific response. Similarly, activation of T cells typically arises through Ag-dependent mechanisms in vivo. The expression profile of infiltrating CD3 cells compared with circulating CD3 cells in patients with LCH is consistent with an activated phenotype, and also supports enrichment of Tregs. Therefore, the mechanisms by which T cells are activated remain to be determined. Another recent study suggested LCH arises through expression of IL-17A by pathologic LCs. In our study, IL-17A expression was not detectable in CD207 cells, as described previously (80). Furthermore, relative expression of IL-17A and IL-21 was significantly decreased in LCH lesion T cells compared with control tonsil T cells, suggesting LCH is not characterized by a Th1/Th17 response.

In conclusion, increased understanding of the biology is essential to improve the treatment of individuals with LCH. However, research in LCH is challenged by rare tissue samples and a lack of reliable in vitro or animal models. We are now able to generate cDNA probe for expression studies from as little as 1500 cells. The results showed surprisingly distinct gene-expression profiles between CD207+ cells from LCH lesions and control epidermal LCs, which suggests that the LCH CD207+ cells are highly differentiated or arise from a distinct cell population. The relative expression of immature myeloid markers from the LCH CD207+ cells further supports a model in which LCH arises from a bone marrow-derived DC precursor rather than from a transformed resident epithelial LC. This study identified several new genes that had not been associated with LCH and that may be important diagnostic markers or therapeutic targets. Genes that encode osteopontin-1, neuropilin-1, and vanin-1, all of which are involved in lymphocyte trafficking and lymphocyte–DC communication, are highly expressed in LCH CD207+ cells. In summary, we propose a new model in which LCH does not arise from epidermal LCs but from misguided myeloid DC precursors. Future studies will determine the definitive reclassification of “histiocytosis X.”

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