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

J Immunol 2010; 184:4557-4567; Prepublished online 10 March 2010;
doi: 10.4049/jimmunol.0902336
http://www.jimmunol.org/content/184/8/4557

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/03/11/jimmunol.0902336.DC1

References

This article cites 79 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/184/8/4557.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 cell 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 immunohistological 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+).
risk multisystem disease (Supplemental Table 1A). Control epidermal LCs were isolated from discarded skin, primarily elective circumscriptions, 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 TissueTronics Cell-Dispersant, a 70-μm-mesh filter. Cells were washed twice with RPMI 1640supplemented 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 Picotoro 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) at 4˚C for 8 h prior to separation of the epidermal layer. 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 circumscriptions 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). Peripheral 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-FITC (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 with CD3-FTTC Ab, as described above.

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 (Agilent, Palo Alto, CA) at the Baylor College of Medicine Microarray Facility. Any samples with a detectable RNA integrity number (14 < 5) were excluded from the study. cDNA amplification was performed with the WT-Ovation Pico System, according to manufacturer’s protocol (NuGen, San Carlos, CA). This is a whole-transcriptome amplification system with which we were able to generate 4–6 μg cDNA from 70 μg to 25 μg of input RNA. Fragmented and biotinylated cDNA samples for the gene chip studies were generated using the FL-Ovation cDNA Biotin Module v2 (NuGen).

**Affymetrix GeneChip assays**

Fragmented and biotinylated cDNA was hybridized to Affymetrix U133 Plus 2.0 chips (Affymetrix, Santa Clara, CA). cDNA was generated from each of the LCH CD207, control CD207, LCH lesion CD3, and peripheral CD3 samples and was used to hybridize onto individual chips. Four tonsil CD3 cDNA were generated, each from five pooled cDNA samples. Chips were hybridized overnight at 45˚C with 60 rpm for 16 h. The chips were processed in the fluidic station using the FS540_004 wash and stain protocol designed by the manufacturer’s instructions. The stained arrays were scanned using the Affymetrix GeneChip scanner 3000. The data files have been deposited at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo; accession number GSE16395).

**Data analysis**

Quality control of 48 Affymetrix U133 Plus 2.0 gene-expression chips was done using the BioConductor package affyQCReport (15). β-Actin and GAPDH ratios, as well as signal distribution, were assessed to determine the outlier cases. Normalization and probe set summarization was done in BRB-ArrayTools (available at http://linus.nci.nih.gov/BRB-ArrayTools.html) using the Robust Multichip Average algorithm. Hierarchical clustering was performed using centered correlation and average linkage. The Significance Analysis of Microarrays algorithm (16) was used for analysis of differential expression, with a false discovery rate < 0.01. Samples were split into three groups for analysis. Group 1 consisted of 13 CD207 tumor samples and 12 CD207 control samples obtained from normal skin. Group 2 consisted of seven CD3 LCH lesion samples and seven CD3 matched paired samples obtained from patients’ peripheral blood. Group 3 consisted of 12 CD3 LCH lesion samples and 4 CD3 pooled controls obtained from tonsil. Heatmaps and other graphics were created using Multi-Experiment Viewer, part of the TM4 Microarray Software Suite (17).

**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 unlabelled 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 LCH CD207− samples. 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 iQ5 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 AFFP, Hs00289335_s1; CCL5, Hs0017457551_m1; CCR1, Hs00174298_m1; CD36, Hs01567188_g1; CD74, Hs00269961_m1; CD44, Hs01027785_m1; FOXP3, Hs00155512_m1; GAS5, Hs00514869_m1; DUSP4, Hs01027785_m1; FOXP3, Hs00203958_m1; GAPDH, Hs0027589911_g1; HLA-DRA, Hs00219578_m1; HOXB7, Hs00207013_m1; HSPA1A, Hs00359147_s1; IL-17A, Hs00174338_m1; IL-2, Hs00174141_m1; IL-8, Hs00174103_m1; MMP1, Hs00899660_g1; MMP9, Hs00234579_m1; NRP1, Hs00082612_m1; PERP, Hs00751717_s1; S100A7, Hs01062488_m1; S100A8, Hs00374263_m1; SMYD3, Hs00253208_m1; SPP1, Hs00950910_m1; TACSTD1, Hs01058950_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 frozen tissue specimens and stored at −80˚C with 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 air-dried at −80˚C until use. After removal from storage, 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, Carpinteria, CA). Endogenous peroxidase was blocked using Dako Block Buffer for 10 min (Dako). Primary Abs were diluted in 1:200 PBS. After primary incubation, slides were washed three times for 5 min each in Dako Wash Buffer between incubations. HRP activity was detected using Dako Link DA + Substrate Chromagen System. Tissue was then stained with hematoxylin, coverslipped, and stored at room temperature. Abs used 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 DAB + Substrate Chromagen System. Tissue was then stained with hematoxylin, coverslipped, and stored at room temperature. Abs used included CD207: mouse monoclonal – ab49730 (Abcam, Cambridge, MA); CD3: mouse monoclonal – ab6999 (Abcam); HLA-DR+DQ: mouse monoclonal – sc59251 (Santa Cruz Biotechnology, Santa Cruz, CA); CTLA4: goat polyclonal – sc6126 (Santa Cruz Biotechnology); Osteopontin: mouse monoclonal – sc1271 (Santa Cruz Biotechnology); VNN1: 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 polyclonal – 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

**FIGURE 1.** Scatter plot of isolated cells. Tissue samples were prepared as described in Materials and Methods. These images represents typical FACS studies from control tonsil (A), peripheral blood from LCH patients (B), LCH lesions (C, E), and control skin (epidermis, D). The scanner was gated on PI cells (living cells) and then CD207+ and CD3+ cells were purified with fluorescent-conjugated Abs. The scatter plots show results (from right to left) with no Ab staining, isotype control, CD3+ and CD207+, and reanalysis of the sorted cells in a purity check. Sorted cell purity ranged from 95.5–99.2%. For the LCH lesions, the CD3/CD207 ratio varied considerably from sample to sample (Supplemental Table IB), but the plot shown in C and E is a typical result and demonstrates the ability to obtain specific cell fractions from LCH lesions.
Cell-specific gene-expression array analyses

CD207<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells and LCH lesion CD207<sup>+</sup> 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<sup>+</sup> cells. Additionally, some genes, such as CCL20, were not found to be significant, despite high levels of expression in some LCH CD207<sup>+</sup> samples 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 (CDC2A, 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>
</tr>
</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>CDK2NA</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>AIF3</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>
</tr>
<tr>
<td>MMP9</td>
<td>15.5</td>
<td>7.6</td>
</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>NRP1</td>
<td>4.8</td>
<td>4.5–9.5</td>
</tr>
<tr>
<td>TNFRSP9</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>
</tr>
<tr>
<td>S100A8</td>
<td>−20.5</td>
<td>(−22.5–26.2)</td>
</tr>
<tr>
<td>EpCAM</td>
<td>−20.4</td>
<td>−38.1</td>
</tr>
<tr>
<td>CDH1</td>
<td>−269.6</td>
<td>(−52.2–123.6)</td>
</tr>
<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>
<td>NA</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Representative genes identified by CD207 gene-expression array studies are listed in the first column. The fold-change (LCH LC versus control skin LC), as determined by real-time PCR, is listed in the middle column. The fold-change, as determined by microarray, is listed in the right column. There are multiple values for some of the array data as the result of multiple probes for different regions of a single gene on the array chips, which are represented by ranges. Changes are more pronounced in the real-time PCR assays, likely because of the lack of signal compression. In the chip studies, even if gene expression is absent, background signal may be interpreted as minimal expression. In general, real-time PCR confirms trends of increased and decreased expression in candidate genes identified by the gene chip studies. Similarly, real-time PCR fails to identify significant differences in some genes previously associated with LCH

NA, no PCR product was amplified in the real-time PCR reaction, suggesting the absence of target template for IL-2 and IL-17.

### 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>
<td>50,031.3</td>
<td>38.9–69.4</td>
</tr>
<tr>
<td>IL-8</td>
<td>572.6</td>
<td>21.6–45.7</td>
</tr>
<tr>
<td>MMP9</td>
<td>219.9</td>
<td>43.6</td>
</tr>
<tr>
<td>HSPA</td>
<td>40.8</td>
<td>15.3–21.8</td>
</tr>
<tr>
<td>FOXP3</td>
<td>24.7</td>
<td>6.7</td>
</tr>
<tr>
<td>HLRAD</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>

Data are organized as described for Table I for array and real-time PCR experiments using cDNA generated from purified CD3 cells.
metastasis/tissue invasion (CEACAM6, MMP1, and TGFβ1), myeloid cell maturation (CD11D, CD13, CD14, CD33, ITG2B2, ITGAX, ITGAM, and CD300F), and lymphocyte trafficking (SPPI, VNN1, NRPI, 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).
Using stringent statistical criteria, 162 probe sets (129 genes) showed significant differences in hybridization between 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 gene transcription in biopsies from different patient groups. 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).

**FIGURE 5.** Models of LC function and LCH. A. Epidermal LCs and dermal CD207+ DCs. Two CD207+ DC populations have been described: epidermal LCs and dermal DCs (71). LCs (black star) are derived from self-renewing radiosensitive precursors (black circle) that migrate to epidermis (white box) during fetal life (white sphere, dashed arrow). To maintain homeostasis after injury or inflammation, monocytes (gray circle) may migrate 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^+ 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 (40).

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. Neuruplin-1, expressed by NRPI, is a membrane-bound coreceptor to a tyrosine kinase receptor both vascular endothelial growth factor and semaphorin molecules. Neuruplin-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). Vanin-1, expressed by VNN1, is a GPI-anchored molecule expressed by perivascular thymic stromal cells that is essential in the migration of hematopoietic progenitor cells to the thymus (78). Mice deficient in vanin-1 are unable to form granulomas in response to Coxiella burnetii infection (79). Increased expression of SPP1, NRPI, and VNN1 by LCH CD207^+ cells suggests a potentially interesting role for these genes in CD207^+ cell-mediated recruitment of T cells to LCH lesions.

Multiple searches for a common Ag in LCH failed to identify an infectious etiology (reviewed in Ref. 4). A recent study found that Tregs were enriched in LCH lesions, as well as in peripheral blood of patients with active LCH. The same study found polyclonal TCR rearrangements in DNA isolated from frozen biopsy samples (56).

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.”

Acknowledgments

We thank Dr. Cliona Rooney for helpful suggestions throughout this project. We acknowledge Christopher Threeton, Michael Cubbage, and Tatiana Goltsova of the Texas Children’s Cancer Center and Hematology Service Flow Facility for excellent technical support. Finally, we also thank our colleagues who helped with collection of the LCH biopsy samples, as well as patients and families for their generous participation in this study.

Disclosures

K.L.M. owns common stock in the Johnson & Johnson Company. Otherwise, the authors have no financial conflicts of interest.

References


