BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups


Langerhans cell histiocytosis (LCH) is a clonal disorder with elusive etiology, characterized by the accumulation of CD207+ dendritic cells (DCs) in inflammatory lesions. Recurrent BRAF-V600E mutations have been reported in LCH. In this study, lesions from 100 patients were genotyped, and 64% carried the BRAF-V600E mutation within infiltrating CD207+ DCs. BRAF-V600E expression in tissue DCs did not define specific clinical risk groups but was associated with increased risk of recurrence. Strikingly, we found that patients with active, high-risk LCH also carried BRAF-V600E in circulating CD11c+ and CD14+ fractions and in bone marrow (BM) CD34+ hematopoietic cell progenitors, whereas the mutation was restricted to lesional CD207+ DC in low-risk LCH patients. Importantly, BRAF-V600E expression in DCs was sufficient to drive LCH-like disease in mice. Consistent with our findings in humans, expression of BRAF-V600E in BM DC progenitors recapitulated many features of the human high-risk LCH, whereas BRAF-V600E expression in differentiated DCs more closely resembled low-risk LCH. We therefore propose classification of LCH as a myeloid neoplasia and hypothesize that high-risk LCH arises from somatic mutation of a hematopoietic progenitor, whereas low-risk disease arises from somatic mutation of tissue-restricted precursor DCs.

Abbreviations used: ECD, Erdheim-Chester disease; FFPE, formalin fixed and paraffin embedded; gDNA, genomic DNA; HSPC, hematopoietic stem and progenitor cell; IF, immunofluorescence; JXG, juvenile xanthogranuloma; LCH, Langerhans cell histiocytosis; qPCR, quantitative PCR; RDO, Rosai-Dorfman disease.

M.-L. Berres, K.P.H. Lim, and T. Peters contributed equally to this paper.
The first recurrent somatic genetic mutation in LCH, BRAF-V600E, was recently reported in 57% of LCH lesions (Badalian-Very et al., 2010). Subsequently, recurrent BRAF-V600E mutations were reported in LCH as well as the related disorder Erdheim-Chester disease (ECD; Sahm et al., 2012; Satoh et al., 2012; Haroche et al., 2013). Case reports of two other LCH patients describe a potential activating BRAF-V600D somatic mutation and a novel germline BRAF mutation (Satoh et al., 2012; Kansal et al., 2013).

In this study, we investigate the clinical significance of the BRAF-V600E molecular signature and identify cells carrying the mutation to further define the cellular origins of LCH. We found that the presence of BRAF-V600E in pathological DCs within LCH lesions was associated with higher risk of refractory or recurrent disease. Importantly, we found that BRAF-V600E expression in circulating cells was also associated with disease severity in patients. Moreover, we demonstrate that BRAF-V600E expression in DC precursors is sufficient to induce an LCH-like phenotype in mice with risk organ involvement, whereas BRAF-V600E expression in differentiated DCs induces an attenuated phenotype. These results support a pivotal functional role of the mutation in LCH pathogenesis. We propose a model in which somatic mutation of BRAF-V600E in hematopoietic progenitors versus differentiated hematopoietic cells defines clinical risk in LCH.

### RESULTS

#### BRAF genotype in LCH patients: frequency and clinical correlations

LCH lesions (n = 130) from 100 patients with LCH were analyzed for the presence of the BRAF-V600E mutation (Table S1). Patients were identified retrospectively by availability regardless of the extent of disease burden (Gadner et al., 2008). Despite clinical heterogeneity, LCH lesions are generally indistinguishable by histology, which led to the notion that the spectrum of clinical manifestations represents a single disorder, histiocytosis X (Lichtenstein, 1953). The designation “Langerhans cell histiocytosis” was subsequently proposed with discovery of cytoplasmic Birbeck granules in the pathological infiltrating DCs in histiocytosis X lesions, a feature shared by epidermal Langerhans cells (Nezelof et al., 1973). Birbeck granules are intracytoplasmic organelles whose role has remained poorly understood since their first identification in 1961 (Birbeck et al., 1961). Recent data revealed that the formation of the Birbeck granules is a consequence of the antigen capture function of a C-type II lectin receptor called langerin, recently named CD207 (Valladeau et al., 2000; Kissenpfennig et al., 2005; Verdijk et al., 2005). Langerin was initially described specifically on human and mouse epidermal Langerhans cells and subsequently found on histiocytosis X lesions, further supporting the epidermal Langerhans cell origin of the disease (Chikwava and Jaffe, 2004).

However, recent discoveries question the model of LCH arising from transformed or pathologically activated epidermal Langerhans cells. The cell-specific gene expression signature in langerin+ DCs within LCH lesions has been shown to be more consistent with immature myeloid DC precursors than epidermal Langerhans cells (Allen et al., 2010). Furthermore, mouse studies demonstrate that langerin is more promiscuous than previously appreciated (Ginhoux et al., 2007). In addition to epidermal Langerhans cells, langerin is also expressed on a subset of DC expressing the integrin CD103 in non-lymphoid tissues (Merad et al., 2008) and its expression is modulated by the tissue environment in which DCs reside (Chang et al., 2010).
with significantly higher frequency of relapse and lower survival (Fig. 1, A and B). The BRAF-V600E mutation was associated with approximately twofold increased risk of relapse/recurrence (hazard ratio 2.17, 95% CI: 1.06–4.46). Although both BRAF-V600E and high-risk status were associated with increased risk of recurrence, BRAF-V600E was not significantly associated with high-risk disease (Table 1). Despite the increased risk of recurrence from BRAF-V600E, the mutation was not associated with any difference in overall survival (Fig. 1, C and D).

BRAF genotype in LCH lesions
In every case where there were multiple lesions, either synchronous or recurrent, from the same patient (n = 16 patients), the BRAF genotype remained fixed, consistent with acquisition of BRAF-V600E as an early event in LCH pathogenesis (Table S2). Concordance between cell-specific Sanger sequencing of cDNA and qPCR of whole lesion gDNA was observed in 47 out of 48 cases. In the single discordant case (Sanger, wild-type; qPCR, V600E), it is possible that decreased allelic expression or incomplete transcriptome coverage of cDNA amplified from RNA may result in occasional false-negative tests with cDNA sequencing. The percentage of BRAF-V600E cells in LCH lesions varied widely from

Figure 1. Clinical status, BRAF genotype, and clinical outcomes. (A) Estimates for refractory or recurrent disease in the study population by disease risk (red: high-risk, blue: low-risk). (B) Estimates for survival based on disease risk (red: high-risk, blue: low-risk). (C) Estimates for refractory or recurrent disease by lesion BRAF genotype (red: wild-type, blue: BRAF-V600E). (D) Estimates for survival based on lesion BRAF genotype (blue: wild-type, red: BRAF-V600E).
0.01–74.5% of lesion cells, with a median of 8.0% (Table S2 and Fig. 2A). The level of detection of commercially available deep sequencing–based diagnostic strategies requires ~5% cells with the BRAF-V600E mutation (Verma et al., 2012). In this series, 42% (34/82) of the LCH lesion samples would fall below that threshold, suggesting that LCH will require more sensitive diagnostic approaches, as BRAF genotype becomes part of clinical risk stratification. In a subset of samples (n = 7), cDNA from CD3+ T cells purified from LCH lesions was analyzed along with purified langerin+ cells. Where 4/7 langerin+ cDNA samples had detectable BRAF-V600E, only wild-type BRAF was identified in all of the matched lesion CD3+ cDNA.

This series included three patients with a biopsy reported with features with LCH, and also with a history of other histiocytic disorders. In two cases, LCH-JXG-098 and LCH-JXG-099, the patients had lesions with mixed populations of histiocytes described as LCH (CD207+, CD1a+, and S100A+) and juvenile xanthogranuloma (JXG; fascin+, Factor XIIIa+, CD207−, and CD1a−). BRAF-V600E was detectable in lesions from one of the two JXG/LCH hybrid patients (LCH-JXG099). Although serial and hybrid JXG and LCH have been reported previously (Stover et al., 2008), to our knowledge this is the first case of BRAF-V600E associated with a histiocytic lesion including features of JXG. In another case (LCH-ECD100), a patient with a history of ECD had a new bone lesion with histology consistent with LCH (CD207+, CD1a+, and S100A+) with the BRAF-V600E genotype. The presence of BRAF-V600E allele in ECD and the observation of serial ECD and LCH lesions have been reported previously (Pineles et al., 2011, 2012; Haroche et al., 2013). In this series, BRAF-V600E was not identified in lesions from additional patients with ECD (n = 2), JXG (n = 7), or Rosai-Dorfman disease (RDD; n = 5). Additionally, BRAF-V600E was not identified in several biopsies from patients evaluated for possible LCH, with subsequent histology consistent with diagnoses other than histiocytic disorders (n = 17; Table S3).

BRAF genotype in circulating blood and BM in LCH patients: frequency and clinical correlations

From the 100 patients with known BRAF genotype, peripheral blood samples were collected from 77 patients with clinically detectable active disease: 67 patients at the time of diagnosis before chemotherapy and 10 patients at time of relapse before...
salvage chemotherapy (Table S1). Circulating cells with \textit{BRAF-V600E} were identified in 17 of the patients (0.02–2.2% of peripheral blood mononuclear cells), all of whom had \textit{BRAF-V600E} identified in biopsy samples (Table S4 and Fig. 2 B). Clinical correlation with risk group shows that among patients with \textit{BRAF-V600E} lesions, circulating \textit{BRAF-V600E} was identified in 100% (12/12) of patients with high-risk disease (clinically documented liver, spleen, or BM involvement by LCH cells) and in 13% (5/39) of patients with low-risk disease. Circulating \textit{BRAF-V600E} cells were not detected in peripheral blood gDNA from any of the patients with wild-type \textit{BRAF} lesion (0/26). The presence of \textit{BRAF-V600E} in circulating patients with active LCH with \textit{BRAF-V600E} lesions therefore correlates with 100% sensitivity and 87% specificity for clinically defined high-risk disease. All of the clinical low-risk patients with circulating \textit{BRAF-V600E} cells (n = 5) had multifocal lesions in more than one organ system (including skin, bone, gastrointestinal mucosa, and pituitary), and this group had significantly higher frequency of recurrent/refractory disease than clinical low-risk patients without circulating \textit{BRAF-V600E} cells detected (hazard ratio 3.31, 95% CI: 0.94–11.70; Fig. 2 C). Clinical risk categories were assigned according to the medical record. It is possible that these patients were incompletely evaluated or had subclinical liver/spleen/BM involvement. For example, patient LCH068 had biopsy-proven skin and bone lesions, but splenic lesions noted on abdominal ultrasound were not biopsied and the patient was regarded as multi-system low-risk by the treating team. Circulating \textit{BRAF-V600E} cells were identified in this patient in this study. Because the initial therapy is the same for low-risk and high-risk LCH patients, the risks of proving liver or spleen involvement in cases where imaging is equivocal may not be warranted. Interestingly, the described splenic lesions resolved after successful therapy. This example highlights the limitations of conventional staging strategies.

Similarly, \textit{BRAF-V600E} was detected in BM aspirate from 100% (7/7) of patients with \textit{BRAF-V600E} lesions with active high-risk LCH (Table S5). qPCR estimated 0.2–2.1% of BM cells in this series with \textit{BRAF-V600E} mutation. Interestingly, pathology reports describe abnormal findings in only 43% (3/7) of these patients, and one with mixed phenotype peripheral lesions was documented as JXG. In most cases, percentage of \textit{BRAF-V600E} cells in BM and peripheral blood were similar (Fig. 2 D).

Serial blood samples were analyzed from patients with circulating cells with \textit{BRAF-V600E} identified at diagnosis or at an early episode of relapse/refractory disease. In 100% (14/14) of episodes where there was no clinical, imaging, or laboratory evidence of active LCH documented in the medical record, the peripheral blood had no detectable cells with \textit{BRAF-V600E}. In 97% (29/30) of episodes of patients with clinically defined active LCH, \textit{BRAF-V600E} was detected in peripheral blood cells. In patients with circulating \textit{BRAF-V600E} cells in peripheral blood at a time of active disease, analysis of circulating blood for \textit{BRAF-V600E} defined active LCH with 97% sensitivity and 100% specificity (Table S4). In one case of a patient who developed symptoms of high-risk LCH at 6 mo of age (LCH075), \textit{BRAF-V600E} cells were identified in multiple lesion samples and peripheral blood at the time of diagnosis; however, \textit{BRAF-V600E} was not identified in a cord blood sample harvested at birth, suggesting that a precursor population was too rare to identify or that the somatic mutation was acquired at a later time (see Fig. 3 B and Fig. 4 B). These data demonstrate that the \textit{BRAF-V600E} “bar code” may be used not only to diagnose high-risk LCH but also may potentially be tested in serial samples over time as a measure of residual disease (Fig. 2 E).

\textbf{BRAF-V600E may be expressed in CD34+ hematopoietic stem and progenitor cells (HSPCs) in LCH patients}

To identify the circulating cells harboring the \textit{BRAF-V600E} mutation, peripheral blood and BM aspirate fractions were analyzed. Samples were selected from available aliquots of viable stored peripheral blood mononuclear cells and BM aspirates from patients with \textit{BRAF-V600E} detected in unsorted blood and BM aspirate (Table 2). Peripheral blood was first sorted into CD14+ monocytes, HLA-DR+CD11c+ classical DCs, BDCA2+ plasmacytoid DCs, and CD3−CD19−CD56−CD14−CD11c− negative fraction (Fig. 3 A). gDNA was then amplified and tested for the presence of \textit{BRAF-V600E}. In all cases where \textit{BRAF-V600E} was identified in the whole peripheral blood sample, it was also detected in CD11c+ DCs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>\textit{BRAF} lesion</th>
<th>Circulating \textit{BRAF}</th>
<th>CD11c+ PB</th>
<th>CD14+ PB</th>
<th>BDCA2+ PB</th>
<th>CD3− PB</th>
<th>CD19+ PB</th>
<th>CD34+ BM</th>
<th>CD14+ BM</th>
<th>\textit{V600E} in CFU assays</th>
<th>Cord blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCH009</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCH014</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCH040</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCH067</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCH068</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCH070</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCH075</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LCH077</td>
<td>V600E</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PB, peripheral blood; BM, BM aspirate.
purified circulating CD3+ T lymphocytes and CD19+ B lymphocytes were also analyzed for the expression of BRAF-V600E in parallel aliquots from samples where BRAF-V600E was identified in peripheral monocyte/DC populations. BRAF was wild-type in CD3+ T cells from all samples. In the CD19+ fractions, BRAF-V600E was identified in some cases (LCH067, LCH068, and LCH070) but was absent from others (LCH040, LCH075, and LCH077). In all of the samples where BRAF-V600E was identified in peripheral CD19+ B lymphocytes, the mutation was also identified in CD34+ HSPCs from BM aspirate. In LCH075, BRAF-V600E was identified in neither CD34+ HSPC from BM aspirates nor circulating CD19+ B lymphocytes (Table 2 and Fig. 4 D). These observations are consistent with the potential of hematopoietic progenitors with BRAF hyper-activation to develop into B cells as well as CD34+ cells, consistent with somatic mutation of the hematopoietic stem progenitor cell. Lineage− CD34+ HSPCs isolated from BM aspirate from two patients with high-risk LCH were used in CFU assays. The percentage of colonies with BRAF-V600E was similar to the percentage of CD34+ cells from BM aspirate carrying the mutation in both cases (Fig. 4 C). Due to the small number of cells, colonies were pooled to perform BRAF genotyping. BRAF-V600E colonies were identified in the macrophage/monocyte colonies from the LCH068 sample. The colony sizes were smaller with insufficient DNA to perform BRAF genotyping on phenotype-specific colonies for LCH067 (unpublished data). Together, these data suggest that BRAF-V600E mutations may arise in hematopoietic stem cells and promote stem cell differentiation into myelomonocytic precursor cells in patients with high-risk LCH.

It remains possible that the circulating cells identified in this series could represent “leaking” lesion or tumor cells rather than LCH precursors. However, the statistically significant frequency in patients with high-risk LCH and failure to identify any circulating cells in patients with large single-lesion tumor burden favor a precursor versus a lesional origin of these cells. To further explore the possibility that circulating cells could represent leaking tumor cells, we performed FACS analysis on a series of blood samples with detectable circulating BRAF-V600E and consistently failed to identify circulating CD207+ cells above background (<0.01%; not depicted).

BM aspirate samples were also analyzed to determine the cellular origins of the BRAF-V600E mutation (Fig. 4 A). They were sorted into CD34+ HSPCs, differentiated CD14+ monocytes, and a negative fraction, which included myeloid and lymphoid precursors as well as CD14-negative leukocytes (Fig. 4 B). In 4/5 BM aspirate samples from patients with BRAF-V600E cells in lesions and high-risk organ involvement (including patient LCH068 who was clinically classified as low-risk but with undefined splenic lesions that resolved with therapy), the BRAF-V600E mutation was identified in CD14+ monocytes, consistent with somatic mutation of the hematopoietic stem progenitor cell. Lineage− CD34+ HSPCs isolated from BM aspirate from two patients with high-risk LCH were used in CFU assays. The percentage of colonies with BRAF-V600E was similar to the percentage of CD34+ cells from BM aspirate carrying the mutation in both cases (Fig. 4 C). Due to the small number of cells, colonies were pooled to perform BRAF genotyping. BRAF-V600E colonies were identified in the macrophage/monocyte colonies from the LCH068 sample. The colony sizes were smaller with insufficient DNA to perform BRAF genotyping on phenotype-specific colonies for LCH067 (unpublished data). Together, these data suggest that BRAF-V600E mutations may arise in hematopoietic stem cells and promote stem cell differentiation into myelomonocytic precursor cells in patients with high-risk LCH.

Purified circulating CD3+ T lymphocytes and CD19+ B lymphocytes were also analyzed for the expression of BRAF-V600E in parallel aliquots from samples where BRAF-V600E was identified in peripheral monocyte/DC populations. BRAF was wild-type in CD3+ T cells from all samples. In the CD19+ fractions, BRAF-V600E was identified in some cases (LCH067, LCH068, and LCH070) but was absent from others (LCH040, LCH075, and LCH077). In all of the samples where BRAF-V600E was identified in peripheral CD19+ B lymphocytes, the mutation was also identified in CD34+ HSPCs from BM aspirate. In LCH075, BRAF-V600E was identified in neither CD34+ HSPC from BM aspirates nor circulating CD19+ B lymphocytes (Table 2 and Fig. 4 D). These observations are consistent with the potential of hematopoietic progenitors with BRAF hyper-activation to develop into B cells as well as...
progressively increased in size with age (Fig. 5 B, bottom), and by 20 wk of age, all mice analyzed also displayed small lesions in the lung (Fig. 5 B, top). Flow cytometry analysis revealed an increased number of mainly MHC II$^+$CD11c$^+$ DCs (Fig. 5 C) in the liver of BRAF$^{600E}$ langerin mice, whereas the number of DCs in the lung and total numbers of macrophages, NK cells, B cells, and T cells in the tissues were unaffected (Fig. 5, C and D). Immunofluorescence (IF) staining further confirmed that a significant proportion of liver tissue–infiltrating cells also expressed langerin (Fig. 5 E). Importantly, we failed to detect anemia or alteration of circulating blood DC numbers in 12- or 20-wk-old BRAF$^{V600E}$ langerin mice (see Fig. 7, B and C).

Our results in patients showing that BRAF$^{V600E}$ expression in early hematopoietic precursors in human patients correlate with high-risk LCH and early involvement of risk organs prompted us to assess whether BRAF$^{V600E}$ expression at earlier stages of DC development can affect LCH phenotype. Thus, we generated another mouse model in which the BRAF$^{V600E}$ conditional allele was expressed under the CD11c promoter. In mice, CD11c is expressed on committed DC progenitors and remains expressed throughout DC differentiation (Merad et al., 2013). In striking contrast to BRAF$^{600E}$ langerin mice, BRAF$^{600E}$CD11c$^+$ mice rapidly
developed an aggressive LCH-like disease phenotype with a penetrance of 100% associated with a decreased lifespan. Along with severe hepatosplenomegaly and lymphadenopathy, organized histiocytic infiltrates were identified in the skin, liver, spleen, and lungs of all mice by 8 wk of age, resulting in a broad destruction of tissue architecture by 16 wk of age (Fig. 6, A–C). Histologically, the histiocytic lesions observed in BRAFV600E<sup>langerin</sup> mice exhibited classical granulomatous organization, including multinucleated giant cell formation which is frequently observed in human LCH (da Costa et al., 2005; Fig. 6 B). These lesions were associated with a massive increase of MHC II<sup>+</sup>CD11c<sup>+</sup> DCs (Fig. 6 D) with a classical DC shape (Fig. 6 E, higher magnification). The vast majority of the CD11c<sup>+</sup> cells accumulating in the tissues also expressed high levels of langerin protein (Fig. 6 E) associated with a dramatic increase of langerin mRNA transcripts in tissues compared with control littermates (Fig. 6 F). As in human LCH lesions, mitoses were not observed in the granulomatous lesions observed in BRAFV600E<sup>CD11c<sup>+</sup></sup> mice (Senechal et al., 2007; Fig. 6 B).

We confirmed that lesional MHC II<sup>+</sup>CD11c<sup>+</sup> DCs isolated from the liver of BRAFV600E<sup>CD11c<sup>+</sup></sup> mice specifically expressed the BRAF-V600E mutation as assessed by mutation-specific qPCR (Fig. 7 A). Importantly, and consistent with the data in patients with high-risk multi-systemic LCH, BRAF-V600E was also present in BM-resident common DC progenitors (CDPs) in BRAFV600E<sup>CD11c<sup>+</sup></sup> mice (Fig. 7 A), whereas it was absent from BM progenitors in BRAFV600E<sup>langerin</sup> mice (not depicted). The BRAF-V600E mutation was restricted to the DC lineage and was absent from early myeloid precursors or multipotent progenitors in BRAFV600E<sup>CD11c<sup>+</sup></sup> mice. Expression of the mutation in DC-restricted precursors was associated with severe anemia, confirming BM involvement in these mice (Fig. 7 C), together with a dramatic expansion of
The granuloma lesions observed in **BRAF**<sup>V600E</sup> **CD11c**<sup>-</sup> mice contained, in addition to massive **CD11c**<sup>-</sup> **langerin**<sup>-</sup> infiltrates, a large number of macrophages, NK cells, B cells, and T cells, with a specific accumulation of regulatory blood circulating DC precursors (pre-DCs) and blood circulating DCs (Fig. 7 B), whereas blood pre-DCs and DC numbers, as well as hemoglobin levels, remained unaffected in **BRAF**<sup>V600E</sup> **CD11c**<sup>-</sup> mice.

The granuloma lesions observed in **BRAF**<sup>V600E</sup> **CD11c**<sup>-</sup> mice contained, in addition to massive **CD11c**<sup>-</sup> **langerin**<sup>-</sup> infiltrates, a large number of macrophages, NK cells, B cells, and T cells, with a specific accumulation of regulatory
DISCUSSION

The etiology of LCH has intrigued and eluded the medical community for the past century as a paradox of a collection of highly variable clinical manifestations connected by common histopathology. The resulting debate on LCH pathogenesis has recently focused on aberrant activation versus malignant transformation of the epidermal Langerhans cell. Previous studies demonstrated that pathological DCs in LCH are clonal (Willman et al., 1994; Yu et al., 1994) and that the BRAF-V600E point mutation occurs with significant frequency in LCH lesions (Badalian-Very et al., 2010; Haroche et al., 2012; Sahm et al., 2012; Satoh et al., 2012). In this study, T cells (Fig. 7, D and E) closely resembling LCH lesions in humans (Hicks and Flaitz, 2005; Senechal et al., 2007). Importantly, these granuloma-like lesions were also associated with a significant increase in several chemokines and cytokines (Ccl2, Ccl5, Il-6, Il-10, IFN-γ, and TGF-β; Fig. 7 F), consistent with the “local cytokine storm” that characterizes human LCH (Allen et al., 2010). Additionally, a local fibrotic response occasionally observed in human LCH granuloma and likely due to high cytokines expression—for example, TGF-β and IFN-γ (de Graaf et al., 1996)—was detected within the granulomas and the surrounding stroma (not depicted).

Figure 7. Expression of BRAF-V600E in early BM-resident DC progenitors in BRAFV600ECD11c mice results in multi-systemic high-risk disease and is associated with high local cytokine expression and the recruitment of additional inflammatory cells. (A) Relative mRNA expression of BRAF-V600E in sorted BM lineage negative sca1+ c-kit+ (LSK), lin- sca1+ c-kit+ CD34+ CD16/32+ granulocyte myeloid progenitors (GMPs), lin- sca1+ CD135+ c-kit+CD115+ monocyte and DC progenitors (MDPs), and lin- sca1+ CD135+ c-kit+CD115+ common DC progenitors (CDPs) and lung/liver CD45+ MHCII+ CD11c+ DCs in BRAFV600ECD11c mice as assessed by mutation-specific qPCR (data normalized to total BRAF expression, no BRAF-V600E expression detected in corresponding cells of control mice, n = 3, pooled data of three independent isolations). (B and C) Relative numbers of viable singlet CD11b+ CD115+ Flt3+ pre-DCs and CD11c+ MHCII+ DCs among circulating viable, singlet CD45+ cells (B) and hemoglobin levels (C) in BRAFV600ECD11c mice, BRAFV600ECD11c mice, and control mice (n = 4–10 per group, pooled data of four independent experiments, each data point represents one mouse). (D) Immunohistochemical analysis of liver and lung tissue sections stained with indicated mAb in BRAFV600ECD11c mice (top) and control littermates (bottom; bars, 50 µm). (E) Absolute numbers of tissue-infiltrating total hematopoietic CD45+ cells, F4/80+ macrophages, total CD3+ NK1.1+ T cells, total CD3+ NK1.1+ CD4+ CD8+ T cells, total CD3+ NK1.1+ CD4+ Foxp3+ T reg cells, total CD19+ B220+ B cells, total CD3+ NK1.1+ NK cells, and total SiglecF+ CD11c+ eosinophils in lung and liver of BRAFV600ECD11c mice and control littermates as assessed by multicolor flow cytometry (n = 4–5 per group, representative of two independent experiments). (F) Local expression of indicated chemokines and cytokines in peripheral liver tissue was assessed by qPCR (data normalized to Gapdh expression, n = 4–5 per group). All data are shown as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
we demonstrate that \textit{BRAF} status of pathological DCs in LCH lesions is fixed in synchronous lesions as well as serial relapse samples, consistent with the \textit{BRAF-V600E} mutation as an early or initiating event in LCH pathogenesis, tipping the scales to classify LCH as a neoplastic disorder. We also demonstrate that \textit{BRAF-V600E} mutation in patients with high-risk LCH is a somatic event that seems to arise in very early myelomonocytic precursors or in stem cells that may be subsequently driven to myelomonocytic differentiation, whereas \textit{BRAF-V600E} mutation in patients with low-risk LCH was not routinely identified in circulating cells, suggesting it may be restricted to differentiated tissue-resident DCs in these patients. We cannot entirely exclude that the \textit{BRAF-V600E}-expressing cells in the blood and BM of high-risk patients might represent circulating lesional cells; however, the statistically significant presence of circulating cells in high-risk patients and infrequent detection in low-risk patients, lack of langerin$^+$ cells in the blood of patients with circulating \textit{BRAF-V600E}-expressing cells, and the identification of the mutation in the CD34$^+$ fraction in the BM render this hypothesis unlikely. CD34 is a major positive marker of human HSPCs and is not expressed by mature blood cells or tissue-restricted myeloid cells (Kondo et al., 2003). Furthermore, the progenitor nature of \textit{BRAF-V600E}-expressing CD34$^+$ cells is greatly supported by the CFU assay performed with sorted CD34$^+$ BM cells of two high-risk patients, which yield a percentage of colonies with \textit{BRAF-V600E} similar to the percentage of CD34$^+$ cells from BM aspirates. This shows the high proliferative potential of mutation-bearing CD34$^+$ BM cells, defining them as colony-forming cells/progenitor cells. As lesional cells display only low proliferation rate (Senechal et al., 2007), contamination of the assay with circulating LCH cells seems implausible.

Using two novel mouse models, we established that the \textit{BRAF-V600E} mutation in myeloid cells results in the formation of LCH-like lesions in mice. Induction of physiological expression levels of \textit{BRAF-V600E} in BM DC progenitors induced a severe LCH-like phenotype with multi-organ involvement and reduced lifespan, whereas expression of \textit{BRAF-V600E} in differentiated DCs resulted in an attenuated phenotype. Consistent with identification of \textit{BRAF-V600E} mutation in patients with high-risk LCH, \textit{BRAF-V600E} expression in hematopoietic progenitors in mice resulted in BM involvement, indicated by severe anemia and expansion of circulating DC precursors and circulating DC, whereas anemia or alteration in circulating DCs was not detectable when the mutation was restricted to the more differentiated DCs. These findings implicate the \textit{BRAF-V600E} mutation as a driver of LCH-like disease in mice. Although these mouse models recapitulate several features of human LCH, the extent to which these results can be translated to human LCH pathology remains a matter for investigation.

Based on our results, we propose a new hypothetical model of LCH in which the initiating cell defines the extent of the disease. The mixed inflammatory lesion that defines LCH clinically is indistinguishable between different LCH clinical risk groups. However, LCH lesions may represent only a superficial downstream manifestation of pathogenesis. If \textit{BRAF-V600E} is the activating event, somatic mutation in a hematopoietic stem cell or myeloid DC restricted precursors may determine progression to high-risk disease, potentially with lesions in BM, liver, spleen, or virtually any organ system. Neoplastic transformation of an early multipotent myeloid precursor could explain the occurrence of coexistent histiocytic disorders (JXG/LCH and ECD/LCH) in the same patients in this study, as well as in others, and the common \textit{BRAF-V600E} mutation in both LCH and ECD (Stover et al., 2008; Pineles et al., 2011; Haroche et al., 2012). Moreover, immature myeloid origins of high-risk LCH are consistent with effectiveness of acute myelogenous leukemia–based salvage chemotherapy with cytarabine/cladribine and clofarabine for patients with recurrent/refractory high-risk LCH (Bernard et al., 2005; Rodriguez-Galindo et al., 2008).

We further hypothesize that if the initiating mutation occurs in tissue-restricted DC progenitors, multifocal low-risk disease could result. Finally, if an initiating mutation arises in a differentiated DC with restricted tissue tropism, low-risk LCH with a single lesion could result. In this model, as pathological DCs from circulating or tissue-restricted precursors migrate to sites of lesion formation, they may acquire CD1a and langerin expression and recruit and activate “innocent bystander” inflammatory cells, including macrophages and lymphocytes, resulting in the formation of characteristic granulomatous LCH lesions mediated, at least in part, by an altered expression of chemotactic cytokines. Based on the misguided differentiation of the pathological DC and their ability to recruit and activate additional inflammatory cells, we propose that the answer to the neoplasia versus inflammation debate is that both processes may be essential to LCH pathogenesis.

The results from this study also revealed some innovative diagnostic tools that will need to be validated in future prospective clinical studies. The qPCR assay was highly sensitive and specific for diagnosis of LCH. In the absence of fresh tissue on which to perform cell sorting, qPCR on whole tissue samples appears to be a robust strategy compared with sequencing strategies for unmanipulated biopsy samples, many of which have pathological DC content <5%. The \textit{BRAF-V600E} mutation is clinically relevant, as LCH patients with \textit{BRAF-V600E} expression within the lesions were approximately twice as likely to have recurrent or refractory disease as patients with wild-type lesions, and detection of \textit{BRAF-V600E} in peripheral blood was 100% sensitive for clinical high-risk disease associated with significant mortality. Patients with clinical low-risk multisystem LCH but evidence of circulating \textit{BRAF-V600E}–expressing cells also had a significantly higher chance of recurrent/refractory disease compared to low-risk multisystem LCH patients without detectable circulating \textit{BRAF-V600E} cells. It is possible that our findings differ with previous reports of absent circulating \textit{BRAF-V600E} cells due to the relative insensitivity of deep sequencing and/or lack of inclusion of patients with \textit{BRAF-V600E} and active high-risk disease in the study cohorts (Sahm et al.,
LCH may arise through expression of *BRAF-V600E* in precursor cells and DCs but may also represent a valuable tool with which to develop and evaluate future therapeutic strategies. In summary, the observation of *BRAF-V600E* in BM progenitor cells and immature circulating cells in patients with high-risk LCH, coupled with the development of LCH-like lesions in mice expressing the BRAF mutation within the myeloid lineage, supports a causative role of *BRAF-V600E* in LCH pathogenesis and progression, consistent with classification of LCH as a bone fide myeloid neoplasia.

**MATERIALS AND METHODS**

**Human studies**

**Subjects.** LCH diagnosis was established by the presence of CD1a+ or CD207+ histiocytes in clinical biopsy specimens along with characteristic histopathology. Clinical data were collected from approved chart reviews. Studies with patient tissue and clinical data were performed according to protocols approved by the Institutional Review Board of Baylor College of Medicine.

**Analysis of clinical variables.** Frequency distributions of demographic and clinical characteristics were tabulated for those with and without the *BRAF-V600E* mutation. The crude odds ratio (OR) and 95% confidence interval (CI) were estimated using unconditional logistic regression for the association between each characteristic and mutation status. Kaplan-Meier survival estimates were calculated for recurrence/relapse and overall mortality. The sensitivity and specificity of circulating cells with *BRAF-V600E* were compared with the clinical assessment of disease risk (i.e., gold standard). All statistical analyses were performed using Intercooled Stata (version 12.1; StCorp LP).

**BRAF-V600E assays: LCH lesions.** gDNA was isolated from frozen whole lesions, or from frozen sections cut from biopsies embedded in OCT blocks using the QIAamp DNA mini and QIAamp DNA micro protocols (QIAGEN), including treatment with RNase A (QIAGEN). When gDNA isolated from scrotes of formalin fixed and paraffin embedded (FFPE) archived biopsy samples were used, the samples were processed with QIAamp DNA FFPE Tissue kit per manufacturer’s protocol (QIAGEN), including treatment with RNase A. Purified gDNA (30 μg/sample) was used in the *BRAF-V600E* qPCR mutation assay (Somatic Mutation Assay for BRAF 476; QIAGEN). BRAF mutation and reference primers were included in each reaction. Duplicate reactions were performed for each sample. All experiments were performed on an iQ5 iCycler (Bio-Rad Laboratories). The means of ΔCt and Ct were calculated to determine ΔCt = C\text{t}^{\text{mut}} - C\text{t}^{\text{ref}}. The ΔCt was compared with a standard curve to estimate the percentage of cells with *BRAF-V600E* alleles. The standard curve was created by making 13 dilutions of gDNA from A375 cell line (American Type Culture Collection), which carries one allele of *BRAF-V600E* and one allele with wild-type *BRAF* per cell, with gDNA from HEK293 cell line (American Type Culture Collection), which carries two wild-type *BRAF* alleles per cell. The ΔCt was plotted against the percentage of cells from A375/HEK293 cell pools (0.1–100% *BRAF-V600E*) from which the gDNA was isolated. For each LCH gDNA sample, the percentage of cells with the *BRAF-V600E* mutation was then calculated based on the standard curve. Independent controls (wild type and 100% *BRAF-V600E* cell lines) were routinely tested with the standard curve and experimental samples.

**Cell-specific mutation analysis was performed in languimeri and CD3+ cells purified from viable LCH lesions. Cell sorting, RNA purification, and cDNA amplification was performed as described previously (Allen et al., 2010). BRAF sequence was analyzed in cDNA from purified cells by PCR amplification, followed by Sanger sequencing. BRAF-V600E was identified in cases where GAG peak was visible, along with wild-type GTG at codon 600. Primers: forward, 5′-AGCTCAATAGGGCGAGAAT-3′; reverse, 5′-AGCTCAATAGGGCGAGAAT-3′.
BRAF-V600E assays: peripheral blood and BM aspirate. Buffy coat from fresh peripheral blood, BM aspirate, and cord blood samples was purified by spinning an equal volume over Histopaque-1077 (Sigma-Aldrich) at 400 g for 30 min. Samples were washed with PBS, and then viable stored long-term in Recovery Cell Culture Freezing Media (Gibco) until they were thawed for gDNA isolation, per protocol described above, or used for sorting experiments. gDNA from sorted cells was purified with the QIAamp DNA Micro kit (QIAGEN), and then amplified with the REPLI-g Midi kit (QIAGEN) per manufacturer’s instructions. qPCR was performed as described above on the amplified gDNA. 

BRAF-V600E: wild-type standard curves were created for amplified gDNA from 13 dilutions of amplified gDNA from A375 cells with amplified gDNA from HEK293 cells.

BRAF-V600E assays: sorting peripheral blood. Purified leukocytes from peripheral whole blood were subjected to staining using anti-CD11c-PE, anti–HLADR-APC-Cy7, lineage antibodies (anti-CD3-Pacific blue, anti-CD19-Pacific blue, and anti-CD56-Pacific blue), anti-CD14-APC (BD), and anti–BDCA2-FITC (Miltenyi Biotech). Thawed samples were blocked for 30 min in FcR-block (Miltenyi Biotech) and stained for 30 min in ice-cold PBS supplemented with 2 mM EDTA and 5% BSA. For purified peripheral blood populations, isolation of Lin+HLADR-CY+CD11c, Lin+HLADR-CD+CD14, Lin+HLADR-BDCA2, and Lin+HLADR+precursor cells were achieved by six-color sorting on an Aria SL2 (BD). B and T cell populations were isolated from select samples using anti-CD3-FITC and anti–CD19-PE-Cy7 (BD), anti–CD8-FITC, and anti–CD4-APC-Cy5 antibodies (BioLegend, BD, or Invitrogen). Subsequently, biotin was deprotected endogenous RNA. All flow cytometry analysis was performed using Flowjo software (Tree Star).

BM CFU assays. Frozen healthy or patient BM samples were stained with anti-CD123–Brilliant violet 421, anti–CD38–FITC, anti–CD190–PE, anti–lineage markers (CD3, CD4, CD8, CD11b, CD14, and GPA)–Tricolor, anti–CD34–PE-Cy7, anti–CD127–biotin, and anti–CD45RA–APC-Cy7 antibodies (BioLegend, BD, or Invitrogen). Subsequently, biotin was detected by streptavidin-APC (eBioscience). CD34+ cells were sorted into different lineages (CD3, CD4, CD8, CD11b, CD14, and GPA)–Tricolor, anti–CD123–Brilliant violet 421, anti–CD38–FITC, anti–CD90–PE, anti–HLADR–APC-Cy7, lineage markers (CD3, CD4, CD8, CD11b, CD14, and GPA)–Tricolor, and anti–CD11b–BV421. In BM long-term cultures, 4 U/ml EPO, 50 ng/ml GM-CSF, and 10 ng/ml Flt3L (PeproTech), and IL-3, 10 ng/ml IL-6, 10 ng/ml IL-11, 10 ng/ml SCF, 50 ng/ml TPO, methylcellulose (H4230; STEMCELL Technologies) containing 20 ng/ml TPO, supplemented with live/dead fixable dead cell stain kit (Invitrogen) before fixation to assess viability. Multiparameter analysis was performed on the LSR II (BD) and analyzed with Flowjo software.

Mouse studies. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Mount Sinai School of Medicine. BrafV600E mice were generated by crossing mice expressing cre recombinase under the control of the murine langerin promoter (C57BL/6 background; provided by B. Clausen, Erasmus University Medical Center, Rotterdam, Amsterdam; Zahner et al., 2011) with heterozygous mice carrying a cre-activated allele of BrafV600E (BrafV600Ecre/wt, C57BL/6 background) in which wild-type Braf is expressed in both alleles in the absence of cre-mediated recombination (provided by M.W. Rosenberg, Yale University, New Haven, CT; Dankort et al., 2007). BrafV600Ecre/wt mice were created by crossing BrafV600Ecre/wt mice with mice expressing cre recombinase under the control of the CD11c promoter (C57BL/6 background; The Jackson Laboratory). All animals were housed under specific pathogen-free (SPF) conditions and sacrificed at the indicated time points. All experiments were controlled using littermates negative for the cre recombinase transgene construct.

Histology, immunohistochemistry (IHC), and IF analysis. Langerin and CD11c staining was performed on acetone-fixed frozen tissues, and all other staining was performed on FFPE tissue at the Histology Shared Resource Facility of Mount Sinai School of Medicine and at the Baylor College of Medicine Pathology Core according to standard operating procedures. For IF staining with anti-langerin and CD11c, fresh tissue was cryopreserved in OCT (Sakura) and stored at 80°C. 8-µm-thick sections were air dried for 1 h and fixed in acetone for 20 min at −20°C. After incubation with 10% goat serum for 1 h, sections were stained overnight at 4°C with rat anti-langerin (clone eBiol31; eBioscience) or Armenian hamster anti-CD11c (clone N418; eBioscience) mAbs. After several washing steps, sections were incubated for 60 min with goat anti-rat antibody labeled with Alexa Fluor 488 (Invitrogen) and goat anti-Armenian hamster labeled with Alexa Fluor 594 (BioLegend) and embedded in ProLong Gold Antifade Reagent with DAPI (Life Technologies). Images were acquired with a confocal microscope (SP5 DM; Leica) and analyzed with ImageJ software (National Institutes of Health).

For the IHC analysis, 4-µm tissue sections were deparaffinized and rehydrated through xylene/absolute alcohol. Endogenous peroxidase activity was blocked by incubating the sections in methanol with 0.6% hydrogen peroxide for 10 min at room temperature. Heat-induced antigen retrieval was performed with citrate buffer, pH 6.0 (Diagnostic BioSystems), in a steamer for 15 min. To block nonspecific staining, Rodent Block M (BioCare Medical) was incubated on the sections for 1 h at room temperature. Sections were stained with anti-CD3 (Abcam), anti–MHC II (BD), anti–FOX3 (BioLegend), or anti–CD68 (Thermo Fisher Scientific) mAbs overnight at 4°C. Application of the primary antibodies was followed by a 30-min incubation with a Rabbit on Rodent/Mouse on mouse/rat on Rodent Polymer-HRP (BioCare Medical) and visualized with DAB (Diagnostic BioSystems) as a chromagen with CAT hematoxylin counterstaining (BioCare Medical).

Flow cytometry analysis. Single cell suspension was obtained from indicated tissues after digestion with 0.4 mg/ml of type IV collagenase (Sigma-Aldrich) at 37°C for 45 min. For liver tissues, nonparenchymal cells were enriched by density gradient centrifugation with 40/70 Percoll (GE Healthcare) for 30 min at 1,100 rpm. BM and blood single cell suspensions were incubated with RBC lysis buffer (BioLegend) for 2 min at room temperature before staining. mAbs specific to mouse CD45 (clone 30F11; BioLegend), MHC II (I-A/I-E; clone M5/114.15.2; BioLegend), CD11c (clone N418; eBioscience), CD103 (clone 2C7; eBioscience), CD11b (clone M1/70; BioLegend), F4/80 (clone CI:A3-1; BioLegend), CD3 (clone 145-2C11; BioLegend), CD4 (clone L3T4; BioLegend), NK1.1 (clone PK136; eBioscience), FoxP3 (clone FJK-16; eBioscience), Sca-1 (clone D7; eBioscience), c-kit (clone 2B8; eBioscience), CD16/32 (clone 2.4G2; BD), CD33 (clone A2F10; eBioscience), CD115 (clone AFS98; eBioscience), CD34 (clone RAM34; eBioscience), and Siglec F (clone E50-2440; BioLegend). FcεRI (clone E50-2440; BD) were purchased from the indicated vendors. For intracellular FoxP3 staining, cells were fixed overnight in Fixation/Permeabilization buffer (eBioscience). Before acquisition, cells were resuspended in PBS/BSA 0.5%/EDTA (2 mM) solution with 1 µg/ml of DAPI to exclude dead cells. FoxP3 staining, cells were stained with live/dead fixable dead cell stain kit (Invitrogen) before fixation to assess viability. Multiparameter analysis was performed on the LSR II (BD) and analyzed with Flowjo software.

qPCR. Total RNA was isolated from tissue using TRIzol reagent (Invitrogen) and transcribed with RNA to the cDNA EcoDry kit (Takara Bio Inc.). qPCR was performed and analyzed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) using SYBR Green PCR Master Mix (Invitrogen) and the following primers: IFN-γ forward, 5’–TCAAGTGCGTGCTGATGTCGAAAGAA-3’; reverse, 5’–TGGGCTCTGCAGGATTTTCATG-3’.
Assessment of mutation-specific BRAF-V600E mRNA expression in isolated cells. To examine the presence of the mutation-specific BRAF-V600E mRNA in peripheral DC and distinct BM progenitors, single cell suspensions from BM and liver were stained for the appropriate surface markers, and individual cell subsets were purified using FACSana (BD) directly into Trizol reagent (Invitrogen). After RNA isolation and reverse transcription with RNA to cDNA, PCR was performed using Taq Universal or Probes (Bio-Rad Laboratories) on a CFX384 Touch Real-Time PCR Detection system. In detail, reaction was performed with a final dilution of 500 nM of the reverse primer 5′-GTACGCTGCGGCGCTACATCGGC-3′, 500 nM of mutation-specific forward primer 5′-TACTGTGCTTGTGGCTACAGCCAGC-3′, which includes the specific nucleotide substitution of the T>A at the 3′ end and an additional mismatch nucleotide (A>G at the third position from the 3′ end), further enhancing the specific detection of mutant transcripts (strategy adapted from Schnittger et al., 2012), and 250 nM of the FAM-labeled hybridization probe 6-FAM-TGACGCTGCTATCGGCCAGGT-GMBNFQ at an annealing temperature 68°C for 45 cycles. Data were normalized to total BRAF expression using the TaqMan Gene Expression Assay (Life Technologies) recognizing the common exons 13–14 of wild-type BRAF and BRAF-V600E.

Online supplemental material

Table S1 shows the BRAF-V600E status and clinical variables of LCH Patients. Table S2 shows the cell-specific BRAF-V600E status of LCH lesions. Table S3 shows the BRAF-V600E status of non-LCH lesions. Table S4 shows LCH peripheral blood analysis: detectable BRAF-V600E in circulating cells and disease activity. Table S5 shows LCH BM aspirate analysis: detectable BRAF-V600E and disease activity. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130977/DC1.

The authors would like to thank the flow cytometry and microscopy shared research facility at Mount Sinai School of Medicine, Munu Bilgi (Baylor College of Medicine, Pathology Core) for expert technical assistance. The authors would like to thank the flow cytometry and microscopy shared research facility at Mount Sinai School of Medicine, Munu Bilgi (Baylor College of Medicine, Pathology Core) for expert technical assistance. The authors would like to thank the flow cytometry and microscopy shared research facility at Mount Sinai School of Medicine, Munu Bilgi (Baylor College of Medicine, Pathology Core) for expert technical assistance.

Submitted: 11 May 2013
Accepted: 13 February 2014


