bjh review

Recent advances in the understanding of Langerhans cell histiocytosis

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Summary

Langerhans cell histiocytosis (LCH) is a proliferative disease of cells that share phenotypic characteristics with the primary antigen presenting cells of the epidermis. Its clinical manifestations are highly variable, extending from very benign forms to a disseminated, aggressive disease that causes significant mortality. Although many of the fundamental pathogenetic features of LCH have been enigmatic, recent advances have led to a much clearer understanding of the disease. In particular, careful molecular analyses of mouse models and human LCH samples suggest that LCH's cell of origin may not be the epidermal LC itself but a myeloid-derived precursor. Advanced genomic technologies have revealed the presence of activating, somatic *BRAF* mutations in the majority of patient specimens. Together, these observations have produced a new picture of LCH as a myeloid neoplasm. These advances are likely to have profound implications for the use of targeted therapeutics in LCH.

Keywords: histiocytosis, *BRAF*, neoplasia, dendritic cell.

Although Langerhans cell histiocytosis (LCH) is a relatively rare disease, it has nonetheless kept a small army of investigators busy for over a century as they try to answer some of the most basic questions about the disorder. Is LCH a neoplasm? What is its cell of origin? Can a single mechanistic model explain its protean manifestations? Recent advances in molecular genetics and the ability to define and track immune cell lineages have substantially deepened our understanding of LCH and have provided some unexpected answers to these questions. These answers are leading to novel treatment approaches.

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The clinical problem

Langerhans cell histiocytosis is a proliferative disease of cells that share phenotypic characteristics with Langerhans cells (LCs), the primary antigen presenting cell of the epidermis. Although usually thought of as an extremely rare disease of childhood, its incidence of 2–5 per million children per year in Western Europe is comparable to that of Hodgkin lymphoma in a similar population (Guyot-Goubin *et al*, 2008; Reiter & Ferrando, 2009). Still, its incidence is about one tenth that of acute childhood leukaemias. The clinical presentation of LCH is highly variable and, historically, three distinct clinical syndromes have been described: eosinophilic granuloma, characterized by the presence of one or more lytic bone lesions in which the proliferating histiocytes are accompanied by a prominent infiltrate of eosinophils; Hand-Schüller-Christian disease, comprising the clinical triad of bone defects, exophthalmos, and polyuria, the latter due to histiocytic infiltration of the pituitary stalk; and, Letterer-Siwe disease, a fulminant disorder marked by hepatosplenomegaly, lymphadenopathy, skin rash, bone lesions, and haematological compromise (Bechan *et al*, 2006; Degar *et al*, 2009).

Although these presentations are quite disparate, the proliferating cells causing their clinical manifestations share similar morphologies and molecular markers (see below). This insight led to unifying the three syndromes under the heading of Histiocytosis X, the X designating uncertainty about the origin of the histiocytes (Lichtenstein, 1953). Later, when these cells were also shown to share many of the features of LCs, the disease was given the more descriptive but less mysterious name of LCH (Nezelof *et al*, 1973). A useful tool for placing LCH into its proper context among the histiocytoses is the World Health Organization's (WHO) 2008 *Classification of Tumours of Haematopoietic and Lymphoid Tissues* which uses cellular lineage as an organizing principle(Swerdlow *et al*, 2008). LCH and Langerhans cell sarcoma are recognized as a category of tumours derived from LCs. This distinguishes LCH from other entities within the broader class of histiocytic and dendritic cell (DC) neoplasms, which include: histiocytic sarcoma (derived from histiocytes),

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intedigitating dendritic cell sarcoma (from interdigitating DCs), follicular dendritic cell sarcoma (from follicular DCs), disseminated juvenile xanthogranuloma (from macrophages or dermal/interstitial DCs), and 'other rare dendritic cell tumours' including fibroblastic reticular cell and indeterminate dendritic cell tumours.

Considered as a single entity, the clinical manifestations of LCH are more properly viewed as existing along a spectrum of disease that may involve a single site, multiple sites in a single organ system, or multiple organ systems. Stratifying patients based on their position on the spectrum has proven useful in determining prognosis and planning therapy. Single system disease, which occurs in about two-thirds of paediatric LCH patients, usually involves bone but can also affect skin or, rarely, lymph nodes. In many cases, LCH of bone is effectively and definitively treated by surgical curettage with or without local instillation of corticosteroid. Because this form of the disease usually resolves with little or no additional treatment, specific therapeutic modalities are generally chosen based on their low likelihood of causing complications. For this reason, radiation therapy, which was more commonly used in the past, is only rarely applied in this setting. Mild systemic chemotherapy is used for children with disease in cranio-facial bones, also called central nervous system risk sites for their increased risk of late neurodegeneration (McClain, 2005; Grois *et al*, 2006); disease in surgically awkward boney sites e.g. the base of the skull, or who are at risk of fracture; and children with multiple sites of bone involvement, so-called poly-ostotic LCH. Bulky single site disease may also be treated with chemotherapy.

Multisystem disease similarly calls for chemotherapy. However, children who do not have involvement of 'risk organs' i.e. bone marrow, liver, or spleen, generally do well and therefore, again, the guiding principal in this population is to use a therapy that is effective but has minimal short- and long-term toxicities. A variety of clinical trials have been performed in this population but the standard of care is the combination of vinblastine and prednisolone. In clinical trials of combination therapies that included etoposide and mercaptopurine along with vinblastine and prednisolone, low risk patients showed impressive response rates of approximately 90% (Gadner *et al*, 1994; Minkov *et al*, 2000). The high risk patients have a lower response rate and poorer outcomes despite adding methotrexate to the regimen. This population, which tends to be younger, experiences a 20% mortality rate due to refractory or progressive disease (Gadner *et al*, 1994, 2001; Minkov *et al*, 2000).

Adults may experience forms of LCH that are very similar to the childhood versions, i.e. single system or multisystem. Presentations may include seborrheic rash, diabetes insipidus, hepatosplenomegaly, or lymphadenopathy. Bone involvement also occurs but with more frequent involvement of the mandible and less frequent involvement of the skull compared to children (Baumgartner *et al*, 1997). The most common single site presentation is isolated pulmonary disease (Vassallo & Ryu, 2004), which occurs predominantly in patients with a smoking history and, unlike the childhood form, the histiocytes comprising the disease are frequently polyclonal. This has led to the presumption that adult pulmonary LCH is fundamentally a reactive inflammatory disease that is quite distinct from childhood LCH. Nonetheless, as described below, adult pulmonary LCH shares some molecular features with the childhood disease that suggest they are more similar than originally thought.

Pathology as a driving force for unification

Lichtenstein's unification of the three clinical forms of 'Histiocytosis X' was the culmination of nearly a decade of observations made by several discerning pathologists, including T.B. Mallory and Sidney Farber (Farber, 1941; Green & Farber, 1942; Mallory, 1942), who noted that the proliferating cells in all of these disorders had the morphology of histiocytes. Although the origin of these cells was obscure [Lichtenstein wrote that they were derived from 'adventitial reticular cells of blood vessels'(Lichtenstein, 1953)]this novel nosological category provided a new way of thinking about these unusual diseases.

Clues to the true origin of the abnormal histiocytes came initially from ultrastructural analyses. Electron microscopy revealed the presence of Birbeck granules in LCH histiocytes (Mierau *et al*, 1982). These pentilaminar, 'racquet-shaped' cytoplasmic organelles are thought to be functionally important for antigen processing and presentation, and appear to be uniquely present in LCs (Birbeck *et al*, 1961), thus suggesting a shared lineage between these cells and the proliferating cells of LCH. Additional ultrastructural studies supported the notion that all of the various clinical forms of LCH are caused by proliferation of LCs (Basset & Turiaf, 1965; Basset *et al*, 1965; Cancilla *et al*, 1967; Tarnowski & Hashimoto, 1967), leading to the suggestion that they all be denoted LCH (Nezelof *et al*, 1973).

Cell surface markers also support this idea. For example, histiocytes in LCH express CD207, also known as Langerin, which is a C-type lectin with binding specificity for mannose-containing sugars (Geissmann *et al*, 2001). Langerin is also expressed on the surface of LCs and associates with Birbeck granules when it internalizes (Valladeau *et al*, 2000). However, Langerin can also be expressed by so-called Langerin-positive DCs (Romani *et al*, 2010) and by DCs in the marginal zone of the spleen (Idoyaga *et al*, 2009), indicating that it is not solely an LC marker. A more specific marker for LCs and pathological histiocytes is CD1a, which has become the gold standard for LCH diagnostics (Harrist *et al*, 1983; Rousseau-Merck *et al*, 1983; Schuler *et al*, 1983).

A unique ontogeny for LCs

Langerhans cells are haematopoietically-derived antigen presenting cells and thus belong in the DC lineage. Of the two

major categories of DC, classical and plasmacytoid, LCs share surface marker expression patterns with classical DCs, namely major histocompatibility complex (MHC) Class II and CD11c and no lineage markers. However, aspects of LC biology, in particular homeostatic mechanisms regulating the number and distribution of LCs, are distinct from those of classical DCs (Ginhoux & Merad, 2010).

To an extent, both DCs and LCs can be shown to be derived from haematopoietic precursors that enter the systemic circulation. After haematopoietic stem cell transplantation, donor cells efficiently replace recipient classical DCs including those that populate mucosal surfaces (Holt *et al*, 1994; Iijima *et al*, 2007; Liu *et al*, 2007, 2009). Studies of transplantation into lethally irradiated mice also demonstrate that LCs are ultimately derived from circulating precursor cells that arise from the bone marrow (Frelinger *et al*, 1979; Katz *et al*, 1979). Similarly, after UV irradiation of the skin, the LC compartment is replenished by circulating Gr-1^{hi} monocytes (Ginhoux *et al*, 2006) in a process that requires the chemokine receptor CCR2 in order to reach the dermis and CCR6 to populate the epidermis (Vanbervliet *et al*, 2002).

However, at most, only 80% of LCs in transplant recipients were of donor origin in those studies, indicating that LCs are somewhat radioresistant, a unique characteristic for this bone marrow-derived lineage, especially compared to classical DCs. But even this 80% replacement may be an overestimate of the contribution that bone marrow-derived precursors make to steady state LC populations. Whole body irradiation is associated with a significant inflammatory response, which can stimulate the emigration of LCs from the skin. Thus the near-wholesale replacement of recipient LCs with LCs derived from donor bone marrow may depend on the presence of inflammation due to graft-*versus*-host disease in allogeneic recipients or irradiation alone in congenic recipients. In contrast, parabiosis models demonstrate that, under steady state conditions with no acute inflammatory stimuli, LCs in the skin are replaced by local proliferation without a requirement for bone marrow-derived precursors (Merad *et al*, 2002). Furthermore, the half-life of LCs under steady state conditions is long, between 53 and 78 d (Vishwanath *et al*, 2006; Kaplan *et al*, 2007), and is consistent with observations that only 1–2% of LCs are actively proliferating [cited in (Merad *et al*, 2002)]. Slow replacement of LCs in mice after depletion by targeted expression of diphtheria toxin supports the idea that local renewal is the primary source of LCs in the absence of inflammation (Kaplan *et al*, 2005). Even under mild inflammatory conditions that preserve the dermal-epidermal junction, such as atopic dermatitis, the primary mechanism of LC renewal appears to be local proliferation (Chorro *et al*, 2009).

These patterns of homeostatic replacement indicate that LCs and classical DCs have different ontogenies. Growth factor and transcription factor dependencies for the development of LCs and classical DCs are also distinct. Unlike classical DCs, the appearance of LCs does not depend on Flt3 or its ligand, FLt3L (Ginhoux *et al*, 2009). Rather, LCs are nearly absent in mice that are null for some growth factors including TGF-β1 (Borkowski *et al*, 1996), growth factor receptors, such as M-CSFR (Ginhoux *et al*, 2006), and lineage-relevant transcription factors, such as ID2 (Hacker *et al*, 2003) and RUNX3 (Fainaru *et al*, 2004). The requirement for ID2 and RUNX3 is consistent with the requirement for TGF-β1 because they are downstream in the signalling cascade initiated by this growth factor. Furthermore, TGF-β1 is expressed in the skin early in embryogenesis at a time prior to the appearance of CD1a and Langerin expression, consistent with the model of LC ontogeny described above.

Finally, phenotypic studies of LC precursors also point to an origin distinct from that of classical DCs. It has been known for some time that LC precursor cells are Langerin- and CD24-negative in the mouse (Chang-Rodriguez *et al*, 2004; Tripp *et al*, 2004). These cells populate the epidermis when the stratum corneum starts to develop but they do not display MHC Class II and Langerin expression until a week after birth (Romani *et al*, 1986; Elbe *et al*, 1989; Chorro *et al*, 2009). Miriam Merad and her colleagues have been primarily responsible for identifying the precursors of these Langerin-negative cells as monocytoid cells (Merad *et al*, 2002; Ginhoux *et al*, 2006, 2009; Mende *et al*, 2006). These cells are CD11b+, F4/80+, M-CSFR+ and CX3CR1+ although, unlike most monocytes, they are also Gr-1-negative. A hint about the origin of these cells comes from the fact that LC-like cells first appear in limb buds at the same time that yolk sac-derived macrophages appear in skin. Furthermore, yolk sac-derived primitive macrophages display the same suite of surface markers as the Langerin-negative precursors, including the absence of Gr-1. The Merad group hypothesizes that yolk sac-derived primitive macrophages migrate to skin during embryogenesis where they proliferate and then differentiate into Langerin-positive LCs (Ginhoux & Merad, 2010). Thus the origin of LCs is quite distinct from classical DCs and this could have important implications for the cell of origin of LCH.

Recent studies on global gene expression patterns in LCH support the notion that the disease may arise from early myeloid precursors rather than mature LCs themselves (Allen *et al*, 2010). Lesional cells sorted for Langerin positivity expressed a group of genes that was much more characteristic of those expressed by early myeloid cells than mature LCs. Ideally, one would like to know whether the pattern of gene expression in Langerin-positive LCH cells matches that of the yolk sac-derived primitive macrophages (or whatever their equivalent in the human might be). If so, it would suggest that the transforming event in LCH might occur in a cell type that precedes the mature LC that pathological LCs phenotypically resemble.

Pathobiology of LCH

Initial attempts to understand the pathobiology of LCH were focused on the immune and inflammatory nature of the LC,

the presumed cell of origin. The primary function of LCs is to use the dendritic processes they extend in their resting state to survey the epidermis for foreign antigens (Steinman & Hemmi, 2006; Shortman & Naik, 2007). The appearance of these antigens usually occurs in association with immune activators, such as cytokines secreted by epidermal keratinocytes (e.g. TNF-α) or pathogen-associated ligands recognized by immune cell receptors (e.g. Toll-like receptor ligands). Upon activation, LCs take up and process antigen. The activated LCs then migrate to regional lymph nodes where they initiate an adaptive immune response by presenting processed antigen to T lymphocytes.

Some of the molecular mechanisms underlying LC migration are understood. In particular, the process appears to be controlled, at least in part, by sequential expression of chemokine receptors on the surface of LCs (Dieu *et al*, 1998; Sallusto *et al*, 1998; Sozzani *et al*, 1998). In their resting state, LCs express the chemokine receptor CCR6 whose ligand, CCL20, is secreted by epidermal keratinocytes. This ligand/receptor interaction is thought to keep resting LCs in the skin. Upon activation, LCs down regulate CCR6 and in its place up-regulate a different chemokine receptor, CCR7, whose ligands, CCL19 and CCL21, are secreted by cells in regional lymph nodes. In the absence of CCR6 expression, activated LCs are no longer anchored in the skin, and expression of CCR7 attracts them to regional lymph nodes where they can encounter and activate T cells.

A feature of some forms of LCH, such as Letterer-Siwe disease, is the presence of pathological LCs in multiple organs at the same time, including both skin and lymph nodes. This observation led to the hypothesis that patterns of tissue infiltration in LCH might be explained by dysregulated expression of chemokine receptors. Two groups have examined this question carefully. One found evidence for simultaneous expression of CCR6 and CCR7 by pathological LCs in a collection of 20 LCH cases from a variety of tissue sites including skin (Fleming *et al*, 2003). A second group examined a similarly large collection of samples and demonstrated CCR6 expression by pathological LCs but found no evidence for co-expression of CCR7 (Annels *et al*, 2003). The reasons for this discrepancy are not clear but, despite their differences with respect to CCR7, both reports support the notion that chemokine receptor expression is abnormal in LCH; CCR6 should not be expressed by activated LCs. Several pharmaceutical and biotechnology companies are actively searching for chemokine receptor antagonists, including drugs that target CCR6 and CCR7, and this may be a promising therapeutic avenue in LCH.

These studies on chemokine receptors are consistent with a substantial literature suggesting that pathological LCs have markers characteristic both of activated and resting LCs. This would suggest a model in which pathological LCs suffer from a maturation defect. On one hand, pathological LCs express several markers consistent with LC activation, including CD2, CD11b, CD24, CD44, CD54, CD58, CD80, and CD86 (Emile *et al*, 1994a; de Graaf *et al*, 1994; Tazi *et al*, 1999). They also express the gamma chain of the GM-CSF receptor (Emile *et al*, 1995) which is not expressed by normal resting LCs and may provide a means whereby ambient GM-CSF, which is present in the sera of patients with LCH (Emile *et al*, 1994b), could contribute to pathological LC activation. Similarly, pathological LCs express β1 and β2 integrins that are not expressed by resting LCs (Ruco *et al*, 1993; Emile *et al*, 1994a; de Graaf *et al*, 1994), and they are missing E-cadherin, which is also down-regulated during LC activation (Tang *et al*, 1993; Geissmann *et al*, 1997).

On the other hand, pathological LCs also share many features of resting LCs. Most prominently, the Birbeck granules that are so characteristic of pathological LCs are found in resting LCs and are lost upon activation (Stossel *et al*, 1990). Although pathological LCs lose E-cadherin expression, similar to activated LCs, they also express VLA-5 and L-selectin, which is more characteristic of resting LCs (de Graaf *et al*, 1995). In addition, despite the expression of potent co-stimulatory molecules, such as CD80 and CD86, pathological LCs are not efficient activators of resting T cells. However, CD40L treatment *in vitro* can make these cells potent antigen presenters (Geissmann *et al*, 2001).

These studies, as well as many others, generally paint a picture of LCH cells that is consistent with some kind of maturation defect. This would argue against LCH being a disease that is driven by an inflammatory stimulus, in which case the proliferating cells would be expected to resemble activated LCs, and rather argue for a disease that is driven by an underlying molecular abnormality that produces a non-physiological phenotype as one of its consequences. The report of CD40L's ability to enhance antigen presentation by pathological LCs *in vitro* (Geissmann *et al*, 2001) is not inconsistent with this idea as it still implies a requirement for exogenous, potentially non-physiological stimuli to achieve maturation.

Is LCH a reactive inflammatory disease?

The idea that LCH might be a disease driven by a molecular abnormality raises a fundamental question about its nature: is LCH a neoplasm or an inflammatory disorder? In support of the inflammatory nature of LCH is the fact that the presumed cell of origin, until recently the LC, is an immune cell that responds to inflammatory stimuli by proliferation and activation. The prominent inflammatory infiltrates that accompany most LCH lesions can also be considered evidence in favour of this pathogenesis. Further, pathological LCs have a benign cytological morphology, their karyotypes are normal, and again, until recently, no recurrent genetic abnormalities had been associated with LCH (Schmitz & Favara, 1998; da Costa *et al*, 2009). Well-documented examples of spontaneous remissions also suggest the possibility that LCH is a fundamentally benign disorder, albeit with occasionally dire outcomes (Broadbent *et al*, 1984; McElligott *et al*, 2008).

If LCH were a reactive inflammatory disease, it might be analogous to the acquired histiocytoses that occur in response to triggering infections (Janka *et al*, 1998). To date, however, no viral genomes have been identified in LCH lesions and there are no epidemiological studies that support an infectious or environmental stimulus for LCH (Willman & McClain, 1998; Nichols *et al*, 2003). Immunohistochemistry and *in situ* hybridization for herpesviral proteins and RNA (Glotzbecker *et al*, 2004), as well as polymerase chain reaction (PCR) for herpesviral nucleic acids (Leahy *et al*, 1993), had provided suggestive evidence of an association with herpesviruses. However, a careful study of the prevalence and titres of antibodies against these viruses demonstrated that they were the same in LCH patients and age-matched controls (Jeziorski *et al*, 2008), supporting earlier studies indicating the absence of an association between LCH and herpesviruses (Jenson *et al*, 2000).

Is LCH a neoplastic disease?

Despite its inflammatory characteristics, the generally benign morphology of its proliferating cells, and the occasional spontaneous remission, some LCH cases behave in a manner that is indistinguishable from an aggressive malignancy. This observation alone suggests that the question of whether or not LCH is a neoplasm merits serious consideration. Although distinguishing between an inflammatory *versus* neoplastic pathogenesis for LCH may seem like a fine point, the difference is not merely semantic. If the disease were inflammatory, then the proliferating cells in LCH would be dividing aggressively in response to some inciting stimulus as yet undefined. In that case, the LCH cells would be fundamentally normal and one might anticipate that their hyperproliferative state could be reversed or mitigated upon removal of the inflammatory stimulus. In contrast, if the disease were neoplastic, then the unrestrained proliferation of pathological LCs would probably be the consequence of alterations in genes that regulate cell division. In that case, LCH would fall into the contemporary cancer treatment paradigm in which drugs directed against the products of those altered genes might produce a significant clinical response.

A substantial amount of effort has been directed toward determining whether or not LCH is a neoplasm. Among the most compelling findings in support of that pathogenesis is the fact that the pathological LCs in the non-pulmonary forms of LCH are clonal (Willman *et al*, 1994; Yu *et al*, 1994). Although clonality is a hallmark of neoplasia, its presence alone would not be sufficient to classify LCH as a neoplasm; recurrent genetic abnormalities would also be required. These have been sought with great diligence but the rarity of LCH has made the task highly challenging. Nonetheless, several surveys of varying degrees of thoroughness have been published. For example, a small study of five patients reported cytogenetic abnormalities (Betts *et al*, 1998) but none were recurrent and a subsequent larger study of 31 cases identified no similar abnormalities (da Costa *et al*, 2009). Furthermore, the latter study demonstrated diploid genomes in all of the examined cases. Array comparative genomic hybridization (aCGH) analysis of seven bone lesions appeared to show widespread copy number alterations and recurrent loss of heterozygosity (LOH) involving loci on several chromosomes (Murakami *et al*, 2002). Another LOH study using PCR demonstrated a higher degree of fractional allelic loss in multisystem and high-risk LCH compared to single system and low-risk disease (Chikwava *et al*, 2007). Again, however, a larger study using aCGH and high density single nucleotide polymorphism arrays detected no significant copy number variations (da Costa *et al*, 2009). Finally, one of the most common molecular abnormalities in LCH is overexpression of TP53 (Weintraub *et al*, 1998) although its basis is unclear because no mutations in *TP53* or abnormalities of MDM2 were detected in the 10 samples examined in that study, nor were any *TP53* mutations found in a later survey (da Costa *et al*, 2009) (but see below).

Point mutations in LCH

Missing from this litany of negative findings is a global assessment of cancer-related point mutations. This is not surprsing given the rarity of the disease and the requirement for fresh frozen tissue by most mutation detection technologies. Although some investigators have had the foresight and persistence to bank frozen material from LCH patients, they are few in number and the amounts of tissue banked on each patient tend to be quite small. Fortunately, LCH researchers are the beneficiaries of advances in genomic technologies that have been directed toward the analysis of formalin-fixed, paraffin-embedded (FFPE) tissues. These technologies have opened the archives of clinical pathological samples to genomic analysis.

One example of a mutation-detection technology that is particularly robust in its ability to analyse FFPE material is the mass spectrometry-based genotyping platform developed by the Sequenom corporation (Tang *et al*, 1995). In this analysis, the genomic region surrounding the locus of a point mutation of interest is amplified by PCR and the product is then used as the template for single or dinucleotide primer extension over the position of the polymorphism. In the case of a sample taken from someone who is heterozygous for the polymorphism, two primer extension products will be generated corresponding to the two alleles. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry is then used to distinguish between the products. The ability of mass spectrometry to separate and identify molecules that vary only slightly in their molecular masses permits extensive multiplexing. One customized version of Sequenom genotyping is called OncoMap (Thomas *et al*, 2007; MacConaill *et al*, 2009). Through carefully organized multiplexing, this assay can test for the presence of over 1000 alleles derived from 140 genes. The alleles tested by OncoMap were culled from the literature based on their relevance to cancer.

Because the amplicons generated as primer extension templates are only 200–400 base pairs in length, Sequenom-based analyses often work robustly on DNA extracted from FFPE material which, although fragmented, is mostly larger than this size (Goelz *et al*, 1985). In a recent study of paediatric low-grade astrocytomas using a version of OncoMap that measures over 400 alleles in 33 cancer-related genes, the sensitivity and specificity of the test on FFPE-derived material was 89·3% and 99·4%, respectively; in comparison, the sensitivity and specificity of the same OncoMap test on fresh frozen astrocytomas was 93·8% and 100% (MacConaill *et al*, 2009).

Frequent *BRAF* mutations in LCH

OncoMap's ability to generate a broad, point mutation-based genotype on archived specimens with great sensitivity makes it an ideal tool for examining rare cancers. Recently, a version of OncoMap that tests 983 mutations across 115 genes was used to examine 61 archived LCH cases (Badalian-Very *et al*, 2010). Remarkably, the activating mutation BRAF V600E was found in 57% of the specimens. Other point mutations were scarce but included a cancer associated mutation in *TP53* (TP53 R175H), a transforming allele in *KRAS* (KRAS G13D), and a candidate transforming allele in *MET* (MET E168D). Of course, given OncoMap's ability to measure only what it sets out to measure, other mutations may have been present in this collection of LCH material. However, none of the nearly 1000 other mutations in this OncoMap panel was detected. Importantly, the presence of BRAF V600E in these archived samples was confirmed by an orthogonal technology, namely pyrosequencing.

The specificity of the appearance of BRAF V600E in the LCs of LCH as opposed to proliferating normal LCs was demonstrated by finding only wild type alleles in samples of dermatopathic lymphadenopathy. And, its specificity for LCH as opposed to other histiocytoses was demonstrated by the absence of BRAF V600E from samples of Rosai-Dorfman disease (sinus histiocytosis with massive lymphadenopathy) and juvenile xanthogranuloma.

The most parsimonious explanation for the presence of this mutation in archived LCH specimens is that it is a transforming oncogene in LCs. However, demonstrating this would require detection of the mutation or its product specifically in the LCs of these clinical specimens that contain a highly heterogeneous cellular population. Unfortunately, despite a pressing need, no mutant-specific anti-BRAF antibody exists which could be used in immunohistochemistry, immunofluorescence, or fluorescence-activated cell sorting. Instead, the presence of BRAF V600E within LCs was inferred by demonstrating that its relative abundance compared to the wild type allele was enriched in CD1a-positive cellular material harvested from these samples by laser-capture microdissection. Furthermore, the proportion of LCs in any individual sample in this collection was directly proportional to the relative abundance of the mutated allele compared to the wild type allele. (Interestingly, the abundance of the mutated allele was about 50% of the abundance of LCs, suggesting the possibility that the mutated allele was present in a single copy per cell, which would be consistent with the properties of a dominant oncogene).

BRAF functions in the signalling cascade that usually begins with activation of a receptor tyrosine kinase and proceeds by phosphorylation steps through Ras to Raf to MEK and ERK kinases, ultimately resulting in modulation of gene expression (Montagut & Settleman, 2009). Based on the finding of activated BRAF in the majority of LCH specimens, it may not have been surprising that phosphorylated MEK and ERK were observed by immunofluorescence specifically within pathological LCs. However, what was unexpected was the presence of MEK and ERK phosphorylation in 100% of examined specimens regardless of *BRAF* mutation status. Another *BRAF*-based mechanism for activation of this pathway is duplication of the *BRAF* locus. This occurs in a significant proportion of paediatric low-grade astrocytomas (Jones *et al*, 2008; Pfister *et al*, 2008) but was not detected by fluorescence *in situ* hybridization in 43 LCH cases (Badalian-Very *et al*, 2010). Nonetheless, the evidence to date indicates that activation of the RAS-RAF-MEK pathway is likely to occur uniformly in LCH. In about 50% of cases, the pathway is activated by oncogenic point mutation of *BRAF*. The basis for pathway activation in the remaining cases is currently unknown but could be a consequence of receptor tyrosine kinase activation by overexpression of the receptor itself or by its ligand. (Amplification of genes or overexpression of their products cannot be detected by OncoMap.) Ligand overexpression may occur in a different cell type and activate the pathway via paracrine means.

Biology of *BRAF* mutations in LCH

As noted above, the spectrum of clinical and pathological behaviours that fall under the heading of LCH is extremely broad. The presence of BRAF V600E can explain some of these. Certainly, the appearance of a transforming oncogene in a LC provides it with a proliferative advantage that could lead to the development of a clonal lesion. In addition, however, some of the features of other diseases in which BRAF V600E is known to play an important role may shed light on other behaviours observed in LCH. For example, nevi are now understood to be an *in vivo* example of oncogene-induced senescence (Michaloglou *et al*, 2005). Expression of BRAF V600E in otherwise normal melanocytes can lead to several rounds of cell division until senescence supervenes as an organism-level protective response to the presence of an activated oncogene. A similar scenario might apply to early LCH lesions that, in the presence of BRAF V600E, might undergo a few rounds of cell division followed by senescence, thus appearing to resolve spontaneously. However, in other cases, again by analogy to melanomas, other genomic abnormalities may enable LCs to

overcome the senescence pathways initially induced by BRAF V600E, thereby leading to an aggressive, Hand-Schüller-Christian- or Letterer-Siwe-like disease. The frequent appearance of TP53 overexpression in LCH (Weintraub *et al*, 1998; da Costa *et al*, 2009) suggests that abnormalities in this pathway may work in concert with BRAF V600E to produce full-blown LCH.

Unfortunately, the reports to date on the prevalence of BRAF V600E in LCH include only minimal clinical information. So far, there appears to be no correlation between the presence of BRAF V600E and clinical behaviours of LCH, such as multi-system *versus* single system disease. Larger studies with more thoroughly annotated specimens will be required before such associations can be ruled out with confidence. Nonetheless, even with the limited clinical information available, the presence of BRAF V600E does correlate with young age. In one study, 76% of tumours in children <10 years of age had BRAF V600E while only 44% of tumours in patients 10 years of age or older had the mutation (Badalian-Very *et al*, 2010).

In that study, the presence or absence of BRAF V600E also did not correlate with anatomic sites of involvement. In fact, surprisingly, the mutation was present in over 40% of the cases of adult pulmonary LCH examined. This is notable because of the traditional interpretation of this disorder as a polyclonal inflammatory disease of adult smokers. However, a careful study of clonality in pulmonary LCH demonstrated clonal disease in 29% of cases (Yousem *et al*, 2001). This frequency is in a range comparable to the 40% of pulmonary cases that had BRAF V600E. But polyclonality in pulmonary LCH still does not exclude a pathogenetic role for *BRAF* mutation. Cigarette smoke may induce the T1799A transversion that underlies BRAF V600E at multiple sites throughout the lungs of susceptible smokers. This 'field defect' may result in many independent clones of transformed LCs that all carry BRAF V600E but, in the aggregate, would be a polyclonal disease. A similar type of polyclonal BRAF V600E-containing disease has been described in cutaneous nevi (Lin *et al*, 2009).

Conclusions

After decades of careful work and molecular characterization, a clearer picture of LCH is beginning to emerge. The fact that LCH is a clonal disease in which the majority of cases carry an activating allele of an authentic oncogene indicates that it is a neoplasm. While some of its clinical manifestations may be related to an accompanying inflammatory state, and while it is possible that some of its behaviour may be exacerbated by inflammatory stimuli, the pathogenesis of a majority of LCH cases lies firmly within the paradigm of a cell-autonomous proliferative disorder that arises from a somatic mutation of a cell proliferation gene.

This insight creates several opportunities both for research and treatment. The LCH field has suffered from the lack of a faithful animal model that could be used to test hypotheses about pathogenesis as well as potential new treatments. The closest example is a transgenic mouse in which SV40 large T antigen is driven by the promoter of CD11c (Steiner *et al*, 2008). Although this transgene transforms conventional DCs, which is a lineage distinct from LCs as noted above, it produces a histiocytosis-like disease. But, in addition to the question of what cellular precursor is being targeted in this model and whether it is relevant to LCH, inactivation of the retinoblastoma pathway has not been documented in human LCH. Now, however, with the identification of BRAF V600E as commonly occurring in LCH, a variety of murine models incorporating this activated oncogene can be imagined and relatively easily designed. This will be an essential next step in demonstrating that mutated *BRAF* is causal in LCH rather than merely a concurrent finding. These models may also be necessary in order to determine which particular precursor cell in LC ontogeny must undergo the oncogenic 'hit' to *BRAF* in order to produce LCH.

Perhaps most exciting is the potential for treatment strategies based on the presence of BRAF V600E. Again, melanoma provides an instructive analogy. Despite the presence of many additional genomic abnormalities in metastatic melanoma, targeted inhibition of BRAF V600E alone is sufficient to produce impressive clinical responses and prolonged survival (Chapman *et al*, 2011). These responses occur specifically in the patients whose tumours bear BRAF V600E. There is every reason to suspect that BRAF V600E-positive LCH patients would also respond to BRAF V600E inhibition. The toxicity profile of the current generation of BRAF V600E inhibitors includes squamous cell carcinomas of the skin, which are likely to be mechanism-based effects of the drugs (Flaherty *et al*, 2010; Poulikakos *et al*, 2010). While these are easily managed in adults who have life-threatening melanomas, their appearance may be less acceptable in children with mild forms of LCH. Clinical trials will have to be carefully planned to optimize the risk/benefit ratio of BRAF V600E inhibition in this population.

For nearly a century, the analysis and understanding of LCH has been a triumph of lumpers over splitters. Careful morphological analysis united the disparate clinical presentations of eosinophilic granuloma, Hand-Schüller-Christian disease, and Letterer-Siwe disease under the heading of Histiocytosis X. Electron microscopy and immune marker studies showed that these were all diseases of LC-like cells. The demonstration of frequent *BRAF* mutations in all of these disorders further unifies them and draws pulmonary LCH into the fold.

Nonetheless, the LCH umbrella covers distinct clinical entities that 'breed true.' Undoubtedly, there are additional genomic abnormalities that account for the disparate clinical behaviours of these LCH subtypes. As more advanced genomic technologies become adapted for the analysis of archived materials, new abnormalities will almost certainly be associated with specific clinical subtypes of LCH. The next phase of LCH research should witness a triumphant re-emergence of the splitters.

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