Immunologic Heterogeneity of Diffuse Large Cell Lymphoma

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The cellular lineage of 57 diffuse large-cell lymphomas (DLCLs) was determined using a panel of monoclonal antibodies directed against lineage-restricted and -associated T, B, and monocyte antigens. The majority (82%) were of B cell lineage as determined by the expression of sIg and/or B1, with the remaining 16% being of T cell lineage and 2%, of monocyte-myeloid lineage. By the expression of other B cell–restricted and –associated antigens, two major and two minor subgroups could be identified. These subgroups expressed the following phenotypes: (1) B1 B4 \( ^{-} \) sIg B2 \( ^{-} \) (51%); (2) B1 B4 \( ^{-} \) sIg B2 \( ^{-} \) (29%); (3) B1 B4 \( ^{-} \) sIg B2 \( ^{-} \) (10%); and (4) B1 B4 sIg B2 (10%). The morphology of transformed lymphocytes, the weak to absent expression of the early B cell antigens B2 and sIgD, and the absence of the late B cell differentiation antigens PCA-1 and PC-1 suggested that these tumors were the neoplastic counterparts of normal B cells at the mid-stages of differentiation. Further support for the notion that B-DLCLs correspond to transformed B lymphocytes was concluded from the observation that B cells could be identified in normal spleen that expressed the cell surface phenotype and morphological appearance of the majority of B-DLCLs.

Despite a decade of intense investigation, the lineage derivation of some diffuse large-cell lymphomas (DLCLs) is still unresolved. Using conventional immunologic techniques, these tumors have been subdivided into three major subgroups based on expression of lineage-restricted markers. Most DLCLs (50% to 80%) express cell surface immunoglobulin (sIg') demonstrating their B cell derivation. Approximately 10% of these tumors lack sIg (sIg') and bind sheep red blood cells (E') and therefore are of T cell origin. Finally, the remaining 10% to 40% are sIg E' and have been considered to be of indeterminate lineage.

Regardless of cellular lineage, the DLCL cell has been thought to correspond to a transformed lymphocyte based on the morphological criteria of increased cell size, abundant cytoplasm, and a large nucleus with prominent nucleoli. In addition to morphology, the in vivo and in vitro evidence that these cells are rapidly proliferating further suggests that these cells correspond to transformed lymphocytes. More recently, a number of laboratories have developed monoclonal antibodies that are reactive with lineage-associated antigens. These studies have identified tumor cell heterogeneity but, to date, have not determined the cellular origin of all sIg E' DLCLs, nor have they provided evidence that B-DLCLs correspond to transformed lymphocytes.

In the present report, we have used a panel of lineage-restricted and -associated monoclonal antibodies to define the lineage and state of differentiation of tumor cells isolated from 57 patients with DLCL. In the studies detailed later, we will show that the majority of sIg' and sIg' DLCLs are of B cell lineage and appear to correspond to a number of discrete differentiation stages preceding and including the transformed B lymphoblast. The observed phenotypic heterogeneity of these B cell lymphomas provides a context in which to study the normal transformed B lymphocyte as well as providing yet another set of criteria with which to study the clinical behavior of this disease.

MATERIALS AND METHODS

Patients and Sample Preparation

Tumor samples from 57 patients with DLCL were obtained from Dana-Farber Cancer Institute, Brigham and Women's Hospital, New England Deaconess Hospital, and Massachusetts General Hospital after appropriate human protection committee validation and informed consent. Patients in this study have not been reported previously. The samples obtained contained greater than 75% neoplastic cells by Wright-Giemsa stain morphology and were classified according to a modified Rappaport system. Tissue specimens were placed in a medium containing 5% fetal calf serum (FCS), finely minced with forceps and scissors, and made into single-cell suspensions by extrusion through a stainless steel mesh. Isolated tumor cells were studied either fresh or cryopreserved in 10% dimethylsulfoxide and 20% FCS at \(-196 \degree C\) in the vapor-phase of liquid nitrogen until the time of characterization.

Monoclonal Antibodies

The preparation and characterization of monoclonal antibodies used in this study have been described. The antigens to which these
antibodies are directed are summarized and referenced in Table 1. All antibodies used in this study were ascites fluid used at saturated binding concentrations. Controls represent isotype-identical non-reactive ascites.

**Indirect Immunofluorescence**

Indirect immunofluorescent staining and flow cytometric analysis has been described. In brief, 1 to 2 x 10^6 cells were treated with 100 µL of a 1:250 dilution (for all antibodies this was a saturating concentration) of the specific or isotype-identical control unreactive monoclonal antibody, incubated at 4 °C for 30 minutes, washed three times, then stained with 100 µL of a 1:40 dilution of a combination of fluorescein-conjugated goat anti-mouse IgM and goat anti-mouse IgG (g/m-FITC) for 30 minutes at 4 °C (Coulter Immunology, Hialeah, Fla). Cells were washed three times and then analyzed for immunofluorescence using a FACS I (Becton Dickinson, Mountain View, Calif). Attempts were made only to examine the malignant cells within each specimen by appropriate size scatter gating. For each sample, 10,000 cells were analyzed using linear fluorescence. A positive reaction was considered when greater than 20% of test cells were more fluorescent than the number of cells positive with isotype-identical control ascites. For each sample, a quantitative assessment of the number of positive cells was made (number of cells reactive with the test monoclonal antibody – the number of cells reactive with unreactive isotype-identical monoclonal antibody/10,000 total cells tested).

**Sources of Human B Lymphocytes**

Normal spleen was obtained from operative specimens of patients not known to have any systemic or malignant disease. Single-cell suspensions were prepared by dissolution with forceps and scissors and extrusion through a stainless steel mesh. Mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation were enriched for B cells by E rosetting and adherence to deplete T cells and macrophages before analysis. Cells were either used fresh or cryopreserved as described.

**Staining of Lymphoid Populations With Anti-B1 and Anti-B2 Monoclonal Antibodies**

Directly biotin-conjugated anti-B2 (Coulter Immunology) and directly fluoresceinated (f/p 6.8) anti-B1 (Coulter Immunology) were ultracentrifuged at 40,000 g for 20 minutes to remove aggregates immediately before staining. In preparation for dual fluorescent cytofluorographic analysis and sorting, splenic mononuclear cells enriched for B cells were first incubated for 20 minutes at 4 °C with directly fluoresceinated anti-B1 (1:100) and directly biotin-conjugated anti-B2 (1:100). After washing, cells were developed with Texas red-TM (Molecular Probes, Junction City, Ore) conjugated to avidin (Calbiochem, LaJolla, Calif) to label free biotin sites. In this manner, all B1-bearing cells were labeled with green emitting dye (fluorescein), all B2-bearing cells were stained with a red emitting dye (Texas red), and cells bearing both the B1 and B2 antigens were stained with both fluorochromes. The percentage of cells expressing either fluorochrome alone or coexpressing both dyes were determined by analysis of at least 10,000 viable cells using a dual laser cell sorter (EPICS V, Coulter Electronics, Hialeah, Fla).

In addition to the dual staining protocol described, appropriate controls for specificity of fluorescein and Texas red staining included the staining of cells with an unreactive monoclonal antibody as a negative control, as well as incubation with either anti-B1 or anti-B2 monoclonal antibodies as positive controls. All three groups were developed with g/m-FITC. Staining was also performed with an unreactive directly fluoresceinated goat anti-mouse immunoglobulin (Coulter Immunology) as a negative control. Staining with Texas red avidin alone was performed as a negative control. In all cases, background red fluorescence did not exceed that from staining with Texas red avidin alone. The reactivity of directly fluoresceinated anti-B1 and directly biotinylated anti-B2 correlated closely with that obtained using indirect techniques (ie, anti-B1 and anti-B2 devel-

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**Table 1. Expression of Lineage-Restricted and Associated Antigens**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Normal Cellular Expression</th>
<th>Stage of B Cell Differentiation</th>
<th>Molecular Weight (kd)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B restricted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>B</td>
<td>Pan B</td>
<td>95</td>
<td>20</td>
</tr>
<tr>
<td>B1</td>
<td>B</td>
<td>Pan B</td>
<td>35</td>
<td>21-22</td>
</tr>
<tr>
<td>B2</td>
<td>Limited B</td>
<td>140</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td>B</td>
<td>Limited B</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Slg</td>
<td></td>
<td>Limited B</td>
<td>IgM-900</td>
<td>25</td>
</tr>
<tr>
<td>T associated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>B, monocyte, activated T, CFU-C</td>
<td>Pan B</td>
<td>29, 34</td>
<td>26</td>
</tr>
<tr>
<td>CALLA</td>
<td>Pre-B, granulocyte</td>
<td>Limited B</td>
<td>100</td>
<td>27</td>
</tr>
<tr>
<td>PCA-1</td>
<td>Plasma cell, granulocyte, activated T</td>
<td>Limited B</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>C3b (57F)</td>
<td>B, erythrocyte, granulocyte, monocyte</td>
<td>Limited B</td>
<td>200</td>
<td>29</td>
</tr>
<tr>
<td>T1</td>
<td>B, T, thymocyte</td>
<td>Limited B</td>
<td>69</td>
<td>30</td>
</tr>
<tr>
<td>T restricted and associated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>T</td>
<td>19</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>T inducer/helper</td>
<td>62</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td>T cytotoxic/suppressor</td>
<td>76</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>T11</td>
<td>T, thymocyte</td>
<td>55</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Myeloid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mo1</td>
<td>Granulocyte, monocyte, natural killer cell</td>
<td>90, 150</td>
<td>32</td>
<td></td>
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</tbody>
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can conclude that the overwhelming majority of these tumors were also of B cell lineage. Thus by the expression of slg and/or B1, tumors expressed the B cell-restricted antigen B1, expressed slg on T cell antigens. However, nine of these tumors were considered to be of "null" cell origin, since they neither expressed B2 nor were B cell derived. Of the 19 slg negative DLCLs, nine uniformly expressed the mature T cell antigens T1 and T3 as well as other T cell antigens, including T11 and T4 or T8, suggesting a T cell lineage for these tumors. The remaining ten DLCLs would traditionally be considered to be of "null" cell origin, since they never expressed slg or T cell antigens. However, nine of these tumors expressed the B cell-restricted antigen B1, suggesting that these tumors were also of B cell lineage. Thus by the expression of slg and/or B1, we can conclude that the overwhelming majority of DLCLs in this study are B cell derived. It is important to note that one patient with DLCL could not be classified as either B or T cell derived. These tumor cells expressed the Ia and Mo1 antigens, suggesting that they were of a myelomonocytic or natural killer cell origin.

Subgroups of B Cell DLCL

With the observation that more than 80% of DLCLs were of B cell lineage, we then attempted to determine whether immunologically defined subgroups could be identified using monoclonal antibodies directed against four B lineage-restricted differentiation antigens (B1, B4, slg, and B2). Using these monoclonal antibodies, 41 patients with B cell (B)-DLCL were studied. All expressed B1, more than 90% expressed either B4 or slg, and only 39% expressed B2 (Table 3). With these markers, two major subgroups of B-DLCLs could be defined. The largest subgroup expressed B1, B4, and slg but lacked B2 (B2-). The other major subgroup expressed all four antigens (B2+). These two subgroups accounted for 80% of all B-DLCLs. Examination of the two major subgroups demonstrated that of the B2- group, 14 of 24 had been treated with combination chemotherapy before phenotypic analysis. Similarly, eight of 15 of the B2+ group were treated previously. Two minor subgroups were noted, each accounting for 10% of all patients, and included the B1-B4 slg B2 and B1-B4 slg B2- phenotypes. In addition, a small number of patients expressed cell surface phenotypes that did not conform to the subgroups presented in Table 3. Specifically, one patient expressed the B1-B4+ slg B2- phenotype and one expressed the B1-B4- slg B2+ phenotype.

Characterization of the B2+ and B2-

Subgroups of B-DLCL

Previous studies on the expression of B cell antigens on B cell–derived tumors have suggested that these tumors correspond to a finite number of normal B cell differentiative steps. The observations that B2 divides B-DLCL into two subgroups suggests that these tumors might correspond to the stages of B cell differentiation where B2 is lost. Our previous model of

<table>
<thead>
<tr>
<th>Expression of B Cell Restricted Antigens</th>
<th>No. of Patients Expressing Phenotype</th>
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<tbody>
<tr>
<td>B1</td>
<td>B4</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
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</table>

Two additional DLCLs are not included in this table but are described in the text.
B cell differentiation suggested that the loss of B2 occurred with the disappearance of cell surface IgD and preceded the acquisition of plasma antigens PCA-1 and PC-1. We have therefore attempted to further define the immunologic heterogeneity of B-DLCL.

Examination of the intensity of expression of the four previously mentioned B cell-restricted antigens defining the subgroups of B-DLCL was then undertaken. Regardless of the subgroup, the expression of the B1 antigen was very strong (Fig 1). Within each tumor cell population, almost all tumor cells (50% to 90%) strongly expressed B1. In contrast, the B2 antigen was detected in only 16 of 41 B-DLCLs and, more importantly, the antigen intensity and number of positive tumor cells (20% to 40%) was much less than was seen for the B1 antigen (Fig 1). The expression of the B4 antigen was intermediate between B1 and B2 in both antigen intensity and number of antigen positive cells (20% to 80%). These observations are consistent with the notion that the B-DLCL correspond to the stage of differentiation where B2 antigen is lost.

In the past, many studies have examined the expression of the heavy chain isotypes of Ig and Ia on DLCLs. In the present study, we examined the subgroups of B-DLCL for the expression of Ia and heavy chain isotype. Whereas the intensity of Ia was strong, the intensity of sIg expression was weak to moderate regardless of the subgroup. Of the 33 sIg+ specimens studied, IgM was the predominant heavy chain expressed (22 of 33), with IgG occurring less frequently (11 of 33). When the 33 sIg+ patients were subdivided into the B2+ and B2− subgroups, similar percentages of IgM and IgG were found in each subgroup. It is important to note that only three patients—two in the B2+ group and one in the B2− group—expressed IgD and they all coexpressed IgM.

Two patients in the B2+ group coexpressed IgG and IgM.

In previous studies, we have shown that the PCA-1 and PC-1 antigens are expressed exclusively at the terminal stages of B cell differentiation, with PCA-1 preceding PC-1 in its expression. A small number of patients—3 of 32 (two B2−, one B2+)—were noted to express PCA-1 only on a fraction of the cells within the tumor specimen (20% to 30%). No tumor expressed the PC-1 antigen. Therefore, the lack of expression of PCA-1 and PC-1 suggests that B-DLCLs do not correspond to the terminal stages of B cell differentiation.

Previous studies of B-DLCLs have noted that up to 40% of these tumors bind EAC rosettes and therefore express receptors for the third component of complement. The B2 antigen has recently been shown to be the receptor for the C3d complement component. The observation that B2 is expressed on only some (16 of 41) B-DLCLs is consistent with these previous studies. In contrast, a monoclonal antibody (57F) directed against the human C3b receptor was found to be reactive with only four of 36 B-DLCLs, and all were with the B2− subgroup.

Finally, it is of interest to note that seven patients (five B2+, two B2−) expressed the common acute lymphoblastic leukemia antigen (CALLA). The intensity of expression of this antigen was weak in contrast to that seen on non-T cell acute lymphoblastic leukemias. It has been previously noted that CALLA is not a leukemia-specific antigen, and it is expressed on the tumor cells of most patients with nodular poorly differentiated lymphocytic lymphoma, nodular histiocytic lymphoma, and Burkitt's lymphomas. In this series, four patients had previously documented nodular lym-
phoma. Three of four patients expressed CALLA and two expressed B2. However, only one patient coexpressed CALLA and B2.

**Normal Cellular Counterpart of B-DLCL**

With the demonstration that most B-DLCLs express Ia, B4, B1, and slg but lack B2, slgD, and plasma cell antigens, we then attempted to identify normal cells that would have both the phenotypic and morphological characteristics of the B-DLCL cell. To identify the normal cellular counterpart of the B-DLCL cells, B cells isolated from normal spleen were dual fluorochrome-labeled by incubating with fluorescein-conjugated anti-B1 and biotin-conjugated anti-B2 developed with Texas red avidin. These cells were then analyzed by dual-laser flow cytometric analysis for the presence of green fluorescence alone (B1), red fluorescence alone (B2), and simultaneous expression of green and red fluorescence (B1 and B2). Three distinct subpopulations were detected: the largest coexpressed B1 and B2 antigens (B1'B2'), a smaller subset of cells bearing B1 alone (B1'B2'), and a minor subgroup expressed only B2 (B1'B2'). Closer examination of the B1'B2' subgroup revealed that the cells could be divided into two populations by the intensity of B2 staining (Fig 2). Cells strongly expressing B2 (B1'B2'H) were separated from those weakly expressing B2 (B1'B2'L) by dual fluorescence sorting. Three populations (B1'B2'H, B1'B2'L, and B1'B2') were then analyzed for size by flow cytometric analysis and light microscopy. As seen in Fig 3A, the B1'B2'H population was uniformly composed of small cells. However, both the B1'B2'L population (Fig 3B) and the B1'B2' subset (Fig 3C) consisted of predominantly small cells with about 10% larger cells. Wright-Giemsa staining of cytospin preparations from these three subpopulations (B1'B2'H, B1'B2'L, and B1'B2') confirmed the observation that a population of larger cells with abundant cytoplasm and prominent nucleoli were present in the latter two subgroups. Given the phenotype of low to absent B2 and the morphology of transformed lymphocytes, these cells can be considered candidates for the normal cellular counterparts of the B-DLCL malignant cell.

**DISCUSSION**

In the present report, the cellular derivation of tumor cells isolated from 57 patients with DLCL was determined by the expression of T-, B-, and monocytic-restricted antigens. A T cell origin could be assigned to nine patients, whereas a true monocytic origin could be assigned only to one. Of the remaining slg' (n = 38) and slg' (n = 9) patients, all expressed the B1 antigen, demonstrating that these tumors were of B cell lineage. Using monoclonal antibodies to B cell-restricted antigens (slg, B1, B2, and B4), two major subgroups (B2' and B2') and several minor subgroups of B-DLCL could be defined. The lack of expression of slgD and
The lineage derivation of some DLCLs is still controversial. Using conventional markers, approximately 40% to 60% of DLCLs express sIg and 10% express E rosette receptors assigning a B or T cell lineage to approximately 2/3 of these tumors. Of the remaining 30% to 50%, some have been shown to express Ia-like antigens and/or receptors for the C3 component of complement. The lineage of these tumors has largely been unresolved, since these markers are not lineage restricted in their expression. Several lines of evidence now support the notion that sIg DLCLs are of B cell origin. Arnold et al demonstrated that of three DLCLs that were either sIg or lacked monoclonality, two demonstrated Ig heavy chain gene rearrangements and one demonstrated light chain gene rearrangement. Although providing compelling evidence for a B cell lineage, the recent observation that occasional T cell ALLs, Sezary cell syndrome, acute nonlymphocytic leukemias, and a number of murine T cell lymphomas also demonstrate Ig heavy chain rearrangements without light chain rearrangements, reveals that heavy chain rearrangement is not restricted to cells of B lineage. Therefore, Ig heavy chain rearrangements, although very suggestive, are not sufficient evidence to assign a B cell lineage derivation of these DLCLs. The demonstration in this report that the “null” cell DLCLs uniformly express B1 and almost all coexpress B4 supports the notion that these tumors are B cell derived. These data are not contradictory to the report by Horning et al, which showed that only 50% of sIg DLCLs expressed B1. In that study, B1 expression was assessed by immunoperoxidase staining in tissue sections, and it appears that some (22%) sIg- DLCLs did not express B1. We attribute this discrepancy to the occasional instability of B1 antigen in tissue sections as opposed to the analysis of the antigen on single-cell suspensions. Therefore, the use of lineage-restricted monoclonal antibodies now has permitted the assignment of a lineage determination of all 57 DLCLs studied.

With the demonstration that the overwhelming majority of DLCLs are B cell derived, we then examined the expression of other B cell–restricted antigens on these tumors. Of the B-DLCLs, 16 of 41 demonstrated weak B2 expression, whereas the remainder were B2 negative. We have recently demonstrated that the B2 antigen arises after the cytoplasmic μ-positive pre-B cell and is lost at the time when cells no longer express sIgD (unpublished observations). The demonstration that B2 is weakly expressed or absent suggests that these tumors correspond to a stage of B cell differentiation in which the B2 antigen is lost. Further support for this notion comes from the observations that all but three of the B-DLCLs do not express sIgD. Moreover, only three of these tumors expressed the PCA-1 antigen and none expressed PC-1, both of which appear at the terminal stages of B cell differentiation. These observations are consistent with the hypothesis that most B-DLCLs are derived from a very narrow window of B cell differentiation corresponding to the time when the B2 antigen is lost.

Using the B cell–restricted antigens B1, B2, B4, and sIg, we have been able to identify two major and two minor subgroups of B-DLCL. The two major subgroups include the B1·B4·sIg·B2 and the B1·B4·sIg·B2· phenotypes. In previous studies, we have demonstrated that normal B1·B4·sIg·B2· cells can be driven in vitro to lose B2 and therefore express the B1·B4·sIg·B2· phenotype. Therefore, the major subgroups of B-DLCL appear to correspond to these stages of normal B cell differentiation. In contrast, the B1·B4·sIg·B2· and the B1·B4·sIg·B2· B-DLCLs do not conform to our model of normal B cell differentiation. We and others have noted previously that a subset of B cell non-Hodgkin’s lymphomas, including some B cell chronic lymphocytic leukemias and nodular poorly differentiated lymphocytic lymphomas lack sIg. At the present time, it is impossible to conclusively state that these subgroups of B-DLCL correspond to normal B cell differentiative steps. Therefore, the question of whether these phenotypic subgroups correspond to subpopulations of normal cells or alternatively represent anomalous, neoplastically induced phenotypes will require further investigation.

With the demonstration that the majority of B-DLCLs appear to express cell surface markers found in the mid-stages of B cell differentiation, we then attempted to investigate whether we could identify and isolate the normal cellular counterparts of the malignant B-DLCL cell. In a recent report, using dual laser flow cytometric analysis, three distinct B cell subpopulations—B1·B2·, B1·B2·, and B1·B2·—could be demonstrated. The B1·B2· subpopulation has been shown to be phenotypically distinct from the B1·B2· population. The B1·B2· cells express IgM and IgD but only weakly express IgG and essentially do not express PCA-1 and PC-1. Conversely, the B1·B2· cells expressed IgM, a minority expressed IgD, and 20% to 30% expressed IgG and PCA-1, suggesting that
the B1' B2' cells are more differentiated. The B1' B2' subpopulation was examined more closely, and the expression of B2 was found to be of either low or high intensity. The B1' B2' (low) and B1' B2' subpopulations were noted to be composed of predominantly small cells admixed with approximately 10% large cells, some of which have the appearance of transformed lymphocytes. In contrast, the B1' B2' (high) consisted of a uniform population of small cells. Although sufficient numbers of cells were not available for a detailed phenotypic reanalysis of the large lymphoblastoid B1' B2' (low) and B1' B2' normal populations, their morphology and expression of B2 suggests that these cells are likely candidates for the normal cellular counterparts of B-DLCL cells.

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