The Diagnostic Value of CD1d Expression in a Large Cohort of Patients With B-Cell Chronic Lymphoproliferative Disorders

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Key Words: Chronic lymphocytic leukemia; CLL; CD1d; B-cell chronic lymphoproliferative disorders; Immunophenotyping

Abstract

Immunophenotyping is indispensable in the differential diagnosis of B-cell chronic lymphoproliferative disorders (B-CLPDs). However, B-CLPDs often show overlapping immunophenotypic profiles and may be diagnostically challenging. CD1d is an HLA class I–like molecule that presents glycolipids to invariant natural killer T cells. Normal mature B cells constitutively express CD1d, but with the exception of some conflicting data, its expression in B-CLPDs is unknown. We demonstrate that in 222 B-CLPD cases, CD1d expression of less than 45% is strongly predictive of CLL (likelihood ratio, 32.3; specificity, 97.4%; sensitivity, 84.1%). In addition, CD1d showed significantly higher staining intensity in splenic marginal zone lymphoma compared with atypical hairy cell leukemia, lymphoplasmacytic lymphoma, and mantle cell lymphoma, thus allowing the discrimination of the former from the latter immunophenotypically overlapping B-CLPDs. It is important to note that in a given patient, CD1d expression on malignant B cells was similar between tissues and remained unaffected by disease stage and treatment status. Our findings strongly argue for the incorporation of CD1d into routine lymphoma panels.

B-cell chronic lymphoproliferative disorders (B-CLPDs) with leukemic manifestation comprise a heterogeneous group of diseases arising from the clonal expansion of mature B lymphocytes at diverse stages of differentiation.1,2 The accurate discrimination of each separate B-CLPD entity is of paramount importance because the prognosis and treatment differ widely for the different types.

Flow cytometry is a powerful tool for the fast and thorough characterization of leukemic B-CLPDs, and it also involves minimal stress for patients.3 However, despite the accumulated knowledge on the expression patterns of normal and malignant B cells and increasingly sophisticated instruments, many B-CLPDs cannot be accurately characterized if based only on flow cytometric data,4 requiring the use of ancillary techniques.5 Beyond the fact that architectural and cytologic features, both not assessable by flow cytometry, are crucial in certain lymphoma subtypes, other reasons are the limited range of B-cell markers and the frequently observed atypical immunophenotypic patterns.

A typical paradigm is the differential diagnosis of atypical, CD23– chronic lymphocytic leukemia (CLL) from mantle cell lymphoma (MCL). Both entities may share comparable clinical and immunophenotypic findings,6 even the absence of cyclin D1 positivity,7 but the clinical course and treatment modalities differ substantially. Moreover, while CD5 positivity typifies CLL and MCL, it is also reported in other B-CLPDs, such as diffuse large B-cell lymphoma, splenic marginal zone lymphoma with or without villous lymphocytes (SMZL), lymphoplasmacytic lymphoma (LPL), and rare cases of follicular lymphoma (FL).8 Because the aforementioned B-CLPDs bear no distinctive antigenic characteristics, their flow cytometric characterization in the
case of CD5 positivity can be particularly difficult. Such cases are often referred to as “unclassifiable” or “not otherwise specified,” or they can be misinterpreted as MCL, potentially leading to erroneous treatment approaches.9 Even in the absence of CD5 positivity, the optimal distinction of B-CLPDs other than CLL, MCL, and hairy cell leukemia (HCL) is often troublesome because the HCL variant (HCL-v), SMZL, and LPL are usually cytometrically indistinguishable.3,10,11

CD1d is a nonpolymorphic HLA class I–like, β2-microglobulin–associated, molecule presenting phospholipids and glycolipids to a subset of immunoregulatory T cells, the natural killer T cells.12 CD1d is expressed widely in normal hematopoietic and nonhematopoietic cells such as thymocytes, monocytes, macrophages, primitive hematopoietic stem cells, keratinocytes, and hepatocytes, whereas normal peripheral blood B cells exhibit constitutive expression of CD1d.12-14 As regards hematopoietic malignancies, myeloid and lymphoid acute leukemias15,16 variably express CD1d, while CD1d is significantly up-regulated in Sézary syndrome.17 Also, monoclonal gammopathy of undetermined significance and newly diagnosed multiple myeloma strongly expressed CD1d, whereas normal peripheral blood B cells exhibit constitutive expression of CD1d.12-14 We performed a detailed flow cytometric study of CD1d expression in B-CLPDs and determined its diagnostic usefulness. We demonstrate for the first time that CD1d can differentiate CLL from other B-CLPDs with high accuracy, and it is also helpful in the discrimination of SMZL from B-CLPDs with overlapping features, such as LPL, HCL-v, and MCL.

Materials and Methods

Flow Cytometric Analysis in B-CLPD Cases

CD1d expression was measured by 4-color flow cytometry in peripheral blood and/or bone marrow samples from 151 patients with CLL, 27 with MCL, 20 with LPL, 11 with SMZL, 14 with HCL, and 5 with HCL-v. The characteristics of the cases are listed in Table I. In some of the cases, CD1d expression was also tested in lymph node and/or pleural fluid samples. All samples were acquired during the same time (2 years) in all subgroups. CD1d expression was measured at first, and all patients were treatment-naive. Diagnoses were established in all cases on the basis of clinical, morphologic, immunophenotypic, and histologic criteria, according to the World Health Organization classification.23

Erythrocyte-lysed whole peripheral blood and bone marrow samples or mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation from lymph nodes and pleural fluid were labeled with CD1d-phycoerythrin (clone CD1d42), CD38-phycoerythrin (clone HB7), CD49d-allophycocyanin (clone 9F10), and a series of B-cell markers, all from BD Biosciences, San Jose, CA. Data acquisition and analysis were performed on a FACSCalibur cytometer.

Table I
Clinical and Hematologic Characteristics of B-CLPD Cases

<table>
<thead>
<tr>
<th>B-CLPD Type</th>
<th>CLL</th>
<th>MCL</th>
<th>LPL</th>
<th>SMZL</th>
<th>HCL</th>
<th>HCL-v</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>151</td>
<td>27</td>
<td>20</td>
<td>11</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Median (range) age at diagnosis (y)</td>
<td>68 (36-85)</td>
<td>67.5 (57-79)</td>
<td>65.5 (45-77)</td>
<td>66 (52-77)</td>
<td>59 (44-85)</td>
<td>74 (51-75)</td>
</tr>
<tr>
<td>M/F</td>
<td>93/58</td>
<td>17/10</td>
<td>—</td>
<td>14/6</td>
<td>3/8</td>
<td>10/4</td>
</tr>
<tr>
<td>NHL histologic features</td>
<td>NA</td>
<td>Classic, 23, blasts, 4</td>
<td>—</td>
<td>SLVL, 4/11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Binet stage</td>
<td>A, 109; B, 30; C, 12</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IgHv mutational status*</td>
<td>Unmutated, 30; mutated, 51</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD38 expression†</td>
<td>High, 37; low, 107</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD49d expression‡</td>
<td>High, 21; low, 54</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

B-CLL, B-cell chronic lymphocytic leukemia; B-CLPD, B-cell chronic lymphoproliferative disorder; HCL, hairy cell leukemia; HCL-v, HCL variant; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; NA, not available; NHL, non-Hodgkin lymphoma; SLVL, splenic lymphoma with villous lymphocytes; SMZL, splenic marginal zone lymphoma.

* Available in 81 cases.
† Available in 144 cases; cutoff value for positivity, >30%.
‡ Available in 75 cases; cutoff value for positivity, >30%.
A, Representative plots of CD1d analysis in various B-cell chronic lymphoproliferative disorders (B-CLPDs). Residual normal B cells are shown in orange and neoplastic lymphocytes in green, the latter being identified by light-chain restriction or the disease-specific marker CD103. Plots are gated on total lymphocytes, and the percentage of CD1d expression in clonal B cells is shown. B, Histogram overlay of 3 illustrative B-CLPD cases. CD1d was expressed more strongly in SMZL (filled histogram) compared with HCL-v (dotted line) and LPL (solid line). CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; HCL-v, HCL variant; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; SMZL, splenic marginal zone lymphoma.

CD1d expression in clonal B cells was more strongly in SMZL (filled histogram) compared with HCL-v (dotted line) and LPL (solid line). Residual normal B cells are shown in orange and neoplastic lymphocytes in green, the latter being identified by light-chain restriction or the disease-specific marker CD103. Plots are gated on total lymphocytes, and the percentage of CD1d expression in clonal B cells is shown. Histogram overlay of 3 illustrative B-CLPD cases. CD1d was expressed more strongly in SMZL (filled histogram) compared with HCL-v (dotted line) and LPL (solid line). CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; HCL-v, HCL variant; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; SMZL, splenic marginal zone lymphoma.

The usefulness of CD1d as a diagnostic marker for CLL was evaluated with receiver operator characteristic curve analysis. The Mann-Whitney U test was used for the comparisons of CD1d expression between CLL and MCL and also among the various CLL prognostic subgroups (IgHV mutated and unmutated, CD38low/high, and CD49dlow/high CLL cases). The Kruskal-Wallis test was used for the assessment of differences in CD1d staining intensity and percentage between B-CLPDs other than CLL. All analyses were performed using SPSS software, version 14.0 (SPSS, Chicago, IL). The study was approved by the local research ethics committee, and informed consent was obtained from all patients.

Results

CD1d in the Differential Diagnosis of CLL

In our patient cohort, the median percentage of CD1d expression in B-CLPDs was as follows: CLL, 19% (range, 0%-100%); MCL, 87% (range, 15%-100%); LPL, 65.5% (range, 53%-98%); SMZL, 100% (range, 86%-100%); HCL, 100% (range, 100%-100%); and HCL-v, 67% (range, 51%-75%). With rare exceptions, 100% of normal polyclonal B cells expressed CD1d. CD1d expression of less than 45% was strongly predictive of CLL (likelihood ratio, 32.3; specificity, 97.4%; sensitivity, 84.1%); with the exception of 2 MCL cases, in all other non-CLL cases, the CD1d percentage of B cells was higher than 45%.
The high diagnostic accuracy of CD1d was further illustrated by the fact that the area under the receiver operating characteristic curve was 0.949 when measured in all patients and 0.923 when determined only in CD5+ cases, indicating an almost ideal diagnostic test. Figure 1A and Figure 1B. It is important to note that malignant cells in MCL expressed considerably higher levels of CD1d compared with CLL ($P < .001$) Figure 1C, whereas all CD5+/CD1dlow/CD23+ cases ($n = 127$) had CLL, thus making CD1d particularly helpful in the differential diagnosis of these 2 immunophenotypically overlapping but clinically diverse disorders. In CLL, CD1d expression was significantly higher in cases with adverse immunophenotypic features, namely CD38 and CD49d positivity ($P = .001$ in both comparisons of positive and negative cases) Figure 2A and Figure 2B, and tended to be expressed more strongly in unmutated disease, without, however, reaching statistical significance Figure 2C.

It is interesting that in contrast with the generally low CD1d expression in CLL, all 5 cases with trisomy 12 expressed high CD1d levels (range, 65%-100%), in line with previous data showing aberrant immunophenotypic features in trisomy 12. In particular, all 5 CLL cases exhibited very high expression of CD1d (>90%, Figure 1C). In 2 of the cases, trisomy 12 was shown by fluorescence in situ hybridization and conventional cytogenetics.

In the first case (a male), findings from the biopsies of lymph node and bone marrow were consistent with a CLL diagnosis, showing a nodular pattern of infiltration in the bone marrow. The case also displayed unmutated $V_H$ genes, typical CLL morphologic features, and 2 aberrant immunophenotypic features, namely low/moderate CD23 (40%) and strong CD20 expression. In the second case (a female), there was an additional cytogenetic abnormality (47,XX,del(8)(p21)[14]/46,XX[6]), mutated $V_H$ genes, and a bone marrow biopsy showing CLL with a diffuse pattern of involvement. There were also atypical morphologic features, including medium-sized cells with cleaved nuclei and no smudge cells, and 1 phenotypic aberration (strong CD20 expression). The third case (a male) was not checked for trisomy 12, but there was no evidence of 17p and 11q23 deletions by fluorescence in situ hybridization and he carried mutated $V_H$ genes. The bone marrow biopsy showed CLL with a nodular pattern of involvement, whereas CD23 expression was low/moderate (35%), and there were atypical morphologic features with mixed cell types and no smudge cells.

In addition to the aforementioned cases, 2 MCL cases (both men) had very low levels of CD1d (<16%, Figure 1C). In the first case, biopsies of lymph node and bone marrow demonstrated classic MCL with 30% Ki-67+ cells, 0.0 0.2 0.4 0.6 0.8 1.0

Sensitivity

1 – Specificity

0.0 0.2 0.4 0.6 0.8 1.0

A

B

C

Figure 1 CD1d is significantly down-regulated in chronic lymphocytic leukemia (CLL) compared with the other B-cell chronic lymphoproliferative disorders (B-CLPDs). A and B. The receiver operating characteristic curve depicts the accuracy of CD1d expression in predicting the diagnosis of CLL in all 228 B-CLPD cases in the study (A) and in only CD5+ cases (B). Area under the curve, A, 0.949; B, 0.923. C. Box plots showing the median CD1d expression in CLL ($n = 151$) and MCL ($n = 27$) (central line). The edges of the box indicate the 25th and 75th percentiles. CD1d displays significantly higher expression in mantle cell lymphoma (MCL). $P < .01$; Mann-Whitney test. © American Society for Clinical Pathology

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and the second case had blastic MCL with heavy bone marrow infiltration and a leukemic picture.

CD1d in the Differential Diagnosis of SMZL vs Other B-CLPDs

Because of the lack of robust disease-specific markers and their overlapping immunophenotypic profiles, distinction among SMZL, HCL-v, LPL, and occasionally, MCL by immunophenotyping is often problematic. We found that the percentage and MFI of CD1d expression were able to differentiate SMZL from HCL-v, LPL, and MCL. The median percentage of CD1d expression in SMZL was 100% (range, 86%-100%), significantly higher than in HCL-v (67%; range, 51%-75%; \(P < .001\)), LPL (65.5%; range, 53%-98%; \(P < .001\)), and MCL (87%; range, 15%-100%; \(P < .001\)). Similarly, the average staining intensity of SMZL, determined as MFI\(_{CD1d}/\text{MFI}_{iso}\), was 11.1 (range, 7.5-32), notably higher than for HCL-v (4.75; range, 2.5-7.5; \(P = .001\)), LPL (7.25; range, 4-15.25; \(P = .004\)), and MCL (8.5; range, 4-14.5; \(P = .014\)). Remarkably bright CD1d staining, the highest among B-CLPDs, was observed in HCL samples (MFI\(_{CD1d}/\text{MFI}_{iso}\), 16.3; range, 11.3-23.6; Image 1A). However, HCL was not included in the preceding comparisons because it bears a unique phenotype and can be easily discriminated from the other B-CLPDs with the current antibody panels. It is important to note that essential for the diagnostic robustness of CD1d was the stability of expression during disease progression and/or relapse after chemotherapy, as shown in B-CLPD cases assessed at various time points (Figure 4), whereas it was equally expressed in various tissues in the same case (Image 2).

Discussion

Flow cytometric immunophenotyping is essential for the differential diagnosis of B-CLPDs and constitutes an indispensable tool for the proper subclassification of these entities according to the World Health Organization classification. Nevertheless, the biologic variability of B-CLPDs, the paucity of disease-specific markers and/or immunophenotypic patterns, and the lack of standardized panels and analysis strategies compromise the diagnostic efficiency of immunophenotyping. Accordingly, although in most cases of typical CLL and MCL the immunophenotypic diagnosis is straightforward, an appreciable proportion of CLL cases fails to be identified with the classical scoring system.

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**Figure 2** CD1d expression in chronic lymphocytic leukemia (CLL) subsets defined by biologic predictors. **A** and **B**, CD1d is expressed significantly more strongly in CD38high (n = 37) and CD49dhigh (n = 21) CLL cases in comparison with CD38low (n = 107) and CD49dlow (n = 54) cases, respectively. **C**, Although not statistically significant, CD1d expression was also higher in unmutated (n = 30) vs mutated (n = 51) CLL. Cases were designated as CD38low and CD49dlow when fewer than 30% of CD5+/CD19+ cells were positive for the former 2 markers. **A**, \(P < .001\); **B**, \(P < .001\); **C**, \(P = .097\); Mann-Whitney test.
resulting in misclassification, while on the other hand, many otherwise typical MCL cases may, in fact, represent CLL. For example, the expression levels of CD23, CD20, FMC7, and surface immunoglobulin are customarily used for the distinction of CLL from MCL, but their discriminatory power is generally low as all of the former molecules often show overlapping expression in CLL and MCL.

CD1d belongs to the CD1 family of proteins and is an MHC-like, glycolipid-presenting molecule with a wide range of tissue expression, including in normal B cells. The mantle and marginal zone of lymphoid organs exhibit intense CD1d staining, whereas germinal centers are CD1d-. CD1d is also detected in acute leukemias of myeloid and lymphoid origin, Sézary syndrome, and multiple myeloma, but aside from a handful of studies, there are no data for its expression in B-CLPDs.

By measuring CD1d expression by flow cytometry in a large cohort of B-CLPD cases, we show for the first time that CD1d has impressive accuracy as a single marker for the diagnosis of CLL because low CD1d expression was virtually restricted to CLL cases. Moreover, when combined with CD23 staining, it can differentiate CLL from MCL with an accuracy.

![Figure 3](image-url) CD1d staining intensity can differentiate splenic marginal zone lymphoma (SMZL) from hairy cell leukemia variant (HCL-v), lymphoplasmacytic lymphoma (LPL), and mantle cell lymphoma (MCL). A and B, Cumulative analysis of CD1d corrected (A) and raw (B) mean fluorescence intensity in SMZL (n = 11), HCL-v (n = 5), LPL (n = 20), and MCL (n = 24) cases. C, Analysis of the percentage of CD1d expression in the same cases. Overall P values: A, P = .001; B, P = .001; C, P < .001; Kruskal-Wallis test. * P < .001 in pairwise comparisons between SMZL and each of the other B-cell chronic lymphoproliferative disorders; Mann-Whitney test. MFI, mean fluorescence intensity; MFI_{iso}, MFI of the corresponding isotype control.

![Figure 4](image-url) CD1d expression in B-cell chronic lymphoproliferative disorders (B-CLPDs) is stable irrespective of disease stage and treatment status. A, Stable CD1d expression in chronic lymphocytic leukemia (CLL; n = 7; diamonds) and other B-CLPDs (n = 3; circles) during the disease course. B, CD1d expression also remained unaltered in patients with CLL (n = 5) who were in Binet stage A for a long period. Notably, the patient with the highest CD1d expression had trisomy 12. TP, time points with intervals of 1-3 years.
of 100%. Consistent with our results, Zheng et al\(^1\) observed a 5-fold reduction of CD1d transcripts in CLL in comparison with normal B cells by using oligonucleotide microarrays. In addition, Rosenwald et al\(^2\) demonstrated a 2-fold increase of CD1d expression in MCL compared with small lymphocytic lymphoma, whereas an immunohistochemical study using a different antibody (clone NOR3.2) showed CD1d positivity in 4 (31%) of 13 CLL cases,\(^2\) a somewhat higher ratio compared with mutational status, whereas although we also observed that the staining intensity of CD1d correlated with its expression, which, in fact, is the strongest among B-CLPDs.

Another limitation of flow cytometry regarding the classification of B-CLPDs is its inability to effectively distinguish marginal zone lymphoma, LPL, and HCL-v.\(^3,4\) Marginal zone lymphomas and LPLs have a nonspecific immunophenotype, and attempts to distinguish the 2 B-CLPDs by using B-cell markers such as CD22, CD25, and CD11c showed inconsistent results.\(^3,8\) A particular diagnostic conundrum is posed by B-CLPD cases with prominent splenomegaly and absence of lymphadenopathy, in which flow cytometric immunophenotyping can be pivotal for the diagnosis because bone marrow biopsy is not always diagnostic.\(^39\) Although HCL can be readily recognized based on its characteristic coexpression of CD103, CD25, and CD123,\(^40\) the discrimination among HCL-v, SMZL, LPL, and even MCL could prove challenging because CD103 and CD123 cannot always distinguish HCL-v from SMZL,\(^40,41\) whereas LPL and SMZL may occasionally express CD5 along with low levels of CD23, thus resembling MCL.\(^8\)

We showed that CD1d can help in the differentiation of SMZL from LPL, MCL, and HCL-v because SMZL exhibits significantly brighter CD1d staining. It is interesting that in contrast with HCL-v, typical HCL also displays strong CD1d expression, which, in fact, is the strongest among B-CLPDs expressed in CLL compared with other B-CLPDs;\(^36\) therefore, we speculate that LEF-1 up-regulation may be at least partially responsible for the low CD1d expression in CLL.

By contrast, another group using flow cytometry reported that CD1d was highly expressed in all 38 CLL cases and could also trigger activation of natural killer T cell lines.\(^21\) It was also shown that the staining intensity of CD1d correlated with mutational status, whereas although we also observed a trend for higher expression in unmutated cases, we were not able to demonstrate a significant association of MFI or a trend for higher expression in unmutated cases, whereas although we also observed that the staining intensity of CD1d correlated with mutational status (Figure 2C). The reason for these discrepancies is not clear, but it is possible that a difference in laboratory protocols or the different clone (CD1d-55) and formulation of the homemade CD1d antibody\(^21\) may have caused the variations in CD1d staining patterns.

The mechanism of CD1d down-regulation in CLL B cells is unknown, but it was recently demonstrated that the nuclear protein lymphoid enhancer–binding factor-1 (LEF-1) acts as a transcriptional repressor of the \(CD1D\) gene.\(^35\) LEF-1 is highly expressed in CLL compared with other B-CLPDs;\(^36\) therefore, we speculate that LEF-1 up-regulation may be at least partially responsible for the low CD1d expression in CLL.

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It has been recently suggested that SMZL and HCL may originate from a circulating IgM+/IgD+/CD27+ B cell derived from the splenic marginal zone that has acquired somatic hypermutations in the absence of germinal center reactions.\(^4\)\(^2\)\(^\text{43}\) On the contrary, the analysis of their immunoglobulin heavy chain (\(\text{IGH}\)) rearrangements indicated that although HCL, SMZL, and HCL-v are indeed distinct disorders, the \(\text{IGH}\) repertoire of HCL-v was closer to that of SMZL than that of HCL.\(^4\)\(^2\)\(^\text{43}\) The staining pattern of CD1d in our B-CLPD cases argues that SMZL and HCL might share a common cell of origin, which potentially arises from the CD1dbright splenic marginal zone B cells\(^3\)\(^4\) and is different from the CD1dintermediate counterpart of HCL-v.

We demonstrated that CD1d represents a valuable diagnostic marker that contributes significantly in the precise characterization of B-CLPDs. Based on CD1d expression, we formulated a diagnostic algorithm that we use in our laboratory \[\small\text{Figure 5}\]. The high predictive accuracy and efficacy of CD1d in the identification of CLL and the differential diagnosis of the other B-CLPDs support its further validation and, eventually, use in routine lymphoma panels.

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\[\small\text{Figure 5}\] An algorithmic approach to the diagnosis and subclassification of B-cell chronic lymphoproliferative disorders by using CD1d levels. CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; HCL-v, HCL variant; IHC, immunohistochemical analysis; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; SMZL, splenic marginal zone lymphoma.

References

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