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Flow Cytometric Detection of the Double-Positive (CD4⁺CD8⁺)/PD-1bright T-Cell Subset Is Useful in Diagnosing Nodular Lymphocyte-Predominant Hodgkin Lymphoma

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Context.—Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is characterized by neoplastic lymphocyte-predominant cells frequently rimmed by CD3⁺/CD57⁺/programmed death receptor-1 (PD-1)⁺ T cells. Because of the rarity of lymphocyte-predominant cells in most cases, flow cytometric studies on NLPHL often fail to show evidence of malignancy.

Objective.—To evaluate the diagnostic utility of PD-1 in detecting NLPHL by flow cytometry, in conjunction with the CD4:CD8 ratio and the percentage of T cells doubly positive for CD4 and CD8.

Design.—Flow cytometric data obtained from cases of NLPHL (n = 10), classical Hodgkin lymphoma (n = 20), B-cell non-Hodgkin lymphoma (n = 22), T-cell non-Hodgkin lymphoma (n = 5), benign lymphoid lesions (n = 20), angioimmunoblastic T-cell lymphomas (n = 6) and T-cell/histiocyte–rich large B-cell lymphomas (n = 2) were analyzed and compared.

Results.—Compared with the other groups, NLPHL showed significantly higher values in the following parameters: CD4:CD8 ratio, percentage of T cells doubly positive for CD4 and CD8, percentage of PD-1-positive T cells, and median fluorescence intensity of PD-1 expression in the doubly positive for CD4 and CD8 subset. Using a scoring system (0–4) based on arbitrary cutoffs for these 4 parameters, all 10 NLPHL cases scored 3 or higher, as compared with only 3 cases from the other groups, producing an overall sensitivity of 100% and a specificity of 96% (72 of 75). Two of the 3 outliers were non-Hodgkin lymphoma, and both showed definitive immunophenotypic abnormalities leading to the correct diagnosis. The remaining outlier was a case of T-cell/histiocyte–rich large B-cell lymphoma.

Conclusions.—The inclusion of anti–PD-1 in flow cytometry is useful for detecting NLPHL in fresh tissue samples, most of which would have otherwise been labeled as nondiagnostic or reactive lymphoid processes.

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Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is histologically characterized by the presence of relatively rare lymphocyte-predominant (LP) cells (originally termed lymphocytic and histiocytic cells) that are surrounded by an abundance of reactive immune cells. Pathologic diagnosis of NLPHL is established by morphologic examination of biopsy tissues and appropriate immunohistochemical studies. Specifically, the rimming of LP cells by CD3-positive T cells, many of which are also highlighted by anti-CD57, is a key finding. In most cases, flow cytometry is not helpful diagnostically, largely because of the paucity of LP cells. In 2006, Rahemtullah et al. examined a cohort of NLPHL using flow cytometry and found a relatively high frequency of benign T cells coexpressing CD4 and CD8, herein referred to as double-positive T cells (DPTCs). In that study, the authors identified that 58% of the examined 24 NLPHL cases contained a relatively higher proportion of DPTCs (ranging from 10% to 38% of the total T cells) whereas DPTCs accounted for only 4% to 6% of the T cells found in reactive lymph nodes and classical Hodgkin lymphoma (CHL). A subsequent flow cytometric study also confirmed that NLPHL carries higher proportions of DPTCs than T-cell/histiocyte–rich large B-cell lymphoma (TCRBL), CHL, and reactive lymph nodes. Nonetheless, in both studies, the finding of a high frequency of DPTCs alone was not sufficiently diagnostic, because a substantial proportion of NLPHL cases did not demonstrate this immunophenotypic feature. In addition to DPTCs, other studies have also suggested that the CD4:CD8 ratio is useful in differentiating NLPHL from reactive lymph nodes and other types of non-Hodgkin lymphoma (NHL). Again, this criterion alone is neither sufficiently specific nor sensitive. A more recent study using 5-color flow cytometry reported that a high percentage of CD57-positive
T cells or DPTCs is highly suggestive of NLPHL, although only reactive lymphoid processes and CHL were included for comparison.

Programmed death receptor-1 (PD-1), normally expressed by predominantly activated T cells, is known to be a crucial immune checkpoint receptor. The PD-1 proteins are commonly expressed on the surface of neoplastic cells and in the tumor microenvironment. Upon the ligation of its ligands, PD-1 transduces an immune-suppressive signal to T cells, and blockade of PD-1 signaling has become an attractive anticancer therapeutic target. In reactive lymph nodes, PD-1–positive T cells are often localized to the periphery of germinal centers, where they are labeled follicular helper T cells, which are believed to play an important role in generating and maintaining the germinal center reaction. In recent years, lymphoid malignancies arising from the neoplastic counterpart of follicular helper T cells have been described, including angioimmunoblastic T-cell lymphoma (AITL), follicular T-cell lymphoma, and a subset of peripheral T-cell lymphoma, all of which have now been included in the World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. In the context of NLPHL, it has been reported that T cells rimming LP cells are also frequently PD-1 positive, and the use of PD-1 immunohistochemistry is useful in the histologic diagnosis of NLPHL. To our knowledge, the question of whether the inclusion of PD-1 in the flow cytometric workup of NLPHL is of diagnostic utility has not been fully addressed.

We hypothesized that the addition of PD-1 in the flow cytometric study panel may result in a higher diagnostic accuracy in detecting NLPHL, particularly in the differentiation between NLPHL and benign lymphoid processes. In the first part of this study, we evaluated 4 flow cytometric parameters, including the CD4:CD8 ratio, the percentage of DPTCs, the percentage of PD-1–positive T cells, and the median fluorescence intensity (MFI) of PD-1 expression on DPTCs. The inclusion of the last parameter (ie, PD-1 expression intensity) was based on previous reports that PD-1bright but not PD-1dim T cells display the phenotype of follicular helper T cells. In the second part of the study, we developed a scoring system based on these 4 parameters and demonstrated that this scoring system is particularly useful in distinguishing NLPHL from benign lymphoid processes by flow cytometry.

**MATERIALS AND METHODS**

**Case Selection**

In our database dated between January 2019 and April 2020, we identified 21 cases that had a histologic diagnosis of NLPHL. Flow cytometry was performed on 10 of these 21 cases during the initial diagnostic workup using the same biopsy samples sent for histologic diagnosis. Using a similar approach, we searched the database for several diagnoses: CHL (20 consecutive cases), B-cell NHL (22 consecutive cases), T-cell NHL (5 consecutive cases), and reactive lymphoid processes (20 consecutive cases). In addition, we identified all cases of TCRBL (2 cases) and AITL (5 cases) in this period. Again, flow cytometry was performed during the initial diagnostic workup using the same biopsy samples sent for histologic diagnosis. In view of the known biological relationship between NLPHL and progressive transformation of germinal centers (PTGC), we specifically sought outside the database time period and identified 2 cases of PTGC to be included for comparison.

**Flow Cytometry**

Except for bone marrow aspiration samples, all cases in this study were excisional biopsy or fine-needle biopsy of lymph nodes or soft tissue masses. Tissues were mechanically disaggregated using a Medimachine system (Becton Dickinson, San Jose, California) to yield single-cell suspensions, which were subsequently filtered via 100-μm filons (Filcon; BD Biosciences, San Jose, California). Our antibody panel included CD2-FITC, CD279/ PD-1-PE (clone PD-1.3), CD56-PE, CD3-APC, CD19-PE, CD4-PE, CD7-APC, CD5-APC-A700, CD8-APC-A750, CD8-PB, and CD45-KO, all of which were from Beckman Coulter (Mississauga, Ontario, Canada). Staining was performed for 15 minutes at room temperature. Red blood cells were lysed using 1× PharmLyse (Becton Dickinson) followed by washing and finally fixation in 0.1% formaldehyde. Stained samples were acquired on a Navios 10-color flow cytometer (Beckman Coulter). Flow-Check Pro and Flow-Set Pro (Beckman Coulter) fluorospheres were run daily to monitor instrument performance. Compensation was set up using the Autosetup scheduler (Beckman Coulter) and monitored by performing a “comp tube” (an in-house laboratory developed tube with mutually exclusive markers to verify correct compensation set up). Flow cytometric data analysis was performed using Kaluza software (Beckman Coulter). Cell viability was tested on every sample using propidium iodide (Becton Dickinson). Single cells were selected by gating on forward-scatter peak versus forward-scatter integral signal. Lymphocytes were identified by light scatter and bright CD45 staining. Only cases with viability greater than 70% were included in this study. For fluorescent dot plots, control gates were set by gating the lower left quadrant around the double-negative (ie, negative for both CD4 and CD8) lymphocyte population and quadrants were linked to similar fluorescent dot-plot pairings.

**Statistical Analysis**

Statistical comparison of the case types found in our search was performed using Student t test with unequal variance and the Kruskal-Wallis test in STATA software version 16 (StataCorp, College Station, Texas).

**RESULTS**

**Case Characteristics**

As described in Materials and Methods, we identified and included 10 NLPHL cases for this study. All 10 cases were subjected to flow cytometric analysis during the initial diagnostic workup, using the same tissue samples sent for histologic diagnosis. There were 9 excisional lymph node biopsy samples and 1 needle core lymph node biopsy. Histologic review of these 10 neoplasms confirmed the presence of LP cells. No evidence of T-cell cytologic atypia was found. In all cases, LP cells carried the immunohistochemical profile typical of NLPHL, characterized by their strong and uniform expression of CD20, PAX5, and CD45 and their weak expression or lack of expression of CD30 and CD15. Prominent rimming of LP cells by CD3-positive T cells, some of which also showed PD-1 and CD57 positivity, was identified in all cases.

The flow cytometry diagnostic comments for these 10 NLPHL cases are summarized in Table 1. No diagnostic immunophenotypic abnormalities were described in 6 cases. An increase in the CD4:CD8 ratio was described in 4 cases. The presence of a DPTC population and/or the finding of increased PD-1 expression in the T-cell population was described in 3 cases. In 1 of these 3 cases, the possibility of T-cell lymphoma was raised by the pathologist. Morphologic descriptions of the cytoplasm preparations accompanying the flow cytometric studies were largely noncontributory, except for 1 case in which...
In group 2, we selected 22 consecutive B-cell NHLs, with the exclusion of TCRBL. Most (20 of 22) samples were derived from excisional lymph node biopsy or needle core biopsy; 2 samples were derived from needle core biopsy of solid organs (ie, parotid gland and thyroid gland). Most of these cases were indolent B-cell lymphomas, including follicular lymphoma (n = 9), marginal zone lymphoma (n = 6), and small lymphocytic lymphoma/chronic lymphocytic leukemia (n = 3). A monotypic B-cell population was identified by flow cytometry in all 22 cases, and the presence of “a few cells suspicious for LP cells” was described.

For comparison, we collected 4 groups of cases collected from the same database, and the characteristics of these groups are summarized in Table 1. Similar to the NLPHL group, all of these cases were subjected to flow cytometry during the initial diagnostic workup, using the same tissue samples sent for histologic examination.

In group 1, there were 20 consecutive CHL cases selected from our database. These samples were derived from excisional lymph node biopsy (n = 11) and needle core lymph node biopsy (n = 9). In all 20 cases, no diagnostic immunophenotypic abnormalities were found in the lymphoid cell population, although an increase in the CD4:CD8 ratio was described in 4 of 20 cases. In the original flow cytometry reports, morphologic examination of the cytospin preparations described the presence of “a few cells suspicious for LP cells” in 5 of 20 cases.

In group 3, we selected 5 consecutive T-cell malignancies. The flow cytometry diagnostic comments described a mixture of small and large lymphocytes. The more specific findings described in the histologic diagnosis. The flow cytometry diagnostic comments described varying proportions of T cells and polytypic B cells, and morphologic examination of the cytospin preparations described a mixture of small and large lymphocytes.

### Table 1. Characteristics of Study Cases

| No. of Cases | Further Classification | Diagnostic Comments in the Flow Cytometry Report |
|--------------|------------------------|-------------------------------------------------|
| NLPHL        | 10                     | NA                                              |
| CHL          | 20                     | NA                                              |
| B-cell NHL   | 22                     | Follicular lymphoma (n = 9) Marginal zone lymphoma (n = 6) Chronic lymphocytic leukemia/small lymphocytic lymphoma (n = 3) Mantle cell lymphoma (n = 1) Diffuse large B-cell lymphoma (n = 3) | Abnormal T-cell immunophenotype detected in 4 of 5 cases of T-cell malignancies |
| T-cell NHL   | 5                      | Anaplastic large cell lymphoma (n = 1) T-lymphoblastic lymphoma (n = 2) Peripheral T-cell lymphoma (n = 2) | Abnormal T-cell immunophenotype detected in 4 cases |
| AITL         | 6                      | NA                                              |
| TCRBL        | 2                      | NA                                              |
| Nonlymphoma  | 20                     | Reactive lymphadenitis (n = 19) Metastatic carcinoma (n = 1) | No diagnostic immunophenotypic abnormalities in all cases |
| PTGC*        | 2                      | NA                                              | PD-1+ DPTCs noted in 1 case, no diagnostic immunophenotypic abnormalities in the other case |

Total Cases 87 NA

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; CHL, classical Hodgkin lymphoma; DPTC, double-positive T cells; NA, not applicable; NHL, non-Hodgkin lymphoma; NLPHL, nodular lymphocyte-predominant Hodgkin lymphoma; PD-1, programmed death receptor-1; PTGC, progressive transformation of germinal centers; TCRBL, T-cell/histiocyte-rich large B-cell lymphoma.

* Cases of PTGC are from outside the study database time period.

the presence of “a few cells suspicious for LP cells” was described.

For comparison, we collected 4 groups of cases collected from the same database, and the characteristics of these groups are summarized in Table 1. Similar to the NLPHL group, all of these cases were subjected to flow cytometry during the initial diagnostic workup, using the same tissue samples sent for histologic examination.

In group 1, there were 20 consecutive CHL cases selected from our database. These samples were derived from excisional lymph node biopsy (n = 11) and needle core lymph node biopsy (n = 9). In all 20 cases, no diagnostic immunophenotypic abnormalities were found in the lymphoid cell population, although an increase in the CD4:CD8 ratio was described in 4 of 20 cases. In the original flow cytometry reports, morphologic examination of the cytospin preparations described the presence of “a few cells suspicious for LP cells” in 5 of 20 cases.

In group 2, we selected 22 consecutive B-cell NHLs, with the exclusion of TCRBL. Most (20 of 22) samples were derived from excisional lymph node biopsy or needle core biopsy; 2 samples were derived from needle core biopsy of solid organs (ie, parotid gland and thyroid gland). Most of these cases were indolent B-cell lymphomas, including follicular lymphoma (n = 9), marginal zone lymphoma (n = 6), and small lymphocytic lymphoma/chronic lymphocytic leukemia (n = 3). A monotypic B-cell population was identified by flow cytometry in all 22 cases, and the diagnosis of a B-cell lymphoma was strongly suggested in the flow cytometry reports.

In group 3, we selected 5 consecutive T-cell malignancies. For comparison, with 1 case of ALK-positive anaplastic large cell lymphoma from an inguinal lymph node excision, 2 cases of peripheral T-cell lymphoma (not further classified) from lymph node cores, and 2 cases of acute T-lymphoblastic lymphoma from neck lymph nodes. Apart from the single case of anaplastic large cell lymphoma, an abnormal T-cell immunophenotype was detected and the diagnosis of a T-cell malignancy was strongly suggested in the flow cytometry reports. By morphology, the anaplastic large cell lymphoma case was reported to show “larger atypical cells with vacuolated cytoplasm and prominent nucleoli.”

In group 4, we selected 20 consecutive cases of non-lymphomatosus samples from the same database. Most samples were derived from excisional biopsy or needle core biopsy of lymph nodes (n = 18); the remaining 2 were needle core biopsy of solid organs (ie, liver and lung). Other than 1 case of metastatic squamous cell carcinoma, all cases were benign reactive lymph nodes, with granulomatous reaction (n = 2) and Castleman-like changes (n = 1) being the more specific findings described in the histologic diagnosis. The flow cytometry diagnostic comments described varying proportions of T cells and polytypic B cells, and morphologic examination of the cytospin preparations described a mixture of small and large lymphocytes.
In the database, we also included all cases of TCRBL (n = 2) andAITL (n = 6). Both cases of TCRBL were needle core lymph node biopsy samples, and no diagnostic immunophenotypic abnormalities were found in either case by flow cytometry. Morphologic examination of cytoplasm preparations from both cases was reported as “mixed lymphoid cells with a scant excess of larger cells with some mild atypia” and “mixed lymphoid cells devoid of significant atypia.” Of the 6 cases of AITL, 3 were derived from needle core lymph node biopsies and 3 were from bone marrow aspirations. An abnormal T-cell immunophenotype was identified in all cases and the possibility of T-cell lymphoma was raised in 4 of 6 cases. One case of AITL was reported as negative by flow cytometry, but in retrospective review, the CD4-positive cell population did show bright PD-1 expression. The other case of TCRBL was also reported as negative by flow cytometry, but the “skewed” CD4:CD8 ratio (ie, 6.0) in B cells was noted.

The 2 cases of PTGC found outside of the database time period were lymph node excisional biopsy samples. One case showed no diagnostic immunophenotypic abnormalities by flow cytometry, with the cytoplasm reported to show “occasional very large cells.” The other case was reported to show “double positive T cells (CD4\(^+\)/CD8\(^+\))” with strong expression of CD1-1” with no other diagnostic immunophenotypic abnormalities by flow cytometry, with the corresponding cytoplasm reported as demonstrating “mixed lymphoid cells with no significant atypia.” The corresponding histologic slides from both cases were reviewed by 2 of the authors (Z.W.C. and R.L.) to confirm the diagnosis of PTGC and exclude the presence of NLPHL.

Flow Cytometric Analysis

For each case, we first identified the CD4:CD8 ratio within the CD3\(^+\) T-cell population. We then assessed the proportion of the DPTCs that were characterized by the CD4\(^+\)/CD8\(^+\) immunophenotype using density plots (Figure 1, A through F), and this was expressed as a percentage of the total CD3\(^+\) T cells. Using the CD4/CD8-1 plot (Figure 1, B), we determined the percentage of PD-1\(^+\) expression within the DPTC population (Figure 1, C). Lastly, the MFI of PD-1 in the DPTC population was determined (Figure 2, A and B).

NLPHL Has the Highest CD4:CD8 Ratio.—As demonstrated in Figure 3, A, the CD4:CD8 ratio within the CD3\(^+\) T-cell population was highest in the NLPHL group (median, 6.0; range, 1.7–14.0), compared with those of the CHL (median, 4.0; range, 0.7–17.4), B-cell NHL (median, 2.9; range, 0.4–7.8), T-cell NHL (median, 2.9; range, 1.0–55.7), AITL (median, 2.0; range, 0.8–3.3), and nonlymphomatous groups (median, 3.5; range, 0.8–9.4). Despite the substantial overlaps, 3 groups were found to be statistically significantly different from NLPHL, including B-cell NHL, AITL, and the nonlymphomatous group (P < .05; Student t test) (Table 2). No significant differences were found between NLPHL and CHL or T-NHL. Of note, the CD4:CD8 ratios of the NHL group as an aggregate did not significantly differ from that of NLPHL. Statistical analysis was not performed for TCRBL because of the low case numbers. Statistical analysis using the Kruskal-Wallis test, which assumes that events are not normally distributed, generated similar conclusions.

Proportion of DPTC Population Is Higher in NLPHL.—As shown in Figure 3, B, the median percentage DPTC of all CD3\(^+\) T cells was 4.5% for the NLPHL group (range, 1.1%–33.4%), which is significantly higher than that of the CHL (median, 0.8%; range, 0.2%–3.8%), T-NHL (median, 1.8%; range, 0.3%–7.1%), all NHL (median, 1.5%; range, 0.3%–27.1%), and nonlymphomatous groups (median, 1.7%; range, 0.4%–7.1%) (P < .05, Student t test) (Table 2). No significant difference was found between NLPHL and AITL (median, 2.6%; range, 0.5%–6.8%). Excluding the TCRBL group, the Kruskal-Wallis test reveals statistically significant differences between NLPHL and all other groups (P < .04). The mean of the 2 cases of TCRBL was 3.8% (range, 1.1%–6.4%).

NLPHL Has a Higher Percentage of PD-1\(^+\) Cells in the DPTC Population.—Excluding the 2 cases of TCRBL, NLPHL was the only group consistently demonstrating greater than 95% PD-1\(^+\) cells within the DPTC population (median, 98.4%; range, 95%–100%) (Figure 3, C). Apart from the 2 cases of TCRBL, this was significantly higher than found in other groups, including the CHL (median, 71.4%; range, 0%–97.4%), T-NHL (median, 86.9%; range, 68.8%–99.2%), AITL (median, 78.9%; range, 28.2%–89.2%), all NHL (86.1%; range, 28.2%–100%), and nonlymphomatous groups (median, 81.3%; range, 43.3%–97.7%) (P < .05, Student t test) (Table 2). The Kruskal-Wallis test revealed similar conclusions. The mean of the 2 cases of TCRBL was 98.9% (range, 98.7%–99.1%).

MFI of PD-1 in the DPTC Population.—The last parameter we examined was the MFI of PD-1 within the DPTC population. As illustrated in Figure 3, D, we found that the MFI of PD-1 in the DPTC group was noticeably higher than that found in the other 4 groups. The MFI was 21.1 (range, 5.5–28.2) in the NLPHL group, which was significantly higher than the MFI of the CHL (median, 2.1; range, 0.8–13.3), B-NHL (median, 9.4; range, 0.9–24.7), AITL (median, 3.1; range, 0.9–14.9), all NHL (median, 8.2; range, 0.9–24.7), and nonlymphomatous groups (median, 3.4; range, 0.9–20.7). The Kruskal-Wallis test also revealed statistically significant differences (P < .01) between NLPHL and the other categories, except for T-cell NHL. The MFI for the 2 cases of TCRBL was 11.2 (range, 8.2–14.1).

Parameters in PTGC Cases.—The results of the 4 above-mentioned flow cytometric parameters appeared to separate NLPHL from the other groups (except for TCRBL and PTGC), there were substantial overlaps, as shown in Figure 3, A through D. Thus, we assessed whether a simple scoring system incorporating these 4 parameters could facilitate the separation between NLPHL and the other groups, with a special interest on possible distinction between NLPHL and the nonlymphomatous group. As summarized in Table 3, arbitrary cutoff points were established for each of the 4 parameters, and one point was assessed for each of the following 4 metrics when it was met: a CD4:CD8 ratio of 5.8 or higher, a percentage of DPTCs of all CD3\(^+\) T cells 2.9% or higher, a percentage of PD-1\(^+\) cells in the DPTC population greater...
than 96%, and MFI for PD-1 expression in DPTCs 5.5 or higher. Thus, the maximum achievable score was 4.

Applying this scoring system, all 10 cases of NLPHL demonstrated a total score of 3 (n = 6) or 4 (n = 4). In contrast, all 20 CHL cases had a score of 2 or less, with 10 scoring 0, 8 scoring 1, and 2 scoring 2. Excluding the 2 cases of TCRBL, only 2 of all NHL cases had a score of 3; none had a score of 4. Specifically, 7 cases scored 0, 17 cases

Figure 1. Flow cytometric gating strategy to determine CD3⁺CD4⁺CD8⁺ double-positive T-cell (DPTC) population. A, Double-positive CD4/CD8 dim population gated on CD3⁺ cells in a nodular lymphocyte-predominant Hodgkin lymphoma case. B, Determination of cutoff for programmed death receptor-1 (PD-1) positivity based on control gating of CD4/PD-1-negative population in total lymphocytes. C, PD-1 positivity applied to DPTC population from (A), using cutoff determined in (B). D through F, Similar results for a classical Hodgkin lymphoma case with low DPTC population. F, PD-1 positivity applied to DPTC population from (D) using cutoff determined in (E). Abbreviations: PB, Pacific Blue; PE, phycoerythrin.

Figure 2. Histograms illustrating programmed death receptor-1 (PD-1) median fluorescence intensity (MFI) of double-positive T cells (DPTCs). A, PD-1 MFI of CD4⁺CD8⁺ DPTCs in a nodular lymphocyte-predominant Hodgkin lymphoma case. B, PD-1 MFI of CD4⁺CD8⁺ DPTCs in a classical Hodgkin lymphoma case with low DPTC numbers. Abbreviation: PE, phycoerythrin.
scored 1, and 2 cases scored 2. Of the 2 NHL cases with a score of 3, the first case was peripheral T-cell lymphoma, not further classified. The diagnostic possibility of a T-cell lymphoma was raised in the flow cytometry report based on the detection of definitive immunophenotypic abnormalities in a T-cell population and the cytologic finding of highly atypical lymphoid cells. The second case was follicular lymphoma. Similarly, the diagnostic possibility of a B-cell NHL was raised based on the detection of a monotypic, CD10-positive B-cell population and the cytologic finding of atypical lymphoid cells. Of the 2 cases of TCRBL, one scored 2 points and the other scored 3 points. Of the 2 cases of PTGC, one scored 2 points and the other scored 3 points.

Using a cutoff score of 3 or higher, we were able to achieve a sensitivity of 100% (ie, 10 of 10 NLPHL cases) and a specificity of 96% (72 of 75 other cases). Of note, we did not include PTGC cases in this calculation because these cases were selected outside of the database used for this study. Importantly, the use of this scoring system reliably separated NLPHL from the nonlymphomatous group.

Figure 3. Graphical box plot representation of data from the 4 parameters examined in this study. A, CD4:CD8 ratio within CD3+ T cells. B, Percentage of double-positive T cells (DPTC) of CD3+ T cells. C, Percentage of DPTC expressing programmed death receptor-1 (PD-1). D, Median fluorescence intensity (MFI) for PD-1 in DPTC. Note: cases of progressive transformation of germinal centers (PTGC) are from outside the study database time period. Including B-cell non-Hodgkin lymphoma (NHL), T-cell NHL, T-cell/histiocyte–rich large B-cell lymphoma (TCRBL), and angioimmunoblastic T-cell lymphoma (AITL). Abbreviations: CHL, classical Hodgkin lymphoma; NLPHL, nodular lymphocyte-predominant Hodgkin lymphoma.
except for PTGC. In both cases of NHL showing a score of 3, the possibility of PTGC was largely excluded based on the abundance of cytologically abnormal lymphoid cells.

**DISCUSSION**

Because of the paucity of neoplastic LP cells, flow cytometric studies of NLPHL frequently reveal no diagnostic immunophenotypic abnormalities in the lymphoid cell population, typically leading to a diagnostic interpretation of “nondiagnostic” or “reactive lymphoid process.” Similarly, cytologic examination of the specimens subjected to flow cytometry often does not show any evidence of malignancy. In the current study, the diagnostic possibility of NLPHL was raised at the initial flow cytometric workup in only 1 of 10 cases. Similar findings have been published in the literature.22 The same diagnostic challenge holds true for CHL, because Reed-Sternberg cells in these cases are typically rare and relatively difficult to detect by flow cytometry and cytologic examination. Therefore, one of the major diagnostic challenges for the flow cytometric workup of suspected lymphoma cases is to differentiate NLPHL/CHL from benign reactive lymphoid processes. A false-negative flow cytometry report, especially when fine-needle aspirate samples are used, could potentially result in a delay in tissue biopsy, diagnosis, and treatment.

Several flow cytometric studies aiming to better differentiate NLPHL/CHL and benign reactive lymphoid processes have been previously published. In this regard, one of the flow cytometric parameters used is the CD4:CD8 ratio, which has been described to be elevated in NLPHL and CHL.24 In the current study, we found that the median CD4:CD8 ratios for NLPHL and CHL were indeed higher than those of the nonlymphoma group (medians, 6.0 and 3.8 versus 3.5, respectively). However, because of its high variability within each diagnostic category, the diagnostic

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In the literature,23 the same diagnostic challenge holds true in only 1 of 10 cases. Similar findings have been published of NLPHL was raised at the initial flow cytometric workup malignancy. In the current study, the diagnostic possibility flow cytometry often does not show any evidence of

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**Table 2. P Values as Compared With Nodular Lymphocyte-Predominant Hodgkin Lymphoma (NLPHL) for Each Diagnostic Category**

| Group               | CD4:CD8 Ratio Within CD3+ T Cells, No. (%) | % DPTC of CD3+ T Cells | % of DPTC Expressing PD-1 | MFI for PD-1 in DPTC, No. (%) |
|---------------------|--------------------------------------------|------------------------|--------------------------|-------------------------------|
| CHL (n = 20)        | .27                                        | .02b                   | .001b                    | .001b                         |
| B-cell NHL (n = 22) | .02b                                       | .05b                   | .001b                    | .01b                          |
| T-cell NHL (n = 5)  | .26                                        | .04b                   | .05b                     | .08                           |
| TCRBL (n = 2)       | NP                                         | NP                     | NP                       | NP                            |
| AITL (n = 6)        | .003b                                      | .06                    | .01b                     | .002b                         |
| All NHL (n = 35b)   | .12                                        | .04b                   | .001b                    | .002b                         |
| Nonlymphoma (n = 20)| .04b                                       | .03b                   | .001b                    | .001b                         |
| PTGC (n = 2a)       | NP                                         | NP                     | NP                       | NP                            |

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; CHL, classical Hodgkin lymphoma; DPTC, double-positive T cells; MFI, median fluorescence intensity; NHL, non-Hodgkin lymphoma; PTGC, progressive transformation of germinal centers; TCRBL, T-cell/histiocyte–rich large B-cell lymphoma.

a One case of follicular lymphoma and 1 case of peripheral T-cell lymphoma.

b Including TCRBL, B-cell NHL, AITL, and other T-cell NHL.

cases of PTGC are from outside the study database time period.

For CHL, because Reed-Sternberg cells in these cases are typically rare and relatively difficult to detect by flow cytometry and cytologic examination. Therefore, one of the major diagnostic challenges for the flow cytometric workup of suspected lymphoma cases is to differentiate NLPHL/CHL from benign reactive lymphoid processes. A false-negative flow cytometry report, especially when fine-needle aspirate samples are used, could potentially result in a delay in tissue biopsy, diagnosis, and treatment.

Several flow cytometric studies aiming to better differentiate NLPHL/CHL and benign reactive lymphoid processes have been previously published. In this regard, one of the flow cytometric parameters used is the CD4:CD8 ratio, which has been described to be elevated in NLPHL and CHL.24 In the current study, we found that the median CD4:CD8 ratios for NLPHL and CHL were indeed higher than those of the nonlymphoma group (medians, 6.0 and 3.8 versus 3.5, respectively). However, because of its high variability within each diagnostic category, the diagnostic

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**Table 3. Scoring System Used to Differentiate Nodular Lymphocyte-Predominant Hodgkin Lymphoma (NLPHL) From the Other Groups**

| Group           | CD4:CD8 Ratio Within CD3+ T Cells ≥5,8, No. (%) | DPTC Expressing PD-1 >69%, No. (%) | DPTC/CD3+ T Cells ≥2,9%, No. (%) | MFI for PD-1 in DPTC ≥5,5, No. (%) | Score Distribution |
|-----------------|-----------------------------------------------|-----------------------------------|---------------------------------|-----------------------------------|-------------------|
| NLPHL (n = 10)  | 6 (60)                                        | 9 (90)                            | 9 (90)                          | 10 (100)                         | 0 0 0 6 4         |
| CHL (n = 20)    | 5 (25)                                        | 1 (5)                             | 2 (10)                          | 4 (20)                           | 10 8 2 0 0       |
| B-cell NHL (n = 22) | 2 (9)                                       | 5 (23)                            | 3 (14)                          | 14 (64)                          | 5 11 5 1 0       |
| T-cell NHL (n = 5) | 2 (40)                                      | 1 (20)                            | 1 (20)                          | 4 (80)                           | 1 1 2 1 0       |
| TCRBL (n = 2)   | 0                                             | 2 (100)                           | 1 (50)                          | 2 (100)                          | 0 0 1 1 0       |
| AITL (n = 6)    | 0                                             | 0                                 | 3 (50)                          | 2 (33)                           | 1 5 0 0 0       |
| All NHL (n = 35b)| 4 (11)                                       | 8 (23)                            | 8 (23)                          | 22 (63)                          | 7 17 8 3 0      |
| All NHL excluding TCRBL (n = 33) | 4 (12)                                     | 6 (18)                            | 7 (21)                          | 20 (61)                          | 7 17 7 2 0      |
| Nonlymphoma (n = 20) | 4 (20)                                     | 1 (5)                             | 3 (15)                          | 6 (30)                           | 11 4 5 0 0      |
| PTGC (n = 2a)   | 0                                             | 1 (50)                            | 2 (100)                         | 2 (100)                          | 0 0 1 1 0       |

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; CHL, classical Hodgkin lymphoma; DPTC, double-positive T cells; MFI, median fluorescence intensity; NHL, non-Hodgkin lymphoma; PD-1, programmed death receptor-1; PTGC, progressive transformation of germinal centers; TCRBL, T-cell/histiocyte–rich large B-cell lymphoma.

a One case of follicular lymphoma and 1 case of peripheral T-cell lymphoma.

b Including TCRBL, B-cell NHL, AITL, and other T-cell NHL.

cases of PTGC are from outside the study database time period.
utility of the CD4:CD8 ratio alone is limited. This is particularly true for CHL, because the median difference between CHL and the nonlymphoma group is relatively small. The second flow cytometric parameter is the size of the DPTC population, which was found to be higher in NLPHL but not CHL.\(^2,28\) In our study, we confirmed this finding, and the median DPTC percentage was significantly higher in NLPHL (4.5%) than in CHL (0.8%) and the nonlymphoma group (1.7%). Again, because of its variability, this parameter in various diagnostic groups, this parameter alone has some diagnostic limitations. Specifically, 1 of 10 NLPHL cases (10%) did not meet the arbitrary cutoff of 2.9% or higher, whereas 3 of 20 nonlymphoma cases (15%) and 3 of 6 AITL cases (50%) met this cutoff. Thus, in contrast to the conclusion of a previous study,\(^9\) we did not find DPTC percentage alone to be sufficient in diagnosing NLPHL by flow cytometry. We believe that the reason for this discrepancy is likely related to the fact that this previous study included only NLPHL, CHL, and benign lymphoid processes. We speculate that the sensitivity and specificity of DPTC percentage would have been lower if NHL (especially T-cell NHL) cases had been included. This limitation was fully acknowledged by the authors of that study.

More recent studies have shown that the LP cells in NLPHL are frequently rimmed by T cells that express markers of follicular helper T cells such as PD-1,\(^19\) CD57,\(^26\) and CXCL-13.\(^1\) Thus, we included PD-1 in our flow cytometry panel used in the initial diagnostic workup of suspected lymphoma cases. We found percentage of PD-1\(^+\) T cells to be useful because this parameter is significantly higher in NLPHL compared with nonlymphoma cases and most NHL cases. In addition, we observed that the PD-1 expression in the DPTC population is appreciably higher than that in the non-DPTC population. In this regard, it has been reported that PD-1\(^{bright}\) T cells display the phenotype of follicular helper T cells that are localized to the germinal centers, whereas PD-1\(^{dim}\) T cells are “exhausted” T cells that are mostly localized in the interfollicular space.\(^20\) Taken together, the PD-1\(^{bright}\) immunophenotype is a relatively specific marker of the follicular helper T cells, which are known to be increased in NLPHL. In support of this concept, the MFI of PD-1 within the DPTC subset is significantly higher than that of all the other groups, with the exceptions of the TCRBL and T-cell NHL groups. Although the 2 parameters related to PD-1 expression (i.e., percentage PD-1\(^+\) T cells and MFI) might be perceived as redundant measurements of the same endpoint, they are actually relatively independent. Specifically, we found that the correlation coefficient between these 2 parameters for the entire cohort is 0.61. We also attempted to use a 3-point scoring system, with either percentage PD-1\(^+\) T cells or MFI omitted, which resulted in decreased sensitivity and specificity (data not shown). Lastly, there were several cases that demonstrated a high DPTC percentage, but with weakly weak PD-1 expression (low MFI), as well as cases in which there was a low DPTC percentage showing relatively strong PD-1 expression (high MFI).

By incorporating all 4 flow cytometric parameters discussed above, our scoring system can be used to greatly facilitate the distinction between NLPHL and the nonlymphoma group, excluding PTGC. Although NLPHL cases consistently had a score of 3 or 4, all 20 nonlymphoma cases had a score of 0 to 2, with the vast majority (15 of 20; 75%) of these cases being 0 or 1. Apart from cases of TCRBL, the 4-point scoring system also allowed us to readily separate NLPHL from all other lymphomatous groups. Under our scoring system, 18 of 20 CHL cases scored 0 or 1 and all cases scored 2 or lower. Excluding the TCRBL group, only 2 of the 33 NHL cases scored greater than 2 (both scored 3), namely 1 case of peripheral T-cell lymphoma and 1 case of follicular lymphoma. In both cases, the overall flow cytometric finding of an immunophenotypically abnormal lymphoid cell population and the morphologic finding of atypical lymphoid cells largely excluded the possibility of NLPHL or a benign lymphoid process. Thus, their relatively high scores should not cause significant diagnostic difficulty.

The observation that TCRBL showed similarities with NLPHL in our 4 flow cytometric parameters and that 1 of 2 TCRBL cases had a score of 2 or more is not surprising, as NLPHL demonstrates morphologic, molecular, and some clinical features that overlap with TCRBL.\(^27\) It is well documented that the distinction between the diffuse variant of NLPHL and TCRBL can be highly difficult, and the term TCRBL-like variant of NLPHL has been used to describe these cases.\(^28\) No specific immunohistochemical markers can be used to discriminate between TCRBL and NLPHL, and the distinction of the diffuse variant of NLPHL from TCRBL is primarily based on the morphologic assessment of the relative extent of nodularity versus diffuseness.\(^27\) To further highlight the relatedness between NLPHL and TCRBL, gene expression profiling and comparative genomic hybridization array studies have demonstrated the molecular similarities between these 2 entities.\(^29,30\) Likewise, the similarities between NLPHL and PTGC are not surprising in view of their biological relationship and immunohistochemical overlaps.\(^22\) Practically speaking, the overlap between NLPHL, PTGC, and TCRBL cases in our scoring system is not of significant concern, as our main objective is to minimize false-negative results through mislabeling these 3 entities as “benign lymphoid process” or “nondiagnostic” in the flow cytometry reports.

Some of the unique immunophenotypic features of the immune cells in NLPHL has shed light into the biology of this disease. In nonneoplastic diseases, DPTC can be expanded in certain reactive conditions, such as viral infections as well as various types of autoimmune diseases, chronic inflammatory conditions, HIV, and Epstein–Barr virus infections.\(^31-36\) Their increase in viral infections suggests the DPTC may represent effector T cells that fight infection. Although traditionally Epstein–Barr virus is more strongly associated with CHL, recent cases of NLPHL demonstrating Epstein–Barr virus infection have been reported.\(^37-39\) which lends support to the notion that viral infection may play a role in the pathogenesis of NLPHL in CHL.

The weak CD8 expression in the DPTC population also has been reported in healthy individuals and immune-deficient patients, and presence of T cells carrying this unique immunophenotype has been postulated to represent chronic stimulation or an emerging lymphoproliferative disorder.\(^40\) Furthermore, DPTCs have been reported to be seen in various cancers, including breast cancer, melanoma, and colon cancer,\(^31\) in addition to NLPHL.\(^2\) The relative abundance of this unique cell population may suggest that the immune cells present in NLPHL may be reactive to underlying stimulations that are yet to be defined.

In conclusion, our data suggest that the inclusion of anti–PD-1 in our flow cytometry panel is helpful in alerting diagnosticians to the possibility of NLPHL in initial
diagnostic workup. This marker is particularly useful when the lymphoid cells in the samples do not show definitive immunophenotypic abnormalities and examination of the cytospin preparation fails to reveal convincing evidence of an abnormal cell population. In situations where only fine-needle aspirate samples are available, the markers discussed in this study may be particularly helpful in deciding whether watchful waiting or tissue biopsy is the more appropriate course of action.

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