Specific Polo-Like Kinase 1 Expression in Nodular Lymphocyte-Predominant Hodgkin Lymphoma Suggests an Intact Immune Surveillance Program

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Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is a rare and relatively indolent B-cell lymphoma. Characteristically, the lymphocyte-predominant (LP) tumor cells are embedded in a microenvironment enriched in lymphocytes. More aggressive variants of mature B-cell and peripheral T-cell lymphomas exhibit nuclear expression of the polo-like kinase 1 (PLK1) protein, stabilizing c-MYC and associated with worse clinical outcomes. We demonstrate frequent expression of PLK1 in the LP cells in NLPHL cases (100%; n = 76). In contrast, <5% of classic Hodgkin lymphoma cases (n = 70) show PLK1 expression within the tumor cells. Loss-of-function approaches demonstrated that the expression of PLK1 promotes cell proliferation and increased c-MYC stability in NLPHL cell lines. Correlation with clinical parameters revealed that the increased expression of PLK1 was associated with advanced-stage disease in patients with NLPHL. A multiplex immunofluorescence panel coupled with artificial intelligence algorithms was used to correlate the composition of the tumor microenvironment with the proliferative stage of LP cells. The results showed that LP cells with PLK1 (high) expression were associated with increased numbers of cytotoxic and T-regulatory T cells. Overall, the findings demonstrate that PLK1 signaling increases NLPHL proliferation and constitutes a potential vulnerability that can be targeted with PLK1 inhibitors. In addition, the findings suggest that an active immune surveillance program in NLPHL may be a critical mechanism limiting PLK1-dependent tumor growth. (Am J Pathol 2023, 19: 1–14; https://doi.org/10.1016/j.ajpath.2023.10.008)

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immunochemotherapy, radiotherapy, and watchful waiting in patients with asymptomatic and nonbulky disease.6,7

Morphologically, NLPHL is characterized by large, atypical lymphocytes with convoluted nuclei [lymphocyte-predominant (LP) cells], set within a background of numerous small lymphocytes.8 Rosettes of T-follicular helper (Tfh) type T cells immediately surround the LP cells, which comprise <5% of total cells.9 In contrast to classic Hodgkin lymphoma (cHL), the tumor cells of NLPHL characteristically express B-cell markers and are usually negative for CD30 expression. However, LP cells may occasionally express CD30 and/or CD15, making the distinction between NLPHL and cHL challenging.10,11 The histologic distinction between NLPHL and T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL), an aggressive B-cell lymphoma variant, also poses difficulties. These arise because one variant morphologic pattern of NLPHL (pattern E) is indistinguishable from THRLBCL, and the neoplastic cells of NLPHL and THRLBCL express a similar immunophenotype.11–13 Sometimes, NLPHL may mimic reactive conditions, such as progressive transformation of germinal centers, particularly when limited tissue is available for diagnosis.

The tumor microenvironment of NLPHL is predominantly composed of T cells with a variable number of background B-cell lymphocytes.7 In contrast to reactive lymphoid tissue, increased PD1+CD4+ T-cell subsets have been detected in NLPHL cases, and Tfh type lymphocytes provide an advantage for LP cell survival via direct paracrine stimulation of IL21 receptor.14 In addition, the presence of FoxP3+CD4+ T-regulatory cells (T-regs) could modulate the number of Tfh cells in the immediate proximity of LP cells,9 further sustaining the survival and growth of the tumor component.

The polo-like kinase 1 (PLK1) protein is a serine/threonine kinase that promotes cell cycle progression and proliferation.15 PLK1 increases the protein stability of c-MYC oncoprotein by preventing its degradation by the ubiquitin-proteosome pathway. Increased expression of PLK1 has been detected in aggressive B- and T-cell lymphomas, and this directly correlates with the expression of c-MYC in high-grade B-cell lymphomas.16 In contrast, the expression of PLK1 is not detected in small B-cell lymphomas, including follicular lymphoma, mantle cell lymphoma, and small lymphocytic lymphoma.17 Therefore, the expression of PLK1 was evaluated in NLPHL cases to delineate further its diagnostic utility of discriminating NLPHL (particularly the variant histologic patterns) from aggressive counterparts.

# Materials and Methods

## Reagents

Reagents used for these studies included antibodies for Western blot analysis, c-MYC, PLK1, and glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technologies, Danvers, MA). Proliferation assays were performed using CellTiter Glo (Promega, Madison, WI) reagent, per the manufacturer’s instructions. Volasertib was purchased from Selleckchem (Houston, TX). Reagents used for immunohistochemistry and immunofluorescence are detailed in upcoming sections.

## Tumor Samples

A retrospective cohort of 76 NLPHL cases was collected from the pathology archives of the University of Illinois at Chicago, University of Chicago (Chicago, IL), Yale University (New Haven, CT), Henry Ford Hospital (Detroit, MI), University of Pittsburgh (Pittsburgh, PA), Rush University (Chicago, IL), Ohio State University (Columbus, OH), and the University of Michigan (Ann Arbor, MI). Hematoxylin and eosin sections, CD20 immunostainings, and PLK1 immunostainings were centrally reviewed by two pathologists (K.I. and C.M.-Z.). All the clinical data were annotated in JMP Clinical software. A retrospective cohort of 70 cases of classic Hodgkin lymphoma was obtained from Henry Ford Hospital, the University of Illinois at Chicago, and the University of Michigan. This study was performed with the approval of the Institutional Review Board of the University of Illinois at Chicago (STUDY2022-0757).

## Immunohistochemistry

All slides were deparaffinized and stained on BOND RX autostainer (Leica Microsystems). For single chromogen PLK1 staining, slides were subjected to antigen retrieval with BOND Epitope Retrieval solutions 2 (Leica Microsystems; number AR9640) for 20 minutes at 99°C and stained using BOND Polymer Refine Detection kit (Leica Microsystems; number DS9800). The non-specific signal was blocked by incubating the slides with peroxide block for 15 minutes and protein block (Background Sniper; Biocare Medical; number BS966) for 15 minutes at room temperature. Slides were stained with anti-PLK1 rabbit monoclonal antibody (1:50; Cell Signaling; number 4531) for 30 minutes, and the signal was detected with polymer–horseradish peroxidase/diaminobenzidine. Slides were counterstained with hematoxylin, dehydrated on Autostainer XL (Leica Microsystems), and mounted with Micromount (Leica Microsystems; number 3801730).

For dual sequential PLK1 and CD30 staining, samples were stained with PLK1, as described above, without the hematoxylin counterstain, followed by staining with anti-CD30 ready-to-use mouse monoclonal antibody (Millipore-Sigma; number AB9130) and BOND Polymer Red Detection Kit (Leica Microsystems; number DS9390). Incubation with CD30 antibodies was for 30 minutes, and the signal was developed with Polymer-AP and Fast Red.

For multiplex immunofluorescence (mIF), a panel consisting of CD3, CD4, CD20, PD-1, PLK1, and FoxP3, and

DAPI was optimized on tonsil and lymphoma samples using BOND RX autostainer and BOND Research Detection System (Leica Microsystems; number DS9455). Opal 6-plex Detection Kit (Akoya Biosciences, Marlborough, MA; number NEL821001KT) was used for detection. For previously optimized targets, monoplex staining slides at the assigned position and with the assigned Opal dye were prepared for each target and spectrally unmixed. The staining patterns were compared with the single chromogen immunohistochemistry performed on the adjacent slide, and the intensity of the signal was also assessed for each target to ensure it was in the desired range. For PLK1, PD-1, and CD20, additional optimization steps, such as serial dilutions, testing of different positions in the multiplex sequence, and stripping controls, were performed to ensure the optimal staining conditions and complete removal of the primary and secondary antibodies after each round of staining. The final multiplex panel information is listed in Table 1. Dual antigen retrieval with BOND Epitope Retrieval Buffers 1 and 2 was performed before the beginning of the staining protocol. BOND Epitope Retrieval Buffer 1 was used for stripping primary and secondary antibodies after each staining round.

Multiplex Immunofluorescence Imaging

Slides were scanned using a PhenolImager HT multispectral microscope (Akoya Biosciences) in MSI mode at 20× resolution. Regions of interest containing tumor cells were manually selected by a pathologist (C.M.-Z. or R.P.) and scanned at 40× resolution. Spectral profiles were optimized using Nuance software (Akoya Biosciences) to ensure the accurate unmixing of each multispectral channel. Multispectral images were analyzed using HALO software (Indica Labs, Albuquerque, NM) and custom-written MATLAB code. A pathologist (C.M.-Z. or R.P.) manually annotated tumor (LP) cells. Quantifying tumor cell PLK1 intensity was performed using Halo Area Quantification Fl Module version 2.3.3. The tumor cell nucleus was defined as the region negative for CD20. The tumor cell cytoplasm was defined as the positive region for CD20. The mean PLK1 intensity was reported for the nucleus, cytoplasm, and whole cell for each annotation region. Images were manually annotated to outline tissue regions and remove artifacts. Nuclear segmentation was performed using a manually trained HALO AI Nuclei Seg classifier (Indica Labs). HALO HighPlex FL module version 4.1.3 (Indica Labs) was used to manually set thresholds for each channel and manually identify cell phenotypes. Cell phenotypes and position coordinates were exported from HALO for spatial analysis. Spatial analysis identifying the immune cells within each tumor microenvironment was performed in MATLAB. The mean width of all immune cells was calculated to be 7.89 μm. The average width of five cells was estimated to be 40 μm. For each tumor cell, the tumor microenvironment was defined as an 80- by 80-μm square with the same center as the tumor cell annotation, excluding the tumor cell annotation region. All immune cells within the tumor microenvironment were identified for each tumor cell.

**Statistical Analysis**

Comparisons of PLK1 expression and tumor microenvironment were conducted in R. Tumor PLK1 expression levels were binned to either high or low levels based on a threshold of 8. This threshold was determined by inspection of density plots of PLK1 levels over the data set: the mean level of PLK1 varied from subject to subject, and the authors observed that threshold 8 was an effective separator of the high and lower distributions. In addition, a value of 8 was close to the median for the data set over all subjects, providing an even split of PLK1 levels. On the basis of the distribution of %PLK1high LP cells in all the cases, the authors determined that a threshold of 20% of PLK1high tumor cells was in the middle. Therefore, the authors dichotomized the cases in predominant PLK1high (>20% of PLK1high LP cells) or not. The authors did that to correlate with the limited or advanced clinical stage and variant patterns.

For each immune cell type, the authors counted the fraction of immune cells in the microenvironment of PLK1 high or low tumor cells. The authors also counted the overall number of PLK1 high or low tumor cells and computed a log2 odds ratio for each immune cell i as follows:

**Table 1** List of Antibodies Used for Multiplex Imaging and Immunohistochemistry Analysis

<table>
<thead>
<tr>
<th>Order</th>
<th>Target</th>
<th>Antibody, manufacturer, catalog no.</th>
<th>Dilution</th>
<th>Opal dye</th>
<th>Opal dye dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PD-1</td>
<td>Cell Signaling, 43248</td>
<td>1:100</td>
<td>620</td>
<td>1:150</td>
</tr>
<tr>
<td>2</td>
<td>PLK1</td>
<td>Cell Signaling, 4513</td>
<td>1:50</td>
<td>650</td>
<td>1:150</td>
</tr>
<tr>
<td>3</td>
<td>CD3</td>
<td>Abcam, 16669</td>
<td>1:100</td>
<td>690</td>
<td>1:100</td>
</tr>
<tr>
<td>4</td>
<td>FoxP3</td>
<td>Cell Signaling, 12653</td>
<td>1:100</td>
<td>570</td>
<td>1:150</td>
</tr>
<tr>
<td>5</td>
<td>CD4</td>
<td>Millipore-Sigma, 104R</td>
<td>1:100</td>
<td>520</td>
<td>1:150</td>
</tr>
<tr>
<td>6</td>
<td>CD20</td>
<td>Leica Biosystem, CD20-L26-CE</td>
<td>1:1000</td>
<td>540</td>
<td>1:150</td>
</tr>
</tbody>
</table>

PLK1, polo-like kinase 1.
Where $PLK_{1\text{high}}$ and $PLK_{1\text{low}}$ are the fraction of immune cell $i$ in the microenvironment of PLK1 high or low tumor cells, $PLK_{1\text{high\text{overall}}}$ and $PLK_{1\text{low\text{overall}}}$ are the overall fraction of PLK1 high or low tumor cells, and $\log 2 OR_i$ is the log2 odds ratio for immune cell $i$.

These statistics were computed in aggregate over all samples. However, high variability in both PLK1 levels and immune cell types from sample to sample was identified. The authors used bootstrapping to compute CIs for the above statistics to quantify the uncertainty in the estimates due to this variability. The authors sampled 20 times, with replacement, from the original grouping of 20 samples and computed $PLK_{1\text{high\text{overall}}}$, $PLK_{1\text{low\text{overall}}}$, plus $PLK_{1\text{high\text{i}}}$, $PLK_{1\text{low\text{i}}}$, and $\log 2 OR_i$ for each immune cell type. The authors repeated this process 1000 times and obtained 95% CIs for each statistic as the 2.5 to 97.5 percentiles over all bootstraps. $P$ values were computed from the CIs for the log2 odds ratios, as described by Altman and Bland. Q values were computed by adjusting $P$ values for multiple testing using the false discovery rate procedure over all the immune cells tested.

**Ethical Approval and Consent to Participate**

This study was performed with the approval of the Institutional Review Board of the University of Illinois at Chicago (STUDY2022-0757). The study was performed in accordance with the Declaration of Helsinki.

**Results**

**PLK1 Expression Is Detected in NLPHL Tumor Cells**

The expression of the PLK1 protein was evaluated by immunohistochemistry in a cohort of NLPHL cases and reactive lymphoid tissue obtained from eight different institutions. The results demonstrate that native expression of PLK1 occurs predominantly within germinal center lymphocytes in benign reactive lymphoid tissues (Figure 1). Interestingly, the PLK1 expression was detected in at least 10% of the LP cells in all the NLPHL cases evaluated ($n = 76$) (Figure 1), and the average percentage of PLK1-positive LP cells was 68% (range, 10% to $\geq 95$%) (Figure 2 and Supplemental Figure S1). PLK1 expression was detected in both the cytoplasmic and nuclear compartments of the LP cells, and at variable intensity (Figure 1). This contrasts with...
aggressive B- and T-cell lymphomas, in which PLK1 exhibits predominantly high-intensity nuclear intensity and decreased cytoplasmic expression (Figure 2 and Supplemental Figure S2). PLK1 expression was also evaluated in de novo T-cell/histiocyte-rich large B-cell lymphomas (n > 5). In these cases, the neoplastic cells also showed variable intense expression of PLK1 in both the nuclear and cytoplasmic compartments (Figure 2) in a pattern indistinguishable from the NLPHL cases. An mIF panel was performed in a subset (n = 21) of the NLPHL cases. The panel included markers identifying populations of CD3⁺PD1⁺ T cells, CD20⁺ B cells, and PLK1⁺ LP cells. Multiplex imaging identified LP cells surrounded by rosettes of CD3⁺PD1⁺ T-cell lymphocytes (Figure 2 and Supplemental Figure S3). In addition, quantitative analysis from the immunofluorescence multiplex analysis demonstrated a linear correlation between total PLK1 staining intensity and the nuclear/cytoplasmic ratio of the PLK1 signal (R² = 0.96; n = 11,358) (Figure 2). This is consistent with previous studies showing increased PLK1 protein stability within the nuclear compartment compared with the cytoplasmic compartment. Overall, the findings demonstrate that PLK1 expression can be detected in LP cells...
in a large subset of NLPHL cases, and that higher levels of PLK1 expression correlate with increased nuclear localization.

PLK1 Expression Is Infrequent in Classic Hodgkin Lymphoma Cases

The histologic distinction between NLPHL and cHL may be problematic when LP cells co-express CD30, or when the morphologic features of Reed-Sternberg (RS) cells overlap with those of LP cells, as in the lymphocyte-rich variant of cHL. Therefore, PLK1 expression was also evaluated in a cohort of cHL cases (n = 70). Dual staining of PLK1/CD30 was used to detect PLK1 expression within RS cells. The analysis demonstrates that 9% of the cHL cases tested included RS cells expressing PLK1 (n = 6) (Figure 3). In these cHL cases, the PLK1+ RS cells comprised <15% of total RS cells analyzed, and exhibited dim, mostly cytoplasmic expression (Figure 3). These findings suggest that PLK1 expression in cHL occurs infrequently compared with NLPHL.

PLK1 Promotes c-MYC Protein Expression and Tumor Growth in NLPHL

PLK1 expression correlates with increased stability of the c-MYC protein and adverse clinical outcomes in high-grade B-cell lymphomas.18,22 Therefore, c-MYC protein expression was evaluated in the DEV (NLPHL) cell line in the presence or absence of the selective PLK1 kinase inhibitor volasertib. DEV cells treated with volasertib showed decreased expression of c-MYC protein (Figure 4). In addition, proliferation assays were performed to evaluate the contribution of PLK1 kinase activity to the growth of NLPHL tumor cells. Treatment with volasertib decreased the proliferation and increased the cell death of DEV cell lines in a dose-dependent manner and in a proportion similar to that achieved in a T-cell lymphoma cell line (MAC1) known to express PLK1 protein (Figure 4 and Supplemental Figure S4). Overall, these findings indicate that PLK1 can promote c-MYC protein levels and the survival of NLPHL cell lines.

PLK1 Expression Correlates with the Clinical Stage of NLPHL

Clinical data, including age at diagnosis, response to therapy, and disease stage, were available for 69 cases in the NLPHL cohort (Table 2). The median age at diagnosis was 41.2 years, and most patients were men [n = 45 (65%)]. Most patients (60%; n = 42) presented with advanced disease stage (stage 3 to 4). Initial management included radiotherapy alone [n = 12 (17%)], chemotherapy alone [n = 46 (67%)], or active surveillance [n = 11 (16.0%)].

Figure 3  A and B: Classic Hodgkin lymphoma (cHL) cases were stained for CD30 (pink) and polo-like kinase 1 (PLK1; brown). Reed-Sternberg (RS) cells (CD30 positive) rarely feature PLK1 expression. C: Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) case stained with the same combination of markers shows few weak CD30-positive cells in the background (immunoblasts) that are negative for PLK1. D: Distribution of classic Hodgkin lymphoma cases with different percentages of PLK1-positive Reed-Sternberg cells. Scale bar = 20 μm (A–C).
The patients who received chemotherapy were treated with CHOP \(^{24}\) [\(n = 24\) (52%)], ABVD \(^{25}\) [\(n = 14\) (30%)], or other regimens \(^{26}\) [\(n = 8\) (17%)]. A small number of cases \(^{27}\) [\(n = 8\) (7%)] transformed into more aggressive lymphomas. The median follow-up period was 48.2 months, and 5-year overall survival was 94%.

Kinase activity of PLK1 is associated with increased c-MYC protein stability and proliferation of NLPHL cell lines (Figure 4). Nuclear localization of PLK1 is required to promote c-MYC protein stability and entry into the cell cycle and correlates directly with increased PLK1 protein stability.\(^{17,21}\) As c-MYC expression is associated with tumor proliferation and aggressive behavior in B-cell lymphomas, the authors sought to evaluate the correlation between the nuclear intensity of PLK1 and clinical parameters of disease progression. On the basis of the PLK1 nuclear staining detected using a multiplex immunofluorescence panel (24 cases and 11,358 LP cells analyzed) (Figure 2), a threshold was set for high or low nuclear PLK1 intensity. Subsequently, the percentage of LP cells with PLK1\(^{\text{high}}\) expression was determined in each case, and the cases were dichotomized using a cutoff of 20%. The clinical stage at diagnosis was available for 21 of the cases analyzed, and based on this threshold, 43% of the cases featured \(<20\%\) LP cells with higher nuclear (not cytoplasmic) intensity PLK1 signals (PLK1\(^{\text{high}}\)). Correlation with nuclear/cytoplasmic ratio of PLK1 signal indicates that PLK1\(^{\text{high}}\) cases predominantly featured nuclear PLK1 signals (Supplemental Figure S5). Correlation with the disease stage demonstrated that 89% (\(n = 9\)) of these cases with \(\geq\)20% PLK1\(^{\text{high}}\) LP cells were diagnosed at an advanced (stage 3 or 4) clinical stage (Fisher exact test, \(P < 0.01\)). In addition, 90% (\(n = 12\)) of the cases with \(<\)20% PLK1\(^{\text{high}}\) LP cells were diagnosed at limited (stage 1 or 2) clinical stage (Fisher exact test, \(P < 0.01\)) (Figure 4). Variant histologic patterns (patterns C, D, E, or F of Fan et al\(^{23}\)) constitute an independent prognostic factor for poor outcomes in NLPHL.\(^{2}\) Correlation between PLK1 intensity and the histologic patterns\(^{23}\) demonstrated that 92% (\(n = 12\)) of the cases with a typical histologic pattern featured \(<20\%\) of
Table 2 Demographics of the Patients Included in the Study

<table>
<thead>
<tr>
<th>Patient demographics</th>
<th>Value (N = 69)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average age, years</td>
<td>41.2</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14 (21.7)</td>
</tr>
<tr>
<td>II</td>
<td>13 (18.8)</td>
</tr>
<tr>
<td>III</td>
<td>25 (36.2)</td>
</tr>
<tr>
<td>IV</td>
<td>17 (24.6)</td>
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<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>45 (65.2)</td>
</tr>
<tr>
<td>Female</td>
<td>24 (34.8)</td>
</tr>
<tr>
<td>Treatment</td>
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<tr>
<td>Chemotherapy</td>
<td>46 (66.6)</td>
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<tr>
<td>CHOP-based regimen</td>
<td>24 (52.2)</td>
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<tr>
<td>ABVD regimen</td>
<td>14 (30.4)</td>
</tr>
<tr>
<td>Other regimen</td>
<td>8 (17.4)</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>12 (17.4)</td>
</tr>
<tr>
<td>Active surveillance</td>
<td>11 (15.9)</td>
</tr>
<tr>
<td>Median follow-up, months</td>
<td>48.2</td>
</tr>
<tr>
<td>Transformation to aggressive lymphoma</td>
<td>5 (7.2)</td>
</tr>
<tr>
<td>5-Year overall survival, %</td>
<td>94</td>
</tr>
</tbody>
</table>

Data are given as number (percentage) unless otherwise indicated.

PLK1\textsuperscript{high} LP cells (Figure 4). Consistent with this finding, 75% (n = 8) of the cases with a variant histologic pattern had $\geq$20% PLK1\textsuperscript{high} LP cells (Fisher exact test, $P < 0.01$), indicating that cases with a high proportion of PLK1\textsuperscript{high} LP cells have variant histologic patterns.

Correlation of PLK1 expression and disease stage was performed in additional NLPHL cases not included in the set analyzed by multiplex immunofluorescence. The percentage of tumor cells with positive immunohistochemical expression of PLK1 was analyzed in 69 cases. Quantitative analysis of PLK1 staining intensity was not performed, as it varied significantly between the PLK1\textsuperscript{high} LP cells in each case analyzed, preventing the use of semiquantitative criteria based on light microscopy observation. On the basis of the distribution of the PLK1\textsuperscript{high} LP-cell percentages, a cutoff of 80% PLK1\textsuperscript{high} LP cells was chosen to define cases as PLK1\textsuperscript{high}\% or PLK1\textsuperscript{low}\%. Using this cutoff, cases with $\geq$80% PLK1\textsuperscript{high} LP cells (PLK1\textsuperscript{high}\%) accounted for 61% of the cases (n = 69). Most cases (72%; n = 43) associated with advanced disease stage at diagnosis had $\geq$80% PLK1\textsuperscript{high} LP cells (PLK1\textsuperscript{high}\%, Fisher exact test, $P < 0.01$). Consistent with this, more than half of cases associated with limited-stage disease at diagnosis (56%; n = 26) had $<80\%$ PLK1\textsuperscript{high} LP cells at diagnosis (PLK1\textsuperscript{low}\%, Fisher exact test, $P < 0.01$). The findings suggest that the percentage of PLK1\textsuperscript{high} LP cells correlates directly with an advanced clinical stage at diagnosis in NLPHL.

Multiplex Analysis Identifies Specific Subsets of Immune Cells within the Immediate Vicinity of LP Cells

mIF panels combined with artificial intelligence algorithms can be used to characterize the complex and dynamic composition of the tumor microenvironment. The specific expression of PLK1 within LP cells in NLPHL facilitates identifying the immediate tumor microenvironment repertoire of LP cells using these techniques. Therefore, a targeted mIF panel was performed to determine the relative frequencies of specific lymphocyte subsets in the immediate neighborhood of LP cells (three-five-cell ratio; 40-µm radius distance) (Supplemental Figure S6). The mIF analysis identified 11,358 LP cells in 21 cases, with 1,138,160 lymphocytes (CD3\textsuperscript{+} or CD20\textsuperscript{+} cells) localized within a 40-µm radius distance (Table 3). The most abundant lymphocytes were T-cells, accounting for 85% of the total lymphoid events. Within this T-cell fraction, T-helper type (CD3\textsuperscript{+}/CD4\textsuperscript{+}) lymphocytes and cytotoxic T cells (CD3\textsuperscript{+}/CD8\textsuperscript{+}) comprised 48% and 32% of events, respectively. T-cell follicular helper-type (CD3\textsuperscript{+}/CD4\textsuperscript{+}/PD-1\textsuperscript{high}/FoxP3\textsuperscript{+}) cells comprised 1% of the total lymphocytes, and T-regs (CD3\textsuperscript{+}/CD4\textsuperscript{+}/CD8\textsuperscript{+}/FoxP3\textsuperscript{+}) represented $<1\%$ of the T-helper type lymphocytes. In addition, double-positive CD4/CD8 populations were detected in nearly all cases, accounting for 12% of total T cells (Table 3).

Different Frequencies of Immune Cells Are Differentially Detected According to PLK1 Levels in LP Cells

The current findings indicate that the degree of nuclear PLK1 expression correlates with the clinical disease stage.

Table 3 Percentage and Total Number of Lymphocyte Subsets Identified Near LP Cells (40-µm Radius)

<table>
<thead>
<tr>
<th>Lymphocyte subset</th>
<th>Immunophenotype</th>
<th>Total number of lymphocyte subsets</th>
<th>% of Lymphocyte subsets</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>CD20\textsuperscript{+}, CD3\textsuperscript{+}</td>
<td>140,611</td>
<td>15</td>
</tr>
<tr>
<td>T cells</td>
<td>CD3\textsuperscript{+}, CD20\textsuperscript{+}</td>
<td>767,933</td>
<td>85</td>
</tr>
<tr>
<td>Double positive, T cells</td>
<td>CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD8\textsuperscript{+}</td>
<td>109,228</td>
<td>12</td>
</tr>
<tr>
<td>Cytotoxic T cells</td>
<td>CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD8\textsuperscript{+}</td>
<td>247,512</td>
<td>27</td>
</tr>
<tr>
<td>Helper type, T cells</td>
<td>CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD8\textsuperscript{+}</td>
<td>431,793</td>
<td>48</td>
</tr>
<tr>
<td>Follicular helper, T cells</td>
<td>CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD8\textsuperscript{+}, PD-1\textsuperscript{high}</td>
<td>5798</td>
<td>1</td>
</tr>
<tr>
<td>Regulatory, T cells</td>
<td>CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD8\textsuperscript{+}, FoxP3\textsuperscript{+}</td>
<td>288</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

A total of 11,358 LP cells were analyzed across 21 cases. LP, lymphocyte predominant.
and NLPHL proliferation. Therefore, the relative frequencies of different subsets of T cells in the neighborhood of LP cells with low or high PLK1 intensity (PLK1^{high} and PLK1^{low}) were determined. Among the different T-cell subtypes, T-regs are characterized by the expression of the transcription factor Fox3 and suppressive activity against the B-cell proliferation.24–27 Consistent with this, an increased frequency of T-regs within a given B-cell lymphoma constitutes a favorable prognostic factor.24 Compared with benign reactive lymphoid tissue, the frequency of T-regs is decreased in NLPHL cases,28 suggesting a regulatory role of this group of T-cell lymphocytes in NLPML. The present analysis demonstrated that CD4^{+} T-regulatory type T cells (CD4^{+}Fox3^{+}) were more frequently detected close to PLK1^{high} LP cells [log2 odds ratio (OR), 3.1; CI, 67%–95%; Fisher exact test, \( P < 0.001 \)] (Figure 5 and Table 4). In addition, a specific subset of T-regs exhibits a high-level expression of PD1. This group of T-regs (follicular regulatory T cells) can suppress the expansion of TFH lymphocytes and germinal center B cells.29 The follicular regulatory T-cell subset accounted for approximately 5% of the T-regs and was more frequently detected in the proximity of PLK1^{high} LP cells; however, this difference did not show statistical significance (log2 OR, 4.41; CI, 44%–95%; Fisher exact test, \( P = 0.02 \)) (Figure 5 and Table 4).

Seemal studies have demonstrated the anti-tumor role of cytotoxic CD8^{+} T-cell lymphocytes in lymphoid malignancies.30 The degree of cytotoxic CD8^{+} T-cell tumor infiltration correlates inversely with advanced tumor stage and poor prognosis in solid tumors and B-cell lymphomas, including NLPHL.9,31–34 Analysis of the miF findings indicated that the frequency of cytotoxic T cells was increased in proximity to PLK1^{high} LP cells (log2 OR, 0.76; \( P < 0.05 \)) (Figure 5 and Table 4). Notably, the cytotoxic activity of CD8^{+} T cells can be abrogated by persistent antigenic exposure, which correlates with the surface expression of the co-inhibitory receptors TIM-3, LAG-3, and PD1.35,36 Therefore, the frequency and localization of these dysfunctional CD8^{+} T cells36 were also evaluated in the NLPHL cases. The results demonstrate that cytotoxic T cells co-expressing TIM-3 and PD1 were nearly absent in the immediate vicinity of most LP cells and accounted for <0.001% of the total CD8 T-cell lymphocytes. The frequencies of additional subsets of cytotoxic T cells were also analyzed. Subsets of cytotoxic T cells co-expressing PD1 or Fox3P (and negative for markers of exhaustion; eg, TIM-3) have been described as enriched in solid tumors. The significance of this group of cytotoxic cells remains to be established, as anti-tumor or protumor effects have been described.35,37–42 In the present NLPHL cases, the subset of PD1^{+/−}TIM-3^{−} cytotoxic T cells and Fox3P^{+}/TIM-3^{−} CD8^{+} T cells appeared at higher frequencies in the vicinity of PLK1^{high} LP cells (Figure 5).

T-helper type T cells liaise with B-cell lymphocytes, induce bacteria killing, and coordinate immune responses against helminths.43–46 A subset of T-helper lymphocytes called TFH lymphocytes co-expresses the chemokine receptor CXCR5 and the immune checkpoint protein PD1 and exhibits a unique gene-expression profile.47–50 TFH lymphocytes provide CD40 ligands to germinal center B cells, an interaction required for B-cell lymphocyte survival and differentiation.31,52 TFH lymphocytes can be identified by their high expression level of PD1 (PD1^{high}). However, a subset of TFH lymphocytes expressing PD1 at relatively low levels (PD1^{low}), co-expressing the co-inhibitory receptor TIM-3, is considered to represent an exhausted TFH subset.53 The spatial proximity between TFH lymphocytes and marginal zone lymphoma cells correlates directly with increased proliferation rates of the lymphoma cells.54 In addition, the increased frequency of PD1^{low} exhausted TFH lymphocytes53,54 is associated with a worse prognosis in follicular lymphoma and diffuse large B-cell lymphoma.53 Analysis of the differential frequencies of TFH pools in the present NLPHL cases did not demonstrate a substantial increase in the frequency of T-helper PD1^{high} lymphocytes in proximity to PLK1^{high} LP cells (log2 OR, 0.37) (Figure 5 and Table 4). In addition, the subset of PD1^{low} TIM-3^{+} TFH lymphocytes represented <0.001% of the total T cells, and no significant differences were observed in the frequency of occurrence of these cells near PLK1^{high} or PLK1^{low} LP cells.

**Discussion**

In contrast to small B-cell lymphomas, the high frequency of expression of PLK1 in NLPHL indicates that up-regulated PLK1 expression may be specific to subsets of lymphoma cells with distinct nuclear structure abnormalities.12,55 Similar to aggressive B-cell counterparts, in NLPHL, the kinase activity of PLK1 can promote the stability of c-MYC protein in NLPHL. In addition, the increased nuclear staining intensity of PLK1 occurs predominantly in NLPHL cases with variant histologic morphologies. These variants represent intermediate stages between NLPHL with typical architectural patterns and lymphoma cells with distinct nuclear structure abnormalities.12,55,56 Therefore, dysregulation of epigenetic mechanisms that mediate the nuclear translaction of PLK1, such as sumoylation,20,22 may contribute to the transformation and progression of NLPHL to aggressive forms. Consistent with prior studies, these findings support the driver role of c-MYC during the growth and expansion of NLPHL.9 This suggests a potential role for evaluating PLK1 staining intensity and percentage positivity as a prognostic marker in NLPHL cases, as higher PLK1 expression levels correlate with advanced disease stage. In addition, on average, patients who transformed to diffuse large B-cell lymphoma had higher expression PLK1 levels at diagnosis than those that did not transform to more aggressive lymphoma. However, these findings were not statistically significant, possibly because of the relatively
small number of transformed patients in this cohort (n = 5). These results support the role of PLK1 in clinical disease progression and transformation. Although most patients with NLPHL have favorable outcomes, the subset with more aggressive disease may benefit from combination therapies, including selective PLK1 inhibitors. However, larger patient cohorts will be necessary for more definitive conclusions.

The findings demonstrate that the characteristic cytoplasmic and nuclear expression of PLK1 in LP cells is distinct from that of more aggressive B-cell lymphomas that commonly feature a strong and predominantly nuclear expression.
expression of PLK1. T-cell/histiocyte-rich large B-cell lymphomas typically have a relatively low percentage of tumor cells (often <5%) and architectural features that mimic a subset of NLPHL cases (NLPHL pattern E). The current cohort also included three NLPHL cases with transformation into aggressive forms.9

The current multiplex immunofluorescence analysis demonstrated an increased frequency of cytotoxic T cells that correlated directly with the proliferation rate (PLK1high) of LP cells. This suggests that a cytotoxic host response persists despite NLPHL-mediated immune evasion. However, these findings are limited to the characterization of immune cells in the immediate vicinity of LP cells. Also, we did not identify different frequencies of cytotoxic T cells when the cases were dichotomized by clinical stage (limited or advanced) or when differentially evaluated in cases with variant histologic patterns. This could be secondary to the limited number of cases analyzed by multiplex immunofluorescence panels. Therefore, further studies with larger case series are required to define more complex immune cell frequency distribution patterns. Expression of FoxP3 within cytotoxic T cells has been associated with potent anti-tumor effects, as FoxP3 facilitates adaptation by helping to efficiently use glucose to maintain effector cytokine functions.60 However, knockout expression of FoxP3 within cytotoxic T cells demonstrated improved anti-tumor functions in models of adoptive T-cell therapy.40 The current findings demonstrated an increased frequency of immunoblasts are negative for PLK1 (Figure 3). Consequently, analysis of PLK1 expression may also aid the evaluation of small biopsy specimens containing suspicious atypical, large lymphocytes.

Another notable finding is the near absence of PLK1 expression in the tumor cells of classic Hodgkin lymphoma cases. Fewer than 15% of RS cells were positive for PLK1 in rare exceptions. In contrast, most NLPHL cases in this study (n = 76) showed 68% PLK1+ LP cells on average, and no single case had <10%. Therefore, PLK1 expression may help distinguish between cHL and NLPHL in difficult cases with atypical CD30 and B-cell antigen expression patterns, combined with other markers, such as STAT6.30 PLK1 expression in normal lymphoid tissue occurs predominantly in germinal center lymphocytes, and CD30+ immunoblasts are negative for PLK1. The fraction and CI of each subset identified close to LP cells with PLK1high and PLK1low intensities are indicated.

### Table 4 Frequencies of T-Cell Subsets Near (40-µm Radius) LP Cells

<table>
<thead>
<tr>
<th>T-cell subsets</th>
<th>Immunophenotype</th>
<th>Total no.</th>
<th>PLK1high</th>
<th>PLK1low</th>
<th>Log2 OR</th>
<th>CI log2 OR, % Freq.</th>
<th>CI log2 OR, % Freq.</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-regs</td>
<td>CD3+, CD4+, CD8−, FoxP3+, PD1low/neg</td>
<td>240</td>
<td>29</td>
<td>3.1</td>
<td>90</td>
<td>67–95</td>
<td>10</td>
<td>5–10</td>
<td>0.000001 ****</td>
</tr>
<tr>
<td>Follicular regulatory T cells</td>
<td>CD3+, CD4+, CD8+, FoxP3+, PD1high</td>
<td>14</td>
<td>5</td>
<td>1.5</td>
<td>73</td>
<td>42–93</td>
<td>26</td>
<td>6–57</td>
<td>0.150000 NS</td>
</tr>
</tbody>
</table>
FoxP3+/CD8+ T cells in the neighborhood of PLK1high LP cells. The significance of this finding remains to be established, as the function of this subset of effector cytotoxic T cells during tumor responses has yet to be delineated. More important, the frequency of exhausted T-helper cells and cytotoxic T cells was not increased in the vicinity of LP cells with PLK1high intensities; in fact, both populations were nearly absent in NLPHL cases. In addition, the relatively increased frequency of T-reg cells around PLK1high LP cells supports a disease model in which the immune-surveillance role of T-reg is preserved.

Consistent with previous studies, TFH-type lymphocytes were detected frequently near LP cells. The oncogenic advantage provided by TFH lymphocytes during the growth of NLPHL has been described previously. The frequency of TFH subsets was not increased near LP cells with a high proliferation rate (PLK1high), which could be secondary to the increased frequency of immunomodulatory cells (such as T-reg) near the PLK1high tumor cells. This finding suggests that LP cells have relatively limited control over the immediate immune surveillance program, which may explain the lower relapse rates and decreased rate of progression of NLPHL compared with aggressive B-cell lymphomas. Because PD-L1 expression occurs in 75% of NLPHL cases, and effector T cells predominate in the tumor microenvironment in low- and high-stage disease and in NLPHL cases with transformation into diffuse large B-cell lymphoma, the therapeutic use of immune checkpoint inhibitors could be a viable strategy for treating NLPHL at advanced stages.

**Author Contributions**


**Disclosure Statement**

M.L.X. receives financial support for consultancy at Treeline Biosciences. The authors declare no other competing financial interests in relation to the work described.

**Supplemental Data**

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2023.10.008.

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