

Chapter 2

Flow Cytometry for Non-Hodgkin and Classical Hodgkin Lymphoma

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Abstract

Multiparametric flow cytometry is a powerful diagnostic tool that permits rapid assessment of cellular antigen expression to quickly provide immunophenotypic information suitable for disease classification. This chapter describes a general approach for the identification of abnormal lymphoid populations by flow cytometry, including B, T, and Hodgkin lymphoma cells suitable for the clinical and research environment. Knowledge of the common patterns of antigen expression of normal lymphoid cells is critical to permit identification of abnormal populations at disease presentation and for minimal residual disease assessment. We highlight an overview of procedures for processing and immunophenotyping non-Hodgkin B- and T-cell lymphomas and also describe our strategy for the sensitive and specific diagnosis of classical Hodgkin lymphoma.

Key words: B cells, B-cell lymphoma, Clonality, Flow cytometry, Hodgkin lymphoma, Light chain restriction, T cells, T-cell lymphoma, T-cell receptor V-beta repertoire analysis

1. Introduction

Immunophenotypic analysis of both non-Hodgkin lymphomas (NHL) and Hodgkin lymphomas is commonly required for proper diagnostic classification and is currently accomplished by immunohistochemistry and flow cytometry (1). Multiparameter flow cytometry is an advanced diagnostic technology that is widely used for the clinical evaluation of complex cellular mixtures such as peripheral blood, bone marrow aspirates, body fluids, lymph node, and other tissue specimens (2). The ability of the technique to perform rapid characterization of numerous cellular populations for the simultaneous expression of multiple cell surface or cytoplasmic antigens on individual cells over several orders of magnitude of

expression has led flow cytometry to be regarded as a “standard of care” for the evaluation of hematopoietic processes in the clinical laboratory.

In flow cytometry, a suspension of cells is injected into a fluid stream under laminar flow conditions (3) resulting in individual cells being directed through a quartz capillary tube (flow cell). Following illumination by one or more light sources, typically lasers, multiple cellular properties may be simultaneously assessed, including light scatter and the expression of surface and/or cytoplasmic antigens (3, 4), when cells are labeled with fluorochrome-conjugated antibodies directed against specific antigens (5). Both liquid (peripheral blood, bone marrow, cerebrospinal fluid, etc.) and tissue specimens are routinely analyzed to provide unique diagnostic information about hematopoietic populations (2). In general, flow cytometry permits the evaluation of multiple antigens on cells in a given experiment, typically 4–6 and increasingly in up to 10 or more antigens (4). Here, we discuss practical aspects of lymphoma diagnosis and classification by flow cytometry. As part of our approach, we assume that an abnormal cell population will have an aberrant immunophenotype as compared to background normal or reactive cells. As such, emphasis is placed on identifying aberrant immunophenotypes in a multiparametric analysis. As there have been numerous articles describing various strategies for flow cytometry, the reader is advised to access other articles in the field to fill deficiencies or intentional omissions herein.

2. Materials

Reagents are generally used as provided by the manufacturer with in-house validation prior to use. However, it is important to titer antibodies for optimal signal-to-noise response under the conditions to be used, and this may result in the use of antibodies at concentrations below that recommended by the manufacturer. The antigens that we target for routine B- and T-cell NHL and classical Hodgkin lymphoma (CHL) analysis are included in Table 1, with specific antibodies and fluorochromes employed using a modified LSRII flow cytometer (Becton-Dickinson).

2.1. Buffers and Cell Staining Reagents

1. PBS–BSA buffer: Dulbecco’s Phosphate-Buffered Saline (GIBCO®) with 3% bovine serum albumin added. PBS contains 2.67 mM KCl, 1.47 mM KH_2PO_4 , 137.9 mM NaCl, and 8.1 mM Na_2HPO_4 .
2. RPMI 1640.

Table 1
Fluorochrome combinations used for lymphoma immunophenotyping at the University of Washington

Antigen	B cells	B-cell				T cell	TCR V-beta	cHL (9-color)	cHL (6-color)
		B-cell add-on for CLL/mantle cell	Add on tube for CLL prognosis	B-cell add-on for hairy cell leukemia	add-on for marginal zone				
CD2						FITC			
CD3						PE-Cy7	PE-Cy7		APC-Cy7
CD4						A594	A594		
CD5	PE-Cy5.5	PE-Cy5.5	PE-Cy5			PE	ECD	APC-Cy7 (or ECD)	
CD7						APC	APC		
CD8						V450	APC-Cy7		
CD10	APC								
CD11c				APC	APC				
CD15								APC	
CD19	PE-Cy7	ECD	ECD	PE-Cy7	PE-Cy7				
CD20	V450							PE-Cy7	PE-Cy7
CD23		PE							
CD25				PE					
CD30						APC-A700		PE	PE
CD34						ECD			
CD38	A594		PE						
CD40								PE-Cy5.5	PE-Cy5.5

(continued)

Table 1
(continued)

Antigen	B cells	B-cell add-on for CLL/mantle cell	Add on tube for CLL prognosis	B-cell add-on for hairy cell leukemia	B-cell add-on for marginal zone	T cell	TCR V-beta	cHL (9-color)	cHL (6-color)
CD45	APC-H7			V450	APC-H7	APC-H7		ECD (or APC-H7)	
CD56						PE-Cy5	PE-Cy5		
CD64								FITC	FITC
CD71								APC-A700	
CD95								PB	APC
CD103				FITC					
HLA-DR							V450		
FMC-7		FITC							
ZAP-70			FITC						
Kappa	FITC				FITC				
Lambda	PE				PE				
V-beta isoform reagents							FITC, PE, and FITC and PE		

PB pacific blue, *FITC* fluorescein isothiocyanate, *PE* phycoerythrin, *ECD/PE-TR* PE-Texas Red, *PECy5* PE-Cyanine-5, *PE-Cy5.5* PE-Cyanine-5.5, *PE-Cy7* PE-Cyanine-7, *A594* AlexaFluor 594, *APC* allophycocyanin, *APC-A700* APC-AlexaFluor 700

3. Lysing/fixation solution: 0.15 mol/L NH_4Cl , pH 7.2 containing 0.25% ultrapure formaldehyde (Polysciences). The solution fixes cells and lyses red blood cells.
4. Medium A and B for cytoplasmic antibody staining are from Invitrogen (Fix & Perm[®]).

3. Methods

3.1. Sample Preparation

Tissue specimens are first disaggregated to create a single-cell suspension:

3.1.1. Disaggregation of Tissue Specimens

1. Mince the tissue with a scalpel in approximately 5 mL of RPMI 1640.
2. Filter the disaggregated cell suspension through a 40 μm filter.
3. Pellet the cells by centrifugation ($550\times g$ for 5 min) and decant the remaining RPMI 1640.
4. Resuspend the cells in PBS–BSA or RPMI 1640, pellet the cells again by centrifugation ($550\times g$ for 5 min), and resuspend in RPMI 1640 to a cell count of 10,000 cells/ μL or less.
5. Add sufficient cell suspension to deliver up to one million cells in a volume of $<200\text{ }\mu\text{L}$.

3.1.2. Cell Surface Labeling of Cell Suspensions

1. Add appropriate fluorescently labeled, titrated antibodies to cell suspension (from disaggregated tissue, bone marrow, blood, etc.), typically 5–20 μL of each antibody, and mix gently. The antibodies may be cocktailed prior to use for efficient delivery.
2. Incubate the labeled cells for 15 min at room temperature (RT) in the dark.
3. Add 1.5 mL of lysing/fixation solution.
4. Incubate for 15 min at RT in the dark.
5. Centrifuge the cells ($550\times g$ for 5 min) and decant the supernatant.
6. Add 3 mL of PBS–BSA, centrifuge ($550\times g$ for 5 min), and decant the supernatant.
7. Resuspend the cells in 100 μL of PBS–BSA.
8. Collect 150,000 events (if possible).

3.1.3. Cytoplasmic Labeling of Cell Suspensions (for ZAP-70 and Bcl-2)

1. Add appropriate fluorescently labeled, titrated cell surface antibodies to cell suspension and mix gently.
2. Incubate the labeled cells for 15 min at room temperature in the dark.
3. After washing the cells twice with PBS–BSA, add 100 μL of Medium A and mix well.

4. Incubate for 15 min at room temperature in the dark.
5. Wash the cells twice with PBS–BSA, add 100 μ L of Medium B, and mix well.
6. Add appropriate amount of cytoplasmic antibody, mix, and incubate the labeled cells for 30 min in the dark at room temperature.
7. Wash the cells twice with PBS–BSA and resuspend the cells in 100 μ L of PBS–BSA.

3.1.4. Sample Preparation for Hodgkin Lymphoma

The sample processing and immunostaining protocols are the same as those used for NHL with the exception that more events should be collected, preferably ~500,000. This CHL assay has only been validated on lymph nodes and thus is only recommended for tissue specimens.

3.2. Gating Strategies, Data Analysis, and Interpretation

3.2.1. B-Cell Analysis

Compensated data files for B-cell NHL are first gated to exclude the so-called doublet events using a plot of forward scatter area versus forward scatter height. The doublet events represent coincident cells in the flow cell and need to be excluded as the antigenic profile derived from these events may result in an apparent composite immunophenotype due to the combined antigenic expression of two or more cells. Subsequently, nonviable events are excluded using forward and side light scatter gating. As cells degenerate, initially forward scatter decreases while side scatter increases and later both decrease in intensity. These nonviable events can be readily excluded by selective gating considering these findings.

The evaluation for B-cell NHL next involves identification of B cells with subsequent evaluation for the presence of surface immunoglobulin light chain-restricted populations with aberrant immunophenotypes (see Note 1). We currently gate lymphocytes with forward and side light scatter (see Note 2), computationally subtract the CD5-positive, CD19-negative T cells, and then isolate the B cells on a plot of CD19 versus side scatter. Data is subsequently evaluated by examining various antigens (Table 1) plotted against each other for both the B cells and lymphocytes. Evaluation of lymphocytes based on forward versus side scatter (in addition to CD19-positive B cells) prevents inadvertent exclusion of B-cell populations that have aberrant loss or decreased expression of CD19 (see Note 3). In general, the normal kappa-to-lambda ratio of the B cells is approximately 1.4; however, sole emphasis on a skewed light chain ratio for identifying an abnormal B-cell population is discouraged due to its poor sensitivity and specificity. Rather, an emphasis is placed on identifying abnormalities of other antigens assessed in these studies (CD5, CD10, CD19, CD20, CD38, and CD45) that are often under- or over-expressed relative to any normal population, helping to separate the abnormal and normal populations. Typical examples of CLL/SLL (Fig. 1), marginal

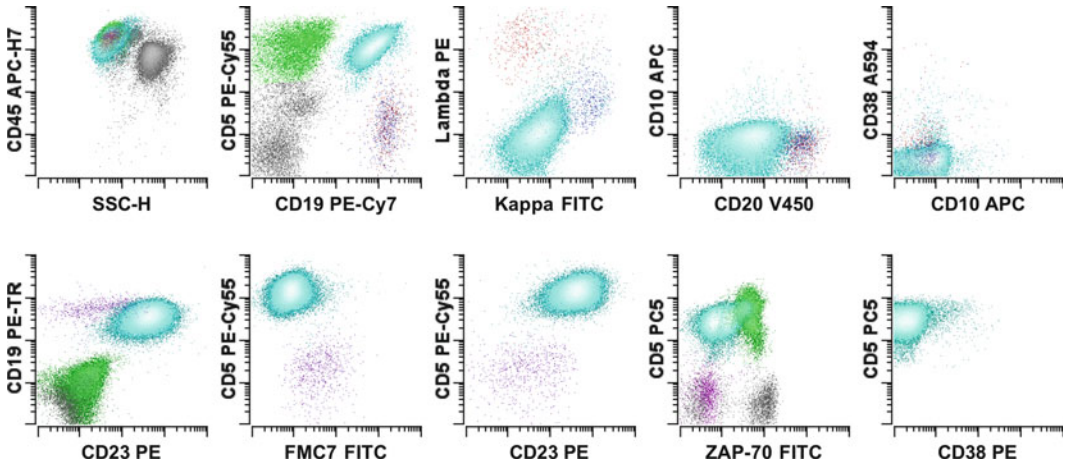


Fig. 1. Flow cytometric characterization of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). The neoplastic population (*cyan*) demonstrates expression of CD45, CD19, CD5, low-level kappa surface light chains, decreased CD20, CD23, and no expression of FMC7 or CD10. The neoplastic population does not express ZAP-70 (positive cells determined by a discriminator which is negative on the normal B cells and positive on the normal T cells) or CD38, features associated with a more favorable prognosis in CLL/SLL (52, 53). The first dot plot of the *top panel* shows all cells, the second shows lymphocytes, and the last three show only B cells. In the *bottom panel* of dot plots, all lymphocytes are shown in the first and fourth plots, while the second, third, and fifth plots show only B cells. A small population of reactive B cells identified by *blue* (kappa expressing) and *red* (lambda expressing) events. Reactive T cells are colored in *green*.

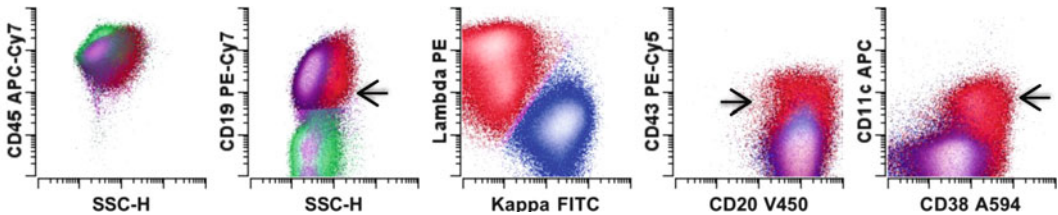


Fig. 2. Characterization of an abnormal B-cell population using CD11c and CD43. The right parotid gland biopsy demonstrated a decreased kappa-to-lambda ratio (0.83) but no other antigenic abnormalities. Characterization of the B cells using CD11c and CD43 demonstrates a lambda-restricted population with expression of these antigens (*arrows*), consistent with involvement of a B-cell lymphoproliferative disorder (marginal zone lymphoma by morphology). T cells are *green* and kappa- and lambda-expressing B cells are in *blue* and *red*, respectively.

zone lymphoma (Fig. 2), hairy cell leukemia (Fig. 3), Burkitt lymphoma (minimal residual disease (MRD)) (Fig. 4), and follicular lymphoma (Fig. 5) are provided. While individual cases vary, a summary of common immunophenotypes of typical B-cell lymphomas is provided (Table 2). A more comprehensive discussion of immunophenotypes in B-cell NHL can be found in the review by Crain and Foon (2).

The approach for evaluating specimens for B-cell NHL MRD is identical to that used for evaluating samples at disease presentation, with the obvious difference being that the abnormal population post therapy can be very small, sometimes <0.1% of the viable cells. In practice, a series of sequential gates to selectively include

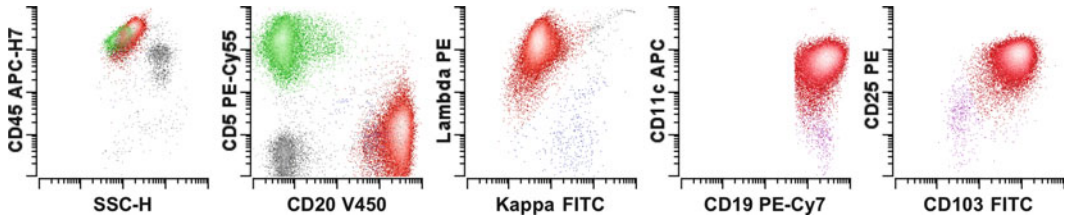


Fig. 3. Immunophenotypic characterization of a case of hairy cell leukemia in the bone marrow. The *first* panel shows all leukocytes, the second all lymphocytes, and the remaining three panels show only B cells. T cells are colored in *green*, kappa-restricted B cells are colored in *blue*, and the lambda-restricted B cells are colored in *red*. The neoplastic hairy cell leukemia population demonstrates increased side light scatter and CD45 expression, bright expression of CD20, lambda light chain expression, and expression of CD11c, CD19, CD25, CD38 (data not shown), and CD103, without CD5 or CD10 (data not shown).

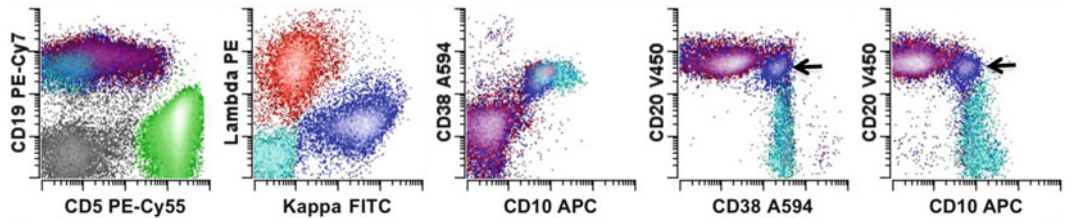


Fig. 4. Minimal residual disease detection of Burkitt lymphoma in the bone marrow. Overall, this bone marrow specimen demonstrates a normal kappa-to-lambda ratio of 1.5. In addition, multiple dot plots do not demonstrate an abnormal B-cell population. However, a small (1% of leukocytes) kappa-restricted B-cell population is noted (*arrows*) with relatively bright expression of CD38 and CD10. This immunophenotype is identical to that identified in the prior presenting mesenteric mass. The first dot plot shows all lymphocytes, while the remaining four panels show B cells. T cells are colored *green*; kappa- and lambda-restricted mature B cells are colored *blue* and *red*, respectively; immature, normal B lymphoblasts are colored *cyan*.

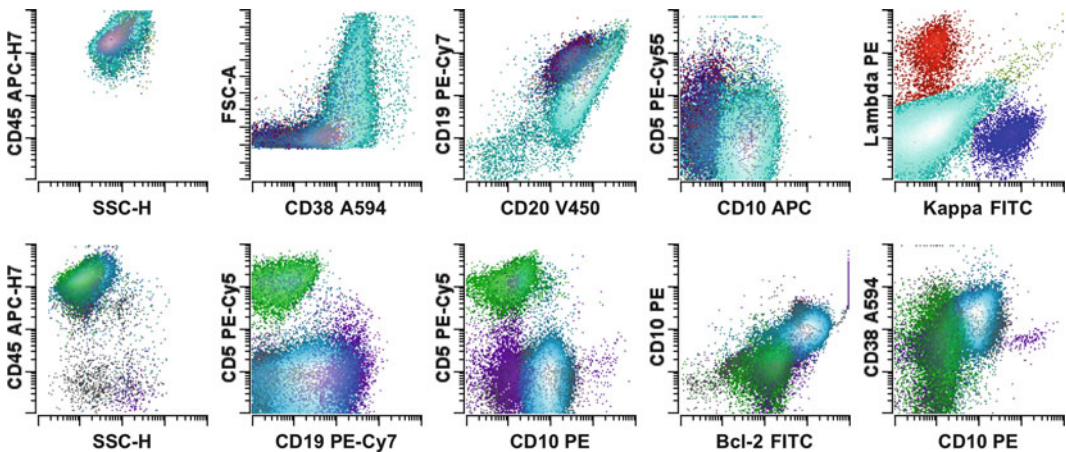


Fig. 5. Immunophenotypic characterization of a case of follicular lymphoma (grade 3). The first dot-plots of both the *top* and *bottom* panels show all leukocytes, the last four in the *top* panel show B cells, and the remaining four plots in the *bottom* panel show lymphocytes. T cells are colored in *green*, kappa-restricted B cells are colored in *blue*, the lambda-restricted B cells are colored in *red*, and neoplastic B cells are colored in *cyan*. The neoplastic cells show expression of CD45, increased forward light scatter, decreased expression of CD19 relative to the reactive B cells, absent surface light chain restriction, and expression of CD10, CD20, bcl-2 (relative to the reactive T cells), and CD38 (normal to slight decreased relative to the expression of a normal germinal center population (29)) and no expression of CD5.

Table 2
Immunophenotypes of common B-cell NHL

B-cell lymphoma	Immunophenotype
Diffuse large B-cell lymphoma	Increased forward and side light scatter with variable expression of CD19, CD20, and monotypic surface light chain. CD10 may or may not be expressed
Follicular lymphoma	CD45, CD19 (decreased), CD20 (decreased), CD10 (increased), CD38 (decreased), monotypic surface light chain, increased BCL-2 as compared to background T cells
Chronic lymphocytic leukemia/small lymphocytic lymphoma	CD45, CD5, CD20 (dim), mono- or bi-typic dim to absent surface light chain expression, CD23 positive, FMC-7 negative, with variable CD38 and ZAP-70 expression
Mantle zone lymphoma	CD45, CD5, CD20 (normal), monotypic surface light chain restriction, CD23 negative, FMC-7 positive
Burkitt lymphoma	CD45, CD20, CD10, CD38 (increased), monotypic surface light chain expression, no over-expression of BCL2
Hairy cell leukemia	CD20 (bright), CD19, monotypic surface light chain expression, aberrant co-expression of CD11c, CD25, CD103, without CD5 and CD10
Marginal zone lymphoma	CD20, CD19, negative for CD5 and CD10. Occasional cases have expression of CD43

and/or exclude various populations may be required to convincingly isolate the abnormal population and evaluate for definite light chain restriction (Fig. 4). Identifying these small populations is facilitated by knowledge of the original immunophenotype of the abnormal B-cell population at presentation (when the population is usually present at significantly increased proportion) preferably using the identical reagent combination used to immunophenotype the presentation specimen. Other caveats related to the analysis of B cells are described in Notes 4–7.

3.2.2. T-Cell Analysis

In general, the flow cytometric gating strategy for T cells is similar to that for B cells. Compensated data files are first gated to exclude doublet events and then nonviable events (see Note 8). T cells are then identified typically by two gating strategies: (1) forward versus side scatter to identify lymphocytes or (2) CD3 versus side scatter to identify T cells. The former approach helps to identify T cells that have aberrant decreased or absent expression of CD3, while the latter identifies T cells using CD3 to permit identification of potentially larger cells that may have increased side scatter (see also Note 9). While the analysis of the B cells rests on identifying an immunophenotypically abnormal B-cell population that is clonal, evaluation of the T cells involves the recognition of an

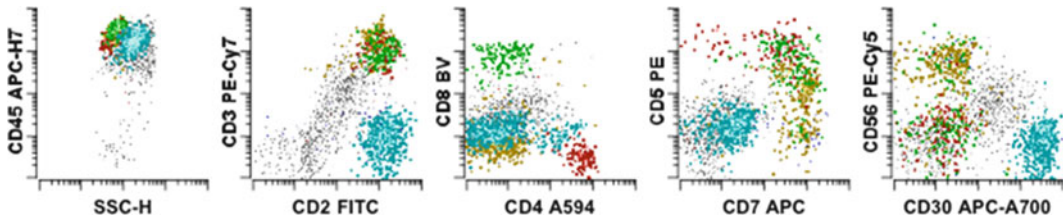


Fig. 6. Example of a CD30+ T-cell lymphoma. An abnormal CD2-positive T-cell population is identified with strong CD30+ expression, CD4 expression on a small subset, and aberrant loss of CD3, CD5, and CD7 without CD8 or CD56 (teal). Reactive CD4, CD8, and CD4/CD8-negative T cells are colored *red*, *green*, and *orange*, respectively. Of note, although the expression of CD2 and possible dim expression of CD4 are not considered lineage specific, subsequent molecular analysis of T-cell receptor gamma gene rearrangement confirms the population is clonal.

immunophenotypically abnormal T-cell population. At some institutions, evaluation of probable clonality of T-cell populations can also be performed using flow cytometry by assessing the T-cell receptor (TCR) V-beta repertoire.

As part of the immunophenotypic evaluation of T cells, it is critical to recognize that numerous reactive T-cell populations may be present in any given sample (see Note 10). These T cells may include memory T cells (decreased CD7 expression), gamma-delta T cells (increased CD3, absent CD4 and CD8 (partial)), and large granular lymphocytes (typically CD8+/CD5 dim/absent) and may be identified in any given patient sample, sometimes in increased proportion (2). Familiarity with this normal spectrum of antigenic variation of reactive T cells is critical to prevent misinterpretation of a reactive population as a malignant clone. On the other hand, it is also possible that a neoplastic, clonal population of T cells may show identical immunophenotypic change as any of these reactive populations. As such, careful clinical and pathologic correlation is required in every case.

With this spectrum of antigenic variation in mind, it is diagnostically fortuitous that the majority of T-cell lymphoproliferative disorders will demonstrate aberrant antigenic expression such that the level of expression of T-cell-associated antigens (CD2, CD3, CD4, CD5, CD7, CD8, and CD45) can be increased, decreased, or completely absent, as compared to the background, normal reactive T cells (6, 7). Multiparametric identification of these antigenic changes consequently permits one to reliably identify an abnormal T-cell population, which in conjunction with histologic findings or clinical data can permit subsequent definite classification. As an example, flow cytometry of a liver core needle sample from a 69-year-old man with multiple liver lesions (Fig. 6) identified an abnormal T-cell population comprising 25.5% of total white cells with increased forward and side scatter and expression of CD2 and CD30 (bright) and with aberrant loss of expression of CD3, CD5, and CD7 without CD8, CD34, or CD56. CD4 may be expressed dimly on a small subset. The immunophenotype of the abnormal

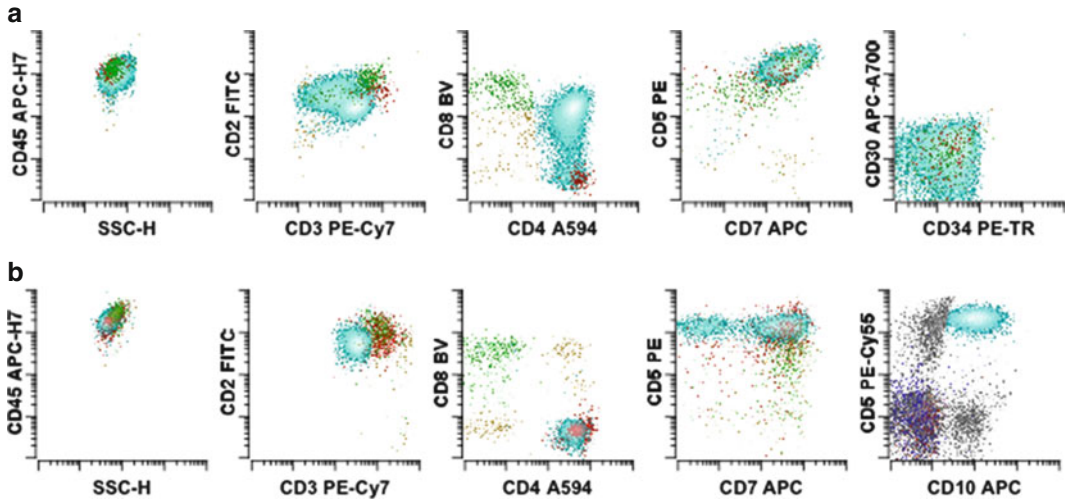


Fig. 7. Examples of other mature T-cell lymphomas. **(a)** T-cell prolymphocytic lymphoma, a population of CD4/CD8+double-positive T cells with decreased expression of CD2, CD3, and CD45, without evidence of CD34 or Td (data not shown), is identified in a peripheral blood sample of a 73-year-old male with a white blood cell (WBC) count of 398,000 and prior cytogenetic studies showing involvement of a complex karyotype, including $\text{inv}(14)(q11.2q32)$. Background reactive CD4+ (red) and CD8+ (green) T lymphocytes are also shown. **(b)** Angioimmunoblastic T-cell lymphoma, a population of CD4+positive T cells (teal) with aberrant expression of CD2 (decreased), CD3 (decreased), and CD10, with variable CD7 and normal expression of CD5 and CD45, is identified in a 91-year-old female peripheral blood sample, comprising 18.6% of total white cells. Background reactive CD4+ (red) and CD8+ (green) T lymphocytes are also shown.

CD45, CD30+ cell population was favored to represent a CD30+ peripheral T-cell lymphoma and a clonal TCR gamma gene rearrangement (by PCR) was identified, providing support for T-cell lineage. Other examples of mature T-cell lymphomas, including T-cell prolymphocytic leukemia and probable angioimmunoblastic T-cell lymphoma, are also shown (Fig. 7). While individual cases vary, a summary of common immunophenotypes of typical T-cell lymphomas is provided (Table 3). A more comprehensive discussion of immunophenotypes in T-cell NHL can be found elsewhere (2). See Note 11 for an additional caveat regarding the analysis of T cells.

At presentation, a common question clinically is whether a T-cell population of interest represents a clonal process or alternatively is a reactive oligoclonal or polyclonal expansion of an immunophenotypically distinct subset. Flow cytometric analysis of TCR V-beta repertoire is a methodology that uses fluorescently labeled anti-TCR V-beta antibodies to determine if the identified T-cell populations represent a diverse or a clonal process (8–11). As provided by the manufacturer, the IOTest® Beta Test Mark assay is a set of 24 antibodies that covers approximately 70% of the normal human TCR V-beta repertoire. These V-beta-specific antibodies are each conjugated to one of the three fluorochromes, FITC, PE, or PE, and FITC conjugate. According to the intended original

Table 3
Immunophenotypes of common T-cell NHL

T-cell lymphoma	Immunophenotype
T-PLL	CD4+/-, CD8-/+ , variable CD3, CD2, CD5, CD7
AITL	CD4+, CD5+, CD10+/-, CD3-/+
Mycosis fungoides/Sezary syndrome	CD4+, variably decreased CD7, CD3, CD5, CD45
PTCL-NOS	CD4+/-, often with loss of CD5 and/or CD7
ATLL	CD4+, CD25+, CD3+, decreased CD7
ALCL	ALK+/-, CD30+, variable loss of T-cell antigens, TIA1/granzyme B+
T-LGL	CD3+, CD4-, CD8+, with decreased CD5 and or CD7

T-PLL T-cell prolymphocytic leukemia, *AITL* angioimmunoblastic T-cell lymphoma, *PTCL-NOS* peripheral T-cell lymphoma, not otherwise specified, *ATLL* adult T-cell leukemia/lymphoma, *ALCL* anaplastic large cell lymphoma, *T-LGL* T-cell large granular lymphocyte leukemia, *TIA1* T-cell intracellular antigen 1

protocol, the assay is run in eight separate tubes such that three V-beta family-specific antibodies (one labeled with FITC, one labeled with PE, and one labeled with FITC and PE) are present in each tube. Through the use of gating reagents (for example CD3, CD4, or CD8), the T-cell population of interest can be isolated to determine if there is evidence for overrepresentation of a particular TCR V-beta isoform relative to normal, a finding that is strongly suggestive of clonality. Numerous studies have demonstrated the utility of this approach for assessing putative T-cell clonality in both the clinical and research environment (8–11). The reader is advised to consult these publications for performing this approach for TCR V-beta analysis.

At our institution, a simple permutation of the original protocol for TCR V-beta repertoire analysis has been developed and used for several years (manuscript submitted). In this modification, instead of 8-tube analysis as intended by the manufacturer, all of the fluorescently labeled TCR V-beta antibodies are combined into a single tube for combined analysis. This modification permits rapid identification of a putative TCR V-beta-restricted T-cell population with emphasis on identifying aberrant antigenic expression through the use of an increased number of T-cell-specific gating reagents (Table 1). As compared to the standard method, this modified approach is fast, can be adopted in any laboratory currently performing routine TCR V-beta analysis, and minimizes the amount of reagent and sample used for analysis, thus permitting application to specimens that are typically hypocellular in nature, such as skin biopsies and cerebrospinal fluid samples. An example of this modified approach is shown in which an expanded large

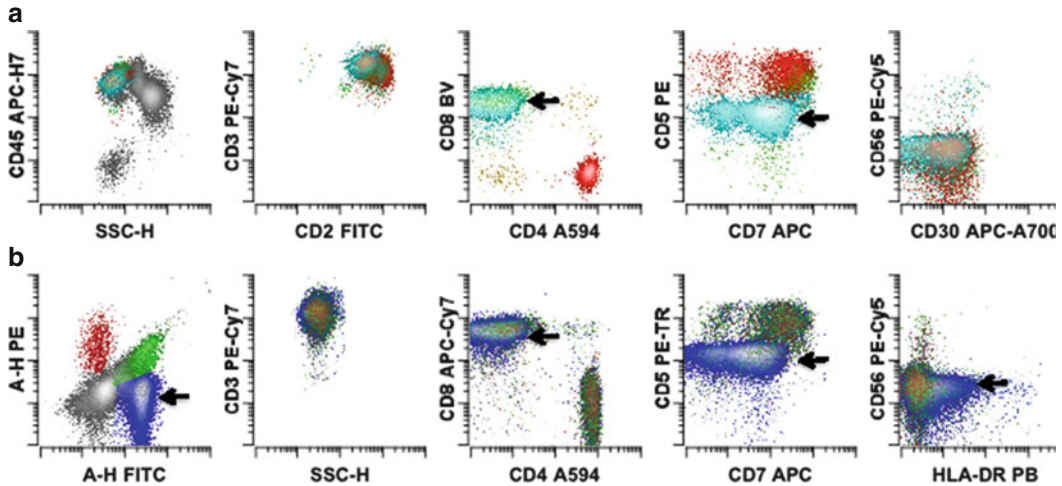


Fig. 8. Example of combined using modified TCR V-beta assay showing clonality of T-LGL population. (a) A CD8+ T-cell population (teal, arrows) is identified with decreased CD5 and CD7 expression as compared to background CD4+ (red) and CD8+ (green) T cells. (b) Subsequent analysis of TCR V-beta expression using the modified assay in which all TCR V-beta antibodies are combined together shows the T-LGL population (blue, arrows) with apparent TCR V-beta restriction limited to an FITC-labeled isoform. By comparison, background, reactive T cells show variable expression of PE, FITC, PE-FITC, and unlabeled TCR V-beta isoforms.

granular lymphocyte population is identified with expression of CD8 and decreased expression of CD5 and CD7 (Fig. 8). Subsequent assessment of TCR V-beta repertoire analysis using a modified approach in which all V-beta antibodies are combined together permits identification of probable FITC-fluorochrome-labeled TCR V-beta isoform. If clinically indicated, molecular analysis of TCR gene rearrangement could be subsequently performed to confirm the presumed clonal nature of this population. In addition, if knowledge of the specific TCR V-beta isoform is important, such as for future minimal residual disease monitoring, the standard TCR V-beta repertoire assay could be performed to permit identification of which V-beta isoform is identified by the PE-labeled anti-V beta antibody.

3.2.3. Hodgkin Cell Analysis

CHL is an unusual type of B-cell lymphoma (12–14) in which the neoplastic Hodgkin and Reed–Sternberg (HRS) cells are rare (<1% of the cells in lymph node) (13, 15); the bulk of the cells in an involved lymph node include reactive lymphocytes, eosinophils, plasma cells, and histiocytes (15); and the HRS bind to non-neoplastic T cells, resulting in HRS cell–T-cell rosettes (16–22). Traditionally, CHL has been diagnosed by morphology and immunohistochemistry (HRS cells demonstrate expression of CD15 and CD30 but lack expression of CD20, CD3, and CD45 (12, 15, 23, 24)). Our recent studies, however, have demonstrated that HRS cells can be directly identified by flow cytometry with high

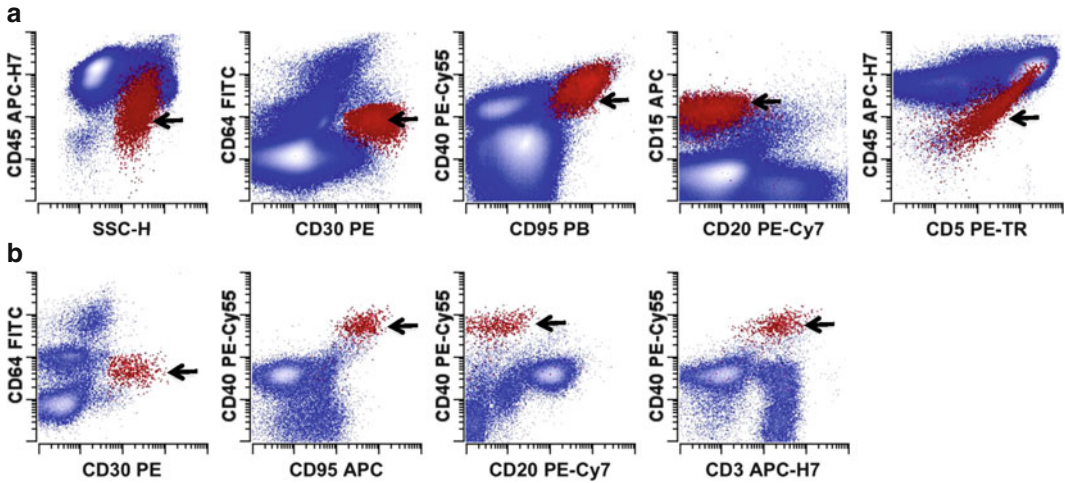


Fig. 9. Representative examples of 9- (a) and 6-color (b) flow cytometry studies of CHL cases. HRS cells (arrows; also shown in red and emphasized) are identified by their absence of expression of CD64 (position of negative determined by control experiments, not shown), expression of CD30, CD40, CD95, and increased side light scatter (SSC-H) compared to normal lymphocytes; all the remaining viable events are in blue. (a) The population of neoplastic HRS cells have expression of intermediate CD15, intermediate to bright CD30, intermediate to bright CD40, variable CD71 (data not shown), and intermediate to bright CD95, without expression of CD64 or CD20. The diagonal relationship between CD45 and CD5 is due to the presence of T cells bound to the HRS cells. (b) Neoplastic HRS cells have expression of intermediate CD30, bright CD40, and intermediate CD95, without expression of CD64 or CD20. CD3 is expressed suggesting some degree of HRS–T-cell rosetting.

clinical sensitivity and specificity (22), allowing this neoplasm to be diagnosed by this technique (22, 25). The interaction of T cells and HRS cells (rosetting) can be directly detected by flow cytometry, as demonstrated by the observation of a composite immunophenotype of the HRS cells and T cells, that is, cells with expression of both HRS-cell and T-cell antigens (22). T-cell–HRS cell rosetting can be disrupted by unlabeled “blocking” antibodies (antibodies that can compete for the binding of the adhesion molecule binding partner (20, 22)), a practice that is useful for purifying HRS cells (22) but is not necessary for diagnostic flow cytometry (25). Reagent combinations are proposed for either 9-color (25) or 6-color (26) flow cytometry platforms (Table 1). In addition, a reactive T-cell population (CD4⁺ T-cell population with CD45^{bright}, CD7^{bright}) has been identified in lymph nodes involved by CHL, a finding that can be used to suggest a diagnosis of CHL (see Note 12) (27).

The gating strategy to identify HRS cells is different from that for non-Hodgkin B- and T-cell lymphomas. The first difference is that because of the relatively increased cell size of HRS and rosetted cells, increased side scatter is used to identify these populations (Figs. 9 and 10); HRS cells are then identified by the requisite expression of CD30, CD40, and CD95 with the absence of

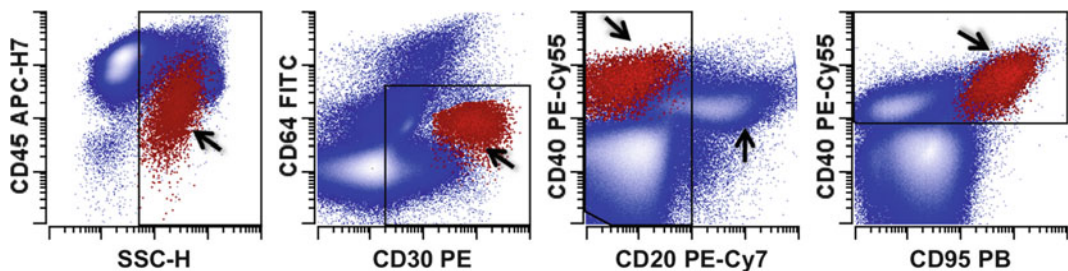


Fig. 10. Gating strategy to identify HRS cells in CHL. HRS cells are in *red* (also identified by *oblique arrows*) and are emphasized, while all other events are in *blue*. The B cells are identified by the *vertical arrow* (CD20, CD40+). Putative HRS cell events must fall in all four gates and form a distinct population. Specifically, the cells must have increased side scatter (1st plot), be CD30+ and have increased auto-fluorescence in the FITC channel compared to small lymphocytes (2nd plot), express no or low CD20 (3rd plot), and express CD40 at or greater intensity than a reactive B cell (4th plot).

Table 4
Criteria for identifying an HRS cell population

1	Express CD30, CD40, and CD95
2	Have increased light scatter properties (compared to background lymphocytes)
3	Lack moderate to bright expression of CD20
4	Lack expression of CD64
5	Represent a discrete cell population in multidimensional projections of the immunophenotypic data with increased autofluorescence as compared to CD30+ reactive immunoblasts

The putative HRS cell population must meet all of the above criteria

strong CD20 expression (25, 26). An HRS cell population must be identifiable in all four gates (Fig. 10) having increased side scatter as compared to background lymphocytes; expression of CD30 and increased autofluorescence, as assessed using the FITC channel; absence of or decreased expression of CD20; and lastly, strong expression of CD40. Of note, the increased autofluorescence seen in HRS cells is critical to distinguishing these from CD30+ reactive immunoblasts, and frequently, CD40 expression on these cells is increased as compared to background cells in the tissue preparation. If properly gated, HRS cells may appear as two subset populations, one with apparent T-cell antigen expression (such as CD3 or CD5) and the second with decreased (but not absent) CD45 but no expression of T-cell antigens due to the presence and absence, respectively, of T-cell rosetting (see Note 13).

A summary of the immunophenotypic criteria (see also Notes 14 and 15) for identifying a putative HRS cell population is provided in Table 4 (22, 25, 26). See Note 16 for a further caveat regarding the analysis of CHL.

4. Notes

1. Some B-cell populations may demonstrate aberrant loss of surface light chains. Evaluation of cytoplasmic light chains may show monotypic restriction in these cases. Aside from hematogones (normal immature B cells in the marrow and rarely in lymphoid tissues) and plasma cells, absence of surface light chain expression usually is considered aberrant antigen expression (28). Note, however, that some normal B-cell populations may exhibit decreased light chain expression (for example, germinal center B cells (1)), and consequently this finding should be interpreted cautiously.
2. Some large cell B-cell lymphomas may be missed if analyzed using the routine gating strategy due to increased light scatter. In every case, it is important to consider flow cytometry-detected events that fall outside the normal expected size range for typical small lymphoid cells based on light scatter properties. Evaluating events with increased scatter properties is helpful to ensure that complete sampling has been achieved.
3. Some B-cell lymphomas may show absent or decreased expression of CD19 (29–31) and/or CD20 (29) and may therefore be inadvertently excluded from analysis using standard gating strategies. To avoid this pitfall, evaluate more than one B-cell antigen and examine their expression on all lymphocytes gated by light scatter.
4. Not all clonal germinal center B-cell populations are neoplastic (32). This not uncommonly occurs in reactive states, particularly Hashimoto's thyroiditis (33). Correlation with other immunophenotypic abnormalities and histologic studies is required to make a determination of malignancy.
5. Not all neoplastic populations that express CD19 represent B-cell lymphomas. B lymphoblastic leukemia/lymphoma (B-ALL) and, rarely, myeloid neoplasms (namely, myeloid neoplasms with t(8;21) (34)) may show expression of CD19. Additional studies are required to completely evaluate these neoplasms (discussion beyond the scope of this paper).
6. Occasionally, dying cells and debris may appear to express B-cell antigens and appear clonal. While the differentiation of debris and authentic abnormal B-cell populations can be challenging, as a general rule, debris binds antibodies nonspecifically, often resulting in a correlated (diagonal) relationship between antigens in multiple 2-dimensional projections of the immunophenotypic data. The exclusion of low forward scatter events, e.g., a viability gate, may significantly reduce apparent nonspecific binding and simplify analysis. However, neoplasms with a high proliferative rate may show

preferential degeneration with loss of forward scatter, e.g., Burkitt lymphoma, so examination of events prior to exclusion by viability gating is important to detect this occurrence.

7. Small clonal B-cell populations that do not represent B-cell lymphoma are relatively common in normal individuals and have been described in the literature (35, 36). Ultimately, flow cytometric evaluation in the context of clinical findings, peripheral blood counts, and tissue morphology is required for the diagnosis of B-cell lymphoma.
8. As with B cells, debris can mimic an authentic abnormal T-cell population. See Note 6 above for B cells for evaluation of this phenomena.
9. Some large T-cell lymphomas may be missed if analyzed using the routine gating strategy due to increased light scatter. In every case, it is important to consider flow cytometry events that fall outside the normal expected size range for typical small lymphoid cells based on light scatter properties. Evaluating events with increased scatter properties is helpful to ensure that complete sampling has been achieved.
10. Not all clonal and immunophenotypically aberrant T-cell populations represent T-cell lymphomas (2). In some situations, clonal populations may arise as part of the normal process of the adaptive immune system, such as in response to cytomegalovirus (37).
11. Some T-cell lymphomas may show aberrant expression of CD19 or CD20 (38, 39). This should not be inadvertently interpreted as a B-cell neoplasm. Correlation with expression of other T-cell antigens (cytoplasmic CD3, CD7, CD4/CD8) and absence of expression of other B-cell antigens (cytoplasmic CD79a, CD19) is recommended.
12. In the absence of specific analysis for HRS cells, the presence of a characteristic, reactive T-cell population may suggest the diagnosis of CHL in the appropriate clinical and histologic context (40, 41). We have noted that increased expression of CD2, CD5, CD7, and CD45 on the CD4+ T cells, increased CD5 and CD45 on the CD8+ T cells, and decreased expression of CD3 on the CD4 and CD8+ T cells is suggestive of CHL (27). The increased expression of CD45 and CD7 on CD4+ T cells (Fig. 11) may be the most useful reactive T-cell finding to suggest involvement of CHL (27).
13. Putative HRS cell populations should be identifiable using the aforementioned gating strategy. To support this diagnosis, one should attempt to identify the presence of a diagonal relationship on a plot of CD45 versus CD3 or CD5. This diagonal relationship occurs due to the presence of varying numbers of T cells rosetting the HRS cells.

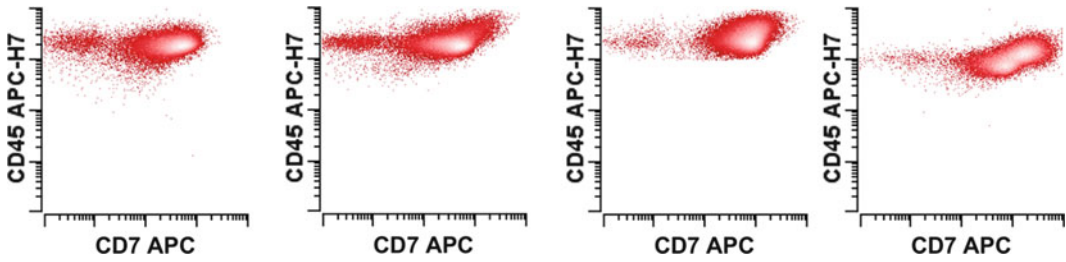


Fig. 11. Reactive T cells in classical Hodgkin lymphoma. CD4+ T cells from CHL demonstrate increased expression of CD45 and CD7 when compared to reactive lymph nodes. 1st dot plot, typical reactive lymph node; 2nd to 4th dot plots, three examples of CHL cases from three different patients.

14. There may be slight variance to the typical HRS cell immunophenotype. For instance, while most HRS cells do not express CD20, low-level expression may occasionally be observed. Further, some HRS cell populations may lack expression of CD15, a finding that correlates with reported immunohistochemical studies (~20% of CHL cases lack expression of CD15 ([42–44](#))).
15. In clinical samples, there may be populations of cells with an apparent immunophenotype that has some resemblance to HRS cells. However, since the immunophenotype does not meet the four basic criteria outlined in Table 4, these events are almost always shown to be a different type of hematologic neoplasm or cellular debris. For example, anaplastic large T-cell lymphoma cells will commonly express CD30 with increased scatter properties. However, its expression of CD40 is usually at a lower level than reactive, background B cells, as also observed in immunohistochemical studies ([45, 46](#)). Diffuse large B-cell lymphoma (DLBCL) may occasionally show expression of CD30; however, the level of expression of CD30 in DLBCL (if present) is relatively lower than that seen for a typical HRS cell population. Further, DLBCL cells will have intermediate to bright expression of CD20 (not dim as compared to HRS cells) with a slight increase in side scatter properties (but not as increased as in CHL). Lastly, nodular lymphocyte predominant Hodgkin lymphoma is a type of Hodgkin lymphoma in which the neoplastic cells, referred to now as lymphocyte predominant (LP) cells, express CD20, CD40, and CD45 without CD30 or CD15 ([47](#)). Although these cells cannot yet be reliably identified by flow cytometry, the expression of CD20 without CD30 would argue against this population from being a putative HRS cell population.
16. It is critical to note that HRS cells may occasionally be identified by flow cytometry in patients with NHL, such as peripheral T-cell lymphoma ([48](#)) and chronic lymphocytic leukemia ([49–51](#)).

In these instances, a concurrent abnormal non-Hodgkin B- or T-cell population can also be identified. Accordingly, the determination of the presence of an HRS cell population should be considered in the context of all the flow cytometry data. Ultimately, the diagnosis of lymphoma requires the integration of all the clinical, morphologic, and immunophenotypic data.

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