Flow Cytometric Evaluation of B-cell Lymphoid Neoplasms

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Evaluation of B-lymphocytes is one of the most well-established clinical applications of flow cytometric immunophenotyping. Using this technique, B-lymphocytes are recognized by expression of lineage-associated antigens; the profile of antigens expressed identifies B-cells at different stages of maturation and belonging to different biologic compartments; neoplastic B-cells can be distinguished from reactive B-cells by demonstration of immunoglobulin light chain restriction or phenotypic aberrancy; and the phenotype of the neoplastic cells can be used to identify one of the distinct disease entities recognized in the World Health Organization classification [1]. However, because of overlapping phenotypes, flow cytometric immunophenotyping is often used in conjunction with other studies as part of a multiparametric approach to the diagnosis of B-cell lymphoid neoplasms [1]. This article addresses general principles of the flow cytometric evaluation of B-cell lymphoid neoplasms, followed by discussion of how flow cytometric data can assist in determining a list of diagnostic possibilities and directing additional testing.

Medical indications for flow cytometric evaluation of B-cells

B-cell lymphoid neoplasms are common malignancies that often involve lymph nodes, bone marrow, and peripheral blood, and may involve extranodal sites and body fluids. The possibility of a B-cell lymphoid neoplasm can be considered in patients presenting with a wide variety of clinical signs, symptoms, and laboratory findings. Flow cytometric evaluation for B-lymphocytes is often of value in the investigation of unexplained lymphocytosis, lymphadenopathy, cytopenias, and effusions. In addition, evaluation of...
plasma cells should be considered if there is monoclonal gammopathy, lytic bone lesions, or hypercalcaemia. Flow cytometric immunophenotyping also supplements morphologic evaluation in the staging of previously diagnosed B-cell lymphoid neoplasms [2–5] and for detection of minimal residual disease following therapy [6].

Paraffin section immunohistochemistry may be used to evaluate many of the same antigens as flow cytometric immunophenotyping, but often provides complimentary information [7]. Paraffin section immunohistochemistry has the advantages of combining morphology and phenotyping and providing some stains that are not currently available by flow cytometry (eg, cyclin-D1). Flow cytometric immunophenotyping offers a more rapid technique that evaluates cell surface as well as intracellular markers and detects many different antigens simultaneously on the same cell. Therefore, flow cytometry is a powerful tool for the detection of surface immunoglobulin light chain restriction and subtle phenotypic aberrancies. However, a negative flow cytometric evaluation may raise questions about whether the sample submitted is representative and if cells have been lost during processing (see section on false negative flow cytometric evaluation).

**Principles of the flow cytometric evaluation of B-cell lymphoid neoplasms**

In the evaluation of clinical specimens for B-cell lymphoid neoplasms, flow cytometric immunophenotyping has several goals: identification of abnormal B-cells though detection of antigen expression that differs from normal B-cells found in that location, characterization of abnormal cells through determination of the antigens expressed and absent (ie, phenotype) development of a list of possible disease entities to be considered, and suggestion of additional diagnostic studies that might be of diagnostic value. These goals are usually met in the clinical laboratory by the application of standard combinations of fluorochrome-labelled antibodies (tubes) that are grouped together into panels. The laboratory constructs tubes and panels that will maximize the sensitivity and specificity of disease detection while minimizing the number of repeated, and therefore nonbillable, antibodies. This is often facilitated by the development of several different panels that address the diseases most frequently found in different specimens or patient types. For example evaluation of bone marrow should include antibodies that can identify normal precursor B-cells; in pediatric specimens, acute lymphoblastic leukemia (ALL) is often a consideration, but chronic lymphocytic leukemia (CLL) would be unlikely, and it might be appropriate to consider hairy cell leukemia (HCL) in peripheral blood and bone marrow specimens but not in all lymph node specimens.

The panels developed in the flow cytometry laboratory often reflect the model of flow cytometry instrument used, including number and types of fluorochromes detected, the specimen and patient mix evaluated by the laboratory (eg, pediatric or adult), whether the institution also evaluates
histology and other tests performed on the specimen, and the preference of those interpreting the results. Table 1 lists some of the antibody combinations used to evaluate B-cell lymphoid neoplasms at the author’s institution.

B-cells are often divided into three broad groups based on their maturity: B-lymphoblasts, mature B-lymphocytes, and plasma cells. These groups correlate with normal cell function and appearance, and each has a neoplastic correlate: acute lymphoblastic leukemia, mature B-cell lymphoid neoplasms, and plasma cell neoplasms. Mature B-lymphocytes can be further divided to cells belonging to different biologic compartments (e.g., follicular germinal center cells). Flow cytometric immunophenotyping of B-cells usually includes evaluation of several different B-cell antigens that have a characteristic pattern of expression at different stages of maturation and in different disease processes.

**B-lineage–associated antigens**

**CD19**

Expression of CD19 is restricted to B-cells and is present throughout B-cell maturation; CD19 appears on lymphoblasts committed to a B-cell lineage and is expressed on all stages of maturation, including normal plasma cells. CD19 is also expressed by most B-cell malignancies, but, unlike normal plasma cells, many plasma cell neoplasms (PCN) are CD19 negative [8]. The intensity of staining for CD19 is often moderately bright and therefore may be used as a gating tool to separate B-cells from other cell types and debris. Alterations in intensity of CD19 staining are seen in some B-cell lymphoid neoplasms, and when present can assist in distinguishing reactive and neoplastic cells (Figs. 1 and 2). For example, the germinal center cells of follicular lymphoma (FL) often demonstrate weaker intensity

<table>
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<tr>
<th>FITC</th>
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<th>Per-CP Cy5.5</th>
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<td>CD138</td>
<td>CD19</td>
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*Abbreviations:* ALL, acute lymphoblastic leukemia; APC, allophycocyanin; CD13&33 is a combination of two antibodies labeled with the same fluorochrome to increase the sensitivity of detection of myeloid antigen expression; CLL, chronic lymphocytic leukemia; Cyto, cytoplasmic staining after permeabilization; FITC, fluorescein, PE; phycoerythrin; Per-CP Cy5.5, peridinin chlorophyll protein Cy5.5 tandem.
staining for CD19 than those of follicular hyperplasia [9]. However, alterations in CD19 intensity are not restricted to, or characteristic of, a particular subtype of lymphoid neoplasm, and occur less frequently than alterations in intensity of CD20 or CD22.

Fig. 1. Chronic lymphocytic leukemia and marginal zone lymphoma (MZL) involving the peripheral blood. Although there is a mixture of kappa and lambda positive cells, further evaluation of CD19 and CD20 intensity of staining identifies two phenotypically abnormal populations of cells, one kappa positive and one lambda positive. CLL indicated in green: (A) CD19+, (C) CD20+ weak intensity, (A) CD5 positive, (B, D) weak intensity lambda light chain restriction, (E) CD23 positive, (F) FMC-7 negative. MZL: (A) CD19+ brighter intensity, (C) CD20+ brighter intensity, (A) CD5 negative, (B, C) surface immunoglobulin kappa light chain restriction, (E) CD23 negative, (F) FMC-7 positive.
CD20

CD20 is expressed by most mature B-cells and is also expressed weakly on a subset of mature T-cells \[10,11\]. B-cell expression of CD20 is more restricted than that of CD19; CD20 is absent from the most immature B-cells and increases in intensity during B-cell maturation. Normal immature

\[\text{Fig. 2. Mantle cell lymphoma. (A) CD19 demonstrates unusually weak staining that could assist in distinguishing from normal B-cells. This CD5 positive phenotype is not characteristic of CLL and requires further investigation: (B) CD20 + moderate intensity, (C) moderate intensity kappa immunoglobulin light chain restriction, (D) CD23 negative, (E) FMC-7 positive. The diagnosis was confirmed by immunohistochemical staining for cyclin-D1.}\]

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B-cells in the bone marrow (hematogones) demonstrate a characteristic maturation pattern with gradual acquisition of CD20 and CD22 in synchrony with loss of CD34 and CD10 (Fig. 3) [12]. In contrast, B-lineage ALL often demonstrates an abnormal pattern of CD20 expression that is out of synchrony with expression of CD34 and CD10 [12,13]. Mature B-lymphocytes demonstrate bright staining for CD20, but normal plasma cells and most PCN are CD20 negative [8]. Therefore, analysis of CD20 expression can assist in determining the stage of maturation of B-lineage cells and recognizing abnormal immature B-cells and plasma cells that deviate from this normal pattern of expression.

Intensity of staining for CD20 varies between subsets of mature B-lymphocytes and often between types of B-cell lymphoid neoplasm. Normal follicle center cells and the cells of FL usually demonstrate CD20 staining that is brighter than other normal B-cells, CLL characteristically has weak intensity staining for CD20 (see Fig. 1), and HCL demonstrates bright staining for CD20 (Fig. 4).

**Fig. 3.** Hematogones. Normal bone marrow B-lineage precursors demonstrating a “maturation pattern” with increasing intensity of staining for CD20 (B, C) and CD22 (D), and transition from CD10 positive and CD38 positive to CD10 negative and CD38 negative (A, B, C).
Documentation of CD20 expression is often required before initiation of therapy targeted at CD20 (eg, Rituxan anti-CD20 monoclonal antibody therapy). Evaluation of surface CD20 expression by flow cytometry is probably a better measure of potential drug binding sites than paraffin section immunohistochemistry that also detects cytoplasmic CD20. Patients who have received anti-CD20 monoclonal antibody therapy may lack B-cell staining for CD20 either due to blocking of detection antibody binding by

Fig. 4. CD10 positive hairy cell leukemia. HCL characteristically demonstrates (A) bright intensity staining for CD20 and CD22, (B) bright intensity CD11c, (C) CD25 positive, (D) CD103 positive, (E) and light chain restriction. CD10 is expressed by approximately 10% of HCL (F).
bound therapeutic antibody or emergence of a CD20 negative population of neoplastic cells [14].

**CD22**

The intensity of staining for CD22 varies during B-cell maturation in synchrony with CD20 and differs between disease processes (eg, characteristically bright intensity in HCL and dim intensity in CLL [see Fig. 4]).

**Immunoglobulin heavy and light chains.** As B-cells mature they initially acquire cytoplasmic immunoglobulin heavy chain \(\mu\) followed by complete surface immunoglobulin composed of both heavy and light chains. Plasma cells lack demonstrable surface immunoglobulin but contain abundant cytoplasmic immunoglobulin. Normal or reactive B-cells usually contain a mixture of cells expressing kappa or lambda. In contrast, B-cell neoplasms are usually composed of a single clone of cells expressing the same light chain (ie, kappa or lambda) [see Fig. 2]. Therefore, evaluation for surface and cytoplasmic immunoglobulin light chains is a key component of the flow cytometric evaluation for B-cell lymphoid neoplasms [see discussion of immunoglobulin light chain restriction and monoclonality]. Although B-cell lymphoid neoplasms differ somewhat in the class of immunoglobulin heavy chain expressed, evaluation of immunoglobulin heavy chains by flow cytometry is usually not necessary for clinical diagnostic purposes.

**CD10**

CD10 is normally expressed by immature B-cells, mature B-lymphocytes from the follicular germinal center, immature T-cells, and neutrophils. CD10 is also characteristically expressed in precursor B-cell ALL, Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), FL [Fig. 5], and rarely in other B-cell lymphoid and plasma cells neoplasms. CD10 expression in FL is often brighter intensity than that of reactive follicular hyperplasia, but weaker intensity than lymphoblastic lymphoma and hematogones [9]. Therefore, anti-CD10 antibodies bound to fluorochromes that give a bright signal (eg, phycoerythrin and APC) should be used in the evaluation for follicular germinal center cells. Although flow cytometric evaluation for CD10 has been reported to be more sensitive than paraffin section immunohistochemistry [15], occasionally FL appears CD10 negative by flow cytometry and positive by paraffin section immunohistochemistry. This discrepancy is probably due to difficulty in manually disaggregating the most brightly CD10 positive lymphoma cells from follicular dendritic meshwork structures and overrepresentation of cells from outside the follicles that have down-regulated CD10 expression [16].

**Distinguishing reactive from neoplastic B-cells**

After B-cells relating to one of the three broad phenotypic stages of B-cell maturation have been identified, their phenotype can be further evaluated
for abnormalities that can help to distinguish normal from abnormal. For all B-cells, this involves evaluation for aberrancies in antigen expression and, in mature B-lymphocytes and plasma cells, evaluation for immunoglobulin light chain restriction.

Kappa and lambda immunoglobulin light chain restriction

In contrast to most normal and reactive populations, neoplasms of mature B-cells usually represent a single clone of cells that express only one light chain (ie, kappa or lambda immunoglobulin light chain restriction) (see Fig. 2). Although often used as a surrogate marker of clonality, light chain restriction has been reported in nonclonal reactive B-cell populations [17]. For example, lambda immunoglobulin light chain restricted nonclonal populations have been identified in tonsillar specimens during childhood [18] and in multicentric Castleman disease [19]. Therefore, it is probably prudent to avoid using the term monoclonal B-cells in a flow cytometric report, and instead use the more accurate term immunoglobulin light chain class restricted B-cells. In addition, some light chain restricted populations...
that are truly clonal are not neoplastic [17]. Clonal populations have been identified in florid follicular hyperplasia especially in patients who are HIV positive [20]. Therefore, light chain restriction does not always represent malignancy, and the results of flow cytometric immunophenotyping should be interpreted in conjunction with other clinical, morphologic, and sometimes genotypic data.

Interpretation of kappa and lambda immunoglobulin light chain staining

A pure population of light chain restricted B-cells is easy to recognize using flow cytometric immunophenotyping and is usually reflected in an abnormal kappa/lambda ratio. However, evaluation of the kappa to lambda ratio will not identify small clonal populations admixed with reactive polyclonal B-cells. A more sensitive approach for the detection of light chain restriction is the separate evaluation of populations of cells that have a distinct phenotype and/or size (see Fig. 1). This approach can be used in the diagnosis of lymphoid neoplasms that have a large number of accompanying reactive cells (eg, marginal zone B-cell lymphoma [MZL]). However, caution should be exercised when evaluating small populations of cells for light chain restriction because reactive B-cells may include small subsets of phenotypically identical cells. Therefore, evaluation for low levels of involvement by B-cell lymphoid neoplasms, such as in the detection of minimal residual disease following therapy, usually involves assessment for abnormal antigen expression rather than the presence of an immunoglobulin class restricted population.

Interpretation of staining for kappa and lambda immunoglobulin light chain can be made more difficult by the presence of nonspecific staining. Nonspecific binding of detection antibodies can occur through association with Fc receptors and adherence of antibody to “sticky” cells, including damaged or dying cells. Binding of antibodies to non-B-cells can be excluded by evaluating only cells that express one or more B-lineage–associated antigen (eg, CD19 or CD20). Nonspecific staining can also be minimized by incubation of cells with a blocking reagent (eg, immune serum) before staining with antilight chain antibodies. Blocking can be employed if nonspecific staining is encountered using conventional staining techniques or routinely in situations whereby nonspecific staining is frequently encountered (eg, HCL).

Some normal B-cell populations lack surface immunoglobulin: lymphoblasts, plasma cells, thymic B-cells. Neoplastic proliferations of these cell populations are also surface immunoglobulin negative: acute lymphoblastic leukemia, PCN, primary mediastinal B-cell lymphoma. Plasma cells can be further evaluated by staining for cytoplasmic immunoglobulin following permeabilization (Fig. 6). In addition, CLL usually demonstrates only low-intensity staining for immunoglobulin probably due to the low density of all components of the membrane B-cell receptor complex; immunoglobulin, CD20, CD22, and CD79a. Other types of lymphoma lack surface
immunoglobulin more sporadically (eg, DLBCL and FL) [21]. Surface immunoglobulin negative FL may derive from the small population of sIg negative B-cells found in normal germinal centers, or might reflect ongoing somatic mutation of the immunoglobulin gene that may produce phenotypically abnormal epitopes no longer recognized by some monoclonal antibodies. Surface immunoglobulin can sometimes be demonstrated in these cases by applying several different monoclonal and polyclonal anti-immunoglobulin light chain antibodies. An alternative approach is to identify other phenotypic abnormalities (eg, bcl-2 staining of CD10 positive B-cells in FL) (see Fig. 5) [22].

Aberrant B-cell phenotypes

In addition to evaluating populations of B-cells for immunoglobulin light chain class restriction, flow cytometric immunophenotyping can be used to identify abnormalities in B-cell antigen expression. This may represent identification of antigens not normally expressed by B-cells (eg, presence of myeloid antigens CD13 or CD33 on B-cells). Aberrant expression of myeloid
antigens is found less frequently in mature B-cell lymphoid neoplasms than ALL and although it has been reported in a many different subtypes, is perhaps found most often in lymphoplasmacytic lymphoma (LPL) [23]. Although CD5 expression on B-cells is often referred to as an aberrant phenotype, there is a small population of normal mature CD5 positive B-cells that is found most often in the peripheral blood but may be seen in lymph node specimens especially in patients who have autoimmune disease [24]. CD5 expression has also been reported in a subset of hematogones [25]. Therefore, interpretation of CD5 expression by B-cells requires evaluation for other abnormalities including immunoglobulin light chain restriction and altered intensity staining for CD20, CD22, and CD79.

Another type of phenotypic aberrancy is abnormal expression of antigens by a subset of B-cells belonging to a biologic compartment (eg, bcl-2 expression on the CD10 positive subset of B-cells). Normal germinal center B-cells and hematogones are usually CD10 positive and bcl-2 negative, whereas bcl-2 is present in most other B-cell subsets. Abnormal bcl-2 expression can be found in most FL (see Fig. 5), some DLBCL, and some B-lineage ALL [22,26]. BL, like normal germinal center B-cells, is usually CD10 positive and bcl-2 negative.

More subtle phenotypic aberrancies include alteration in intensity of staining of B-lineage–associated antigens. For example, FL often demonstrates the following phenotypic aberrancies that can assist in distinction from reactive follicular germinal center cells: decreased intensity of staining for CD19 and brighter intensity CD10 [9].

Significance of small populations of abnormal B-cells

In the staging of patients who have previously characterized lymphoid neoplasms, identification of even a small population of phenotypically abnormal cells can be used to identify involvement by a neoplasm, particularly if the phenotype matches that of the original diagnostic material. However in patients who have no previous diagnosis of a lymphoid malignancy, the significance of a small population (less than 5% of the total cells analyzed) of phenotypically abnormal B-cell is less clear [27]. The best-documented example of this is identification of small clinically insignificant CLL-like populations in peripheral blood and bone marrow specimens from older patients [28]. Small abnormal populations with other phenotypes have been also been reported in peripheral blood and bone marrow specimens and are not necessarily associated with a diagnosable neoplasm [27]. Therefore, if a small population of phenotypically abnormal B-cells is identified in a patient who has no previous diagnosis of a lymphoid neoplasm, it should not be used to establish a new diagnosis of malignancy, but correlated with the morphologic and other findings. However, if the presence of lymphocytosis or an abnormal lymphoid infiltrate cannot be established, further clinical investigation for a lymphoid neoplasm may be warranted.
False-negative flow cytometric evaluation

Occasionally flow cytometric evaluation fails to detect an abnormal population of B-cells in a specimen involved by a B-cell lymphoid neoplasm. There are several possible explanations including sampling error, cell loss during processing, paucity of neoplastic cells, and the presence of cell populations that are difficult to identify.

Allocation of appropriate material for flow cytometric studies is rarely an issue in liquid specimens, but becomes essential for tissue samples because the infiltrate of interest might not involve the entire specimen or might demonstrate variation from area to area. Therefore, fresh tissue should be evaluated, usually by reviewing touch imprints, to determine what is representative. Touch imprints should then be submitted to the laboratory for comparison with the cells present following processing.

The frequency of cell loss during processing for flow cytometric studies varies with the cell type and the processing procedure. Large lymphoid cells and plasma cells appear to be lost easily during processing, particularly following manual disaggregation of tissue specimens. Comparison of smears or touch imprints prepared from the fresh specimen with a cytospin prepared from the specimen stained can help to confirm the presence of the cells of interest.

Some tumors contain few neoplastic cells (eg, the T-cell/histiocyte rich variant of DLBCL) or many admixed reactive B-cells (eg, MZL). Although, it is important to acquire enough events to detect small populations of abnormal cells, most clinical laboratories cannot routinely acquire the numbers required for minimal residual disease detection on all specimens (eg, 500,000 to 1 million events). Therefore, a compromise is often made (eg, 30,000 to 50,000 events) with acknowledgment of the limitations, and recommendation for correlation of flow cytometric results with the morphologic and other findings.

Populations of abnormal B-cells may be present but not recognized on flow cytometric immunophenotypic studies. Examples of populations that are easily overlooked include B-cells that are negative for CD20 (eg, following therapy with Rituxan) [14] and B-cells lacking demonstrable surface immunoglobulin [21]. The following strategies can be used to avoid overlooking elusive populations: characterize all cells present in the specimen, not just those that are CD20 positive; evaluate more than one B-cell–associated antigen; and thoroughly assess all cell populations for phenotypic aberrancies (eg, bcl-2 expression in CD10-positive cells).

Classification of B-cell lymphoid neoplasms

The World Health Organization classification of mature B-cell lymphoid neoplasms separates distinct disease entities that are, for the most part, related to normal stages of B-cell maturation and normal mature B-cell
compartments (eg, precursor B-cell ALL, PCN, FL, mantle cell lymphoma [MCL]) [1]. Although this classification scheme emphasizes a multiparametric approach with use of morphologic, phenotypic, and genotypic data, most of the diseases can be diagnosed with evaluation of morphology and phenotype alone. The World Health Organization blue book for the classification of tumors of the hematopoietic and lymphoid system lists the characteristics of each disease entity, including the typical phenotype [1]. The next section outlines how flow cytometric data can be used to formulate a list of diagnostic possibilities, and how the phenotype can direct further work-up of a B-cell lymphoid neoplasm. PCN and B-cell non-Hodgkin lymphoma and chronic lymphoid leukemia are discussed separately because there are often differences in the clinical presentation that allow selection of an appropriate flow cytometric panel.

B-cell non-Hodgkin lymphoma and chronic lymphoid leukemias

Flow cytometric evaluation of mature B-cell lymphoid neoplasms involves identification of B-cells, recognition of immunoglobulin light chain restriction or other phenotypic abnormalities, and further characterization to identify a phenotype characteristic of a disease entity. B-cell expression of CD5 and CD10 can be used to identify four broad groups of mature B-cell lymphoid neoplasm: CD5(+/−)CD10(-), CD5(-)/CD10(+), CD5(+)/CD10(+), CD5(-)/CD10(-). For each group, additional flow cytometric data in combination with morphology can limit the diagnostic possibilities and direct the use of additional ancillary studies (Table 2).

CD5(+/−) CD10(-)

B-cell lymphoid neoplasms positive for CD5 and negative for CD10 include CLL, prolymphocytic leukemia (PLL), MCL, and less frequently MZL, DLBCL, and LPL. In addition, it should be remembered that a small population of normal B-cells are CD5 positive [24]. Most cases of CLL have a characteristic phenotype that allows distinction from other CD5-positive B-cell lymphoid neoplasms: CD23 positive (often moderate to strong intensity), FMC-7 negative, CD20 weak intensity, CD22 weak intensity, CD79a weak intensity, surface immunoglobulin weak intensity (see Fig. 1) [28]. However, this phenotype is not entirely specific for CLL, and therefore, review of morphology is recommended to help confirm a diagnosis of CLL and exclude other possibilities such as DLBCL and PLL.

Cases with a CD5 positive B-cell phenotype that is not characteristic of CLL are more difficult to identify with certainty [29–32]. Although variant phenotypes have been described in CLL (eg, brighter intensity CD20, brighter intensity surface immunoglobulin, weaker or absent CD23, and presence of FMC-7), additional work-up should be performed on these atypical cases to exclude other CD5 positive B-cell lymphoid neoplasms.
The large cells of DLBCL and PLL can usually be recognized by morphologic evaluation, but the distinction between CD5 positive small B-cell lymphoid neoplasms (eg, MCL, MZL, and LPL) is more difficult.

Although phenotypic features characteristic of MCL have been reported (CD5 positive, CD20 bright intensity, surface immunoglobulin bright intensity, CD23 negative or only weak intensity, FMC-7 positive), they cannot be relied on to make a definitive diagnosis (see Fig. 2). Therefore, any CD5 positive small B-cell lymphoid neoplasm with a phenotype atypical for CLL should be further evaluated for the diagnostic features of MCL (ie, over-expression of cyclin-D1 protein or the translocation t(11;14)(q13;q32) leading to the CCND1 gene rearrangement). Several groups have attempted to develop a flow cytometric assay for cyclin-D1, but most lack sensitivity, and the one more sensitive method that uses an enzymatic amplification step seems to lack specificity in the distinction between CLL and MCL [33]. Therefore, for CD5 positive B-cell lymphoid neoplasms with a phenotype that is not characteristic of CLL, it would be prudent to allocate tissue for fluorescence in-situ hybridization or paraffin section immunohistochemistry.

Phenotypic variants of CLL can also be difficult to distinguish from CD5 positive MZL by flow cytometric immunophenotyping. Although MZL is usually CD5 negative, CD5 positive MZL does occur and has been suggested to more frequently involve the peripheral blood and bone marrow [34]. In contrast to CLL, MZL is usually CD23 negative (see Fig. 1). However, reliable distinction from CLL and other CD5 positive B-cell lymphoid neoplasms often involves morphologic evaluation of lymphoid tissues and paraffin section immunohistochemical staining. On histologic sections, MZL lacks the proliferation centers that are characteristic of CLL/small lymphocytic lymphoma and often demonstrates a distinctive association with, and over-growth of, benign follicular germinal centers. In addition, some MZL demonstrate plasmacytic differentiation as demonstrated by cytoplasmic immunoglobulin light chain restriction. Although plasmacytic differentiation can assist in the distinction of MZL from typical CLL, it also raises the possibility of other subtypes of B-cell lymphoid neoplasm that may demonstrate plasmacytic differentiation (eg, LPL and CLL with plasmacytic or plasmacytoid differentiation). Identification of characteristic genetic abnormalities can assist in the diagnosis of some extranodal MZL [eg, t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21)] involving the same bands by classical cytogenetics as the BCL-2 gene rearrangement but involving the MALT1 gene, and deletion 7q31 in splenic MZL [35]. However, like CLL, many cases of MZL do not have a unique genotype. Therefore, if a definitive distinction cannot be made, a diagnosis of a CD5 positive small B-cell lymphoid neoplasm should be rendered with discussion of the diagnostic possibilities.

Approximately 5% of LPL is reported to be CD5 positive [36,37]. CD23 is usually negative and, when positive, often demonstrates weak, variable,
<table>
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<td>CD5(+), CD10(+)</td>
<td>Chronic lymphocytic leukemia</td>
<td>CD20(d), CD22(d), sIg(d), CD23(+), FMC-7(-)</td>
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<td>Mantle cell lymphoma</td>
<td>CD20(i), slg(i), CD23(-/+), FMC-7(±)</td>
<td>Cyclin-D1 IHC, t(11;14)/CCND gene rearrangement</td>
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<td>Prolymphocytic leukemia</td>
<td>CD20(i), slg(i), FMC-7(±), CD5 (±)</td>
<td>Large cells prominent nucleoli</td>
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<td>Marginal zone B-cell lymphoma</td>
<td>CD23(-), CD11c(±), CD103(-/+)</td>
<td>Exclude blastic MCL</td>
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<td>Sometimes clg</td>
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<td>Growth around and into follicles, maybe</td>
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<td></td>
<td>Lymphoplasmacytic lymphoma</td>
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<td>bcl-2(+)</td>
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<td>Some follicular growth, t(14;18)/BCL-2 gene rearrangement</td>
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<td></td>
<td>Burkitt lymphoma</td>
<td>CD10(b), CD43(+), bcl-2(-)</td>
<td>Uniform intermediate size cells c-MYC gene rearrangement, Ki-67 ~ 100%</td>
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<tr>
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<td>Hairy cell leukemia</td>
<td>CD20(b), CD22(b), CD11c(b), CD25(+), CD103(+), slg(i)</td>
<td>Confirms characteristic morphology</td>
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Additional ancillary studies:
- Confirm characteristic morphology
- Cyclin-D1 IHC, t(11;14)/CCND gene rearrangement
- Large cells prominent nucleoli
- Exclude blastic MCL
- Growth around and into follicles, maybe plasmacytic, t(11;18), t(1;14), t(14;18)/MALT-1 gene rearrangement
- Small cells, subset plasmacytic
- Primarily PB and BM
- Uniform intermediate size cells c-MYC gene rearrangement, Ki-67 ~ 100%
- Confirm characteristic morphology
| CD5(+) | Follicular lymphoma | — | Some follicular growth, t(14;18)/BCL-2 gene rearrangement |
| CD10(+) | — | Diffuse growth, large cells |
| Diffuse large B-cell lymphoma | Mantle cell lymphoma | CD20(i), sIg(i), CD23(-/+), FMC-7(+) | Cyclin-D1 IHC, t(11;14)/CCND gene rearrangement |
| Burkitt lymphoma | CD10(b), CD43(+), bcl-2(-) | Uniform intermediate size cells c-MYC gene rearrangement, Ki-67 ~ 100% |
| CD5(-), CD10(-) | Hairy cell leukemia | CD20(b), CD22(b), CD11c(b), CD25(+), CD103(+), sIg(i) | Confirm characteristic morphology |
| Marginal zone B-cell lymphoma | | | |
| Diffuse large B-cell lymphoma | Follicular lymphoma | CD10(-) | Some follicular growth, t(14;18)/BCL-2 gene rearrangement |
| Mantle cell lymphoma | CD5(-) | CD20(i), sIg(i), CD23(-/+), FMC-7(±) | Cyclin-D1 IHC, t(11;14)/CCND gene rearrangement |

+ = usually positive, ± = often positive, -/+ = maybe positive, - = usually negative.

*Abbreviations:* b, bright intensity; BM, bone marrow; d, dim intensity; i, intermediate intensity; PB, Peripheral blood.
staining. Therefore, LPL usually does not demonstrate a phenotype characteristic of CLL. The distinction of LPL from other CD5 positive small B-cell lymphomas is often more difficult. LPL does not have a characteristic genotype, and therefore, diagnosis often involves recognition of a component of plasma cells with cytoplasmic immunoglobulin light chain restriction and exclusion of MCL by immunohistochemistry or cytogenetic testing. LPL and MZL share many features. Although there is no clear consensus over how to distinguish these two entities, evaluation of the histologic features and distribution of disease may help [36]. LPL is primarily a disease of the peripheral blood and bone marrow.

A subset of DLBCL is CD5 positive. Although some of these cases represent large cell transformation of CLL (Richter transformation) or blastoid variants of MCL, there is a small subset of de-novo CD5 positive DLBCL [38] and extremely rare cases of intravascular large B-cell lymphoma. Although it is uncertain if de-novo CD5 positive DLBCL is a distinct entity, it seems to have some genotypic differences from other DLBCL and may be associated with a worse prognosis [38]. Therefore, after identification of a large cell CD5 positive B-cell lymphoid neoplasm, it is important to recommend additional testing for MCL, consider the possibility of transformation of CLL, and raise the possibility of a de-novo DLBCL.

Prolymphocytic lymphoma may arise through transformation of CLL or de-novo. Although in comparison to CLL, prolymphocytoid transformation is often accompanied by increased intensity CD20 staining, increased intensity surface immunoglobulin, acquisition of FMC-7, and decreased staining for CD5, some cases of PLL demonstrate a phenotype typical for CLL [39]. The phenotype of de-novo PLL is also variable, and therefore, flow cytometric data should not be interpreted in isolation. PLL is usually recognized by its morphologic appearance with large cells containing moderately abundant basophilic cytoplasm and a single prominent central nucleolus, and is often associated with rapid development of a high white blood cells count (often greater than \(100 \times 10^3/\mu L\)), hepatomegaly, and splenomegaly. However, it is important to exclude other large B-cell lymphoid neoplasms, particularly a blastoid variant of MCL [30,40].

\(CD5(-) \ CD10(+)\)

DLBCL and FL (see Fig. 5) represent the most frequent CD10 positive, CD5 negative mature B-cell lymphoid neoplasms, followed by BL. CD10 positive HCL is uncommon but represents approximately 10% of all HCL and can be easily overlooked if the appropriate antibody combinations are not included in an initial flow cytometric screening panel (see Fig. 4) [41]. CD10 expression in other types of lymphoma is unusual with only a few reports of CD10 positive LPL [36], and rare CD10 positive MZL and MCL. CD10 expression is not restricted to B-cells but also occurs
on subsets of mature T-cells and precursor T-cell lymphoblasts and neutrophils.

DLBCL is a heterogeneous category that includes a subset with a CD10 positive germinal center–like phenotype. CD10 positive DLBCL may be difficult to distinguish from BL (see discussion of BL) and FL composed of many large cells (ie, higher-grade FL). When a mature CD10 positive B-cell phenotype is identified by flow cytometry, the possibilities of FL and DLBCL should be further evaluated by morphology. Although on histologic sections the diffuse growth pattern of DLBCL can readily be distinguished from the nodular growth pattern of FL, this distinction is often not possible in fine needle aspirate, body fluid, peripheral blood, and bone marrow specimens. Mean fluorescence intensity of CD71 staining has been reported to help distinguish FL (low intensity) from DLBCL and BL (higher intensity) [42]. However CD71 intensity did not differentiate FL grades. This might be considered a deficiency because FL grade 3 of 3 is often treated in a similar fashion to DLBCL. FL and DLBCL also overlap in their genotype with the translocation t(14;18)(q32;q21) identified in approximately 20% of DLBCL and 70% to 95% of FL.

Accurate identification of BL is essential because it is usually treated with a different therapeutic regimen than other neoplasms composed of mature B-lymphocytes and ALL. Flow cytometric immunophenotyping can provide a useful screen for BL by identifying B-cells that are CD10+, CD5-, bcl-2-, often CD43+, surface immunoglobulin positive, TdT negative, and CD34 negative. However, this phenotype is not specific for BL. A significant subset of CD10 positive DLBCL are bcl-2 negative, rare cases of ALL demonstrate a more mature surface immunoglobulin positive phenotype, and a few cases of BL have a variant bcl-2 positive phenotype. Although, further evaluation for the c-MYC gene rearrangements can assist in the diagnosis of BL, c-MYC rearrangements are also present in approximately 5% of DLBCL. Therefore, the distinction between BL and DLBCL usually requires a multiparametric approach including evaluation of the phenotype, morphologic appearance, and the proliferative index. BL is usually composed of a more uniform population of intermediate size cells with basophilic cytoplasm, often cytoplasmic vacuoles, variable numbers of nucleoli, a Ki-67 proliferative index approaching 100%, and an isolated c-MYC rearrangement. DLBCL is more heterogeneous but is usually composed of more pleomorphic large cells; a lower Ki-67 proliferative index; and variable genotype that may include rearrangements of c-MYC, BCL-2, or BCL-6; and may demonstrate more than one rearrangement. DLBCL with c-MYC and BCL-2 rearrangements is associated with a particularly poor prognosis [43]. ALL usually demonstrates other features of immaturity such as blastic morphology or expression of CD34 and TdT.

Approximately 10% of HCL is CD10 positive but is morphologically and clinically similar to CD10 negative HCL and usually treated in the same way (see Fig. 4) [41]. Therefore, HCL should be considered when a CD10
positive, CD5 negative phenotype is identified by flow cytometric evaluation, especially if there is bright intensity staining for CD20, CD22, and surface immunoglobulin, lack of staining for CD38, and expression of FMC-7. The possibility of CD10 positive HCL can usually be established by demonstration of other phenotypic features characteristic of HCL: CD11c+ bright intensity, CD25 positive, and CD103+ (see Fig. 4).

**CD5(+) CD10(+)**

Mature B-cell lymphoid neoplasms expressing CD5 and CD10 are uncommon [44-47]. This group includes several different subtypes of lymphoma (in order of incidence): DLBCL, FL, MCL, CLL, BL, ALL, and rare individual reports of other mature B-cell malignancies. Morphologic evaluation can assist in the identification of lymphoid neoplasms composed of small cells (FL, MCL, CLL) from those composed of larger cells (DLBCL and BL) and blastic malignancies (ALL). Flow cytometric immunophenotyping provides only limited additional information, and further classification usually requires genotypic studies. Evaluation for CD43 staining can help distinguish FL (characteristically CD43 negative) from CLL and MCL (CD43 positive). BL is also usually CD43 positive but bcl-2 negative, and DLBCL is CD43 and bcl-2 variable. FL, MCL, and CLL are all bcl-2 positive. Although a phenotype that is typical for CLL, in all regards other than CD10 expression, probably represents CLL, evaluation for BCL-2 gene rearrangement by molecular diagnostic or fluorescence in situ hybridization studies may assist in excluding FL. Evaluation for cyclin-D1 staining and/or t(11;14)(q13;q32) translocation are important for consideration of CD10 positive MCL, and c-MYC translocation for CD5 positive BL.

**CD5(-) CD10(-)**

DLBCL includes a phenotypically heterogeneous collection of lymphomas that must be distinguished from FL, other lower-grade lymphoma, MCL, and BL. Phenotyping plays only a limited role in this distinction, which usually requires morphologic and sometimes genotypic evaluation. In addition, information about the clinical presentation is essential for the diagnosis of mediastinal large B-cell lymphoma [48]. Mediastinal large B-cell lymphoma, as recognized in the World Health Organization classification, often has distinctive morphologic features and may have an unusual surface immunoglobulin negative, CD30 positive phenotype, but cannot be reliably distinguished from DLBCL using flow cytometric immunophenotyping [48].

HCL has a distinctive phenotype that permits diagnosis and detection of low levels of disease following therapy: CD20 bright intensity, CD22 bright intensity, CD11c bright intensity, CD25 positive, CD103 positive, sIg intermediate to bright intensity, FMC-7 positive, CD23 negative, CD5 negative,
CD10 negative (see Fig. 4). This phenotype is more sensitive and specific for the diagnosis of HCL than staining for tartrate resistant acid phosphatase. Several additional markers have been reported to have utility in the distinction between HCL and small lymphoid B-cell malignancies (CD123 positive in HCL, CD27 negative in HCL, annexin A1 positive in HCL) [49]. Although, these additional markers are rarely required if material is available for flow cytometric immunophenotyping, they may have utility in paraffin section immunohistochemistry. Occasionally classical HCL deviates from this characteristic phenotype [50,51]. CD10 positive HCL has already been discussed. Other immunophenotypic variations reported include lack of CD103, lack of CD25, and staining for CD23 [51]. These phenotypic variations should be distinguished from HCL variant (HCLv) [50]. The term HCLv has been used to describe cases with an unusual combination of morphologic, clinical, and phenotypic findings. HCLv presents with a higher white blood cell count, lacks accompanying monocytopenia, is composed of cells that resemble those of HCL but demonstrates prominent nucleoli, often lacks staining for tartrate resistant acid phosphatase, and is negative for CD25 but otherwise phenotypically similar to classical HCL. However, the existence of HCLv is debated, and it has been questioned if some of these cases represent splenic MZL [50].

MZL usually has a CD5 negative, CD10 negative phenotype, and is composed predominantly of small cells [52]. A diagnosis of MZL is often established by identification of characteristic morphologic features and exclusion of other small lymphoid B-cell neoplasms (eg, CD10 negative FL, CD5 negative MCL, and HCL). In peripheral blood and bone marrow aspirate specimens, the morphologic features of MZL are often less distinctive than those present in lymphoid tissues and may overlap those of HCL. In particular, circulating villous lymphocytes have been described in splenic MZL involving the peripheral blood. The distinction of MZL and HCL is made more difficult by overlapping phenotypes: MZL is often CD11c positive and may be positive for CD103 [50,51]. However, MZL usually demonstrates weaker more variable staining for CD11c than HCL, lacks the combination of CD103, CD11c, CD25, and lacks bright intensity staining for CD20 and CD22. Although a specific genotype has not been described in HCL, deletion 7q31 has been identified in some cases of SMZL.

LPL is a disease that is often difficult to distinguish from other small lymphoid B-cell neoplasms, in particular MZL [36,37]. It is composed predominantly of small lymphoid cells with varying degrees of plasmacytic differentiation and usually accompanied by a monoclonal serum and/or urine protein. However, the boundaries of this disease, and criteria used to distinguish from other lymphoid neoplasms with plasmacytic differentiation, are not well defined. Therefore, there is some variation in the cases included in this category in the literature. Approximately 60% to 80% of cases have a CD5 negative, CD10 negative, CD23 negative phenotype [36,37]. Many cases express CD11c and CD25, but they are usually CD103 negative [36,37].
Although CD10 negative FL [16,53] and CD5 negative MCL [54] are recognized and would fall in the CD5 negative CD10 negative group, the possibility of CD5 negative CLL is debated [55]. CD5 negative MCL can be recognized by staining for cyclin-D1 or genotypic studies for the t(11;14)(q13;q32). CD10 negative FL can be recognized by genotypic studies for the BCL-2 gene rearrangement. Part of the difficulty in recognizing CD5 negative CLL is lack of a specific genotypic marker for CLL.

**Plasma cell neoplasms and monoclonal gammopathy of undetermined significance**

PCN include several disease entities: plasmacytoma, plasma cell myeloma and variants, plasma cell leukemia, amyloidosis, and immunoglobulin light and heavy chain diseases. Diagnosis of a PCN requires identification of plasma cells; demonstration of an abnormal phenotype; distinction from a lymphoid neoplasm with plasmacytic differentiation; and further classification using a combination of morphologic, laboratory, radiologic and other clinical findings. Flow cytometric immunophenotyping is a useful tool for the identification and characterization of plasma cells and lymphocytes, but is of less value in plasma cells quantitation [6,8]. Even using sensitive techniques, flow cytometric immunophenotyping often identifies fewer plasma cells than paraffin section immunohistochemistry of biopsy sections. This discrepancy seems to reflect a combination of sampling differences, loss of plasma cells during processing for flow cytometric studies, and difficulty encountered in including all plasma cell populations in the flow cytometric analysis. However, although flow cytometric studies may not accurately quantify plasma cells, they have been used successfully in the detection of minimal residual disease following therapy [6].

**Plasma cell associated antigens**

Two antigens are commonly used to identify plasma cells: CD38 and CD138. In addition to plasma cells, CD38 is expressed on a wide variety of cell types: hematogones, some mature B-cells, activated T-cells, and myeloid cells. Although the combination of bright intensity CD38 and lack of staining for CD45 distinguishes plasma cells from most other CD38 positive cells, it is not entirely specific, and its sensitivity is limited by staining of a subset of PCN for CD45. Expression of CD138 is more restricted than that of CD38, being found only on plasma cells and some carcinoma. However, CD138 is not as sensitive as CD38 in the detection of plasma cells, is often negative in neoplastic plasma cells circulating in the peripheral blood, and may be lost with a delay in cell processing. Therefore, the combination of CD138, CD38, and CD45 has been proposed to be a more effective approach to the identification of plasma cells [8]. In addition to staining for CD38 and CD138, PCN are usually CD45 negative, CD20 negative,
CD19 negative, surface immunoglobulin negative, and express cytoplasmic immunoglobin heavy and light chains.

**Phenotypic aberrancies in plasma cell neoplasms**

An abnormal phenotype is identified in most PCN and allows distinction of neoplastic plasma cells from normal plasma cells, and PCN from lymphoma with plasmacytic differentiation (eg, LPL). Normal plasma cells demonstrate a CD19 positive and CD20 negative phenotype. Most PCN demonstrate a CD19 negative and CD20 negative phenotype. In contrast, the vast majority of B-cell lymphoid neoplasms are CD19 positive and CD20 positive. Although a few PCN are CD19 positive, they can be distinguished from B-cell lymphoid neoplasms by absence of staining for CD20. Approximately 10% of PCN express CD20, almost always in the absence of CD19. CD20 expression in myeloma has been associated with a more "lymphoid" appearance, presence of the translocation t(11;14) and cyclin-D1 protein staining by paraffin section immunohistochemistry [8].

Aberrant CD56 expression is identified in many cases of myeloma [8]. CD56 negative myeloma seems to more frequently involve the peripheral blood and may meet the criteria for plasma cell leukemia [56]. Approximately 20% of PCN demonstrate staining for CD117. CD117 is more typically thought of as a marker of immature myeloid cells and mast cells. Therefore, if a plasma cells marker is not included in the analysis, the presence of a population staining for CD117 could be misinterpreted [8]. PCN may also demonstrate expression of myeloid antigens and CD10, both of which have been described rarely in normal plasma cells.

**Monoclonal gammopathy of undetermined significance**

The phenotype of monoclonal gammopathy of undetermined significance (MGUS) overlaps that of PCN. Although by definition MGUS has less than 10% plasma cells in the bone marrow, flow cytometric immunophenotyping cannot reliably distinguish this from a PCN because of the problems encountered in the accurate quantitation of plasma cells. It has been suggested that identification of an admixture of phenotypically normal (CD19 positive, CD56, negative, polytypic cytoplasmic light chain expression) and abnormal plasma cells are more characteristic of MGUS than PCN [57].

**References**


