Flow Cytometry of B-Cell Neoplasms

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INTRODUCTION

The role of flow cytometry in the evaluation of B-cell neoplasia has been well established for several decades. It has expanded, however, from an early focus on classification of leukemic disorders in blood or bone marrow to far broader applications in the identification, classification, prognostication, and follow-up of many different disorders in virtually any body site. Consequently, the literature pertaining to this topic is voluminous and complex; therefore, this review focuses mainly on the general approach to and major issues in the flow cytometric evaluation of this group of disorders.

KEYWORDS

- Flow cytometry
- Immunophenotypic aberrancy
- Light chains/clone
- Chronic lymphoproliferative disorders
- Small B-cell lymphomas
- Aggressive B-cell lymphomas

KEY POINTS

- Light chain restriction or absence of light chain expression is evidence of clonality for most mature B-cell neoplasms; however, assessment of light chains by flow cytometry has technical, specimen source, and disease-specific challenges.
- Most B-cell neoplasms show immunophenotypic aberrancy, when compared with their normal cell counterparts.
- Chronic lymphocytic leukemia/small lymphocytic lymphoma is defined by its immunophenotype: CD5(+), CD10(−), CD20 dim(+), CD23(+), FMC7(−), CD79b(−), CD200(+), surface immunoglobulin(dim+).
- Hairy cell leukemia can be distinguished from its morphologic mimickers with CD25, CD103, and CD123.
- Double-hit lymphomas have a characteristic immunophenotype, showing diminished CD19, CD20, and light chain expression and positivity for CD10 and CD38 (bright).

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GENERAL APPROACH AND TECHNICAL ISSUES

The diagnosis of neoplasia by immunophenotypic methods can be divided into 2 general categories: demonstration of clonality and demonstration of antigenic differences from normal counterparts (aberrancy). The former, accomplished through analysis of surface light chain expression, is most often emphasized in the assessment for mature B-cell malignancies. This approach is not applicable, however, to most B-lymphoblastic processes and the small proportion of mature B-cell malignancies that lack surface light chain expression. In these scenarios, the demonstration of deviations from normal patterns forms the primary basis of flow cytometric diagnosis.

Light Chain Analysis

The classic approach to light chain analysis involves gross gating on lymphocytes or B cells, enumerating the percentage of cells expressing either kappa or lambda, often by quadrant analysis, and calculating a kappa to lambda ratio. If the ratio is within normal limits, a B-cell clone is presumed to be absent. If the ratio exceeds the normal range, a clonal process is assumed to be present. The normal range for kappa to lambda ratios given for gross B-cell populations is variable, but a typical range is 0.5 to 3.0.1–5 From a technical standpoint, a few issues are worth mentioning. First, it is essential that kappa and lambda be assessed in the same tube and visualized in a bivariate plot. Light chains often demonstrate significant nonspecific fluorescence, and bivariate plots of these mutually exclusive antigens allow more precise discrimination of kappa and lambda expressing cells. Second, B cells should be gated as precisely as possible, preferably with more than one pan-B-cell antigen, to avoid noise in the light chain analysis. Finally, the kappa and lambda clusters should be defined as precisely as possible; gross quadrant analysis may not be sufficient. When this more rigorous approach is applied, the normal kappa to lambda ratio in most tissues shrinks to approximately 1.0 to 2.0.3

Even with precise gating on B cells, this approach can lack sensitivity. Clonal B-cell populations may be outnumbered by reactive, polyclonal B cells (in many extranodal marginal zone lymphomas, for example) (Fig. 1), and the kappa to lambda ratio may be normal despite the presence of a clone. Fortunately, lymphoma cells in most cases are not only light chain restricted but also deviate from normal B cells by expression of or intensity of expression of one or more antigens.6–9 These deviations create variably distinct populations in virtual high dimensional space; precise analysis of these populations demonstrates true light chain restriction (as opposed to light chain skew) and, therefore, confident diagnosis of neoplasia (by both clonality and aberrancy) (see Fig. 1). Kappa to lambda ratios become almost beside the point, other than as a trigger to perform more detailed analysis when skewed. Implicit in this concept is the fact that the more antigens assessed per tube (ie, the higher the dimensionality of the data), the easier it will be to discriminate populations. However, sufficiently robust analysis techniques are required for these discriminations; gross gating is not sufficient. Ideally, an iterative approach that maximally uses the multidimensional data available should be applied. One such approach (sometimes termed cluster analysis) has been repeatedly demonstrated to effectively and precisely discriminate population clusters in high dimensional analysis.10

Finally, it should be mentioned that physiologic clones exist. Given that a lymphocyte’s normal response to antigen is clonal expansion, this should not be surprising. Specifically, germinal center populations in lymph nodes with florid follicular hyperplasia may show a skewing of the kappa to lambda ratios or even the appearance of light chain restriction.3,11,12 Key in differentiating physiologic from lymphomatous B-cell
clones in these circumstances is that the physiologic clones will lack immunophenotypic aberrancy, in contrast to their neoplastic counterparts (Fig. 2). Therefore, it can be argued that in the lymphoid realm, aberrancy is a more direct and specific expression of the neoplastic phenotype than clonality.

**Light Chain Negative Lymphomas**

It has been argued that lymphoma cannot be diagnosed without light chain expression. Two basic arguments have been proposed to support this proposition. The first is that demonstration of clonality is necessary to establish a diagnosis of lymphoma. This argument, of course, is clearly not the case, as anyone who has made a histologic diagnosis of lymphoma without the aid of clonality studies (which is the norm) can readily attest. The basis of the histologic diagnosis of lymphoma is morphologic aberrancy that exceeds acceptable limits. By analogy, the immunophenotypic diagnosis of lymphoma is based on aberrancy that exceeds acceptable limits.

A more compelling argument is that lack of light chain expression in B cells is not strictly aberrant. Specifically, normal germinal center cells downregulate surface immunoglobulin. However, when specifically discriminated from nongerminal center cells (for example, based on a combination of CD20 and CD38 or CD10 and CD38), they show a pattern of limited polytypic antigen expression rather than a total absence of surface light chain (see Fig. 2B). Consequently, a complete lack of light chain expression can be used as an indication of a lymphomatous proliferation. Also, of course, germinal center cells will show otherwise normal patterns of antigen expression for that subset (see Fig. 2B). It is also worth noting at this point that lack of light chain expression may represent a technical artifact. Specifically, B cells in serous body fluids often seem to lack light chain expression. This lack of expression can...
be mitigated by technical maneuvers, such as 37°C incubation and/or increasing the amount of light chain reagent (Fig. 3). Finally, it has been demonstrated that some lymphomas will seem to not express light chains with one set of light chain reagents but be clearly restricted with another.15

**Other Analysis Considerations**

As mentioned earlier, gross B-cell gating with determination of kappa/lambda ratios may be insufficient to identify small populations. Cluster analysis with color eventing (arbitrary assignment of colors to simultaneously visualized populations) can help identify subtle findings. When kappa and lambda clusters are assigned different colors, lack of complete overlap of these clusters in other fluorescent plots will usually be evident when an abnormal population is present (see Fig. 1). It is also important to

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**Fig. 2.** (A) This cervical lymph node from a 19-year-old man demonstrates a 13% population of cells with a germinal center immunophenotype; they are CD10(+) but also bright for CD19, CD20, and CD38 when compared with primary follicle/mantle zone cells (blue, 39%). Note, however, that most of this population expresses kappa light chain (kappa to lambda ratio of 14:1). However, histology revealed conventional florid follicular hyperplasia, with no morphologic indication of lymphoma. Such a light chain skew is rarely seen in florid follicular hyperplasia. (B) In this typical case of florid follicular hyperplasia, the germinal center cells (violet) demonstrate the same immunophenotypic characteristics as those illustrated in (A), with the exception of a pattern of partial polytypic light chain expression. (C) Contrast both (A) and (B) with this example of follicular lymphoma in which there was a prominent background polytypic B-cell population. The neoplastic cells (red) constituted 4.8% of events; although they are CD10(+), they differ from normal germinal center cells by underexpression of CD19 and CD38 (in addition to being light chain restricted). Dim CD19 is a common finding in follicular lymphoma.
not rely strictly on scatter gating or CD45/side scatter gating but also to review ungated fluorescence plots to identify populations that scatter in unusual locations, have diminished CD45 expression, or do not form distinct clusters. For example, heavily vacuolated cells may have higher side scatter than expected for lymphocytes and mimic monocytes or granulocytes in scatter plots. These considerations are particularly important for lymphoblastic processes, which frequently do not reside in the CD45/side scatter blast gate, either because of small cell size or diminished or absent CD45 expression.

Finally, a few words regarding the criteria for calling antigens positive or negative is warranted, as this can affect both classification and prognostication. Many practitioners use a threshold of 20% of events beyond a negative control, although this is arbitrary; some investigators have suggested 10% might be more appropriate on precisely gated neoplastic populations. It is certain, however, that the optimal threshold will vary based on choices of antibodies, fluorochromes, instrument settings, and processing protocols; in most situations it is impractical or impossible to validate such thresholds. Therefore, this will likely remain arbitrary and, thus, represent a challenge for harmonization across laboratories. More important, perhaps, than the threshold used is the negative control that is used. Some investigators use internal negative populations (eg, T cells as a negative control for B-cell antigens). This practice is appropriate when the populations being compared have similar physical characteristics (eg, both are small lymphocyte populations) but not when they differ. Large cells, for example, have higher autofluorescence than small cells in some fluorescence channels, necessitating the use of autofluorescence controls (no antibodies), isotype controls, or fluorescence minus one controls, with the same populations gated in the control and the experimental tubes (ensuring an apples-to-apples comparison) (see Fig. 5).

**SMALL B-CELL LYMPHOMAS AND CHRONIC LYMPHOPROLIFERATIVE DISORDERS**

**Classification**

The application of flow cytometry to the classification of neoplasms of small B cells ranges from providing supportive information (eg, in the case of follicular lymphoma [FL]) to delineating immunophenotypes that are essentially definitional (eg, in the cases of chronic lymphocytic leukemia/small lymphocytic lymphoma [CLL/SLL] and...
hairy cell leukemia). The immunophenotypic profiles of the various small B-cell malignancies are provided in Table 1.

**Chronic lymphocytic leukemia/small lymphocytic lymphoma versus mantle cell lymphoma**

The diagnosis of CLL/SLL has, for practical purposes, become an immunophenotypic one, given the lack of defining genetic markers. Although characteristic lymph node histology may substitute for a complete immunophenotype, in blood and bone marrow the disease definition rests on flow cytometry. The defining immunophenotype is CD5(+), CD10(−), CD19(+), CD20(dim+), CD22(dim+), CD23(+), FMC7(−), surface immunoglobulin(dim+) (Fig. 4). In 1994, Matutes and colleagues proposed a scoring system that incorporated 5 markers: CD5, CD22, CD23, FMC7, and slg. A score of 1 was assigned when the proper immunophenotypic pattern was seen and 0 when it was absent; CLL/SLL was diagnosed when the score was 4 or 5. The investigators reported an 87% sensitivity and greater than 99% specificity for the diagnosis of CLL/SLL. The same group later reported that the classification was improved with the addition of CD79b in the scheme or the replacement of CD22 with CD79b (CD79b is negative in most cases of CLL/SLL and positive in most other small B-cell disorders). This modification improved the accuracy of the scoring system from 92% to 97%.

The proportion of CLL/SLL cases that show atypical immunophenotypic features varies across studies. This variation likely relates to both differences in definition

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**Table 1**

Typical immunophenotypic features of small B-cell disorders

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<tr>
<th>Disorder</th>
<th>Typical Immunophenotype (Key Diagnostic Features Italics)</th>
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<tr>
<td>CLL/SLL</td>
<td>CD5(+), CD10(−), CD11c(−/+, CD19(+), CD20(dim+), CD22(dim+), CD23(+), FMC7(−), CD79b(−), CD200(+), surface immunoglobulin(dim+))</td>
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<tr>
<td>Mantle cell lymphoma</td>
<td>CD5(+), CD10(−), CD19(+), CD20(+), CD22(+), CD23(−), FMC7(+), CD79b(+), CD200(−), surface immunoglobulin(+)</td>
</tr>
<tr>
<td>FL</td>
<td>CD5(−), CD10(+), CD19(dim+), CD20(+), CD23(+/-), FMC7(+), CD38(+)</td>
</tr>
<tr>
<td>Lymphoplasmacytic lymphoma</td>
<td>CD5(−), CD10(−), CD11c(+), CD19(+), CD20(+), CD22(+), CD23(+/-), FMC7(+), CD79b(+), CD200(−)</td>
</tr>
<tr>
<td>Extramedullary marginal zone lymphoma</td>
<td>CD5(−), CD10(−), CD11c(−), CD19(+), CD20(+), CD23(−), FMC7(−)</td>
</tr>
<tr>
<td>Nodal marginal zone lymphoma</td>
<td>CD5(−), CD10(−), CD11c(−), CD19(+), CD20(+), CD23(−)</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>CD5(−), CD10(−), CD11b(bright+), CD19(+), CD20(+), CD22(bright+), CD23(−), FMC7(+), CD25(−), CD103(+)</td>
</tr>
<tr>
<td>Splenic marginal zone lymphoma</td>
<td>CD5(−), CD10(−), CD11c(+/−), CD19(+), CD20(+), CD22(bright+), CD23(−), FMC7(+), CD25(−), CD103(+)</td>
</tr>
<tr>
<td>Hairy cell leukemia variant</td>
<td>CD5(−), CD10(−), CD11b(bright+), CD19(+), CD20(+), CD22(bright+), CD23(−), FMC7(+), CD25(−), CD103(+)</td>
</tr>
<tr>
<td>Splenic diffuse red pulp small B-cell lymphoma</td>
<td>CD5(−), CD10(−), CD11c(moderately bright+), CD19(+), CD20(+), CD22(moderately bright+), CD25(−), CD103(−/+)</td>
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(eg, what constitutes dim vs bright slg) as well as the lack of a clear gold standard for the diagnosis of CLL/SLL. Clearly, a minority of CLL/SLL cases can deviate from the typical pattern for each of the antigens described earlier, as detailed in Table 2. Obviously, immunophenotypic variability is problematic for a disease that effectively has an immunophenotypic definition, and it complicates the distinction of CLL/SLL from mantle cell lymphoma (MCL), the other major CD5(+) small B-cell disorder as well as from uncommon CD5(+) variants of other small B-cell disorders that are usually CD5(-).21–29 MCL, although CD5(+) like CLL/SLL, differs immunophenotypically in that it demonstrates strong CD20 and surface immunoglobulin (that is, similar intensity of expression as normal B cells), and is characteristically CD23(-) and FMC7(-).30–34 However, some MCLs may be CD23(+) (though usually of dim intensity) and/or FMC7(-), further complicating this immunophenotypic distinction.35–41 Consequently, investigators have sought additional markers to aid in the discrimination of CLL/SLL from other small B-cell disorders. CD200 has been shown to be
moderately brightly expressed in virtually all cases of CLL/SLL, including cases with an otherwise atypical immunophenotype, whereas it is dim or negative in greater than 95% of MCLs.\textsuperscript{39,42–45} Note that CD200 is not useful to distinguish CLL/SLL from marginal zone lymphomas, which show a wide range of CD200 expression.\textsuperscript{46} Expression of CD11c has been shown to have a high negative predictive value for MCL, although the literature indicates a low sensitivity (27%) for CLL/SLL.\textsuperscript{47} In the authors’ experience, most CLL/SLL cases express partial or variable CD11c (see Fig. 4), whereas MCLs usually completely lack expression of this antigen (Kroft SH, 2017, unpublished data). Finally, it is worth noting that occasional CD5(+) small B-cell disorders are unclassifiable.\textsuperscript{35}

**Hairy cell leukemia versus splenic small B-cell lymphomas**

Hairy cell leukemia (HCL), like CLL/SLL, has been traditionally defined based on immunophenotype. The recent discovery of a highly sensitive and specific genetic marker for this disease, BRAF V600E mutation,\textsuperscript{48–50} has provided a new gold standard for the diagnosis of this entity. Nevertheless, flow cytometric immunophenotyping remains the first diagnostic modality used in this disorder; the specificity of the immunophenotype and other clinicopathologic features arguably obviates proceeding with molecular analysis to make this diagnosis. The importance of making an accurate diagnosis of HCL should also be stressed, given the availability of very specific and highly effective therapy for this disorder.\textsuperscript{51}

The classic immunophenotype of HCL was established in 1993 by Robbins and colleagues\textsuperscript{52}: strong expression of CD19, CD20, and surface immunoglobulin; very bright coexpression of CD11c and CD22; and expression of CD25 and CD103 (Fig. 5). More recently it has been recognized that CD123 expression and bright CD200 are also characteristic features of HCL.\textsuperscript{42,53–55} Additionally, HCL is usually CD23(−) and FMC7(+).\textsuperscript{56} This immunophenotype describes most HCL cases. However, deviations from this classic pattern have been described. Chen and colleagues\textsuperscript{57} described a lack of CD25 in 3% of cases, a lack of CD103 in 6%, and expression of CD23 in 17%. Notably, no individual case showed more than one of these uncommon features.

Most cases of HCL are CD5(−) and CD10(−). However, CD10 is expressed in 10% to 20% of HCL cases; these do not seem to differ in other respects from CD10(−) cases.\textsuperscript{58} Rare cases of CD5(+) HCL exist, but too few have been reported to assess whether they differ clinically from those with a classic immunophenotype.\textsuperscript{27,52,59,60}

The main differential diagnoses with HCL, both clinically and pathologically, are the group of splenic small B-cell lymphomas, which includes splenic marginal zone lymphoma (SMZL), HCL variant (HCV), and splenic diffuse red pulp small B-cell lymphoma (SDRPL). The first is a moderately well-characterized clinicopathologic entity, whereas

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<th>Classic Antigen Pattern</th>
<th>Percentage of Cases Deviating</th>
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<tr>
<td>CD20 dim-positive</td>
<td>11%–38%\textsuperscript{20,174–177}</td>
</tr>
<tr>
<td>CD22 dim-positive</td>
<td>0%–8%\textsuperscript{7,8,20,175}</td>
</tr>
<tr>
<td>CD23 positive</td>
<td>3%–5%\textsuperscript{20,175}</td>
</tr>
<tr>
<td>FMC7 negative</td>
<td>7%–14%\textsuperscript{20,174,177}</td>
</tr>
<tr>
<td>CD79b negative</td>
<td>5%–18%\textsuperscript{20,177,178}</td>
</tr>
<tr>
<td>Surface immunoglobulin dim-positive</td>
<td>5%–42%\textsuperscript{20,175,176}</td>
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the other two remain provisional entities in the World Health Organization’s (WHO) classification. All of these typically CD5(-)/CD10(-) disorders show some immunophenotypic features suggestive of HCL, and all need to be distinguished from HCL because of poor response to agents that are highly active in that disorder. Whether they need to be (and, in fact, reliably can be) distinguished from one another is unclear now, and it is difficult to pin down their immunophenotypes precisely because of overlapping clinicopathologic features and lack of good gold standards for diagnosis. Nevertheless, some generalizations can be made.

The closest immunophenotypic mimic of HCL is HCV. This entity characteristically shows bright coexpression of CD11c and CD22 and expression of CD103 (Fig. 6). However, the lack of CD25 and CD123 will distinguish it from HCL. SMZLs typically coexpress CD11c and CD22 but do not show bright coexpression of these markers. CD25 is expressed in a minority of cases, and both CD103 and CD123 will be negative. SDRPL is the least well characterized of all of these entities and has been reported to have heterogeneous immunophenotypic features. Although CD11c and CD22 may be brightly coexpressed, they tend to be less bright than in HCL. Although CD103 is expressed in approximately 40%, and CD123 dimly expressed in 16%, CD25 is negative in most reported cases.

**Other classification issues**

Expression of CD10 is an immunophenotypic hallmark of FL (see Fig. 2). However, roughly 10% of low-grade FLs lack expression of this antigen by flow cytometry. Conversely, other low-grade B-cell processes that are characteristically CD10(-) may occasionally be CD10(+). As mentioned earlier, this is a feature of 10% of HCLs; but rare cases of CLL/SLL, MCL, and lymphoplasmacytic lymphoma may express CD10.
Just as CD10 defines FL, CD5 is a defining feature of CLL/SLL and MCL. However, roughly 10% of MCLs lack CD5.\textsuperscript{41,73} CD5(CD\textsubscript{0}) CLL/SLL probably exists,\textsuperscript{74} but establishing this diagnosis is extremely difficult.

Finally, it is worth mentioning that immunophenotypic variation in lymphomas may be seen across different tissue sites and over time in individual patients, although these are uncommonly of sufficient magnitude to affect classification.\textsuperscript{40,75–77}

**Prognostic Markers**

*Chronic lymphocytic leukemia/small lymphocytic lymphoma*

By far the most work on the impact of immunophenotypic features on prognosis has been performed in CLL/SLL. In the wake of the discovery that lack of somatic hypermutation in Ig VH genes was associated with poor outcomes in CLL/SLL,\textsuperscript{78,79} it became desirable to identify an easily measurable surrogate laboratory test for mutational status. CD38 was immediately identified as a candidate, with expression associated with an unmutated status.\textsuperscript{79} Additional investigation clearly identified CD38 expression as an adverse prognostic indicator in CLL/SLL, although it has turned out not to be a useful surrogate for Ig VH mutation.\textsuperscript{80–84} Furthermore, because expression of CD38 often does not show an all-or-none pattern of expression, thresholds for the percentage of cells expressing CD38 beyond a control must be established. Unfortunately, optimal thresholds vary based on the technical differences, such as processing protocols, reagent choices, and instrument settings; thus, different investigators have used different values.\textsuperscript{80,83–91} This difference essentially means that thresholds cannot be blindly adopted from the literature for clinical use. Yet, few laboratories have the ability to establish their own thresholds based on outcome data. Finally, concern has been expressed that CD38 levels can change during the

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**Fig. 6.** HCL variant. This CD5(CD\textsubscript{0})/CD10(CD\textsubscript{0}) leukemia (red) demonstrates bright CD11c and CD22 and is predominantly positive for CD103. However, it is CD25(CD\textsubscript{0}). Also, compare the light scatter properties with the HCL illustrated in Fig. 5. Normal B cells are illustrated in blue.
course of the disease, although this has not been a consistent finding. It also has been shown that the level of CD38 expression depends on the tumor microenvironment.

After identification of the differential expression of the gene encoding the T-cell signaling protein ZAP-70 between mutated and unmutated CLL/SLL (expression being associated with lack of somatic mutation), investigators began assessing this protein by flow cytometry and suggested that it predicted mutational status in most cases. As in the case of CD38, ZAP-70 was found to be a less useful surrogate for mutational status than originally hoped (~75% concordance); but it is clearly a potent prognostic factor. In fact, there is some evidence that it is a stronger predictor of outcome than mutational status. However, as in the case of CD38, the continuous expression patterns of ZAP-70 in CLL/SLL require the establishment of thresholds; various approaches to establishing these have been proposed. As with CD38 though, thresholds have proven to be extremely different to standardize across laboratories. The utility of ZAP-70 expression by flow cytometry as a clinical laboratory test (as opposed to a research test) is further compromised by the lability of this protein; expression seems to depend on the type of specimen handling and the time to processing. Ultimately, ZAP-70 by flow cytometry seems to lack sufficient robustness to be suitable as a routine clinical test; consequently, many clinical laboratories have discontinued testing for this biomarker.

Most recently, expression of CD49d has been investigated and found to be a strong, independent indicator of more aggressive disease. Importantly, this marker seems to be analytically stable, and stable over time in individual patients; in most cases expression is either bright or absent, presumably allowing for a more robust, reproducible clinical assay.

Finally, it should be noted that these 3 immunophenotypic markers show correlations with one another but have been demonstrated to provide independent prognostic information. Therefore, approaches using combinations of the markers have been proposed.

Other small B-cell processes
Little information is available on the impact of immunophenotype on the clinical course in other small B-cell neoplasms. Kelemen and colleagues reported that CD23 expression was associated with better outcomes in MCL. Similarly, CD23 expression in FL has been linked to better outcomes. Recently, CD38 has been reported to be a poor prognostic marker in HCL.

Minimal Residual Disease
Flow cytometry has emerged as an effective means of monitoring minimal residual disease (MRD) following therapy in CLL/SLL, MCL, and HCL and should be applicable to other small B-cell disorders, such as FL. The details of the approach to MRD analysis and clinical utility in these disorders is beyond the scope of this review.

AGGRESSIVE MATURE B-CELL NEOPLASMS
Flow cytometry plays a smaller role in aggressive B-cell non-Hodgkin lymphoma than in small B-cell neoplasms. However, flow cytometry is used as a first-line diagnostic modality in many centers; productive flow cytometry analyses may eliminate the need for subsequent immunohistochemistry in many cases. Flow cytometry is particularly useful in the case of fine-needle aspirations, in which material may
not be available for immunohistochemical evaluation. Finally, as described later, immunophenotypic features detected by flow cytometry may be of value in prognostication or prediction of genetic lesions.

**Diffuse Large B-Cell Lymphoma**

Although it commonly stated that DLBCL is difficult to diagnose by flow cytometry because of losses in processing, in the authors’ experience, most DLBCLs can be identified by flow cytometry, independent of the site of involvement. DLBCLs most commonly show expression of CD19, CD20, CD22, and bright CD45. In one report, diminished CD20 expression by flow cytometry was described in roughly 15% of all DLBCLs and was notably discordant with strong positivity by immunohistochemistry in this subset. Further, this decreased CD20 expression was associated with a poorer prognosis, though cytogenetic and molecular factors were not studied in this analysis. Most DLBCLs show monotypic light chain expression by flow cytometry; however, nearly a quarter of cases lack both kappa and lambda surface staining. CD23 is expressed in approximately one-third of DLBCL and FMC7 in two-thirds (unpublished observations). CD200 is observed in 22% of cases.

Immunophenotypic subsets of DLBCL have prognostic and therapeutic relevance. CD10 expression may be observed in 30% to 60% of DLBCLs by flow cytometry and/or immunohistochemistry, with expression of this marker establishing germinal center origin per the Han criteria. Of note, the Han criteria are based on immunohistochemical assessment; flow cytometry may be more sensitive for the detection of CD10 than immunohistochemistry in DLBCL. CD5 expression is present in approximately 5% to 10% of DLBCLs and may indicate either de novo CD5(+) DLBCL or a Richter transformation of CLL/SLL. The de novo subset has been shown to have a poorer outcome compared with CD5(-) DLBCLs. Immunophenotypically, the large cell lymphomas arising out of CLL/SLL have similar antigen expression patterns to the CLL/SLL.

**Burkitt Lymphoma**

Burkitt lymphoma (BL) has the following immunophenotype by flow cytometry: CD5(-), CD10(+), CD19(+), CD20(+), CD38 bright(+), surface light chain(+), and terminal deoxynucleotidyl transferase (TdT)(-). Several groups have described patterns of antigen expression helpful in distinguishing BL from CD10(+). Of note, bright CD38 expression on CD10(+) B-cell neoplasms, at a similar intensity to plasma cells, is predictive for MYC rearrangements generally, with enhanced specificity when there is expression of FMC7 in the absence of CD23.

**High-Grade B-Cell Lymphoma with MYC and BCL2 and/or BCL6 Rearrangements (Double/Triple-Hit Lymphomas)**

In the WHO’s 2016 classification of tumors of hematopoietic and lymphoid tissues update, large B-cell lymphomas with MYC and BCL2 and/or BCL6 rearrangements and a mature immunophenotype will all be classified in one category, independent of morphology. Approximately 5% to 10% of DLBCLs are double-hit lymphomas (DHLs) with MYC and BCL2/BCL6 rearrangements. These high-grade neoplasms have a poorer prognosis compared with DLBCLs without such rearrangements; therefore, their identification is critical to clinical management. Multiple studies have
examined clinical, morphologic, genetic, and immunophenotypic features of these lymphomas in an effort to identify characteristics predictive of the presence of these rearrangements and, therefore, optimize cytogenetic testing for maximum cost-effectiveness.146,147

Flow cytometry can play a role in predicting the double or triple hits. Most DHLs are CD10(+) and, therefore, of germinal center origin. They have the following characteristic immunophenotype, as compared with normal resting or germinal center B cells, as confirmed across multiple studies: diminished CD19 (30%–67%), diminished CD20 (36%–70%), diminished CD19 and CD20 (28%–56%), and decreased CD45 (30%–45%) expression (Fig. 8).148–150 Surface light chain is also dim or absent in 47% to 67% of DHLs.148,150 Finally, bright CD38 expression is also characteristic.139,148

**B-LYMPHOBLASTIC LEUKEMIA/LYMPHOMA**

The diagnosis of B-lymphoblastic leukemia/lymphoma (B-ALL) is established by blast immunophenotyping via flow cytometry or immunohistochemistry, with flow cytometry being the preferred modality given its increased sensitivity. A thorough, diagnostic immunophenotype by flow cytometry is recommended for assigning B-lineage specificity, excluding mixed phenotype leukemias, predicting cytogenetic or molecular abnormalities, and monitoring disease with MRD assessment. Recommended antibodies at diagnosis include cytoplasmic TdT, myeloperoxidase, CD22, IgM, and CD79a; surface CD10, CD19, CD20, CD22, CD34, CD38, CD45, kappa, and lambda; myeloid antigens, such as CD11b, CD13, CD14, CD15, CD33, CD64, and CD65; and T-cell antigens.151,152

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**Fig. 7.** BL in a tissue biopsy. The BL cells (red) are larger in size (by forward scatter) than mature B cells (blue) and are CD5(−), CD10(+), CD19(+), CD20 bright(+), CD23(−), FMC7(+), CD38 bright(+), and kappa light chain restricted. Bright CD38 expression has been associated with MYC rearrangements.
B-ALLs have a characteristic immunophenotype, showing expression of CD19, HLA-DR and cytoplasmic CD22, CD79a, and TdT in nearly all cases. Most B-ALLs also express CD10 (75%–90%), CD38 (85%), and CD34 (80%). When compared with their normal counterpart, hematogones, leukemic B-lymphoblasts show immunophenotypic aberrancy in nearly all cases (Fig. 9). Such aberrancies can manifest as asynchronous antigen expression (such as coexpression of CD34 and CD20), lineage infidelity (such as expression of myeloid or T-cell antigens), or underexpression/overexpression of normally expressed antigens (see discussion of the immunophenotype of hematogones, the normal counterpart of B-ALL, later). Among the most frequent aberrancies are uniform or continuous expression of TdT or CD34 (73%–93%), overexpression of CD10 (61%–86%), and underexpression of CD38 and CD45 (52%–80%). Aberrant myeloid antigen expression may be observed in up to 86% of B-ALLs when multiple antigens are tested. Light chain expression is rarely observed. Notably, it is well documented that B-ALL blast immunophenotypes can change to varying degrees over time and/or as a result of therapy (30%–78% of cases).

The major immunophenotypic differential diagnosis of B-ALL in bone marrow is hematogone hyperplasia. Hematogones can be recognized by their highly reproducible maturation pattern toward mature B cells (see Fig. 9; Fig. 10). The most immature forms are CD10(bright+), CD19(+), CD20(−), CD22(moderately+), CD38(moderately bright+), CD45(dim+), TdT(+), CD123(−), and CD34(+). As they mature, hematogones slightly downregulate CD10, slightly upregulate CD19 and CD38, upregulate CD45 and CD123 expression, lose CD34 and TdT, and progressively gain CD20. Acquisition of polytypic surface immunoglobulin is seen during hematogone maturation; interestingly, this begins before CD20 is acquired. Deviations

Fig. 8. DHL in bone marrow. DHL cells (red) show enlarged size by forward scatter, positivity for CD10, bright expression of CD38, and frequent underexpression of CD19, CD20, and light chain. Normal B cells are illustrated in blue.
from this maturation pattern represent immunophenotypic aberrancy and are evidence for a neoplastic proliferation of lymphoblasts. Other markers that have been shown to have characteristic expression patterns in hematogones and, thus, potential utility in distinguishing B-ALL from hematogones are CD58, CD81, CD123, and BCL-2.\textsuperscript{160–163}

Hematogones may be arbitrarily divided into 3 maturational stages.\textsuperscript{159} Stage 1 comprises the CD20(–), CD34(+), TdT(+) subset, stage 3 the most mature subset, with CD20 expression at or exceeding that seen in mature B cells, and stage 2 everything in between stages 1 and 3. Normally, stage 2 hematogones predominate, averaging roughly two-thirds of the hematogone population, with the remaining one-third equally divided between stages 1 and 3.\textsuperscript{153} However, both right shifts and left shifts may be seen in hematogone maturation in reactive states. However, the qualitative maturation pattern will remain unchanged.

The concomitant presence of hematogones and neoplastic blasts is unusual at B-ALL diagnosis but may be occasionally seen at the time of recurrence (see Fig. 9). However, these populations should be easily distinguished based on the invariant maturation pattern of hematogones coupled with the various aberrancies seen in B-ALL. Follow-up analyses for residual B-ALL require an antibody panel designed to interrogate the most common B-ALL aberrancies relative to hematogones. A detailed discussion of minimal residual disease analysis in B-ALL is presented in Aaron C. Shaver and Adam C. Seegmiller’s article “B Lymphoblastic Leukemia Minimal Residual Disease Assessment by Flow Cytometric Analysis,” in this issue.

**Fig. 9.** Recurrent B-ALL with hematogones. The neoplastic B-lymphoblasts (red) show aberrant bright CD10 and CD34 expression with dim CD38 and CD45 expression, as compared with the hematogones (green), which show a normal, reproducible maturation pattern (also see Fig. 10). CD13 is additionally expressed aberrantly by the neoplastic B-lymphoblasts. Mature B cells are illustrated in blue.
Some B-ALL immunophenotypes are predictive of recurring cytogenetic and molecular abnormalities and, thus, prognosis. In the good cytogenetics category, blasts usually express CD10, CD22, CD34, and CD123 in hyperdiploidy states and CD10(bright), CD27, CD34, and CD13 and/or CD33 in t(12;21) B-ALLs. BM Blasts with MLL rearrangements show expression of NG2 (highly specific), CD15, CD34, and TdT and have diminished CD10 and CD24 (Fig. 11). Most Philadelphia chromosome(+) B-ALLs express bright CD10, CD25, TdT and CD34 and show CD13 and/or CD33 expression. Finally, B-ALLs with the t(1;19) characteristically show negativity for both CD10 and CD34 in the blasts.
Fig. 11. B-ALL with MLL gene rearrangement. The neoplastic B-lymphoblasts (red) show multiple aberrancies, including bright, uniform CD34 and CD22 expression, lack of CD10, partial CD20 expression, and CD15 positivity. The lack of CD10 and expression of CD34 and CD15 is characteristic MLL-rearranged B-ALL. Mature B cells are illustrated in blue.

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