V. Acute leukemia

Evaluating a sample for an acute leukemia

Acute leukemia is a neoplasm of immature myeloid or lymphoid cells characterized by a block in maturation, usually at the stage of an early progenitor (blast), with an associated increase in proliferation leading to an expansion of immature cells. Immature lymphoid cells or lymphoblasts are expanded in acute lymphoblastic leukemia (ALL), while immature myeloid cells, including myeloid progenitors, abnormal promyelocytes, monoblasts, or promonocytes, are expanded in acute myeloid leukemia (AML). In most cases, a diagnosis of AML requires a morphologic blast percentage of 20% or greater in the blood or marrow. For ALL, a similar cutoff is preferred but not required for diagnosis. ALL, more commonly T-cell ALL, may primarily involve tissues, and when this is the primary manifestation, this process is called lymphoblastic lymphoma. AML may involve the tissues as well and when present is designated myeloid sarcoma.

By flow cytometry, acute leukemia is typically first recognized on a CD45 versus side scatter (SSC) plot as an expansion of cells with low CD45 and intermediate SSC. Lymphoid blasts may have a slightly lower SSC in comparison with the slightly higher SSC commonly seen on myeloid blasts or the increased SSC characterizing APL.

Figure V-1: CD45 versus side scatter for ALL, AML, and APL. (A) Cells falling into the CD45 versus side scatter (SSC) defined blast gate (black circle) comprise less than 5% of the white blood cells by flow cytometry in a normal resting marrow. Acute leukemias are often first recognized as an expansion of cells occupying the CD45 versus SSC defined blast gate. (B) Acute myeloid leukemia (AML) typically shows intermediate SSC with decreased CD45, and (C) acute lymphoblastic leukemia (ALL) typically shows low SSC with decreased CD45. It should be noted that not all blast equivalents occupy the blast gate. (D) For instance, the abnormal blast equivalent seen in acute promyelocytic leukemia (APL) is an abnormal promyelocyte that typically has a very high SSC, approximating that of maturing myeloid cells.
teristic of abnormal promyelocytes, which may approximate that of granulocytes (Figure V-1). Other cell types, including basophils, plasma-cytoid dendritic cells, and hypogranular neutrophils, may also fall into the “blast gate” as defined by CD45 versus SSC; therefore, more specific markers are required for definitive blast identification. Such markers should enable one to confirm immaturity of the population of interest, define lineage, and define the population of interest as immunophenotypically aberrant and different from normal progenitor cells, including normal myeloid progenitors or B-cell precursors (hematogones) present in the marrow or T-cell precursors (thymocytes) present in the thymus. For myeloid blast equivalents, a combination of CD34 and CD117 is often employed to define immaturity, while antigens including CD5, CD7, CD11b, CD11c, CD13, CD14, CD15, CD16, CD33, CD38, CD56, CD64, CD65, HLA-DR, and MPO may be used to define lineage and demonstrate aberrancies in antigen expression. Markers including CD10, CD19, CD20, CD34, CD38, immunoglobulin light chains, and TdT achieve these goals for B cells, and markers including CD1a, CD2, CD3 (surface and cytoplasmic), CD4, CD5, CD7, CD8, CD10, CD34, and TdT may be employed in evaluating T cells. The assignment of lineage is a principal component of the diagnosis of acute leukemia. A relatively small number of antigens are sufficiently lineage associated to allow definitive lineage assignment when present and are discussed in detail in conjunction with Case 35.

Flow cytometry is also useful in enumerating blast populations, particularly in the blood. However, blast enumeration in bone marrow is complicated by two principal factors: hemodilution and under-representation of nucleated red blood cells. The former is invariably present in marrow specimens and increases in amount with increasing volume of marrow aspirate and decreased aspirability of the marrow, eg, fibrosis. The latter is dependent on the method of processing and the instrument detection threshold used for specimen acquisition. In addition, it is important to recognize that blasts are defined by morphology and that the immunophenotypic equivalent is the sum of populations as diverse as stem cells, CD34+ progenitors, B-cell progenitors, promyelocytes, monoblasts, and promonocytes. For these reasons, morphology, rather than flow cytometry, should be used for blast enumeration in the marrow when historic or World Health Organization criteria are applied.

Cases 25 through 36 highlight several examples of acute leukemias and related disorders.

References
Case 1

Case presentation

Clinical history
A 56-year-old man presents to his primary care provider with a painless lump in his left inguinal area that he first noticed 3 months ago. The lump appears to have increased slightly in size since that time. The patient has no other findings on review of systems and has no significant past medical history. Physical exam is notable only for the presence of left inguinal adenopathy. Concurrent laboratory findings are unremarkable. A lymph node biopsy is performed and sent for flow cytometry and morphologic evaluation.

Flow cytometric data (Figure 1-1)

Figure 1-1: Flow cytometry of lymph node biopsy. (A) A dot plot of all viable cells demonstrating CD45 versus side scatter (SSC) shows a dominant population of lymphocytes that comprise the bulk of the specimen. (B) These lymphocytes are composed of an admixture of CD5+ T cells (green) and CD19+ B cells. (C) The B cells show an increased kappa-to-lambda ratio due to an expanded population of kappa restricted B cells that express CD10 with a high level of CD20 (D) and without CD5 expression. A gate is drawn around this abnormal population, which colors the population magenta (E) and defines the abnormal cells as expressing intermediate level kappa light chain (F). The flow cytometric findings, in conjunction with concurrent lymph node morphology, were diagnostic of a low-grade follicular lymphoma.
Diagnosis
Follicular lymphoma.

Discussion
Follicular lymphoma (FL) is a B-cell lymphoma consisting of germinal center B cells that accounts for approximately 20% of all lymphomas. FL is predominantly a disease of adults, with a median age of onset in the sixth decade. Diagnosis has historically rested on the demonstration of neoplastic follicles by morphology. Although morphology is essential to confirm architecture and determine cell size, flow cytometry has made many contributions in defining and characterizing the neoplastic population, and in distinguishing FL from both reactive processes and other B-cell lymphomas.

Follicular lymphoma usually consists of a proliferation of clonal CD19+ and CD20+ B cells that coexpress CD10. The expression of CD19 is often decreased in FL as compared to normal B cells and follicular hyperplasia. Light chain restriction is the norm; however, in occasional cases, light chain expression may be downregulated or even absent. In such cases, evaluation of cytoplasmic light chain expression or evaluation for BCL2 overexpression on the population of interest may be helpful in establishing the diagnosis (see Figure 1-2). Similarly, CD10, although positive in the majority of cases, may be negative in a minor subset of cases. In the absence of CD10 expression, morphology generally brings FL into the differential diagnosis. In such cases, demonstration of BCL6 expression by immunohistochemistry may be useful in establishing the germinal center cell origin of the neoplastic population, and FISH for a BCL2 or BCL6 translocation may

Figure 1-2: Follicular lymphoma with absent surface light chain expression. (A) Kappa and lambda staining on the B cells shows a B-cell population with absent surface light chain expression (magenta). (B) This abnormal population expresses CD10 and variable CD20. The size of the population (70% of the white blood cells), absence of CD38 expression (C), and low level of CD19 expression (D, E) are all highly suggestive of follicular lymphoma, with the demonstration of high-level BCL2 expression (F) confirming the diagnosis.
be considered to confirm the diagnosis. FL is typically negative for CD5; however, rare CD5+ cases have been reported, with one study suggesting that this immunophenotype may be more frequently seen in the morphologic “floral” variant. The majority of FL overexpress BCL2 as a result of the t(14;18)(q32;q21) IGH/BCL2 translocation. BCL2 overexpression can be identified by immunohistochemistry or flow cytometry and is seen in approximately 85% to 90% of low-grade FL (grade 1 to 2) but is less common (approximately 50%) in intermediate-grade (grade 3) FL.

The differential diagnosis in FL includes reactive follicular hyperplasia and B-cell lymphoma of a different subtype.

In reactive follicular hyperplasia, reactive germinal center B cells are relatively expanded and may be identified as a CD10+ B-cell population detectable by flow cytometry. In contrast to FL, these populations typically show polyclonal light chain expression and increased levels of CD38 expression. In some cases, reactive follicle center cells may show down-regulation of light chain expression, giving the impression of light chain negativity. Reactive follicle center populations may even show light chain restriction, complicating distinction from FL. In some cases, demonstration of an additional antigenic abnormality such as decreased CD19 or CD38 (both more common in FL) may help in distinguishing a reactive follicle center cell population from FL (Figure 1-3). Of note, CD20 levels are reported to be slightly lower in FL as compared to reactive follicular hyperplasia. Additionally, demonstration of BCL2 overexpression by flow cytometry can provide more definitive evidence...
of the neoplastic nature of a germinal center B-cell population. In equivocal cases, correlation with morphology is generally helpful in distinguishing these diagnostic considerations.

Table II-1 from chapter II, *B-cell neoplasms*, highlights immunophenotypic patterns that may help in distinguishing FL from other B-cell lymphomas composed of small to intermediate sized B cells. In addition to follicular lymphoma, CD10+ B cells may also characterize diffuse large B-cell lymphoma (DLBCL) and are typical in Burkitt lymphoma (BL). In some cases, higher forward scatter (FSC) may alert one to the diagnosis of DLBCL; however, there is overlap between the FSC characteristics of cells comprising DLBCL and FL. This overlap in FSC occurs not only because FL cells (of any grade) often have a higher FSC than reactive lymphocytes, but also because FSC of DLBCL cells may not always provide a reliable indicator of cell size. Correlation with morphology is required to definitively distinguish between FL and DLBCL. In contrast to BL, FL typically has a low level of expression of CD38 and is BCL2 positive in most cases. Further, both DLBCL and BL are more likely than FL to express high levels of CD71. Despite these differences, again, to definitively distinguish these entities, correlation with morphology is required and cytogenetics and/or FISH may be necessary.

In addition to establishing a diagnosis of follicular lymphoma, flow cytometry can serve as a useful adjunct to bone marrow staging in

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**Table II-1**

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**Figure 1-4: Low-level marrow involvement by follicular lymphoma.** Figure 1-4 demonstrates flow cytometry of the staging marrow from the patient whose diagnostic lymph node biopsy is represented in Figure 1-2. (A) A dot plot demonstrating CD45 versus side scatter demonstrates a distribution of cells that would be expected for the marrow with no increase in lymphoid cells. (B) In evaluating the B cells specifically, the kappa-to-lambda ratio is noted to be normal overall. (C) However, the CD10^+^ B cells include a population without expression of CD38 (highlighted in magenta), an abnormal finding suggesting involvement by follicular lymphoma (FL). This population comprises 0.4% of the white blood cells (A-F), is noted to express CD10, decreased CD19, and absent light chain expression identical to the patient’s known FL. Notably, this abnormal B-cell population differs from hematogones (shown in yellow and aqua) in that it has a lower level of CD10, CD19, and CD38 and a higher level of CD20 and CD45.
FL. As discussed in chapter I, *Introduction*, B cells in normal marrow range from hematogones or B-cell precursors to mature B cells. Early hematogones express low-level CD45 with bright CD10 and CD38 without CD20 or surface light chains. Through the process of B-cell maturation, these cells acquire CD20 and polyclonal surface light chain expression and lose both CD10 and CD38. Given the tightly regulated pattern of antigen expression associated with normal B-cell development, an abnormal FL population can be isolated at very low levels given the distinctive immunophenotype noted above (see Figure 1-4). The sensitivity of flow cytometry in evaluating staging bone marrows for FL is debated in the literature, with some studies suggesting detection rates lower than morphology \(^{11,12}\) (marrow core biopsy), and others suggesting that flow cytometry may have greater sensitivity than morphology\(^3\); however, it should be noted that most of the studies in the literature use a relatively small number of fluorochromes with relatively few events acquired and rely on a skewed kappa-to-lambda ratio, strategies which reduce the sensitivity of the assay. Additionally, the sensitivity of flow cytometry may be limited by sampling error as lymphoid infiltrates in FL involving the marrow are often paratrabecular and associated with fibrosis. Given these considerations, flow cytometry and morphology of the core biopsy are considered complimentary for bone marrow staging in FL.

**Take-home points**

- Follicular lymphoma is generally composed of CD10+ B cells showing clonal light chain expression without CD5 expression.
- Follicular lymphoma can usually be distinguished from follicular hyperplasia on the basis of expression of markers including CD19 and CD38, which are both lower in FL than in follicular hyperplasia.
- In difficult cases, demonstration of BCL2 overexpression by flow cytometry may be helpful in confirming a diagnosis of FL.

**References**

Case 2

Case presentation

Clinical history
A 63-year-old man with no significant past medical history undergoes a routine CBC that shows a leukocytosis with a WBC of 13,000 cells per microliter. A peripheral blood smear shows an absolute lymphocytosis with smudge cells. The hemoglobin and platelet counts are within normal limits. A sample of peripheral blood is sent for flow cytometry

Flow cytometric data (Figure 2-1)

Figure 2-1: Peripheral blood flow cytometry. CD45 versus side scatter (SSC) gating on all viable cells is shown in the upper left panel, while the remaining panels show CD19+ B cells. Flow cytometry of the peripheral blood shows that lymphocytes predominate and account for approximately 60% of the white blood cells. The B cells are composed predominantly of a clonal population expressing low-level kappa light chain restriction, with expression of CD5 and CD23 with low CD20 without FMC-7. The normal background B cells are highlighted with black circles and show a normal level of CD20 expression and normal levels of kappa and lambda light chain, which are expressed in a polyclonal fashion. CD10 is absent on the population of interest (data not shown). The findings are diagnostic of chronic lymphocytic leukemia.
Discussion

Chronic lymphocytic leukemia (CLL) is the most common chronic lymphoid leukemia seen in adults. When present in tissues, this process is called small lymphocytic lymphoma (SLL). In tissues biopsies, CLL/SLL accounts for 6.7% of non-Hodgkin lymphomas. The diagnosis of CLL/SLL is often suspected by morphology and should be confirmed by demonstrating a typical immunophenotype. In addition, immunophenotyping provides prognostic information in CLL/SLL.

Chronic lymphocytic leukemia/small lymphocytic lymphoma is a neoplasm of mature B cells that characteristically express B-cell markers, demonstrate clonality, and show aberrant expression of CD5 and CD23. CD19 is typically positive in CLL/SLL, as is CD20; however, CD20 expression is characteristically low, as is light chain expression. CD10 is negative in CLL/SLL, allowing distinction from follicular lymphoma (see chapter II, B-cell neoplasms, Table II-1). CD5 expression is characteristic of both CLL/SLL and mantle cell lymphoma. These two malignancies can typically be distinguished on the basis of expression of CD23 by CLL, and low-level expression of both CD20 and surface light chain in CLL. Additionally, FMC-7 is usually negative in CLL/SLL and may be helpful in distinguishing these entities. Although CLL/SLL is typically clonal, occasional biclonal cases have been described. Although such cases may have a near normal kappa-to-lambda ratio, aberrant expression of antigens including CD5, CD20, CD23, and CD24 may be present.

![Flow cytometry plots](image)

**Figure 2-2: Biclonal chronic lymphocytic leukemia/small lymphocytic lymphoma.** Chronic lymphocytic leukemia may occasionally have a major or minor biclonal component. In some cases this may lead to a kappa-to-lambda ratio that approximates normal. In the case illustrated in this figure, the kappa-to-lambda ratio was ~3:1. However, the majority of the B cells coexpressed CD5 and CD23 with low CD20 (CD10 and FMC-7 were absent [data not shown]). Of interest, the abnormal cells show differing levels of CD5 expression. CD5 is bright on the lambda-restricted subset (red) and intermediate on the kappa-restricted subset (blue). T cells are shown in green.
surface light chain will allow recognition of biclonal CLL/SLL (Figure 2-2). The presence of a second clone in addition to the primary CLL/SLL clone may be seen in some cases. This finding raises the possibility of a composite lymphoma or, possibly, CLL/SLL with transformation to large cell lymphoma (Richter’s transformation). The first of these possibilities may be considered in cases when a second population expressing a light chain that differs from that of the CLL/SLL (Figure 2-3) is noted, while the presence of a second population with forward scatter (FSC) characteristics of large cells (see Case 7) raises the latter possibility.

Figure 2-3: CLL/SLL with a secondary lymphoid malignancy. Flow cytometry from this lymph node shows a dominant population of lymphoid cells composed of a mixture of T cells (green) and B cells. The B cells show a normal kappa-to-lambda ratio, but light chain expression is bimodal and two populations are notable for aberrant antigen expression. A population is noted in the upper middle panel that expresses lambda light chain (red) with aberrant coexpression of CD5 and decreased CD20. A second abnormal population is most notable in the upper right panel that expresses kappa (blue) light chain with aberrant expression of CD10 and decreased CD19. Gates are drawn around both populations and colored aqua and orange respectively. The aqua colored population expresses low CD23 without FMC-7 (data not shown), suggesting chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). The orange population shows intermediate size by forward scatter (FSC) and low to absent CD38 without CD23, suggesting follicular lymphoma, although large B-cell lymphoma would also be a consideration. Concurrent morphology demonstrated a composite lymphoma consisting of both CLL/SLL and follicular lymphoma.
It is important to note that establishing a diagnosis of CLL/SLL requires not only demonstrating a population with the typical immunophenotype, but, in the absence of significant extramedullary disease, establishing that abnormal cells comprise greater than $5 \times 10^9$ cells/L in the peripheral blood. Patient’s who do not meet this numeric criteria are noted to have monoclonal B-cell lymphocytosis (MBL). MBL is seen with a frequency ranging from 3.5% to 12% of healthy adults, depending on the age of the population assayed and the sensitivity of the flow cytometric method used.\(^3\) For patients not meeting this numeric cutoff, correlation with clinical findings (including an evaluation for lymphadenopathy) will be required to distinguish between overt CLL/SLL and MBL.

In addition to establishing a diagnosis, flow cytometry also provides prognostic information in CLL/SLL. CLL/SLL has a variable likelihood to progress, with some patients doing well without therapy for years and others progressing despite therapy. Evaluation of expression of CD38\(^6\) and ZAP70\(^7,8\) by flow cytometry provides useful prognostic information in CLL/SLL, with information provided by analysis of these two variables being complementary.\(^9\) Expression of CD38 by greater than 30% of CLL/SLL cells is associated with a poorer prognosis in CLL/SLL, as is expression of ZAP70 by greater than 20% of CLL/SLL cells. ZAP70 analysis by flow cytometry can be fraught with technical limitations and should

**Figure 2-4: Evaluation of ZAP70 expression.** All plots show all lymphocytes. The upper panel (A) shows ZAP70 data from the case depicted in Figure 2-1, while the lower panel (B) depicts ZAP70 data from a different case of chronic lymphocytic leukemia (CLL). All dot plots show data from the lymphocyte gate, with T cells shown in green and the abnormal B-cell population shown in blue. T cells normally express ZAP70 (as do NK cells, shown in red, highlighted with red arrow) and are used to define the boundary between positive and negative for ZAP70 expression. When present, normal B cells (shown in pink in the lower panel and highlighted with the pink arrow) should be negative for ZAP70 and can be used as a negative internal control. In the case shown in the upper panel, less than 20% of the CD5\(^+\) B cells express ZAP70, while in the lower panel, greater than 20% of the CD5\(^+\) B cells express ZAP70. Expression of ZAP70 on greater than 20% of the neoplastic cells is associated with a poorer prognosis in CLL.
only be performed in an experienced laboratory. Methods for evaluating ZAP70 expression have been described elsewhere and are beyond the scope of this publication. Figure 2-4 highlights several important features to consider in interpreting a ZAP70 assay.

**Take-home points**

- Chronic lymphocytic leukemia/small lymphocytic lymphoma is composed of mature B cells expressing CD5 and CD23 with low-level CD20 and low-level surface light chain.
- A definitive diagnosis of CLL/SLL, in the absence of significant extramedullary disease, requires demonstrating the presence of greater than $5 \times 10^9$ abnormal cells/L in the peripheral blood. Below this numeric threshold, and in the absence of significant extramedullary disease, patients are considered to have monoclonal B-cell lymphocytosis.
- Evaluation of CD38 and ZAP70 can provide prognostic information in CLL/SLL.

**References**