Overview: Flow cytometry of B-lymphoblastic leukemia and immature B-cells

B-cells are a subset of lymphocytes that are produced in the bone marrow and develop from early pro-B to pre-B stages before migrating into blood and lymphoid tissue. Most B-cell malignancies have a phenotype by flow cytometric analysis that recapitulates a normal subset found in bone marrow, lymphoid tissue, or blood, albeit often with different levels of marker expression when compared to normal.

Normal B-cells maturing in the bone marrow at the pro-B-cell stage are positive for the early lymphoid markers CD10, CD19, CD34, CD38, and terminal deoxynucleotide transferase (TdT) among others. These early cells are known as type I hematogones and have low-level expression of CD45 (Figure 1 A-D, light blue cells) and lack expression of CD20. As the B-cells mature, there is a loss of CD34 and TdT, decrease in CD10 expression, and a gradual increase in CD20 and CD45. CD38 is fairly constant at this stage (type II hematogones; Figure 1 A-D, yellow-green cells), during which immunoglobulin heavy gene rearrangement occurs. In the next stage of development, the immature B-cells lose CD10, downregulate CD38, and express increasing levels of CD20 and CD45. At this stage. the immunoglobulin light chain genes are rearranged, and surface IgM can be detected (type III hematogones; Figure 1 A-D, dark green cells). Figures 2 and 3 show the differences in morphologic appearance between hematogones and lymphoblasts, while Table 1 outlines the surface marker expression differences between them.

The flow cytometric findings from a typical case of B-lymphoblastic leukemia/lymphoma, from a 59-year-old woman with leukocytosis and 91% circulating blasts, is shown in Figure 4. The neoplastic cells are dimly positive

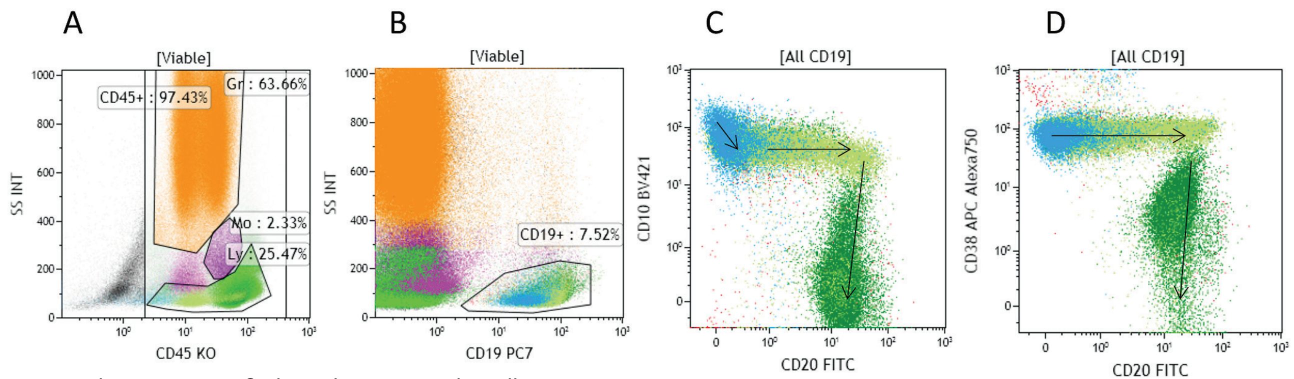

Figure 1. Flow cytometric findings during normal B-cell maturation.

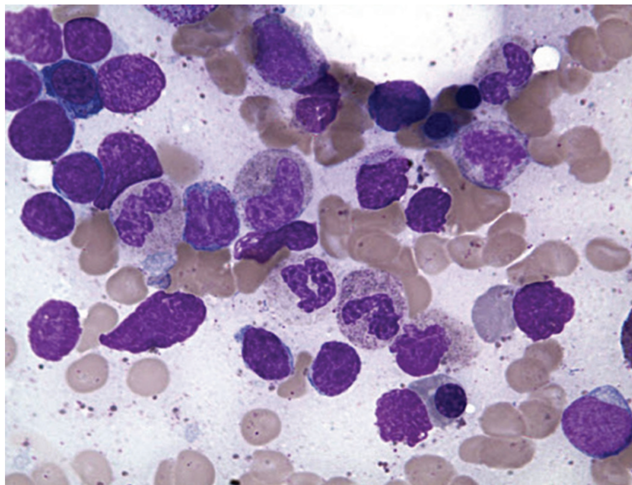

Figure 2. Hematogones are 10 to 20 μm in diameter, with smaller cells predominating, and have round or oval nuclei with homogeneous condensed chromatin without nucleoli and a very high nuclear-to-cytoplasmic ratio.

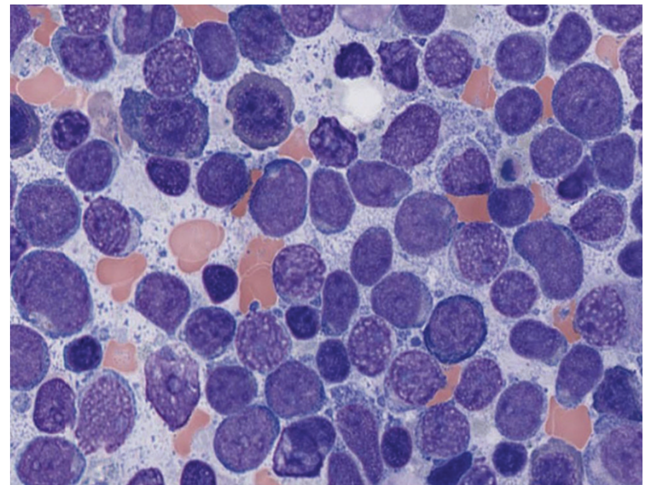

Figure 3. B lymphoblasts in a case of B-lymphoblastic leukemia/lymphoma. The lymphoblasts are small to intermediate in size with round to irregular nuclear outlines, heterogeneous lacy chromatin, nucleoli, scant to moderate weakly basophilic cytoplasm, and a high nuclear-to-cytoplasmic ratio. In some cases, the lymphoblasts are small, with very scant cytoplasm, or may have cytoplasmic pseudopods (hand mirror cells).

Table 1. Hematogones vs B-lymphoblastic leukemia/lymphoma	
Hematogones	**B-lymphoblastic leukemia**
Intermediate/variable CD45 expression	Absent, intermediate, or bright CD45 expression
Variable CD10 expression	Bright or absent CD10 expression
Variable CD38 expression	CD38 expression may be weak or absent
Variable CD20 expression	Positive, variable, or absent CD20 expression
Subset CD34 expression	Positive or negative CD34 expression
Absence of myeloid antigen expression	May express one or more myeloid antigens

Table 2. Specific immunophenotypic findings in B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities
t(9;22); *BCR-ABL1*: CD19+, CD10+, TdT+; frequent myeloid antigen positivity (**CD13, CD33**); poor prognosis
t(v;11q23.3); *KMT2A* rearrangement: CD19+, **CD10-**, CD24- pro-B phenotype; **CD15+**; poor prognosis
t(12;21); *ETV6-RUNX1*: CD19+, CD10+, CD34+, CD9-, **CD20-**; frequent myeloid antigens (**CD13**); favorable prognosis
Hyperdiploidy: CD19+, CD10+, CD34+; **CD45 often absent**; favorable prognosis
t(1;19); *TCF3-PBX1*: CD19+, CD10+, mu heavy chain +; CD9+, CD34-; poor prognosis
BCR-ABL1-like: CD19+, CD10+, **CRLF2+**

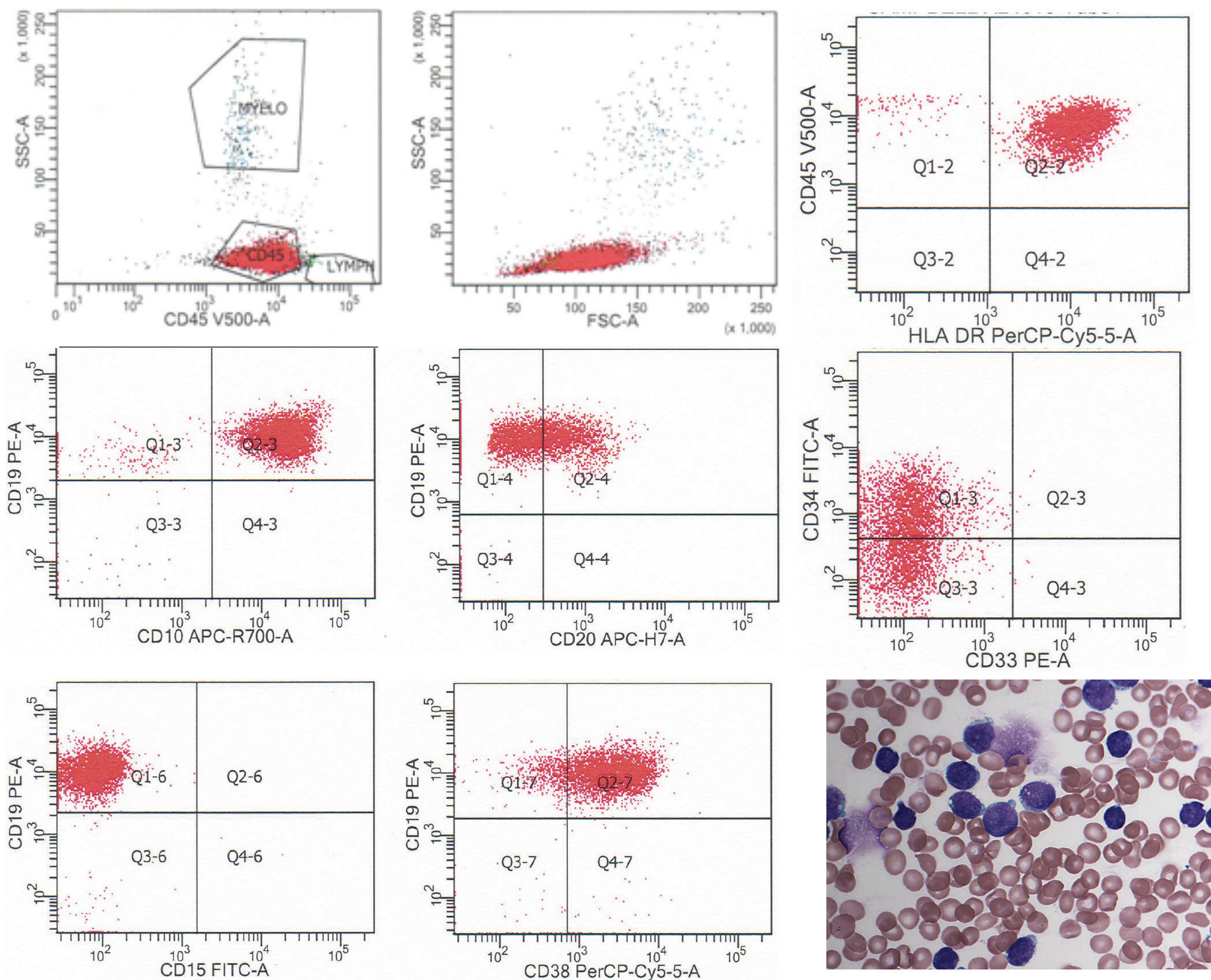

Figure 4. Typical immunophenotypic and morphologic findings in a case of B-lymphoblastic leukemia/lymphoma.

for CD45, CD34, and CD20, positive for HLA-DR, CD38, coexpress B-cell markers CD10 and CD19, and are negative for myeloid antigens CD15 and CD33. In contrast to normal hematogones, the neoplastic cells are uniformly positive for CD10 and CD38. Additionally, certain surface marker expression patterns have been associated with prognostic outcome in subsets of B-lymphoblastic leukemia/lymphoma (Table 2).

As the B-cells migrate from the bone marrow to peripheral lymphoid tissue, naïve B-cells in the interfollicular area

are strongly positive for CD19 and CD20 and negative for more immature markers. These cells either undergo apoptosis or become short-lived plasma cells. Some will go on to enter the germinal center as centroblasts, which mature into centrocytes, while others undergo apoptosis. These germinal center cells reacquire CD10 and undergo hypermutation of the immunoglobulin variable genes and class switching from IgM to IgG or IgA. Post germinal center cells may become memory B-cells, with a subset acquiring CD5 (a surface marker usually associated with T-cells), or long-lived plasma cells, which express high levels of CD38, with reduced levels of CD19 and loss of CD20. Surface immunoglobulin is expressed from the naïve B-cell stage to the memory B-cell stage, but is not present on plasma cells. There are other surface markers present on B-cell populations, which are described in the section of the atlas on B-cell lymphoproliferative disorders.

Having a clear understanding of normal B-cell maturation is critical in identifying abnormal B-cell populations in cases of leukemia and lymphoma, particularly in patients who are monitored for remission status or who have been treated with monoclonal antibody therapy.

The cases in this section will outline where "difference from normal" is used as a basis to determine the presence or absence of a B lymphoid malignancy, in conjunction with morphology, clinical findings, and other laboratory findings.

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Case 1

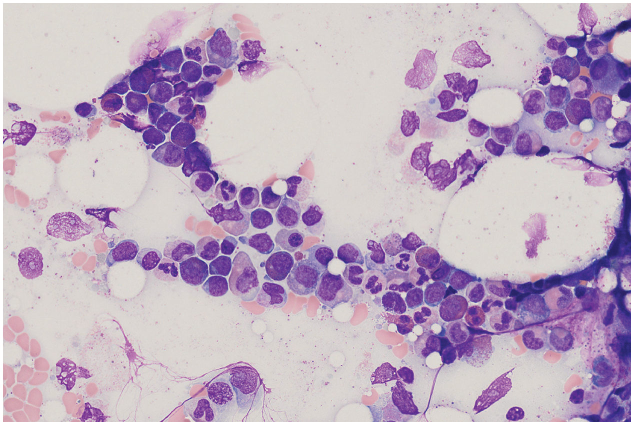
History

The patient is a 4-year-old boy who presented to the emergency department with scalp nodules. A bone marrow aspirate was submitted for flow cytometric immunophenotyping.

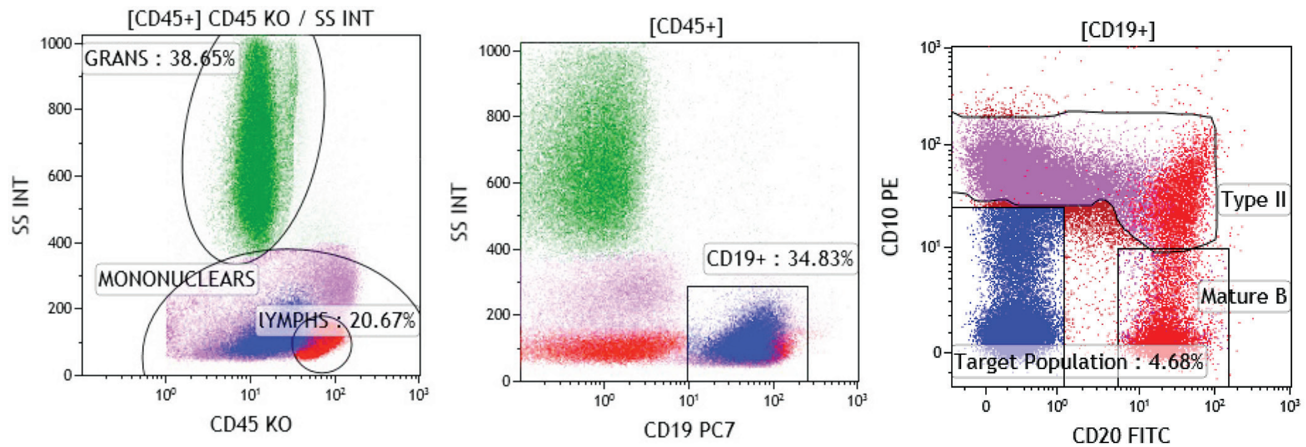
Laboratory results

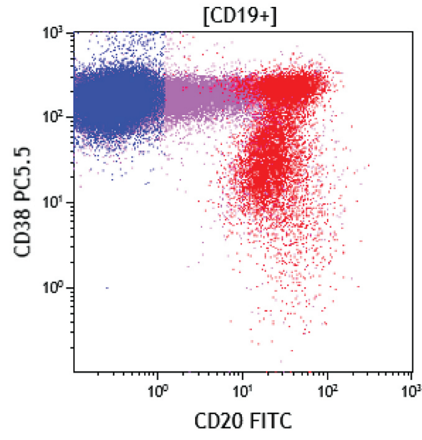
WBC	14.6 x10^9/L	[normal range 3.5-10.0 x 10^9/L]
Hemoglobin	11.0 g/dL	[normal range 11.5-16.0 g/dL]
MCV	82 fL	[normal range 80-98 fL]
Platelets	142 x10^9/L	[normal range 150-350 x 10^9/L]

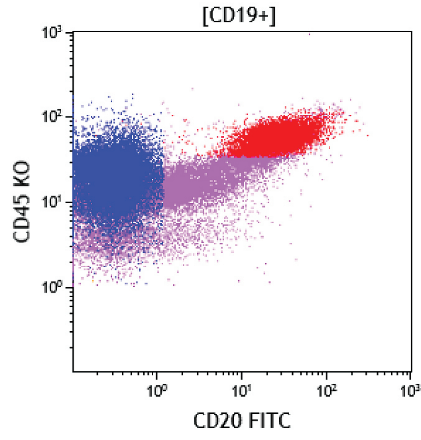
Photomicrograph
(bone marrow aspirate; Wright-Giemsa stain)



Representative flow cytograms




[CD19+]
CD38 PC5.5
CD20 FITC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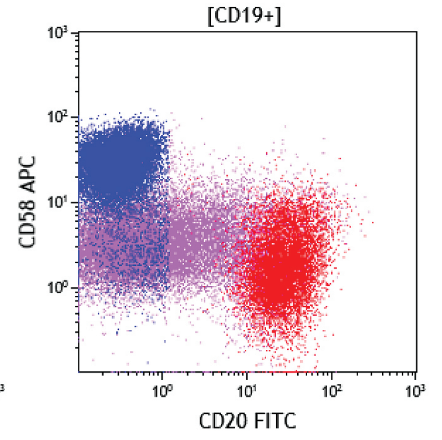
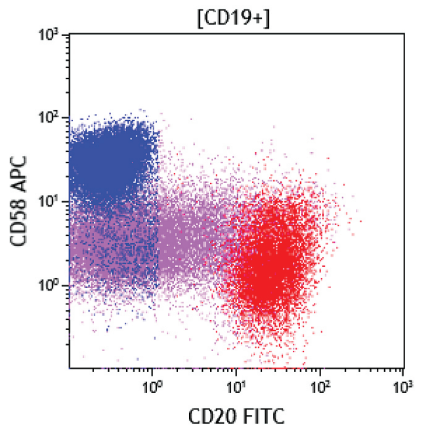
CD45 KO
CD20 FITC


[CD19+]
CD58 APC
CD20 FITC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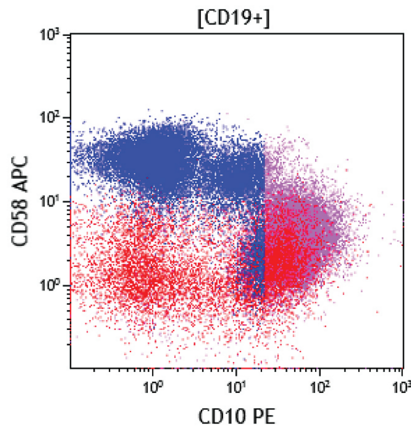
CD20 FITC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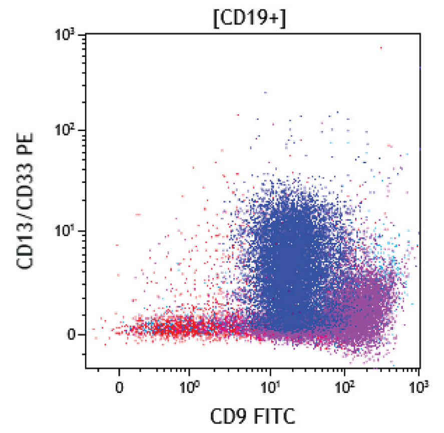
CD58 APC
CD10 PE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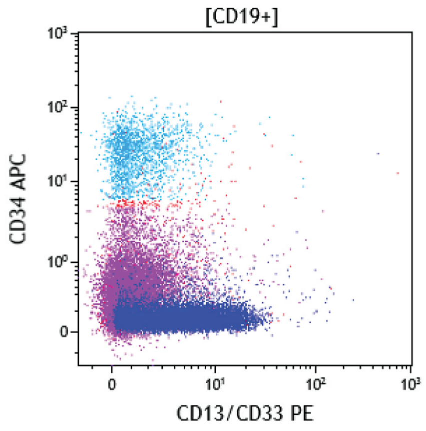
CD13/CD33 PE
CD9 FITC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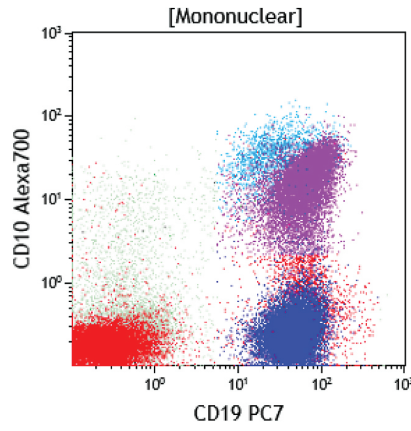
CD34 APC
CD13/CD33 PE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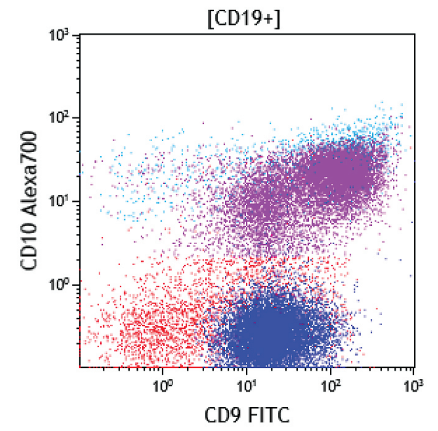
CD10 Alexa700
CD19 PC7


[CD19+]
CD10 Alexa700
CD9 FITC

Summary of flow cytometry findings (participant consensus)

Positive: CD9, CD19, CD38, CD45, CD58
Negative: None
Nonconsensus: CD10, CD20, CD13/CD33, CD34

Favored interpretation

	Participants (207)	
	Number	%
B-lymphoblastic leukemia	**109**	**52.7**
Hematogone hyperplasia	78	37.7
Normal specimen/no evidence of neoplasia	6	2.9
Acute myeloid leukemia (AML)	1	0.5
Advise laboratory to regate primary data	2	1.0
AML, with aberrant T-cell expression	11	5.3

Discussion

This sample came from the bone marrow of a 4-year-old boy with scalp nodules. A CBC showed mild leukocytosis with mild anemia and mild thrombocytopenia. The Wright-Giemsa–stained bone marrow aspirate smear showed a full maturation spectrum in erythroid and myeloid lineages with an increase in blast forms. Many of the blasts were small in size with a high nucleus-to-cytoplasm ratio, small nucleoli, and scant basophilic cytoplasm. The morphology is concerning for acute leukemia.

The flow cytometry data from the patient's bone marrow is somewhat complex, with populations of abnormal B lymphoid blasts (colored blue), normal hematogones (colored light magenta), early hematogones (colored light blue), and a mixture of mature B-cells and late hematogones (colored red). The abnormal B lymphoid blast population was also labeled "Target Population" in one of the plots, with a given percentage of 4.68%. The mixed populations resulted in difficulty in interpreting antigen expression patterns by survey participants, with several of the antigens not reaching an 80% consensus.

The abnormal blue target population expresses slightly weak CD9, weak CD13/33, CD19, bright CD38, intermediate CD45, and bright CD58, with a subset expressing CD10. There was no expression of CD20 or CD34. These findings are consistent with an abnormal B lymphoblast population. The absence of expression of CD10 and CD20 is suggestive of B-lymphoblastic leukemia/lymphoma (B-ALL/LBL) with a *KMT2A* gene rearrangement. In this case, the CD13 and CD33 are "stacked antibodies," where the two antibodies are labeled with the same fluorochrome (phycoerythrin). Stacking allows additional markers to be used with the limited number of detectors available on a flow cytometer. We cannot determine whether CD13 or CD33 is actually positive, but the presence of either on a B lymphoblast population is abnormal.

The presence of a background normal hematogone population allows for direct comparison between the abnormal cells and a normal immature (hematogone) B-cell population. The lack of CD10 in the majority of abnormal cells is abnormal. The expression of CD9 is slightly weaker, and the expression of CD58 is brighter compared with the normal hematogones. The normal hematogone population has variable expression of CD20 and no expression of CD13/33. A subset of hematogones expresses CD34. Many laboratories may not be familiar with CD9 or CD58, but both are useful in detection of minimal residual disease (MRD) in B-lymphoblastic leukemia.

The presence of an abnormal lymphoblast population is consistent with bone marrow involvement by B-ALL/LBL. A total of 52.7% of participants gave the intended response of B-ALL, whereas 37.7% of participants thought the results indicated a normal hematogone population. This case was clearly very challenging, and many participants included comments highlighting some of the difficulties. The blast percentage was low in this case, which makes rendering a final diagnosis difficult. Although it is unusual to diagnose B-ALL/LBL without ≥20% blasts, the abnormal immunophenotype allows for definitive diagnosis. The morphology was intended to show a higher percentage of blasts, suggesting possible hemodilution of the flow sample. Subsequently, the patient's scalp nodules were biopsied and showed B-lymphoblastic lymphoma, supporting the diagnosis. Some laboratories commented that they only deal with adult cases and that some of the markers used were unfamiliar. Similar analyses are performed on adult cases, particularly when minimal residual disease (MRD) flow is ordered; thus, the Diagnostic Immunology and Flow Cytometry Committee thinks that it is useful for interpreting laboratories to be aware of the markers, even if they do not routinely interpret them. A more straightforward case of B-ALL/LBL is provided in the *Overview: Flow Cytometry of B-lymphoblastic Leukemia and Immature B-cells* section.

Some laboratories commented that additional antigens, such as cytoplasmic terminal deoxynucleotidyl transferase (TdT), would be helpful in rendering a diagnosis. This is true, particularly in a case such as this one that lacked expression of CD34. Typically, a broader panel of antibod-

ies would be used on diagnostic material, including surface immunoglobulin, additional B-cell antigens (such as CD22 and CD79a), additional myeloid antigens, and intracellular antigens, including cytoplasmic myeloperoxidase. The age of the patient, the intermediate expression of CD45, along with the bright expression of CD38 and CD58, were all consistent with an immature B-cell population. The abnormalities in the immunophenotype, although somewhat subtle, were sufficient to distinguish the population as B-ALL/LBL.

ALL/LBL is primarily a disease of childhood, with 75% of patients being less than 6 years old. B-ALL/LBL comprises about 80% to 85% of lymphoblastic leukemias and 10% of lymphoblastic lymphomas, with the rest being of T-cell lineage. Children with trisomy 21 have increased risk of B-ALL/LBL. There is no minimal blast percentage needed to diagnose ALL/LBL, although most cases of B-ALL/LBL will exhibit greater than 20% blasts, and current World Health Organization guidelines suggest a diagnosis of B-ALL/LBL should be avoided when blasts are fewer than 20%. By definition, all cases of B-ALL have bone marrow involvement, typically also with peripheral blood involvement. Extramedullary involvement is most common in the central nervous system, lymph nodes, spleen, liver, and testes. B-LBL most frequently involves the skin, soft tissue, bone, and lymph nodes. In a patient with a mass lesion and lymphoblasts in the bone marrow, the distinction between B-lymphoblastic leukemia and B-lymphoblastic lymphoma is arbitrary.

B-ALL/LBL can be categorized on the basis of the degree of blast differentiation. The earliest stage is precursor B-lymphoblastic leukemia (pro-B-ALL), which expresses CD19, cytoplasmic CD79a, cytoplasmic CD22, and nuclear TdT. The intermediate stage (common B-ALL) has CD10 expression. The most mature stage (pre-B-ALL) shows expression of cytoplasmic mu chain and occasionally surface heavy chain (without light chain). B-ALL/LBL should also be categorized by genetic abnormalities because prognosis and treatment can be affected. The presence of t(9;22)(q34.1;q11.2) (*BCR-ABL1*) imparts the worst prognosis, although treatment with tyrosine kinase inhibitors may improve outcomes. Other frequent genetic aberrations include the following:

- Rearrangement of *KMT2A* (11q23.3) with a number of partners (poor prognosis, especially when <6 months of age)
- t(12;21)(p13.2;q22.1) (*ETV6-RUNX1* translocation, very favorable prognosis)
- Hyperdiploidy (favorable prognosis)
- Hypodiploidy (poor prognosis)
- t(5;15)(q31.1;q32.2) (*IGH-IL3* translocation, no effect on prognosis)
- t(1;19)(q23;p13.3) (*TCF3-PBX1* translocation, increased risk of central nervous system relapse)
- *BCR-ABL1*–like gene expression profile (without translocation, typically poor prognosis)
- Amplification of a portion of chromosome 21 (iAMP21, poor prognosis)

It is essential to design flow cytometry panels to maximize detection of aberrant antigen expression. Complete characterization of the blasts by flow cytometry is critical to allow for future MRD detection. Detection of B-ALL/LBL requires knowledge of the normal immunophenotype of all stages of hematogones because comparison with the normal maturation pattern is the most efficient way to detect abnormal blasts. Aberrant expression of myeloid or T-cell markers is also useful in diagnosing B-ALL/LBL. Targeted therapies such as anti-CD19 chimeric antigen receptor–T-cells add complexity to follow-up, not uncommonly requiring additional markers that are not routinely used to identify B lineage cells.

Major teaching points

- B-ALL/LBL is primarily a disease of childhood. Patients with trisomy 21 are at increased risk.
- Unlike acute myeloid leukemia, there is no minimal blast percentage needed to diagnose ALL/LBL, although most cases of B-ALL/LBL will exhibit greater than 20% blasts.
- B-ALL/LBL can be characterized by degree of differentiation (defined by expression of surface and cytoplasmic antigens at different stages of hematogone maturation) and by genetic aberration, if present.
- Diagnosis of B-ALL/LBL is most efficiently performed by comparing the population of interest to normal, maturing hematogones. B-ALL/LBL will typically exhibit absent, intermediate, or bright CD45 expression rather than the variable CD45 expression seen in hematogones. CD58 may be abnormally persistent and/or brighter in B-ALL/LBL than in normal hematogones. CD9 and CD81 are typically bright in early maturation and lost later; abnormally weak expression can indicate malignancy.
- Aberrant myeloid and T-cell antigen expression are useful in identifying malignant B-cell precursors.

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