4

Flow Cytometry in the Diagnosis of Leukemias

Weijie Li

Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, University of Missouri-Kansas City School of Medicine, Kansas City, USA

Author for correspondence: Weijie Li, Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, Kansas City, USA. Email: wli@cmh.edu

Cite this chapter as: Li W. Flow Cytometry in the Diagnosis of Leukemias. In: Li W. editor. *Leukemia*. Brisbane (AU): Exon Publications. Online first 22 Aug 2022.

Doi: https://doi.org/10.36255/exon-publications-leukemia-flow-cytometry

Abstract: Leukemia is a group of hematologic malignancies characterized by the proliferation of abnormal lymphoid or hematopoietic cells in the bone marrow and frequent involvement of peripheral blood and other organs. Leukemia can be classified as acute or chronic based on its rate of progression and specified as one of the many subtypes with other information incorporated according to the WHO classification. Common leukemias include acute lymphoblastic leukemia, acute myeloid leukemia, chronic lymphocytic leukemia, and chronic myeloid leukemia. With the tremendous improvement in instrumentation and reagents during the past several decades, flow cytometry has become a powerful immunophenotyping tool, and plays a critically important role in the diagnosis of various leukemias. Flow cytometry can quickly identify the abnormal cell population, characterize its phenotype, give lineage classification, make the diagnosis, or narrow down the differential list. It can also assess the clonality of a mature B-cell or T-cell population, and determine DNA ploidy, which is also very useful for making diagnosis or predicting prognosis. Correlation with morphology, clinical information, and sometimes cytogenetic/molecular findings is always necessary for accurate

In: Li W (Editor). *Leukemia*. Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, University of Missouri-Kansas City School of Medicine, Kansas City, USA. ISBN: 978-0-6453320-7-0. Doi: https://doi.org/10.36255/exon-publications-leukemia

interpretation of flow cytometry results. This chapter provides an overview of the principles and the significant roles of flow cytometry in the diagnosis of leukemias.

Keywords: flow cytometry; leukemia; acute lymphoblastic leukemia; acute myeloid leukemia; chronic leukemia

INTRODUCTION

Leukemia is a heterogeneous group of hematologic malignant neoplasms characterized by the proliferation of abnormal lymphoid or hematopoietic cells in the bone marrow (BM) and frequent involvement of peripheral blood (PB), spleen, lymph node, and other organs. Leukemia can be classified as acute or chronic based on its rate of progression. Acute leukemia progresses fast, and patients' condition deteriorates quickly without treatment. In contrast, chronic leukemia progresses slowly, and the patients may stay with the disease for a long period of time even without treatment. Acute leukemia is the malignancy of lymphoid or hematopoietic precursors (blasts). Based on cell origin, acute leukemia can be classified as acute lymphoblastic leukemia, acute myeloid leukemia, or acute leukemia of ambiguous lineage. These acute leukemias can be further classified into many subtypes with the incorporation of more clinical, phenotypic, and genetic information. Chronic leukemia is the malignancy of mature or maturing lymphoid or hematopoietic cells. Most of the maturing myeloid malignancies present as chronic leukemia. Although cases of many types of lymphomas exist with extensive BM and/or PB involvement, the terminology of "leukemia" is only used in the names of a few mature B-cell and T/NK cell neoplasms, which usually show predominant BM and/or PB involvement. Leukemia is diagnosed based on clinical information and laboratory testing results.

Flow Cytometry (FCM) is a technology capable of providing rapid multi-parameter analysis of single cells or particles. It measures simultaneously physical characters (size and complexity) and multiple surface/ intracellular markers of single cells, which allows for rapid and accurate phenotypic characterization of a cell population. Together with histomorphology and other information, multi-parameter FCM can quickly diagnose leukemia and accurately classify leukemia as a subtype as defined by World Health Organization (WHO) classification (1–3). FCM study can also assess the clonality of mature B-cells by evaluating immunoglobulin (Ig) light chain expression, and the clonality of mature αβ T-cells by evaluating T-cell receptor (TCR) Vβ repertoire or TCR β chain constant 1 (TRBC1) expression (4, 5). In addition, FCM can readily measure DNA content and determine DNA ploidy of a cell population, which is also helpful in making diagnosis and/or predicting prognosis. The fast turnaround time of FCM testing allows for the quick diagnosis or timely selection of the proper other ancillary tests.

Compared with immunohistochemistry (IHC), FCM is not only faster but also capable of easily and efficiently correlating multiple parameters on a single cell. Moreover, FCM can detect the antigen intensity and aberrant antigen expression pattern. The major disadvantage of FCM is the lack of correlation with histomorphology. In addition, FCM requires viable fresh samples, and decreased viability

of certain neoplasms often precludes accurate FCM analysis. This chapter provides an overview of the principles and the significant roles of FCM in the diagnosis of leukemias.

PRINCIPLES AND KEY ELEMENTS OF FLOW CYTOMETRY

With the tremendous improvement in instrumentation and reagents during the past several decades, FCM has become a very powerful tool applied in many research or clinical fields for a wide variety of purposes (6, 7).

Flow cytometers

Flow cytometers consist of three main systems: fluidics, optics, and electronics (Figure 1A). The fluidics system is responsible for transporting samples to the flow cell where the cells form a single file stream and cross the laser beam. A stream of solution (sheath fluid) surrounding the specimen is introduced into the instrument at higher pressure so that the cells in the specimen form a roughly single file stream due to the phenomenon called hydrodynamic focusing. The cells or particles in the single file stream travel through the interrogation point where they are illuminated by light from one or more lasers. Eventually, the fluid stream is drained into a waste container (Figure 1A). The optics system consists of light sources (lasers), lenses, filters, and detectors. Lasers illuminate the cells or particles, and dichroic mirrors (DMs) and optical filters (OFs) are used to direct the resulting light signals to the appropriate detectors. DMs allow the light of corresponding wavelengths to pass while reflecting the light of other wavelengths. OFs further narrow the wavelengths reaching a detector. The detectors are usually photomultiplier tubes (PMTs) or photodiode arrays (PDAs) that convert the signals to electrical impulses, which are measured and converted to digital information by the electronics system. The digital information is collected and interpreted by the analysis software (6). The connected computer system directly interfaces with the flow cytometer and controls its functions. Data analysis can be performed either on the computer connected to the flow cytometer or on other computers that have access to the data.

Specimens and reagents/ antibody panels

Specimens suitable for FCM analysis include PB, BM, cerebrospinal fluid, serous effusions, fine needle aspirations (FNAs), and fresh unfixed tissue. PB and BM aspirates must be anticoagulated. Sample preparation for FCM analysis varies according to the specimen types and the antigens to be analyzed. The cell suspension generated by mincing tissue should be filtered to remove large particles that may clog the cytometer tubing and/or bind antibodies non-specifically. In any specimen containing a large amount of PB, the red blood cells should be removed through a lysis process using either a commercially available reagent or a homemade ammonium chloride solution before running the sample in a flow cytometer.

The key reagents for FCM studies are fluorochrome-conjugated antibodies against cell surface, cytoplasmic or nuclear antigens. Table 1 lists the antigens

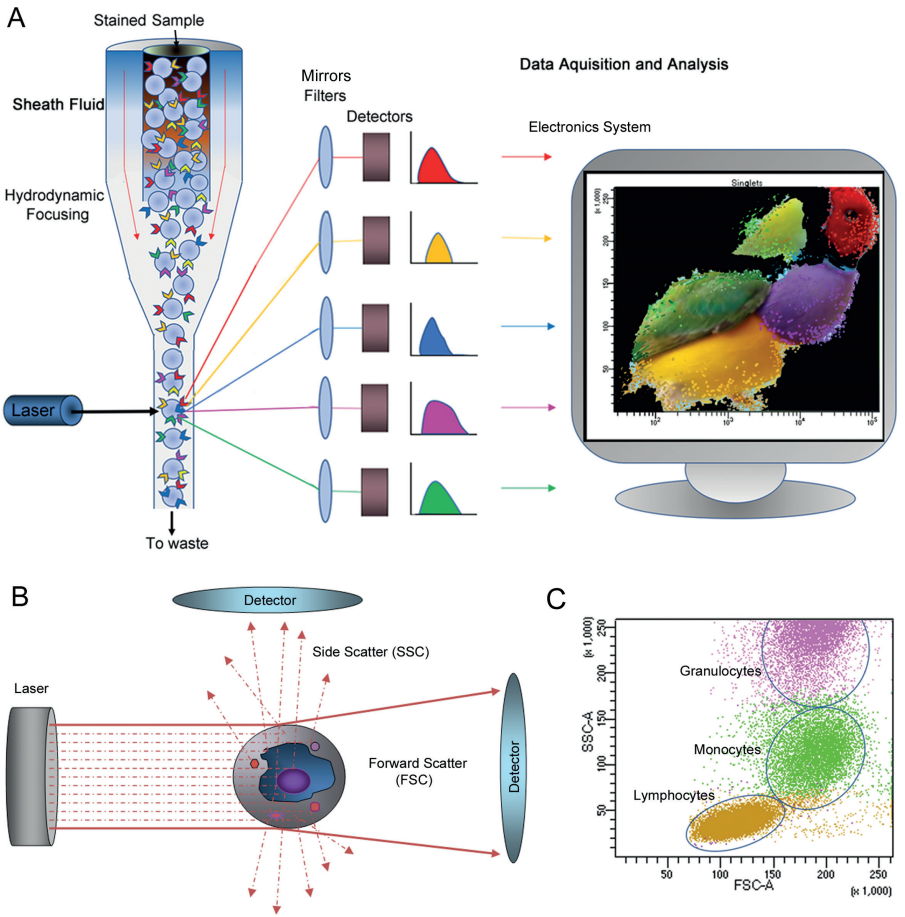

Figure 1. Schematic diagram of how flow cytometry works and light scatter of cells. A, After a liquid sample with stained cells is injected into a flow cytometer, the cells are forced to form a single file stream through hydrodynamic focusing. The cells in a single file stream are illuminated as they travel through a laser beam, and the optical and fluorescence signals of each cell are generated and detected by proper detectors that convert photons to electrical impulses. The electronics system measures the electrical impulses and converts these signals to digital information that is gathered and analyzed by specific software in a computer. **B**, Illustration of forward light scatter (FSC) and side light scatter (SSC) of a cell. **C,** Differentiation of peripheral blood leucocytes according to forward and side scatter properties.

commonly evaluated in the workup for hematologic malignancies. How many antibodies and how many tubes to run for a sample are dependent on the clinical indication and what type of flow cytometer used. A single-laser flow cytometer can evaluate three to five antigens simultaneously besides the light scatter properties. A flow cytometer with more lasers can evaluate more antigens at the same time. Eight to ten-color diagnostic antibody panels for hematologic neoplasms are commonly used currently in the United States. The combination of antibodies in

TABLE 1 **Surface or intracellular antigens commonly assessed by flow cytometry for lineage classification and immunophenotyping of hematolymphoid malignant neoplasms**

Lineage	Antigens
Stem cells	CD34, CD38, CD45
B cells	CD5, CD10, **CD19**, **CD20**, **CD22**, CD23, CD25, CD34, CD38, CD43, CD45, **CD79a**, CD103, CD200, FMC2, **cIgM**, **Kappa**, **Lambda**, LEF1, TdT
Plasma cells	CD19, CD20, CD38, CD45, CD56, CD117, CD138, **cKappa**, **cLambda**
T cells/NK cells	CD1a, **CD2**, **CD3**, CD4, **CD5**, CD7, **CD8**, CD10, CD16, CD25, CD26, CD30, CD34, CD45, CD56, **αβ-TCR**, **γδ-TCR**, TdT, **TRBC1**
Myelomonocytic cells	CD4, CD7, CD10, CD11b, CD11c, **CD13**, **CD14**, CD15, CD16, **CD33**, CD34, CD36, CD38, CD45, CD56, **CD64**, **CD65**, CD71, CD117, CD123, **cMPO**, **cLyso**, HLA-DR
Erythroblasts	CD34, CD36, CD38, CD45, CD71, CD117, **CD235a**
Megakaryoblasts	CD33, CD34, CD38, **CD41**, **CD42**, CD45, **CD61**, CD117, HLA-DR

The antigens in bold are lineage-specific markers. cIgM, cytoplasmic IgM; cKappa, cytoplasmic kappa; cLambda, cytoplasmic lambda; cLyso, cytoplasmic lysozyme; cMPO, cytoplasmic peroxidase; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase; TRBC1, TCR β chain constant 1.

one tube should be properly designed according to the antigens on the cells of interest, fluorochrome characteristics and clinical purpose. In general, the antibody against a weakly expressed antigen should be conjugated with a bright fluorochrome to increase the chance of detecting this antigen. Color compensation to minimize the spillover between different fluorochromes is very important in multicolor tubes. For diagnostic purposes, antibodies are typically combined as panels to answer specific questions about a cell population (8, 9). The screening panels should evaluate sufficient antigens to distinguish a neoplastic cell population from normal/reactive cells with a high degree of sensitivity.

Light scatter and fluorescence signals

Light scatter and fluorescence are the two types of signals generated and collected for a cell as it travels through the laser beam. Light scatter is used to measure the physical properties of a cell. Forward scatter (FSC) refers to the light scattered at narrow angles to the laser beam, and it is collected by a detector positioned in line with the laser beam on the opposite side of the sample stream (Figure 1B). The FCS is proportional to the surface area or size of the cell. Side scatter (SSC) refers to the light refracted or reflected by cytoplasmic organelles or nuclei of the cells, and it is collected by a detector positioned at approximately 90º to the laser beam. The SSC is proportional to the granularity and internal complexity of the

cell (Figure 1B). Together, FSC and SSC signals allow for the separation of cells in a specimen containing heterogeneous cell populations, e.g., three types of leukocytes (granulocytes, monocytes, and lymphocytes) in PB readily separated based on FSC and SSC characters (Figure 1C). When a cell crosses the laser beam, the fluorescent dye or fluorochrome attached to the cell is excited by light of a specific wavelength (excitation wavelength), then it absorbs the energy, gets excited, and emits light (fluorescence) at a longer wavelength (emission wavelength). In a flow cytometer, the fluorescent signals are detected by PMTs after being filtered by DMs and OFs. The intensity of the fluorescent signal is proportional to the expression level of the antigen, to which the corresponding fluorescently labeled antibody binds.

Data acquisition and analysis

Data acquisition refers to the data collection process by which the stained cells are running through a flow cytometer and illuminated by laser light, and the resulting signals are detected by the optics system. The number of cells, also known as events, required for evaluation is dependent on the purpose of the test and the nature of the specimen. For example, in the evaluation of a PB sample with predominantly leukemic cells, a relatively low number of viable cells (e.g., 20,000/tube) acquired is enough to characterize the phenotype of the leukemia. In contrast, minimal residual disease (MRD) detection usually requires a much larger number of viable cells (e.g., >500,000/tube) acquired to increase the sensitivity for detecting a very small population of residual malignant cells.

FCM data analysis requires specially designed software. Each flow cytometer usually has an associated software for data acquisition and analysis. Independent flow cytometry software, such as FCS Express, and FlowJo, with more functions is also commercially available. The flow cytometry data can be displayed as histograms or dot plots. The histograms is used for displaying a single parameter, while the dot plot is used to display two parameters and each dot represents one cell or particle. The stronger the signal is, the further along each scale the data are displayed. An antigen is called positive when its associated fluorescent signal is above the negative control (background signal). Since most of the specimens contain heterogeneous cell populations, an internal negative control is easily found and used for most antigens. However, due to the possible difference of nonspecific binding and autofluorescence levels, internal control may be misleading. Adding nonspecific Ig of the same isotype may work as better negative control and is used in some FCM tests.

Gating refers to the process of isolating cell populations within a heterogeneous cell sample according to their physical and phenotypical features, and searching for the abnormal cell population. Because normal leukocytes and hematopoietic precursors typically show consistent and reproducible patterns on SSC vs CD45 dot plot, SSC vs CD45 gating is the most commonly used initial step to separate various PB or BM cell populations. An abnormal cell population is identified based on the difference(s) of its phenotype from normal. These differences include: (i) abnormal increases or decreases of antigens normally expressed on the cells of interest; (ii) homogeneous expression patterns of antigens that normally show variable expression patterns; (iii) asynchronous antigen expression; and

(iv) aberrant expression of antigens of other cell types. To appreciate these abnormalities, the flow cytometrists must be familiar with the normal antigen expression pattern of all the cell populations in the specimen (10).

It is very important to have a strict quality assurance (QA) practice in an FCM laboratory to maintain consistency in FCM results. Quality control (QC) measures must be performed and documented regularly to ensure optimal instrument performance. Any change in the reagents, panels, instruments, or protocols must be carefully validated to make sure that the change does not affect the results. Clinical FCM laboratories should enroll in the available proficiency testing programs for the tests they are performing to make sure that their results are comparable to others.

FLOW CYTOMETRY IN THE DIAGNOSIS OF ACUTE LEUKEMIAS/ LEUKEMIAS OF BLASTS

Acute leukemia is a hematologic malignancy composed of abnormally proliferating lymphoid or hematopoietic precursors (blasts). The definition of acute leukemia is the presence of a significant number of abnormal blasts in BM according to the WHO classification (3). A value of ≥20% myeloblasts of total nucleated cells is required for making the diagnosis of acute myeloid leukemia (AML). Based on the incoming 5th edition of WHO classification (2), the 20% threshold is not needed for AML diagnosis if the blasts carry *PML::RARA, RUNX1-RUNX1T1, CBFB-MYH11, DEK::NUP214, RBM15::MRTFA, KMT2A* rearrangement, *MECOM* rearrangement, *NUP98* rearrangement, or *NPM1* mutation. In many treatment protocols, a value of ≥25% lymphoblasts is needed to make the diagnosis of acute lymphoblastic leukemia (ALL). If the abnormal precursors are predominantly localized in tissue, forming a solid mass lesion with no significant BM involvement, the diagnosis should be lymphoblastic lymphoma (LBL) or myeloid sarcoma depending on the blast type. LBL shares the same morphology, immunophenotype and biology with their ALL counterpart, so LBL and corresponding ALL are combined as one group (ALL/ LBL) by the current WHO classification (1, 3). FCM analysis can efficiently identify abnormal blast populations, give blasts lineage classification, aid in further WHO classification, and sometimes provide prognostic information (11, 12)

Identification of blast population

The identification of a large blast population, such as the ALL cases in Figure 2 and AML cases in Figure 3, is usually straightforward. Blasts usually show low SSC and dim to intermediate CD45 expression, and thus fall in the so-called blast region (low SSC and dim CD45) on CD45 vs SSC dot plot (13). However, not all the cells in the blast region are blasts. Basophils and plasmacytoid dendritic cells can also be found here, and normal B-cell precursors (hematogones) and early myeloid precursors are present here as well (10). The leukemic cells of certain acute leukemias may be seen outside of the blast region, e.g., acute promyelocytic leukemia (APL) typically showing intermediated to high SSC (Figure 3B). Therefore, careful evaluation of antigen expression profile is always necessary for the identification or

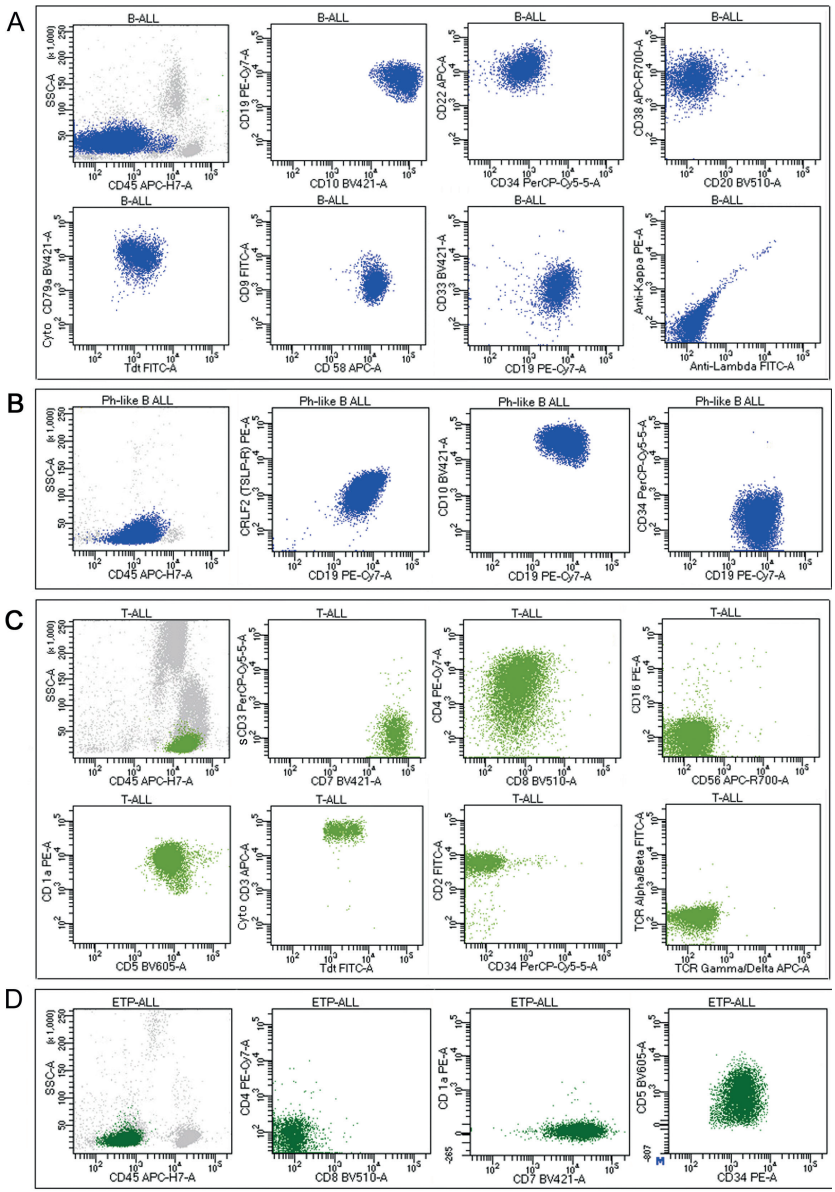

Figure 2. Acute lymphoblastic leukemia. Representative dot plots of acute lymphoblastic leukemia (ALL). **A**, Precursor B-cell ALL (B-ALL, blue) showing low side scatter, dim to negative CD45, expression of CD19, CD10, CD34, CD22, CD38, cytoplasmic (Cyto) CD79a, TdT, CD9, CD58, CD33, and no expression of CD20, kappa, and lambda. **B,** Philadelphia-like (Ph-like) B-ALL (blue) showing expression of CRLF2. **C,** T-cell ALL (T-ALL, green) showing low side scatter, intermediate to bright CD45, expression of CD7, CD4, CD8, CD1a, CD5, Cyto CD3, TdT, CD2, and no expression of surface CD3 (sCD3), CD16, CD56, CD34, TCR alpha beta, and TCR gamma delta. **D,** Early-T-precursor (ETP) ALL (dark green) being positive for CD7, CD34, CD5 (dim and partial), CD45 (dim to negative), and negative for CD4, CD8 and CD1a.

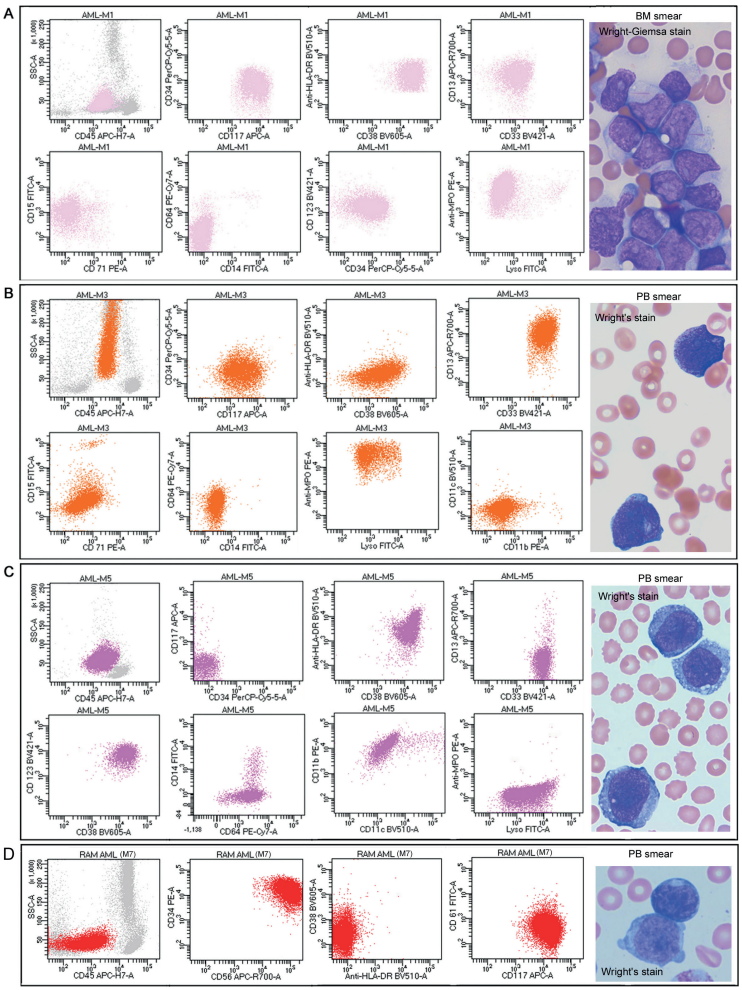

Figure 3. Acute myeloid leukemia. Representative flow cytometry dot plots and cytomorphology of acute myeloid leukemia (AML). **A,** AML-M1 (light pink) showing low side scatter, dim CD45, expression of CD34 (dim), CD117, HLA-DR, CD38, CD13, CD33, CD15, CD123 and myeloperoxidase (MPO), and no expression of CD71, CD64, CD14 and lysozyme. The blasts are large with irregular nuclei, fine chromatin, prominent nucleoli, abundant cytoplasm with thin single Auer rods and no or sparse granules. **B,** AML-M3 (acute promyelocytic leukemia) (orange) showing intermediate to high side scatter, intermediate CD45, expression of CD117, CD38, CD13, CD33, CD15 (dim), CD64 (dim and partial), CD71 (partial), MPO, lysozyme (partial), and no expression of CD34, HLA-DR, CD14, CD11b, and CD11c. The leukemic cells are large with round or irregular nuclei, abundant large azurophilic granules and multiple Auer rods (lower cell). **C,** AML-M5 (magenta) showing low to intermediate side scatter, intermediate to bright CD45, expression of HLA-DR, CD38, CD33, CD123, CD64, CD11b, CD11c, lysozyme, CD13 (small subset), CD14 (small subset), and no expression of CD117, CD34, and MPO. The blasts are large with round or oval nuclei, fine chromatin, inconspicuous nucleoli and abundant basophilic cytoplasm. **D,** AML-M7 with RAM phenotype (RAM-AML, red) being positive for CD34, CD56 (bright), CD117, CD61 (dim), CD38 (dim to negative), CD45 (dim to negative), and negative for HLA-DR. The blasts are medium to large in size with round or oval nuclei, fine chromatin, inconspicuous nucleoli and cytoplasmic blebs.

separation of the abnormal blast populations, especially in the specimens with a low number of blasts. Blasts often differ from mature cells by expressing immature markers and lacking antigens typically present on mature cells. For example, myeloblasts can be distinguished from mature or maturing myeloid cells by the expression of immature markers such as CD34 and CD117, and the absence of maturation markers such as CD11b, CD15, and CD16. Neoplastic blasts often have an abnormal phenotype that is different from that of normal hematopoietic precursors. Phenotypic abnormalities include altered expression level or pattern of antigens, aberrant expression of markers of another cell lineage, and asynchronous or dyssynchronous expression of antigens. For example, compared with hematogones (normal B-cell precursors in BM), leukemic cells of B-cell ALL (B-ALL) commonly show increased CD10, decreased CD38, aberrant expression of myeloid marker CD13 or CD33, and dyssynchronous co-expression of CD34 and CD20. The knowledge of normal precursor antigen expression patterns is especially important for evaluating post-treatment specimens, which commonly contain normal regenerative precursors and very low-level residual leukemia (14, 15).

Assignment of blast lineage

Accurately and quickly classifying an acute leukemia case as AML, B-ALL or T-ALL is very important for the timely selection of appropriate treatment. Leukemic blasts often show antigen expression profile resembling certain cell type and therefore can be assigned to that lineage by FCM analysis. As shown in Figure 2, lymphoblasts of ALL usually show low SSC and phenotype of precursor B cells or precursor T cells. Leukemic blasts from de novo B-ALL are almost always positive for CD19, cytoplasmic CD22 and cytoplasmic CD79a, and negative for surface Ig. Most of the B-ALL cases are positive for surface CD22, CD10, CD38, TdT and CD24. CD34 is commonly expressed, but its expression is quite variable, and often dim or partial. CD45 is usually dim and can be completely negative. CD20 is commonly negative or partially positive. B-ALL cases can be subclassified based on developmental stage as demonstrated by immunophenotyping, which is of little clinical significance nowadays and not required to be mentioned in pathology reports. For example, the majority of B-ALL cases express CD10 and are called common ALL (intermediate developmental stage); the B-ALL cases negative for CD10 are classified as pro-B ALL (early stage); the B-ALL cases expressing cytoplastic μ chain are classified as Pre-B ALL (late stage). Leukemic blasts from B-ALL can aberrantly express myeloid antigens (usually CD13 and CD33, occasionally CD15), and frequently show abnormal expression patterns of some non-specific markers such as CD58 and CD9 (Figure 2A), which are commonly included in the FCM panel for B-ALL MRD detection. Leukemic blasts of T-ALL are always positive for cytoplasmic CD3, usually positive for TdT, CD7, CD38, CD45 (dim), and variably positive for other pan T-cell antigens (surface CD3, CD2, CD4, CD5, CD8), T-cell receptor, CD10, and other immature markers (CD1a, CD34 and CD99) (Figure 2C). Some T-ALL cases can aberrantly express CD56, CD79a, CD117, or myeloid markers such as CD13 or CD33. In some cases, especially in the specimens collected after treatment, the immature markers can be very dim or completely absent, and the immature nature has to rely on detection of the absence of surface CD3, dual CD4/CD8 positivity or dual CD4/CD8 negativity (11). Early T-cell precursor T-ALL (ETP-ALL, Figure 2D) is defined

by a characteristic phenotype: CD8-, CD1a-, CD5- or dimly+ in <75% blasts, and positive for at least one of the myeloid or stem cell markers (CD11b, CD13, CD33, CD34, CD65, CD117, HLA-DR). ETP ALL is considered a high-risk T-ALL subtype, especially in adult patients (16), and it is listed as a separate entity in the WHO classification (1, 3).

The morphology and phenotype of AML cases vary greatly according to the differentiation direction and maturation level of the blasts. Leukemic blasts of AML with minimal differentiation (FAB-M0) or AML with no maturation (FAB-M1, Figure 3A) usually show low SSC, express early hematopoietic precursor antigens (CD34, CD38, and HLA-DR) and lack antigens associated with myeloid and monocytic maturation, such as CD11b, CD14, CD15, and CD65. Blasts express at least two myeloid-associated antigens, such as CD13, CD33 and CD117. CD38 and/or HLA-DR expression may be decreased. Rare cases may show asynchronous expression of CD11b or CD15. Myeloperoxidase (MPO) is usually positive in AML-M1, negative or minimally positive in AML-M0 by FCM. Blasts can aberrantly express CD7, or rarely other lymphoid markers. The leukemic blasts of AML with maturation (FAB M2) usually express multiple myeloid antigens associated with granulocytic differentiation, such as MPO, CD11b, CD13, CD15, CD33, and CD65. HLA-DR, CD34, CD38 and CD117 are usually positive but can be partially negative. Hypergranular variant of APL (FAB-M3) (Figure 3B) usually shows intermediate to high SSC and is characterized by absence or minimal expression of HLA-DR and CD34, and lack of leukocyte integrins CD11a, CD11b and CD18. CD13, CD33, CD64, CD117 and MPO are usually positive, while the granulocytic maturation markers CD15 and CD65 are usually negative or only weakly positive. In cases with microgranular morphology, there is a frequent expression of CD2 and CD34 by at least a portion of the leukemic cells. Leukemic blasts from AML with monocytic differentiation variably express the myeloid antigens CD13, CD15, CD33 (often bright), and CD65; they always express some monocytic markers such as CD14, CD64, CD4, CD163, CD11b, CD11c, CD36, and lysozyme. Most cases express HLA-DR, CD38, and CD123. CD34 and CD117 are positive in a subset of cases. MPO is often negative in acute monoblastic leukemia (FAB-M5a, Figure 3C), but may be partially positive in acute monocytic leukemia and acute myelomonocytic leukemia. Glycophorin A is the lineage-specific marker for acute erythroid leukemia (FAB M6), which is usually also positive for CD71, CD36, and CD117, and negative for CD34 and HLA-DR. CD41, CD61 and CD42b are specific markers for acute megakaryoblastic leukemia (FAB M7), which is often negative for CD34 and HLA-DR. The abnormal megakaryoblasts are usually positive for CD36, and often express CD13, CD33, and CD117, and may aberrantly express CD7, CD4 or CD56. Surface staining of CD41 and CD61 is less specific than their cytoplasmic staining due to possible adherence of platelets to blasts leading to false positive interpretation (17).

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) expresses CD4, CD56 (bright), CD36, CD38, CD43, CD45, CD71, HLA-DR, as well as plasmacytoid dendritic cell (PDC) associated markers (CD123, CD303, CD304, TCF4, TCL1) (2, 18). CD45 expression may range from dim to moderate. SSC of the tumor cells is usually low. CD7 and CD33 are relatively commonly expressed. TdT is positive in approximately one-third of the cases, and occasional cases may express CD117. Based on the WHO diagnostic criteria (2), BPDCN can be diagnosed in the presence of CD123 and one other PDC-associated marker in addition to CD4 and/or

CD56, or the presence of three PDC-associated markers and the absence of CD34 and other cell type-specific markers including CD3, CD14, CD19, lysozyme, and MPO. Given the presence of other hematologic malignancies with similar phenotypes, correlation with histomorphology, clinical information and IHC studies is always necessary to make a definitive diagnosis of BPDCN.

Lineage classification in some cases can be difficult. There are ~4% of acute leukemia cases with no overt evidence of differentiation along a single lineage. These cases can be separated into two groups: acute undifferentiated leukemias and mixed-phenotype acute leukemias (MPALs, Figure 4). MPAL may have one population of blasts expressing antigens belonging to two lineages (biphenotypic, Figure 4B), or have two distinct blast populations (bilineal, Figure 4C). There are defined criteria for assigning more than one lineage to a leukemic cell population for WHO classification (Figure 4A) (2). To qualify for B-cell lineage, the leukemic blasts with strong CD19 expression should have strong expression of at least one of the following: CD10, CD79a, and cytoplasmic CD22, or the leukemic blasts with weak CD19 expression should have at least two of these three other B-cell markers. For CD19 expression to be considered as strong, the intensity of CD19 in at least part of the leukemic blasts should reach 50% of hematogone level. CD3 (cytoplasmic or surface) is the only lineage-defining marker for T-cell, and its presence at a high intensity (at least partially >50% of the mature T-cell level) is enough to classify the blasts as T-cell lineage. Myeloid lineage is determined by the expression of cytoplasmic MPO, which can be detected by FCM, IHC stain or cytochemical stain. Monocytic lineage is determined by the diffuse expression of at least two of the following: nonspecific esterase, CD11c, CD14, CD64, and lysozyme. The most common combination is a myeloid lineage with a T-cell or B-cell lineage. Other combinations are also possible. No clear cut-off values on the percentage of positive blast cells have been given for these lineage-defining markers. MPO may be present at a low level on a small portion of leukemic lymphoblasts. Without other myeloid markers, this kind of MPO expression should not be interpreted as myeloid and lymphoid MPAL (3, 19). Acute undifferentiated leukemias lack antigens specific for any cell lineages as mentioned above, but often express HLA-DR, CD34, and CD38, and may express TdT.

Enumeration of blasts

FCM study can determine the precise percentage of blasts in the samples. However, the percentage of blasts by FCM often differs from that by the gold standard method — manual differential count of aspirate smears. There are several possible explanations for this discrepancy. First, the BM aspirate specimens submitted for FCM analysis are often not the first draw and contain fewer bone marrow spicules with greater dilution with PB. Second, there are likely unlysed erythrocytes in the sample, which increase the denominator for calculating blast percentage. Third, some blasts may be phenotypically difficult to capture and not included in the blast population. These are all possible reasons why blast percentage by FCM is lower than that by manual differential count. The blast percentage by FCM can also be higher due to decreased denominator resulting from the loss of nucleated erythroid precursors during the RBC lysis process. In addition, FCM analysis can identify neoplastic cells that might be missed by manual count because of atypical morphology or suboptimal smears. Moreover, the manual count may include

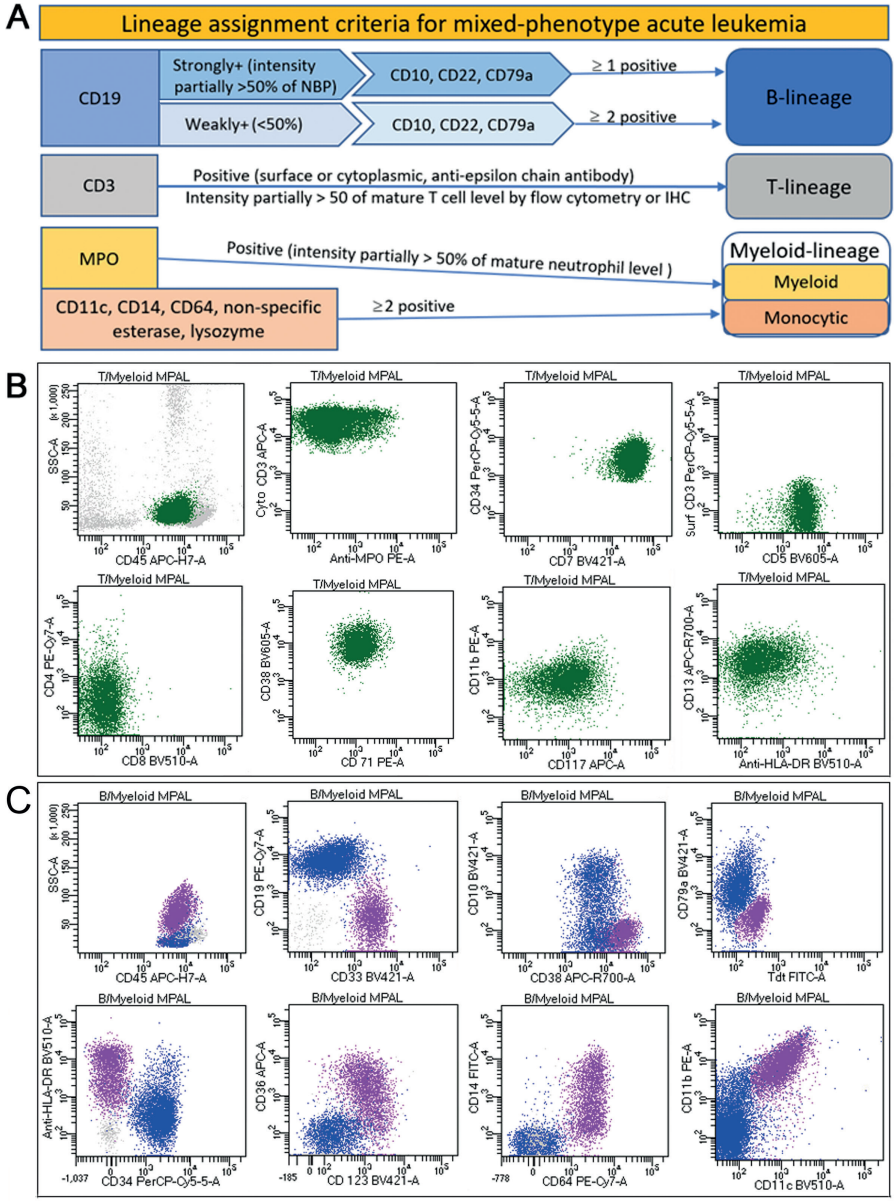

Figure 4. Mixed-phenotype acute leukemia (MPAL). A, WHO lineage assignment criteria for MPAL. NBP, normal B-cell precursor; MPO, myeloperoxidase. **B,** Biphenotypic T-cell/Myeloid MPAL showing one blast population (green) positive for cytoplasmic (Cyto) CD3, MPO (partial), CD7, CD34, CD5, CD4 (partial), CD38, CD71, CD11b, CD117, CD13, HLA-DR (partial), and negative for surface (Surf) CD3 and CD8. **C**, Bilineal B-cell/Monocytic MPAL showing two distinct blast populations: one (purple) with low to intermediate side scatter, positive for CD33, CD38, HLA-DR, CD36 (partial), CD123, CD64, CD14 (partial), CD11b and CD11c, and negative for others; the other (blue) with low side scatter, positive for CD19, CD38, CD10 (partial), CD79a, CD34, CD11b (partial), and negative for others.

some normal immature precursors, such as hematogones, as leukemic blasts. Therefore, it is necessary to perform both FCM and manual count for blast enumeration and investigate the cause of significant discrepancies.

Predicting cytogenetic abnormality and prognosis

Currently, cytogenetic/molecular testing is critical for further WHO classification and risk stratification of acute leukemias. The prognostic value provided by FCM immunophenotyping is most likely attributed to its prediction of certain cytogenetic/molecular subtypes. For example, CD10- B-ALL is commonly seen in B-ALL with *KMT2A* rearrangement, which is also commonly positive for CD15 and has a very poor prognosis. The detection of CRLF2 expression (Figure 2B) is associated with *CRLF2* gene alteration that is commonly seen in Philadelphia-like ALL and associated with poor prognosis (20). AML with t(8; 21)(q22;q22) commonly expresses B-cell markers such as CD19 and is associated with a favorable prognosis (21). Aberrant expression of CD2 in APL is associated with *FLT3–ITD* (22). However, FCM immunophenotyping should not be considered a surrogate tool for the detection of these subtypes due to the lack of specificity and sensitivity. As mentioned above, ETP-ALL (Figure 2D) is defined by the typical early-T-precursor immunophenotype and is associated with poor clinical outcomes in adult patients (16). AML with RAM phenotype (Figure 3D) is defined by the characteristic phenotype (bright CD56 expression, dim to absent CD38, dim to absent CD45, and absent HLA-DR), and is associated with a very poor prognosis (23).

FCM can measure DNA content and assess DNA ploidy for a cell population. This process involves staining cells with a DNA-binding fluorescent dye and then analyzing fluorescent signals of the cells of interest. A histogram is displayed with different ploidy patterns based on the DNA content of the cells in the sample (Figure 5). DNA ploidy is used for further classification of B-ALL and provides

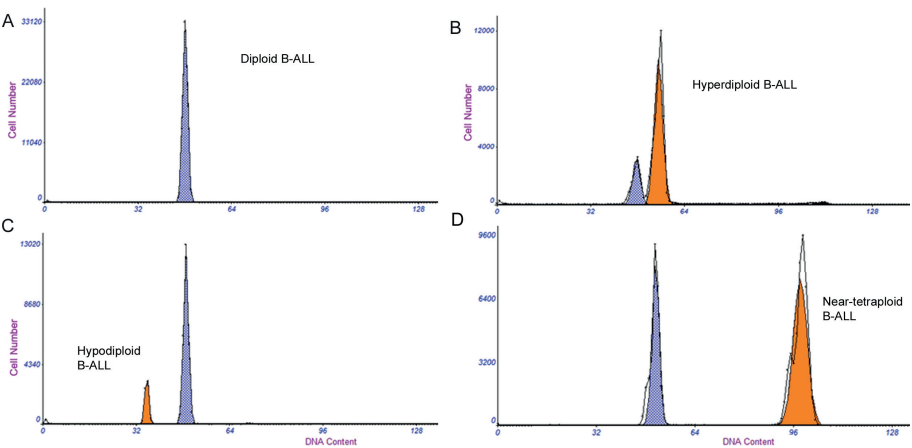

Figure 5. DNA ploidy histogram of precursor B-cell acute lymphoblastic leukemia (B-ALL). **A**, DNA diploid B-ALL (leukemic cells not separated from normal blood cells). **B,** DNA hyperdiploid B-ALL (orange color peak, DNA Index = 1.16). **C,** DNA hypodiploid B-ALL (orange color peak, DI = 0.73). D. Near-tetraploid B-ALL (orange color peak, DI = 1.90).

important prognostic information. High hyperdiploidy in B-ALL (usually DNA index ≥1.16, Figure 5B) is associated with a very favorable prognosis, while hypodiploidy (DNA index <1, Figure 5C) is associated with poor prognosis (1).

FLOW CYTOMETRY IN THE DIAGNOSIS OF MATURE B-CELL LEUKEMIAS

Mature B-Cell neoplasms consist of a heterogeneous group of lymphoid neoplastic diseases derived from B cells, which can be briefly divided into two groups: Hodgkin lymphoma (HL) and non-Hodgkin lymphoma/leukemia /lymphoproliferative disorders (non-HL). Although many of the non-HLs can have a leukemic phase with significant BM and/or PB involvement, the "leukemia" term is only used in the names of a very limited number of non-HLs, i.e., chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL) and Splenic B-cell lymphoma/leukemia with prominent nucleoli (1). FCM is a very powerful tool for diagnosing non-HLs. FCM analysis can efficiently identify an atypical lymphoid population, differentiate neoplastic proliferation from reactive proliferation, characterize the phenotype of the neoplastic cell population, and assist in WHO classification. The neoplastic B cells can be distinguished from normal/reactive B cells by the identification of two main types of phenotypic abnormalities: immunoglobulin light chain restriction and aberrant antigen expression. These two types of abnormalities are present in most of the non-HL cases and can be efficiently identified by FCM analysis. Due to the large size, low proportion of tumor cells and T-cell resetting on tumor cells of HL, FCM is not useful in diagnosing HL.

Immunoglobulin light chain restriction/ B-cell clonality assessment

Neoplastic B cells express only one class of light chain (i.e., kappa or lambda) (Figure 6 A-D), whereas normal or reactive B cells always consist of a mixture of kappa-expressing B cells and lambda-expressing B cells (Figure 6E). The finding of only one class of light chain (light chain restriction) indicates a monoclonal B-cell proliferation. Light chain restriction may occasionally be seen in nonneoplastic reactive B-cell populations (24); therefore, it should not be considered equivalent to monoclonality, or by itself diagnostic of neoplasia. It should always be interpreted in combination with clinical information, morphology, and sometimes cytogenetic or molecular testing results. Identification of a large population of light chain-restricted B cells is usually straightforward, and it is reflected in an abnormal kappa-to-lambda ratio (Figure 6A-D). However, in the specimens with lots of admixed reactive B cells, evaluations of the kappa-to-lambda ratio may fail to identify a small clonal population. To increase the sensitivity, separate evaluations of B-cell subsets with distinct phenotypes and/or light scatter characters should be performed. When evaluating small populations of B cells for light chain restriction, caution should be exercised because reactive B cells may include small subsets of phenotypically identical cells. Some germinal center B (GCB) cells may show the absence of both classes of light chain, or may show one class of light chain excess, and they should not be interpreted as an abnormal CD10+ B-cell population (Figure 6F). It is important to include a prewash step to avoid false

negative results due to soluble antibodies in the samples interfering with the binding of fluorochrome-conjugated antibodies. It should be noted that B-lymphoblasts, plasma cells and thymic B cells are negative for surface Ig, and so are their neoplastic counterparts: B-ALL, plasma cell neoplasms, and primary mediastinal large B-cell lymphoma.

Aberrant B-cell antigen expression

FCM analysis can identify phenotypic deviations of mature B-cell neoplasms from normal antigen expression patterns of B-cells. The most easily recognized phenotypic aberrancy is the presence of antigens not normally expressed by B cells. For example, CD5, a T-cell antigen, can be aberrantly expressed in CLL and mantle cell lymphoma (MCL). It should be noted that a small population of mature B cells normally express CD5. These mature CD5+ B cells are often found in PB but may also be detected in lymph nodes or other tissue specimens, especially in pediatric patients or patients with autoimmune diseases (25). Therefore, interpretation of CD5 expression by B cells needs the correlation with other phenotypic features. Another type of phenotypic aberrancy is the abnormal expression of antigens not typically present in the B cells of a particular subset. For example, normal GCB cells are Bcl-2-, whereas Bcl-2 is positive in most other B-cell subsets. Abnormal Bcl-2 expression is found in most follicular lymphoma (FL) cases, which are derived from GCB cells and express GCB markers CD10 and BCL6. Other phenotypic aberrancies include alteration in the expression level of B-cell-associated antigens. For example, CLL often demonstrates decreased CD20, significantly decreased or absent FMC7, and detectable CD23 (17).

Classification of mature B-cell leukemias/ lymphomas

FCM immunophenotyping plays an important role in the classification of mature B-cell neoplasms. Each type of these diseases has some unique or relatively specific phenotypic features, and FCM findings in combination with histomorphologic features can make the final diagnosis in most cases. Most mature non-plasma cell B-cell neoplasms are positive for B-cell markers CD19, CD20, CD22, CD79a, and surface Ig (sIg), but the expression levels of these markers vary among the neoplasms. CLL is the most common leukemia in the Western world and is characterized by the clonal proliferation of abnormal mature CD5+ B cells in BM, PB, spleen and lymph nodes. CLL has a characteristic phenotype: CD20+ (weak), CD22+ (weak), CD23+ (often moderate to bright), FMC-7-, and sIg+ (weak with light chain restriction). This phenotype is enough to make the diagnosis in conjunction with the typical morphology in the majority of the cases. CD200 is consistently expressed in CLL, but not in MCL. The addition of CD200 in the panel has increased the sensitivity to identify CLL, even for the atypical cases (26). Lymphoid enhancer-binding factor 1 (LEF1) is highly expressed in CLL, but not detected in most other B-cell neoplasms, so it is considered a highly specific marker for CLL (27, 28). Including LEF1 in the FCM panel will increase the sensitivity and specificity for diagnosing CLL. MCL, another CD5+ small lymphocytic neoplasm, can also have significant PB involvement and should be differentiated from CLL. MCL usually shows a phenotype easily distinguished from CLL by FCM: CD20+ (moderate to bright), sIg+ (moderate to bright), CD23- or only

weakly+, FMC-7+, CD200-, LEF1-. The phenotype of MCL is more variable than that of CLL; hence, additional studies are usually needed to confirm the diagnosis. These studies include IHC stain for cyclin-D1 on paraffin section, cytogenetic study to identify the translocation t(11; 14)(q13;q32), or fluorescence in situ hybridization (FISH) analysis for *CCND1* gene rearrangement.

HCL is a rare indolent neoplasm of small mature B cells with characteristic hairy projections. It usually involves PB and diffusely infiltrates the BM and splenic red pulp. HCL has a very distinctive phenotype: CD20+ (bright), CD11c+ (bright), CD25+, CD103+, CD22+ (bright), sIg+ (intermediate to bright with light chain restriction), FMC-7+, CD23-, CD5-, and CD10-. This phenotype together with the characteristic morphology is enough for making the diagnosis of HCL. Occasionally classic HCL may show some deviations from this characteristic phenotype, e.g., CD10+, CD23+, CD103- or CD25-. The presence of phenotypic variations does not necessarily qualify the case as the HCL variant (vHCL) (29). The term vHCL, included in the new entity of splenic B-cell lymphoma/leukemia with prominent nucleoli in the 5th edition WHO classification, has been used to describe the cases with an unusual combination of morphologic, clinical, and phenotypic findings (1). vHCL usually presents with leukocytosis with no monocytopenia, and shows tumor cells with more variable morphology, often lacking the staining for TRAP, and negative for CD25, Annexin A1 and CD200. Marginal zone lymphoma (MZL) is usually distinguished from HCL by the lack of combined positivity for CD11c, CD25 and CD103, and the lack of bright staining for CD20 and CD22. Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of B-cell lymphomas variably positive for B-cell markers with light chain restriction. High FSC is usually present in DLBCL and it reflects the large size of the tumor cells and can be used as a feature to distinguish DLBCL from small mature B-cell neoplasms. It is worth remembering that occasionally any subtype of these B-cell neoplasms can have cases with an atypical phenotype. Correlation with morphologic features, clinical information, and sometimes cytogenetic/molecular findings is always required for accurate interpretation of FCM results.

Enumeration of clonal B cells

FCM numeration of clonal B cells, either through single-platform by using quantification beads or two-platform by using WBC count from hematology analyzer, is important to distinguish monoclonal B-cell lymphocytosis (MBL) from CLL. For the diagnosis of CLL, there must be a monoclonal B-cell count $\geq 5 \times 10^9$/L, with the characteristic morphology and phenotype of CLL in the PB. Individuals with a clonal CLL-like cell count $< 5 \times 10^9$/L and without lymphadenopathy, organomegaly, or other extramedullary disease are considered to have MBL. MBL is defined as the presence of a clonal B-cell population less than 5×10^9/L in the PB without other evidence of lymphoma or lymphoproliferative disorder. Most of the MBL cases present with the immunophenotype of CLL. Depending on the B-cell count, MBL can be classified as low count ($<0.5 \times 10^9$/L) or high count ($>0.5 \times 10^9$/L). Low-count MBL can be detected in up to 5% of adults over 40 years of age. Low-count MBL and high-count MBL have different prognoses, and some different biological and genetic characteristics (30). High-count MBL progresses to CLL at a rate of 1% to 2% per year, while low-count MBL rarely progresses to CLL (3, 30).

Diagnosing plasma cell leukemia

Plasma cell neoplasm (PCN) results from the expansion of a clone of abnormal terminally differentiated B-cells-- plasma cells. Plasma cell leukemia (PCL) is a rare form of PCN in which clonal plasma cells account for >20% of total leukocytes in PB. Identification of a large clonal plasma cell population with restricted cytoplasmic light chain expression is the basis for the diagnosis of PCL. CD38 and CD138 are two commonly used markers for the identification of plasma cells. CD38 is expressed by a wide variety of cell types at a lower intensity than plasma cells, while CD138 is more specific for plasma cells but is less sensitive. The neoplastic plasma cells often demonstrate an abnormal CD19- CD20- phenotype, which is different from most normal CD19+ CD20- plasma cells and CD19+ CD20+ mature B-cell neoplasms. Aberrant CD56 expression is identified in most cases of PCN (31). Decreased CD38 and/or CD138, abnormal gain of CD28, and loss of CD27 are common in PCN, and some PCN cases express CD117 (32). Although FCM is very useful in identifying abnormal plasma cell populations and making a distinction between plasma cell neoplasms and other mature B-cell malignancies, the diagnostic utility of FCM is limited by its difficulties in enumerating plasma cells. FCM usually identifies fewer plasma cells than manual count on the smears or IHC stain on biopsy sections. This discrepancy likely results from sampling difference, loss of plasma cells during sample processing, or a combination of both.

FLOW CYTOMETRY IN THE DIAGNOSIS OF MATURE T/NK CELL LEUKEMIAS

Mature T-cell or NK-cell neoplasms consist of a heterogeneous group of neoplastic diseases derived from T cells or NK cells. The mature T-cell or NK-cell neoplasms with “leukemia” in their names include T-prolymphocytic leukemia (T-PLL), T-large granular lymphocytic leukemia (T-LGL), NK-large granular lymphocytic leukemia (NK-LGL), adult T-cell leukemia/lymphoma (ATLL), aggressive NK-cell leukemia (A-NKL). Other T/NK cell neoplasms, such as Sezary syndrome (SS), also have leukemia presentation or leukemic phase even though they don’t have “leukemia” in their names. FCM is very useful in the diagnosis and classification of mature T/ NK-cell neoplasms. However, it is often more difficult to identify phenotypically abnormal mature T- or NK-cells than abnormal mature B cells, and FCM immunophenotypic features are usually not specific or diagnostic for any subtype of T/ NK-cell neoplasms.

Abnormal mature T-cell or NK-cell population can often be identified by FCM study through the detection of aberrant antigen expression. With the recent availability of specific antibodies against TRBC1, the clonality of an alpha beta T-cell population can also be assessed by a one-tube FCM assay (4). The application of clonality assessment by TRBC1 expression has increased the sensitivity and specificity of FCM analysis in the identification of an abnormal mature T-cell population.

Identification of restricted populations of T cells or NK cells/ clonality assessment

Alteration of CD4/CD8 ratio in T cells is not a useful indicator of clonality or neoplasia since it can be seen in many reactive or medical conditions. However, a significant deviation of the CD4/CD8 ratio from normal can raise concern for the presence of an abnormally restricted T-cell population. For example, an increased CD4/CD8 ratio in PB can lead to further evaluation of the expression of CD26 and CD7 on CD4+ T cells. Significant loss or absence of CD7 and/or CD26 in the CD4+ T-cell population points to the diagnosis of SS. The traditional FCM method for T-cell clonality assessment is to evaluate TCR Vβ repertoire. Normal T-cell populations are composed of a mixture of cells expressing variable Vβ family subtypes, while T-cell neoplasm is the expansion of a clone of T cells with restricted Vβ expression. Since there are more than 20 functional Vβ families and many more subfamilies, this FCM test for TCR Vβ repertoire is complex and labor intensive, and it consumes a large volume of samples and reagents with limited sensitivity. Therefore, it is not widely used in clinical FCM laboratories. FCM assessment of T-cell clonality is significantly improved with the recent advent of the antibody specific for TRBC1, one of two mutually exclusive TCR β-chain constant regions. Normal /reactive alpha beta T cells include a mixture of cells expressing TRBC1 or TRBC2 (TRBC1-) (Figure 6K), while the clonal T-cell population is either TRBC1 positive (Figure 6G-J) or negative. This FCM assay has been tested in many mature alpha beta T-cell neoplasms and proved very useful (4, 5). The panel should include antibodies for TCR αβ and/or TCR γδ besides TRBC1 because TRBC1 is normally negative in γδ T cells, which can serve as an internal negative control (Figure 6L). This assay is not useful for γδ or CD3-negative mature T-cell neoplasms due to the absence of the TCR β chain.

NK cells have no TCR, and hence cannot be assessed for clonality by using the TCR Vβ / TRBC1 FCM test or PCR for TCR gene rearrangement. FCM analysis of NK-receptor (NKR) expression has been developed to seek evidence of NK-cell clonality (33). Normal and reactive NK-cell populations express a variety of NKRs, whereas neoplastic NK-cell populations express a more restricted NKR repertoire.

Aberrant T-cell antigen expression

T-cell neoplasms often demonstrate altered expression of T-cell markers, which is often characterized by the complete loss of one or more pan-T-cell antigens. Rare extreme cases can lack multiple or all T-cell-associated antigens, showing a “null” phenotype, which can make cell lineage determination very difficult. CD5 and CD7 are the most frequently lost antigens in T-cell neoplasms. It should be noted that a small population of non-neoplastic CD7- T cells is well recognized in PB, BM and skin biopsy specimens, and CD7- T cells may expand in some reactive conditions, such as benign dermatoses. In addition, small subsets of T cells may lack CD5, e.g., CD3+CD5- γδ T cells. Some mature T-cell neoplasms can be dual CD4/8- or dual CD4/8+, which is more commonly seen in T ALL/LBL and can also be seen in some normal/reactive T-cell subsets (10, 34).

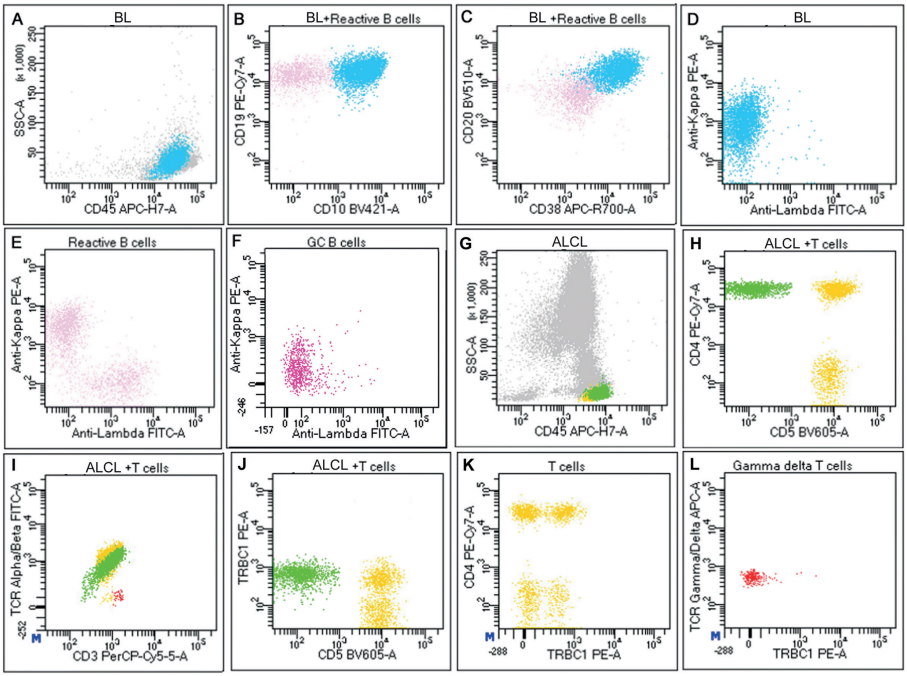

Figure 6. Assessment of clonality of B-cells and T-cells by flow cytometry. Burkitt leukemia (BL, blue) expressing CD45 (**A**), CD19 and CD10 (**B**), CD20 and CD38 (**C**), and showing kappa restriction (**D**). Reactive B cells (light pink) being polytypic (**E**). Germinal center B cells (magenta) showing kappa predominance and partial double-negativity for both kappa and lambda in a thyroid FNA sample from a patient with autoimmune thyroiditis. (**F**). Anaplastic large cell lymphoma (ALCL, green) being CD45+ (**G**), CD4+ and CD5-(**H**), TCR alpha beta+ and CD3+ (**I**), and expressing TRBC1 only (monotypic, **J**). Reactive CD4+ and CD4- T cells (golden) containing mixed TRBC1+ and TRBC1- populations (polytypic, **K**). Gamma delta T cells (red) always negative for TRBC1 (**L**).

The altered Altered staining intensity of T-cell antigens is more frequent than complete loss of them in T-cell and NK-cell neoplasms. However, these changes are more subtle and must be distinguished from the normal or reactive T/NK-cell subsets normally with these variations (10, 34). T/ NK-cell neoplasms can also aberrantly express antigens of other cell types, such as CD15, CD13, and CD33. Again, it should be noted that small subsets of normal/reactive T/NK cells can have markers more specific to other cell types, e.g., subsets of NK cells expressing CD19, CD33 or CD117 (10).

Classification of mature T-cell or NK-cell leukemias/ lymphomas

Although phenotypic heterogeneity exists in nearly all of the T-cell neoplasms, the common or typical features can be identified in most of the cases by FCM. SS and mycosis fungoides (SS/MF) commonly have a CD3+ CD4+ CD26- CD7- T-cell phenotype, which is not specific for these diseases and therefore should

be interpreted in conjunction with the clinical presentation and morphologic features. CD8+ MF exists and is more commonly seen in the pediatric population with a hypopigmented feature. Although SS and MF share lots of similarities in morphology and phenotype, they have different cell origins and should be considered two distinct diseases (35). The normal counterparts of SS tumor cells are circulating central memory T cells, while the counterparts of MF tumor cells are skin-resident memory T cells. ATLL can have a similar CD3+ CD4+ CD7- phenotype to SS/MF, but it usually shows strong CD25 expression and is positive for human T-cell leukemia virus-1 (HTLV-1), the etiologic agent of ATLL. Anaplastic large cell lymphoma (ALCL) primarily involves lymph nodes and skin but may involve BM, PB and any other sites with variable phenotype and morphology. ALCL is commonly CD4+, CD2+, CD25+, usually positive for cytotoxic granule-associated proteins and EMA, and often lacks multiple other T-cell antigens. IHC stains are usually required for the definite diagnosis of ALCL. CD30 is always positive, diffusely, and strongly with membrane and Golgi staining pattern. The diagnosis of ALK+ ALCL is established based on the expression of ALK-1 protein or the identification of *ALK* gene rearrangement. T-LGL usually demonstrates a CD8+ T-cell phenotype with a frequent decrease of CD5 and/or CD7 expression. Most T-LGL cases express CD57, CD16, cytotoxic granule-associated proteins granzyme-B, perforin and TIA-1, while the expression of CD56 is uncommon. A diagnosis of T-LGL is often established by combining this phenotype with morphologic and clinical features. Hepatosplenic T-cell lymphoma (HSTL) is frequently CD4-CD8- (occasionally CD4-CD8+) with the expression of CD56 and no expression of CD57 and CD5. Most HSTL cases express TCR γδ, demonstrate a nonactivated cytotoxic phenotype with expression of TIA-1 but no granzyme-B and perforin, and frequently demonstrate the cytogenetic abnormality of isochromosome 7q (36). T-PLL has a mature T-cell phenotype with expression of surface CD3 and most other T-cell antigens including CD2, CD5, and CD7. T-PLL is frequently CD4+, or dual CD4/8+, negative for CD25 and cytotoxic granule-associated proteins. A diagnosis of T-PLL can be established in most cases by the correlation of immunophenotype with morphology, clinical features and/or demonstration of *TCL1A/B* gene rearrangement. Enteropathy-associated T-cell lymphoma (EATL) is usually negative for CD4, CD5 and CD8, positive for CD3, CD7, CD103, and cytotoxic granule-associated proteins TIA-1, granzyme-B, and perforin. EATL is commonly associated with celiac disease. Monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL) shares some similarities in phenotype with EATL, but it is commonly CD8+, CD56+ and shows no clear association with celiac disease (3, 34, 37). As expected, the phenotype of PTCL, NOS is quite heterogeneous and often overlaps with other mature T-cell neoplasms. Other specified T-cell neoplasm entities must be excluded before a diagnosis of PTCL, NOS can be made.

NK-cell neoplasms, including NK-LGL, A-NKL, extranodal NK/T cell lymphoma (ENK/TCL), and EBV+ NK-cell lymphoproliferative diseases of childhood, have some overlapping phenotypic features, and it is important to distinguish the more aggressive neoplasms (A-NKL and ENK/TCL) from those with a more indolent course. A-NKL and ENK/TCL share many features, including an NK cell phenotype and the presence of EBV. They are usually positive for CD2, CD56, EBV, and cytotoxic granule-associated proteins TIA-1, granzyme-B, and perforin.

Surface CD3 is negative, but cytoplasmic CD3-epsilon is usually positive. CD4, CD5 and CD57 are usually negative (38). The distinction between them is usually made on clinical grounds. A-NKL presents with BM involvement, circulating neoplastic cells, cytopenias and constitutional symptoms, while ENK/TCL is a tissue-based mass lesion, commonly seen in the nasal cavity and surrounding area, and demonstrating angiocentric and angiodestructive growth pattern. NK-LGL, previously named chronic lymphoproliferative disorder of NK cells, is characterized by a persistent increase of NK cells in PB without an identified cause. NK-LGL is rare, not associated with EBV, and usually shows a chronic clinical course with many similarities with T-LGL. By FCM study, NK-LGL usually demonstrates a mature NK-cell phenotype with dim or absent CD2, CD7 and CD57, and restricted or abnormal NK-cell receptor expression (34).

FLOW CYTOMETRY IN THE DIAGNOSIS OF MATURE OR MATURING MYELOID LEUKEMIAS

Maturing myeloid neoplasms are a diverse group of neoplasms composed of neoplastic hematopoietic precursors at different maturation stages. Most of these diseases present as chronic leukemia. The diagnosis of these diseases is based on the combination of clinical information, blood cell counts, histomorphology of BM, and cytogenetic/molecular testing results. Although the aberrant phenotype of hematopoietic precursors is commonly detected in these neoplasms (39, 40), the diagnostic value of FCM immunophenotyping in these neoplasms is limited, and the significance of identifying phenotypic aberrancies in these diseases is not well-characterized. For the cases of most myeloproliferative neoplasms, FCM study is mainly used for the quantification of blasts to assist in detecting accelerated phase or transformation to AML. The advantages and limitations of FCM blast quantification are the same as those mentioned above in acute leukemia, and FCM cannot replace manual blast count.

CONCLUSION

FCM analysis plays a critically important role in the diagnosis of leukemias and other hematolymphoid malignancies. It can efficiently identify abnormal cell populations, differentiate neoplastic cell populations from normal/reactive cell populations, characterize the phenotype, give lineage assignments, and provide important information for WHO classification.

Conflict of Interest: The author declares no potential conflicts of interest with respect to research, authorship and/or publication of this manuscript.

REFERENCES

1. Alaggio R, Amador C, Anagnostopoulos I, Attygalle AD, Araujo IBO, Berti E, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms. Leukemia. 2022;36(7):1720–1748. https://doi.org/10.1038/s41375-022-01620-2
2. Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. Leukemia. 2022;36(7):1703–1719. https://doi.org/10.1038/s41375-022-01613-1
3. Swerdlow SH, Campo E, Harris NL, et al (eds). World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. Revised 4th ed. International Agency for Research on Cancer; Lyon, France: 2017.
4. Berg H, Otteson GE, Corley H, et al. Flow cytometric evaluation of TRBC1 expression in tissue specimens and body fluids is a novel and specific method for assessment of T-cell clonality and diagnosis of T-cell neoplasms. Cytometry B Clin Cytom. 2021;100(3):361–369. https://doi.org/10.1002/cyto.b.21881
5. Horna P, Shi M, Olteanu H, Johansson U. Emerging Role of T-cell Receptor Constant β Chain-1 (TRBC1) Expression in the Flow Cytometric Diagnosis of T-cell Malignancies. Int J Mol Sci. 2021 Feb 12;22(4):1817. https://doi.org/10.3390/ijms22041817
6. Adan A, Alizada G, Kiraz Y, et al. A. Flow cytometry: basic principles and applications. Crit Rev Biotechnol. 2017;37(2):163–176. https://doi.org/10.3109/07388551.2015.1128876
7. McKinnon KM. Flow Cytometry: An Overview. Curr Protoc Immunol. 2018;120:5.1.1–5.1.11. https://doi.org/10.1002/cpim.40
8. Wood B. 9-color and 10-color flow cytometry in the clinical laboratory. Arch Pathol Lab Med. 2006;130(5):680–90. https://doi.org/10.5858/2006-130-680-CACFCI
9. Wood BL, Arroz M, Barnett D, et al. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: optimal reagents and reporting for the flow cytometric diagnosis of hematopoietic neoplasia. Cytometry B Clin Cytom. 2007;72 Suppl 1:S14–22. https://doi.org/10.1002/cyto.b.20363
10. Li W, Morgan R, Nieder R, et al. Normal or reactive minor cell populations in bone marrow and peripheral blood mimic minimal residual leukemia by flow cytometry. Cytometry B Clin Cytom. 2021;100(5):590–601. https://doi.org/10.1002/cyto.b.21968
11. DiGiuseppe JA, Wood BL. Applications of Flow Cytometric Immunophenotyping in the Diagnosis and Posttreatment Monitoring of B and T Lymphoblastic Leukemia/Lymphoma. Cytometry B Clin Cytom. 2019;96(4):256–265. https://doi.org/10.1002/cyto.b.21833
12. Weir EG, Borowitz MJ. Flow cytometry in the diagnosis of acute leukemia. Semin Hematol. 2001;38(2):124–38. https://doi.org/10.1016/S0037-1963(01)90046-0
13. Borowitz MJ, Guenther KL, Shults KE, et al. Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis. Am J Clin Pathol. 1993;100(5):534–40. https://doi.org/10.1093/ajcp/100.5.534
14. Loken MR, van de Loosdrecht A, Ogata K, et al. Flow cytometry in myelodysplastic syndromes: report from a working conference. Leuk Res. 2008;32(1):5–17. https://doi.org/10.1016/j.leukres.2007.04.020
15. McKenna RW, Asplund SL, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) and neoplastic lymphoblasts by 4-color flow cytometry. Leuk Lymphoma. 2004;45(2):277–85. https://doi.org/10.1080/1042819031000151950
16. Jain N, Lamb AV, O'Brien S, et al. Early T-cell precursor acute lymphoblastic leukemia/lymphoma (ETP-ALL/LBL) in adolescents and adults: a high-risk subtype. Blood. 2016;127(15):1863–9. https://doi.org/10.1182/blood-2015-08-661702
17. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood. 2008;111(8):3941–67. https://doi.org/10.1182/blood-2007-11-120535
18. Julia F, Dalle S, Duru G, et al. Blastic plasmacytoid dendritic cell neoplasms: clinico-immunohistochemical correlations in a series of 91 patients. Am J Surg Pathol. 2014;38(5):673–80. https://doi.org/10.1097/PAS.0000000000000156

19. Guy J, Antony-Debré I, Benayoun E, et al. Flow cytometry thresholds of myeloperoxidase detection to discriminate between acute lymphoblastic or myeloblastic leukaemia. Br J Haematol. 2013;161(4):551–5. https://doi.org/10.1111/bjh.12277
20. Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N Engl J Med. 2014;371(11):1005–15. https://doi.org/10.1056/NEJMoa1403088
21. Chen X, Cherian S. Acute Myeloid Leukemia Immunophenotyping by Flow Cytometric Analysis. Clin Lab Med. 2017 Dec;37(4):753–769. https://doi.org/10.1016/j.cll.2017.07.003
22. Takenokuchi M, Kawano S, Nakamachi Y, et al. FLT3/ITD associated with an immature immunophenotype in PML-RARα leukemia. Hematol Rep. 2012;4(4):e22. https://doi.org/10.4081/hr.2012.e22
23. Eidenschink Brodersen L, Alonzo TA, Menssen AJ, et al. A recurrent immunophenotype at diagnosis independently identifies high-risk pediatric acute myeloid leukemia: a report from Children's Oncology Group. Leukemia. 2016;30(10):2077–2080. https://doi.org/10.1038/leu.2016.119
24. Kroft SH. Monoclones, monotypes, and neoplasia pitfalls in lymphoma diagnosis. Am J Clin Pathol. 2004 Apr;121(4):457–9. https://doi.org/10.1309/RR8AXUWM9FYX178A
25. Shirai T, Hirose S, Okada T, et al. CD5+ B cells in autoimmune disease and lymphoid malignancy. Clin Immunol Immunopathol. 1991;59(2):173–86. https://doi.org/10.1016/0090-1229(91)90016-4
26. Sandes AF, de Lourdes Chauffaille M, Oliveira CR, et al. CD200 has an important role in the differential diagnosis of mature B-cell neoplasms by multiparameter flow cytometry. Cytometry B Clin Cytom. 2014;86(2):98–105. https://doi.org/10.1002/cytob.21128
27. Amador-Ortiz C, Goolsby CL, Peterson LC, et al. Flow cytometric analysis of lymphoid enhancer-binding factor 1 in diagnosis of chronic lymphocytic leukemia/small lymphocytic lymphoma. Am J Clin Pathol. 2015;143(2):214–22. https://doi.org/10.1309/AJCPT32JDFIGHFHJ
28. Menter T, Dirnhofer S, Tzankov A. LEF1: a highly specific marker for the diagnosis of chronic lymphocytic B cell leukaemia/small lymphocytic B cell lymphoma. J Clin Pathol. 2015;68(6):473–8. https://doi.org/10.1136/jclinpath-2015-202862
29. Cessna MH, Hartung L, Tripp S, et al. Hairy cell leukemia variant: fact or fiction. Am J Clin Pathol. 2005;123(1):132–8. https://doi.org/10.1309/8QYTYQ1CLQMHQ9CL
30. Strati P, Shanafelt TD. Monoclonal B-cell lymphocytosis and early-stage chronic lymphocytic leukemia: diagnosis, natural history, and risk stratification. Blood. 2015;126(4):454–62. https://doi.org/10.1182/blood-2015-02-585059
31. Lin P, Owens R, Tricot G, Wilson CS. Flow cytometric immunophenotypic analysis of 306 cases of multiple myeloma. Am J Clin Pathol. 2004;121(4):482–8. https://doi.org/10.1309/74R4TB90BUWH27JX
32. Raja KR, Kovarova L, Hajek R. Review of phenotypic markers used in flow cytometric analysis of MGUS and MM, and applicability of flow cytometry in other plasma cell disorders. Br J Haematol. 2010;149(3):334–51. https://doi.org/10.1111/j.1365-2141.2010.08121.x
33. Morice WG, Kurtin PJ, Leibson PJ, Tefferi A, Hanson CA. Demonstration of aberrant T-cell and natural killer-cell antigen expression in all cases of granular lymphocytic leukaemia. Br J Haematol. 2003;120(6):1026–36. https://doi.org/10.1046/j.1365-2141.2003.04201.x
34. Craig JW, Dorfman DM. Flow Cytometry of T cells and T-cell Neoplasms. Clin Lab Med. 2017;37(4):725–751. https://doi.org/10.1016/j.cll.2017.07.002
35. Campbell JJ, Clark RA, Watanabe R, et al. (2010). Sezary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. Blood. 116:767–71. https://doi.org/10.1182/blood-2009-11-251926
36. Yabe M, Miranda RN, Medeiros LJ. Hepatosplenic T-cell Lymphoma: a review of clinicopathologic features, pathogenesis, and prognostic factors. Hum Pathol. 2018;74:5–16. https://doi.org/10.1016/j.humpath.2018.01.005
37. Chen C, Gong Y, Yang Y, Xia Q, Rao Q, Shao Y, et al. Clinicopathological and molecular genomic features of monomorphic epitheliotropic intestinal T-cell lymphoma in the Chinese population: a study of 20 cases. Diagn Pathol. 2021;16(1):114. https://doi.org/10.1186/s13000-021-01173-5

38. El Hussein S, Patel KP, Fang H, Thakral B, Loghavi S, Kanagal-Shamanna R, et al. Genomic and Immunophenotypic Landscape of Aggressive NK-Cell Leukemia. Am J Surg Pathol. 2020;44(9):1235–1243. https://doi.org/10.1097/PAS.0000000000001518
39. Kern W, Bacher U, Schnittger S, et al. Multiparameter flow cytometry reveals myelodysplasia-related aberrant antigen expression in myelodysplastic/myeloproliferative neoplasms. Cytometry B Clin Cytom. 2013;84(3):194–7. https://doi.org/10.1002/cyto.b.21068
40. Ouyang J, Zheng W, Shen Q, et al. Flow cytometry immunophenotypic analysis of Philadelphia-negative myeloproliferative neoplasms: Correlation with histopathologic features. Cytometry B Clin Cytom. 2015;88(4):236–43. https://doi.org/10.1002/cytob.21215