Acute lymphoblastic leukemia (ALL), a malignancy of immature lymphoid cells, comprises approximately 80% of pediatric acute leukemias and 20% of adult cases [1]. The current World Health Organization (WHO) classification for malignancies of hematopoietic and lymphoid tissues [2] recognizes two diagnostic entities: precursor B-cell ALL and precursor T-cell ALL, both of which encompass a spectrum of clinical, morphologic, immunophenotypic, cytogenetic, and molecular features. Although Burkitt’s lymphoma/leukemia is considered a mature B-cell neoplasm in the WHO classification [2], in the earlier French-American-British (FAB) system [3], this tumor was included among the ALLs (designated L3 ALL) and also is discussed in this article.

In current clinical practice, flow cytometric immunophenotyping (FCI) plays an important role in the diagnosis of patients who have ALL. The morphologic characteristics of lymphoblasts may be indistinguishable from those of the myeloblasts in many cases of acute myeloid leukemia (AML), and treatment protocols for ALL and AML differ significantly. Because FCI enables rapid (within hours), typically unambiguous characterization of leukemic blasts with respect to lineage, this modality has become a routine component of the diagnostic work-up of patients who have suspected acute leukemia. Moreover, complete immunophenotypic characterization of the lymphoblasts at diagnosis may predict associated
cytogenetic and molecular abnormalities and serve as a reference point for the detection of residual disease after therapy.

Gating

Samples submitted for FCI are varied, and may include blood, bone marrow, lymph node, extranodal tissues, and body fluids. Although the diagnostic sample in patients who have ALL often contains a preponderance of leukemic blasts, in many cases, samples submitted for FCI contain varying proportions of neoplastic and benign (normal) cells. Because FCI is multiparametric, it is ideally suited for the analysis of such heterogeneous samples and permits the simultaneous distinction from non-neoplastic cells of the leukemic blasts and the description of their composite immunophenotype.

Historically, the isolation of a cellular population of interest for detailed characterization by FCI, or gating, relied on the intrinsic light scatter properties of that population. For instance, in most cases of ALL, the lymphoblasts are contained within a mononuclear cell gate, described by low to intermediate forward-angle light scatter and low right-angle light scatter (SSC) (Fig. 1A). Because lymphocytes and monocytes (and in bone marrow, erythroid precursors) also are contained within the mononuclear cell gate, however, the quality of the composite immunophenotype described depends on the proportion of neoplastic cells within the gate (Fig. 1B). When CD45 fluorescence intensity is plotted against SSC, however, leukemic blasts characteristically are more dimly CD45+ than mature lymphocytes, and, in some cases, completely negative for CD45 (Fig. 1C–E) [4].

Although lymphoblasts generally are more dimly CD45+ than mature lymphocytes, there is substantial heterogeneity in CD45 fluorescence intensity among (and sometimes within) cases of ALL. In precursor B-cell ALL, CD45 expression may range from undetectable (see Fig. 1C) to slightly dimmer than that of mature lymphocytes (Fig. 2). (In this regard, quantitative CD45 fluorescence intensity is an independent prognostic factor in childhood precursor B-cell ALL [5].) CD45 expression in cases of precursor T-cell ALL commonly is brighter than that seen in precursor B-cell ALL (see Fig. 2C) and may even overlap with that of mature lymphocytes. In Burkitt’s lymphoma, CD45 fluorescence intensity is comparable to that of mature lymphocytes, in keeping with the maturational state of the neoplastic B cells in that disorder (see Fig. 2D).

Reagents

The assignment of lineage (ie, B, T, or myeloid) in acute leukemia is one of the principal diagnostic applications of clinical flow cytometry. To accomplish this objective, a panel of fluorochrome-conjugated antibodies, including antibodies recognizing B-lymphoid, T-lymphoid, myeloid, and
Fig. 1. Conventional mononuclear cell gate (A) contains a mixture of cells positive for CD7 and CD19 (B). CD45/SSC gating (C) resolves blasts, which are CD19+ (D), from lymphocytes, most of which are CD7+ (E).
nolineage-related antigens, is applied in combinations deemed to be informative. A panel of antibodies is required, because no single surface membrane antigen is entirely sensitive or specific for a particular type of acute leukemia (reviewed in Refs. [1,6–9]). In some cases, evaluation of cytoplasmic antigens also may be indicated.

Precursor B-cell acute lymphoblastic leukemia

CD19 and CD22 are the B-lineage–related antigens expressed most commonly on the cell membrane in cases of precursor B-cell ALL; in general, CD19 is brightly positive, whereas membrane expression of CD22 tends to be dim. Each of these antigens also may be positive in AML, however. Although CD20 shows somewhat greater specificity for precursor B-cell ALL than CD19 or CD22, it is less sensitive than these antigens. The common ALL antigen, CD10, and progenitor cell antigen, CD34, are positive in most cases of precursor B-cell ALL, but neither is lineage specific. Similarly, HLA-DR and the nuclear antigen, terminal deoxynucleotidyl transferase

Fig. 2. Examples of CD45/SSC gating in precursor B-cell ALL (A, B), precursor T-cell ALL (C), and Burkitt’s lymphoma/leukemia (D).
(TdT), are positive in virtually all (although not all) [10] cases of precursor B-cell ALL but are not informative with respect to lineage.

The most common challenge with respect to lineage assignment in precursor B-cell ALL is the expression of one or more myeloid antigens in a significant minority of cases. (In this regard, although myeloid antigen expression may correlate with the presence of recurrent translocations in precursor B-cell ALL [discussed later], the weight of current evidence argues against myeloid antigen expression, per se, as a prognostic factor in either B-lineage or T-lineage ALL [11,12].) In most cases, such “aberrant” myeloid antigen expression is dim or partial (in contrast with the bright, homogeneous expression usually observed with CD19, for instance) and should not be interpreted as evidence of ambiguous lineage (Fig. 3). In the small proportion of cases in which lineage remains uncertain on the basis of surface membrane antigens, however, cytoplasmic CD22, a sensitive and specific marker of B-lineage differentiation, may be evaluated. Although some have suggested CD79a as a sensitive and specific cytoplasmic marker for B-cell lineage [13],

![Fig. 3. (A–D) Precursor B-cell ALL with dim/partial myeloid antigen expression, gated on blasts using CD45/SSC.](image-url)
this antigen also has been detected in some cases of precursor T-cell ALL [14] and AML [15,16]; on balance, therefore, cytoplasmic CD22 may be superior to cytoplasmic CD79a for this application.

Burkitt’s lymphoma/leukemia

Acute leukemias of B-lineage may be categorized with respect to their expression of immunoglobulin molecules (reviewed in Ref. [6]). Specifically, precursor B-cell ALL is characterized by lack of surface membrane immunoglobulin heavy and light chains and is divisible into early precursor B-cell (cytoplasmic $\mu^-$) and pre-B-cell (cytoplasmic $\mu^+$) types, whereas mature B-cell ALL is defined by surface membrane expression of a complete immunoglobulin molecule. In general, the immunologic category of “mature B-cell ALL” corresponds with L3 ALL, as defined in the FAB classification [3], and with Burkitt’s lymphoma/leukemia, as defined in the WHO classification [2].

Cytoplasmic and surface membrane immunoglobulin expression cannot be relied on solely, however, to the exclusion of other diagnostic features (eg, morphology, immunophenotype, or cytogenetics) in the diagnosis and classification of B-lineage ALL. For instance, cases with a precursor B-cell immunophenotype that, nonetheless, display FAB-L3 morphology and a MYC translocation are described; these are believed equivalent to Burkitt’s lymphoma/leukemia [17]. Moreover, cases of otherwise typical precursor B-cell ALL, distinguished only by monotypic surface membrane immunoglobulin light chain expression, are reported in children and adults [18–20]. Finally, there are cases of B-lineage ALL with neither FAB-L3 morphology nor a MYC translocation that express cytoplasmic and surface $\mu$ heavy chain, without an accompanying light chain; these cases, termed, transitional pre-B-cell ALL, seem to respond well to standard therapy for precursor B-cell ALL [21].

In addition to the clinical, morphologic, and cytogenetic features that distinguish Burkitt’s lymphoma from precursor B-cell ALL [2], there are substantial immunophenotypic differences between these two entities. CD45 expression in Burkitt’s lymphoma generally is brighter than that of precursor B-cell ALL and more closely approximates that of mature B cells. Markers of lymphoid immaturity (ie, CD34 and TdT) are negative, whereas mature B-cell antigens that may be negative or only partially positive in precursor B-cell ALL (eg, CD20 and CD22) are homogeneously and brightly positive. CD10 typically is positive, and (discussed previously) surface membrane immunoglobulin expression (including monotypic light chain expression) is detectable in almost all cases.

Precursor T-cell acute lymphoblastic leukemia

CD2 and CD7 are the most commonly expressed cell membrane antigens in precursor T-cell ALL, but they also are positive in a significant minority
of cases of AML; surface CD3 and CD5 are more specific, although less sensitive [1,6,7,9]. Cytoplasmic CD3 is sensitive and specific for T lineage. This reagent may be useful particularly in cases with minimal expression of T-lineage surface membrane antigens and cases in which myeloid antigen expression complicates lineage assignment. With respect to nonlineage-related antigens, HLA-DR usually is negative in precursor T-cell ALL, in contrast with its nearly uniform expression in precursor B-cell ALL. CD10, CD34, and TdT are positive in variable proportions of cases, although in general, any of these is more likely to be negative in precursor T-cell ALL (in particular those with immunophenotypic evidence of maturation) than in precursor B-cell ALL.

The cortical thymic antigen, CD1a, and T-cell subset antigens, CD4 and CD8, may be expressed in a variety of patterns and have been used (in conjunction with other antigens) to subclassify cases of precursor T-cell ALL that recapitulate different stages in T-cell maturation, including: early/prethymic (CD1a−, CD3−, CD4−, and CD8−), middle/thymic (CD1a+, CD3−, CD4+, and CD8+) (Fig. 4), and late/mature (CD1a−, CD3+, CD4+, or CD8+). In practice, however, many cases of precursor T-cell ALL do not conform precisely to any of these stages. Nonetheless, studies in children and adults suggest that composite immunophenotypes reflective of maturation in precursor T-cell ALL may be associated with a more favorable prognosis, independent of other risk factors, relative to cases with an early/prethymic immunophenotype [22,23].

**Immunophenotypic-genotypic correlations**

In precursor B-cell ALL, certain composite immunophenotypes are shown to correlate to different extents with recurrent karyotypic or molecular abnormalities. For example, the composite immunophenotype, CD9+, CD10+, CD19+, with no more than partial CD20 expression, and complete absence of CD34, which describes less than 10% of cases of precursor B-cell ALL overall, is a highly sensitive (although not completely specific) indicator of the t(1;19)(q23;p13) [24]. In contrast, CD10 is negative or expressed only partially in greater than 95% of cases of childhood precursor B-cell ALL with the t(4;11)(q21;q23); the specificity of a CD10−/partial+ immunophenotype for the t(4;11) is improved substantially (albeit at the expense of sensitivity) when there also is at least partial expression of CD15 and at least a subset of CD24− blasts [25]. A similar composite immunophenotype, CD10−, CD15+, CD19+, CD24−/partial+, CD65s+, has been described in association with precursor B-cell ALL harboring the t(4;11) in adults [26,27]. An additional immunophenotypic-genotypic correlate is that most cases of pediatric and adult ALL harboring the t(4;11) express the chondroitin sulfate proteoglycan recognized by monoclonal antibody 7.1 [27,28].
Other immunophenotypic-genotypic associations in precursor B-cell ALL have been described, but generally these are characterized by a lower sensitivity or specificity than those described previously. Expression of CD13 or CD33 is more frequent in cases of childhood precursor B-cell ALL.
ALL harboring a cryptic t(12;21)(p13;q32) (the most common recurrent translocation seen in pediatric ALL) than in cases without a detectable fusion transcript [12,29]; however, a threshold for myeloid antigen positivity with a high specificity for the translocation seems to have an unsuitably low sensitivity (approximately 25%) [30]. In a different study, a composite CD10+ precursor B-cell immunophenotype with absence or only partial expression of CD9 and CD20 was found to have a sensitivity for the t(12;21) approaching 90% and a specificity approximating 70% [31]. In adult precursor B-cell ALL with a BCR/ABL translocation, CD25 positivity is reported more frequent compared with BCR/ABL− ALL, but there seems to be significant overlap between BCR/ABL+ and BCR/ABL− cases with respect to CD25 expression [32]. In another series, however, all 12 cases of adult precursor B-cell ALL with a BCR/ABL translocation were homogeneously positive for CD10 and CD34, with heterogeneous, generally dim CD38 and aberrant CD13 expression, a composite immunophenotype observed in less than 5% of the BCR/ABL− cases (pediatric and adult) included in the study [33].

**Minimal residual disease—introduction**

In addition to its more traditional roles in the diagnosis and classification of ALL, FCI is useful in the evaluation of response to therapy. In patients who have been treated and are considered to be in remission by standard morphologic criteria (ie, less than 5% bone marrow blasts), small numbers of residual leukemic cells, nonetheless, are detectable in some patients when sensitive immunophenotypic or molecular methods are used. In principle, the detection of such minimal residual disease (MRD) might identify patients at elevated risk for relapse and afford an opportunity to intervene with more precisely risk-adapted therapy.

The two techniques used most widely for the detection of MRD in ALL are FCI and polymerase chain reaction (PCR) amplification of antigen receptor genes. Although the sensitivity of PCR-based methods may exceed that of FCI (0.01% or 1 in 10,000 cells), several studies have shown remarkable concordance between these two methods [34–36]. Moreover, there are several practical advantages of FCI over PCR; specifically, FCI is more rapid, has the ability to discriminate viable from apoptotic cells, and permits direct quantification of the target cells (as opposed to the extrapolation inherent in amplification-based methods) [37].

**Minimal residual disease—detection by flow cytometry**

Although specific methods for the flow cytometric detection of MRD (sample preparation, antibody combinations, and so forth) vary among investigators; the fundamental principle underlying the detection of MRD by
FCI is that leukemic cells differ from their normal counterparts in their qualitative or quantitative expression of one or more antigens (reviewed in Refs. [37–41]). For example, in patients who have ALL, aberrant myeloid antigen expression may complicate lineage assignment at the time of diagnosis; however, such aberrant myeloid antigen expression also serves as an immunologic marker of neoplastic lymphoblasts (Fig. 5). Similarly, coexpression of TdT and cytoplasmic CD3 or a thymic T-cell immunophenotype (eg, CD1a+, CD4+, CD8+) (Fig. 6) would be abnormal in bone marrow or peripheral blood and might, therefore, be useful in the immunophenotypic detection of MRD in T-lineage ALL. (In this context, peripheral blood and bone marrow specimens seem to harbor similar levels of MRD in patients who have T-lineage ALL but not precursor B-cell ALL [42].)

Because of the immunophenotypic diversity characteristic of ALL, antibody combinations that are informative in one case may not provide useful information in another. Although complete immunophenotypic characterization at the time of diagnosis yields one or more targets suitable for

Fig. 5. MRD (approximately 1%) in precursor B-cell ALL with aberrant CD13 and CD33. Leukemic blasts are highlighted in black; normal marrow elements are shown in gray.
subsequent MRD detection in greater than 90% of cases of ALL [37], the stability of such leukemia-associated immunphenotypes has an impact on the likelihood of obtaining a falsely negative MRD result because of immunophenotypic drift or shift. Such changes in immunophenotype at relapse may occur [43–45], but they generally do not preclude the immunophenotypic detection of MRD, provided the use of multiple antibody combinations and an appropriate degree of flexibility in the analysis of the flow cytometric data [46–49].

Because the detection of aberrant or leukemia-associated immunophenotypes requires at least prior knowledge of the composite immunphenotype (and, ideally, the original flow cytometric data to permit direct comparison), this approach to MRD detection is difficult in cases in which the diagnostic immunophenotypic data are not available at the time of the MRD assay. An alternative approach to MRD detection in precursor B-cell ALL that does not require any previous immunophenotypic data is the evaluation of potential deviations from normal B-cell maturation [50]. In patients who have B-lineage ALL, MRD must be distinguished from normal B-cell precursors,
or hematogones. Although the percentage of hematogones in the bone marrow varies with age and several other clinical variables [51], the immunophenotypic pattern of B-cell maturation is precise (Fig. 7) [50,51], and in the majority of cases of precursor B-cell ALL, diminished or increased expression (relative to normal B-cell precursors) of several antigens, including CD10, CD19, CD22, CD34, CD38, CD45, and CD58, is detectable in at least a subset of blasts (Fig. 7) [50,52].

The exquisite sensitivity of normal B-cell precursors to glucocorticoids and other chemotherapeutic agents may facilitate the detection of MRD at specific times during therapy [53]. In a series of patients undergoing remission induction for T-lineage ALL, normal B-cell precursors (defined

Fig. 7. MRD (approximately 2%) in precursor B-cell ALL. Gated leukemic blasts with abnormally bright CD10 (A) and CD58 (B) are highlighted in blue. Gated normal B-cell precursors from a control patient are illustrated for comparison in yellow. (C) MRD (approximately 0.2% to 0.3%) in precursor B-cell ALL. Gated leukemic blasts with diminished CD38 expression are highlighted in red. Gated normal B-cell precursors from a control patient are illustrated for comparison in yellow. (D) MRD (approximately 0.05%) in precursor B-cell ALL. Gated leukemic blasts with diminished CD10 are highlighted in green. Gated normal B-cell precursors from a control patient are illustrated for comparison in yellow.
immunologically as CD19+ cells with coexpression of CD10 or CD34) were not detectable at levels above the sensitivity of a standard flow cytometric assay (ie, 0.01%) on day 19 of induction therapy [53]. In patients who have precursor B-cell ALL (greater than 95% of which are CD19+ with coexpression of CD10 or CD34) [53], cells detected at day 19 of remission induction with this immunophenotype, therefore, would be expected to represent leukemic cells rather than normal B-cell precursors. In keeping with this prediction, a simple three-color flow cytometric assay for CD19+ cells with CD10 or CD34 coexpression performed at day 19 correlated well with a more complicated four-color assay designed specifically to identify leukemia-associated immunophenotypes [53].

The sensitivity of MRD detection by FCI generally is one abnormal cell in 10,000 (0.01% or 10^-4). It is essential to recognize, however, that to achieve this level of sensitivity, many more events must be acquired than required for routine immunophenotyping. As an example, to recognize a cluster of events in a dot plot as a distinct population of abnormal cells, a minimum of 20 to 30 such events must be present. Assuming that these events are present at a frequency of 10^-4, a total of 200,000 to 300,000 total events must be acquired in this example to permit the detection of MRD.

**Minimal residual disease—clinical implications**

A substantial body of evidence exists that the detection of MRD by FCI has independent prognostic value in patients who have ALL. Despite differences in chemotherapeutic regimens, times at which MRD is measured, and immunophenotypic protocols, studies of the prognostic value of MRD detection by FCI in pediatric and adult ALL have yielded remarkably concordant findings. In an early study from St. Jude Children’s Research Hospital, the presence of detectable (0.01% or greater) MRD in bone marrow samples from children who had ALL in morphologic remission was predictive of a greater risk for relapse at the end of remission induction and at all other time points measured during the first year of continuation therapy [54]. Moreover, the risk for relapse increased progressively with the percentage of MRD detected, and this relationship between MRD level and risk for relapse persisted after exclusion of patients who had low-risk and high-risk disease [54]. Similarly, in a multicenter study of children treated with the ALL–Berlin-Frankfurt-Munster 95 protocol, the presence and level of MRD at day 33 and week 12 of induction were predictive of relapse independent from conventional risk factors [55]. In a third pediatric study, MRD status, but not age, white blood cell count, or cytogenetic group persisted in multivariate analysis as a significant risk factor for relapse [56]. Further, rapid clearance of leukemic cells, as determined by lack of detectable MRD on day 19 of remission, identifies a cohort of children who have ALL whose risk for relapse is extremely low [57]. Even in children who have
ALL in first relapse, MRD status after reinduction remains an independent risk factor for subsequent relapse [58]. Fewer studies have addressed the prognostic significance of MRD detection by FCI in adults who have ALL, but in this population also, MRD detection seems to be an independent risk factor for relapse [59,60].

By identifying prospectively patients who have comparatively lower or higher risk for relapse, the immunophenotypic detection and quantification of MRD in patients who have ALL holds the potential to allow more precise risk-adjusted treatment. As yet, there are no data demonstrating that tailoring therapy on the basis of MRD status improves outcome in higher-risk patients or reduces toxicity without sacrificing efficacy in lower-risk patients. MRD status by FCI at day 29 of induction therapy, however, has been incorporated into a risk- and response-based classification scheme currently used by the Children’s Oncology Group to stratify patients for different postinduction therapies [61]. As the data from these and other such trials mature, a more complete understanding of the clinical implications of the detection of MRD by FCI in patients who have ALL should emerge.

References


