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# Flow Cytometry in the Diagnostic Laboratory Workup of Acute Lymphoblastic Leukemias

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## Abstract

Acute lymphoblastic leukemias (ALLs) are hematological neoplasms characterized by clonal proliferation of lymphoid blasts, which can be B- or T-cell type. Flow cytometric immunophenotyping is an integral component in establishing blast lineage during the diagnostic workup of ALLs, aiding in appropriate therapy, prognostication, and monitoring of the disease. The current review focuses on the utility of flow cytometry in the workup of ALLs, including the usefulness of various antibodies and pitfalls in diagnosis.

**Keywords**

- acute lymphoblastic leukemia
- flow cytometry
- B-ALL
- T-ALL

## Introduction

Multiparametric flow cytometry is an indispensable tool for the diagnosis and subclassification of acute lymphoblastic leukemia (ALL). Accurate classification of ALLs into B- or T-cell types is crucial for the optimal choice of therapeutic regimens that varies based on the ALL subtype. The antigenic expression profile, particularly the immunophenotypic aberrancies by the blasts deviating from those encountered during normal hematopoiesis, aids in the differentiation of the blasts from their normal benign counterparts. The panel of antigens for clinical testing has evolved from 4 to 13 colors or more thanks to the substantial development in antibody clones, the fluorochrome conjugate options, and a wide variety of lasers that have dramatically increased the number of antigens that can be simultaneously studied. In this article, we attempt to discuss the strategy and approach to the classification of ALL into B- or T-cell subtypes and the evolution of consensus groups for antigen/antibody/fluorochrome selection, choice of reagents, sample processing methodology for appropriate diagnosis, and classification.

## B-Acute Lymphoblastic Leukemias

B-lymphoblastic leukemia/lymphoma or B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common malignancy seen in childhood. Approximately 75% of BCP-ALL cases occur in children under 6 years of age.[1] However, it shows a bimodal age distribution, with a small peak occurring during the fifth decade of life.[2] BCP-ALL is diagnosed by morphology combined with immunophenotyping, typically done by multiparametric flow cytometer (MFC). Immunophenotyping is essential for differentiating BCP-ALL from acute leukemias of other lineages, like T-lymphoblastic leukemia (T-ALL), acute myeloid leukemia (AML), mixed phenotype acute leukemia (MPAL), etc.

The flow cytometric diagnosis of BCP-ALL is based on identifying an expanded population of immature B cells showing immunophenotypic aberrancies that help distinguish the leukemic blasts from normal B-cell precursors, or hematogones.[3] These aberrancies are in the form of increased or decreased intensity of expression of an antigen on the leukemic blasts compared to the normal counterparts or gain of antigen expression, which are not specific to B-lineage.[4] Knowledge of the spectrum of antigenic expression on the B-progenitor cells during development is essential to differentiate them from B-leukemic blasts.[5] The normal B-progenitors show the expression of certain antigens in a sequential, tightly regulated manner.[3] The B-progenitor cells are derived from common lymphoid progenitor cells in the bone marrow, and they undergo three stages of maturation to become mature B-lymphoid cells. These three stages of

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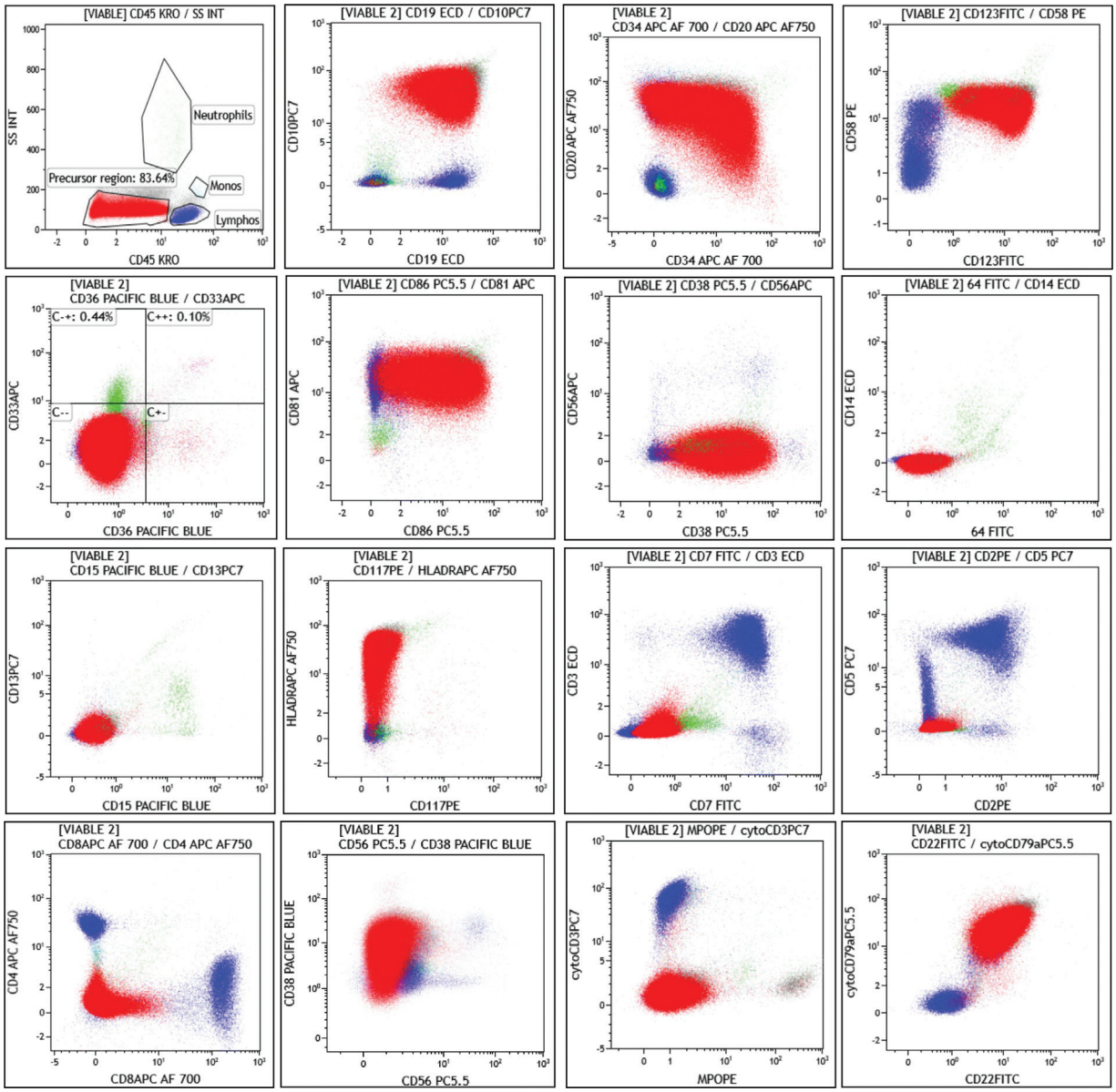


**Fig. 1** Flow cytometric dot plots of a case of BCP-ALL. The blasts (*red population*) are SSC low, CD45 dim to negative, positive for CD19, CD10, CD34, CD20, CD58, CD123, CD81, CD86, CD38, HLA-DR, CD22, and CytoCD79a. T-cell markers like CD3, CD7, CD5, CD2, CD4, CD8 and NK cell marker like CD56, and, myeloid markers like CD13, CD15, CD33, CD26, CD117, CD14, CD64 and MPO are negative.

cells are called hematogones type I (early/pre-B-I), hematogones type II (intermediate/pre-B-II), and hematogones type III (late/transitional B-cells).[6] The three stages of hematogones show a stepwise increase in the intensity of expression of CD45 and CD20 while showing a stepwise decrease in intensity of CD34 and CD10, which are finally lost at the late stage. CD19 expression is the lowest in stage I, and increases significantly in stage II, with an eventual mild reduction in mature B cells. Mature B cells show loss of CD34, CD10, and TdT, bright expression of CD20 and CD45, and a surface expression of polytypic immunoglobulins.[5] ►**Fig. 1** shows flow cytometric dot plots of a BCP-ALL patient.

The B-leukemic blasts show maturation arrest at any stage and deviation from the normal sequential immunophenotypic maturation kinetics seen in hematogones.[6–8] Other than this, over- or underexpression of markers like CD123, CD58, CD81, CD86, CD73, CD200, CD9, and CD304 can be seen in the B-leukemic blasts, as shown in ►**Table 1**. Additionally, they may express aberrant lineage markers like CD13, CD33, CD66c, CD15, CD56, and CD7 that aid in differentiating the B-leukemic blasts from hematogones.[4,6,7,9–17]

In 1995, the European Group for the Immunological Characterization of Leukemia (EGIL) proposed guidelines for diagnosing and subclassifying BCP-ALL based on immunophenotyping by flow cytometry.[18] According to the EGIL guidelines, B-lineage markers (CD19, CD20, CD22, and CD79a) and immaturity markers (CD34 and TdT) are used to diagnose BCP-ALL. Expression of CD10 and cytoplasmic and surface immunoglobulin M (IgM) are used to subclassify BCP-ALL into four subgroups based on their level of maturation (►**Table 2**).

**Table 1** Aberrant antigen expression in BCP-ALL compared to hematogones[a]

| Antigen | Aberrancy |
|---|---|
| TdT | Negative/uniform expression |
| CD34 | Negative/uniform expression |
| CD45 | Negative/uniform expression |
| CD10 | Negative/overexpression (uniform bright) |
| CD20 | Uniform expression or negative |
| CD22 | Negative/under expression |
| CD38 | Under expression |
| CD19 | Under/overexpression |
| CD73 | Overexpression |
| CD58 | Overexpression |
| CD86 | Overexpression |
| CD123 | Overexpression |
| CD200 | Overexpression |
| CD81 | Under expression |
| CD304 | Aberrant expression |
| CD9 | Overexpression |
| CD44 | Overexpression |
| CD13, CD33, CD66c, CD15, CD7, and CD56 | Lineage aberrant markers |

Abbreviations: BCP-ALL, B-cell precursor acute lymphoblastic leukemia; TdT, terminal deoxynucleotidyl transferase.
[a]Please note that all cases of BCP-ALL may not have these aberrancies, and in many cases individual antigens may have expression profile similar to hematogones.

In 2001, the World Health Organization (WHO) introduced a drastic shift in the classification of acute leukemia, including associated genetic abnormalities in the workup for subclassification of BCP-ALL. With an improved understanding of cytogenetic and molecular abnormalities associated with BCP-ALL, and due to their prognostic and therapeutic implications, a complete subtyping and risk stratification of BCP-ALL now require a plethora of molecular tests.

However, since certain specific genetic abnormalities are associated with the expression of particular antigens on the leukemic cells, MFC-based immunophenotyping may provide clues about the genotype[12] and thus help decide which molecular tests to do first.

In 2012, the EuroFlow Consortium put forward guidelines for optimal eight-color antibody panels to diagnose various hematological neoplasms, including BCP-ALL.[8] They initially suggested using an acute leukemia orientation tube (ALOT) for lineage identification (►**Table 3**). After confirmation of B-lineage by ALOT tube, further markers for additional characterization are studied in four eight-color tubes. All four tubes contain three backbone markers (CD45, CD19, and CD34) to identify and gate the B-leukemic blasts. The rest of the five antibodies are used to distinguish hematogones from B-leukemic blasts to subclassify the blasts based on their level of maturation, and to identify leukemia-associated immunophenotype (LAIP), which is especially useful in minimal residual disease (MRD) assessment during follow-up. The four-tube eight-color panel recommended by the EuroFlow Consortium for the characterization of B-ALL is shown in ►**Table 3**. With technological advancements in flow cytometry, 10-colour (and higher) panels are now commonly used for diagnostic purposes. The obvious advantage is that fewer tubes (e.g., single-tube assays) can now be utilized by incorporating a sufficient number of LAIP markers.

Specific markers have been found to be associated with the BCP-ALL genotype in various studies (highlighted in ►**Table 4**). The expression of CD66c is positively associated with *BCR-ABL1* rearrangement and a few cases of hyperdiploidy. It is rarely seen associated with *KMT2A* and *PBX1* rearrangements.[12,19,20] CD13 and CD33 (myeloid markers) are found to be aberrantly expressed in *BCR-ABL1* translocated BCP-ALL.[21] The NG2 expression on the surface of B-leukemic blasts is strongly associated with *KMT2A* rearrangement.[22,23] Also, *KMT2A* rearrangement shows loss of CD10 and aberrant expression of CD15.[12,24]

CD9 is found to be positively associated with the *TCF3::PBX1* rearrangement. The *TCF3::PBX1* rearranged BCP-ALL also shows a pre-B phenotype with complete loss of CD34, and is positive for cytoplasmic IgM.[23,25] CD123 is found to be aberrantly overexpressed in BCP-ALL with hyperdiploid karyotype. At the same time, its expression is suppressed in association with the *ETV6::RUNX1* rearrangement.[12,26] Overexpression of the CRLF2 (also known as TSLPR) antigen on the surface of B-leukemic blasts is associated with Ph-like B-ALL, and it is found to be associated with *IKZF1* deletion.[27,28]

Another increasingly important role of flow cytometry in the diagnostic workup of BCP-ALL is the analysis of the ploidy

**Table 2** EGIL immunophenotypic subclassification of B-ALL

| Subclassification | CD10 | Cytoplasmic IgM | Surface IgM |
|---|---|---|---|
| B I (pro-B) ALL | Negative | Negative | Negative |
| B II (pre-pre-B or common B) ALL | Positive | Negative | Negative |
| B III (pre-B) ALL | Positive | Positive | Negative |
| B IV (mature B) ALL | Negative/positive | Negative/positive | Positive |

Abbreviation: ALL, acute lymphoblastic leukemia; EGIL, European Group for the Immunological Characterization of Leukemia; IgM, immunoglobulin M.

**Table 3** EuroFlow consortium recommended antibody panel for B-ALL

| Tubes | Fluorochromes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PacB | AmCyan | FITC | PE | PerCPCy5.5 | PECy7 | APC | AF700 |
| ALOT Tube | CyCD3 | CD45 | CyMPO | CyCD79a | CD34 | CD19 | CD7 | SmCD3 |
| BCP-ALL Tube 1 | CD20 | CD45 | CD58 | CD66c | CD34 | CD19 | CD10 | CD38 |
| BCP-ALL Tube 2 | SmIgk | CD45 | CyIgμ | CD33 | CD34 | CD19 | SmIgM and CD117 | SmIgλ |
| BCP-ALL Tube 3 | CD9 | CD45 | NuTdT | CD13 | CD34 | CD19 | CD22 | CD24 |
| BCP-ALL Tube 4 | CD21 | CD45 | CD15 and CD65 | NG2 | CD34 | CD19 | CD12 3 | CD81 |

Abbreviations: A-LOT, acute leukemia orientation tube; BCP-ALL, B cell precursor acute lymphoblastic leukemia.

**Table 4** Genotypic associations of a few BCP-ALL antigens detected by MFC

| Marker | Genotype association |
|---|---|
| CD66c | Positively associated with *BCR-ABL1* translocation<br>Rarely seen in *KMT2A* and *PBX1* rearrangement |
| CD13 and CD33 | Positively associated with *BCR-ABL1* translocation |
| CD15, NG2, and loss of CD10 | Strongly associated with *KMT2A* rearrangement |
| CD9 | Positively associated with *TCF3::PBX1* rearrangement |
| CD123 | Overexpressed in BCP-ALL with hyperdiploidy<br>Underexpression associated with *ETV6::RUNX1* |
| CRLF2 | Positively associated with Ph-like BCP-ALL |

Abbreviations: BCP-ALL, B-cell precursor acute lymphoblastic leukemia; MFC, multiparametric flow cytometer.

status of the blasts. It is well established that BCP-ALL cases with high hyperdiploidy have better outcomes, while low-hypodiploid and near-haploid patients have poorer outcomes. Although conventional cytogenetics (CC) is the gold standard for ploidy analysis, many studies have shown that flow cytometry–based DNA ploidy analysis correlates well with CC. Various nucleic acid binding dyes (propidium iodide, DAPI, acridine orange, etc.) have been used for this purpose. Recent studies have found that FxCycle Violet is an excellent DNA selective dye excited by the violet laser, which allows simultaneous six- to seven-color immunophenotyping (using blue and red lasers) to separate the blasts from the background normal population and thus accurately assess the ploidy status of the abnormal B-lineage blasts in BCP-ALL.[29,30]

In recent years, immunotherapy has emerged as another important tool in the treatment of BCP-ALL, and it is often guided by flow cytometric immunophenotyping. A study from MD Anderson Cancer Center showed higher 3-year overall survival with the addition of rituximab (monoclonal antibody [moAb] against CD20) in patients younger than 60 years.[31] Another study showed that the addition of rituximab led to a longer event-free survival and lower frequency of relapses in adult Ph-negative BCP-ALL cases.[32] Both these studies used rituximab in patients whose blasts showed at least 20% CD20 expression. Ofatumumab, another anti-CD20 moAb, has been used with good results in patients in whom CD20 expression was as low as 1%.[33]

CD19 is expressed ubiquitously in BCP-ALL. Immunotherapy targeting CD19, in the form of bispecific T-cell engager (BiTE) called band anti-CD19 chimeric antigen receptor T cells (CARTs), has emerged as a promising agent in relapsed refractory BCP-ALL. CD19-negative blasts have been described in relapses following treatment with Blinatumomab and CART, and pose a challenge to their detection using MFC. CD22 is also expressed in a vast majority of BCP-ALL, and Inotuzumab ozogamicin (directed against CD22) has also shown good results in refractory BCP-ALL.[34]

## T-Acute Lymphoblastic Leukemia

T-acute lymphoblastic leukemias (T-ALLs) account for approximately 15% of the pediatric ALLs and 25% of the adult ALL groups.[35] T-ALLs are defined by surface or cytoplasmic CD3 (CyCD3) expression. The markers of the T-cell lineage are CD1a, CD2, CD3, CD4, CD5, CD7, and CD8. CD7 expression is usually bright and uniform in patients with T-ALL. CD7 is highly sensitive for T-ALL; however, it is nonspecific as it is also aberrantly frequently expressed in blasts of AML.[36] The expression of other markers like CD1a, CD2, CD3 (surface

**Table 5** EGIL classification of T-ALL

| Markers | Pro-T-ALL | Pre-T-ALL | Cortical T-ALL | Medullary T-ALL |
|---|---|---|---|---|
| CD1 | – | – | ++ | – |
| CD2 | + | ++ | ++ | ++ |
| SmCD3 | – | – | – (except in SmCD3+ subtypes, ++) | ++ |
| CyCD3 | ++ | ++ | ++ | ++ |
| $CD4^-/CD8^-$ | ++ | + | – | – |
| $CD4^-/CD8^+$ | – | ± | ± | ± |
| $CD4^+/CD8^-$ | – | ± | ± | + |
| $CD4^+/CD8^+$ | – | – | + | ± |
| CD5 | – | ++ | ++ | ++ |
| CD7 | ++ | ++ | ++ | ++ |

Abbreviations: EGIL, European Group for the Immunological Characterization of Leukemia; T-ALL, T acute lymphoblastic leukemia.
Note: –, positive in <10% of the T-ALLs; ±, positive in 10–25% of the T-ALLs; +, 25–75% of the T-ALLs are positive; ++, positive in >75% of the T-ALLs.

membrane [Sm]), CD4, CD5, and CD8 is variable depending on the maturational stage of the blasts.[37] Markers like CD2, CD5, and CD7 correspond to most immature T cells; however, they lack lineage specificity. CyCD3 is a marker constantly expressed by T-ALLs and is lineage specific with no cross-reactivity, appearing at the early stages of maturation. A combination of CyCD3 and SmCD3 is helpful as it identifies a $CyCD3^+/SmCD3^{-/dim}$ population, most frequently observed in patients of T-ALL.[8] Using anti-CD3 epsilon chain antibody is important and as per available literature, the CD3 clone S4.1 gives increased autofluorescence, whereas UCHT-1-PE gives the highest specific fluorescence intensity for intracellular staining.[38,39]

Maturation abnormalities observed in T-ALLs serve to differentiate abnormal T-lymphoblasts from normal thymic cells. According to the degree of thymic differentiation, the EGIL classified T-ALLs into four major subgroups.[18] These subgroups included pro-T-ALL (T-1), pre-T-ALL (T-2), cortical T-ALL (T-3), and medullary T-ALL (T-4; ►**Table 5**). Immature T-ALLs, $CD1^-/CD7^+$, constitute the pro-T-ALLs, and those expressing CD2 and CD5 represent the pre-T-ALL subtype. Cases positive for CD1 and frequently dual positive for CD4/CD8 form the cortical type, and the ones with $SmCD3^+/CD1^-$ form the mature T-ALL subgroup. Flow cytometry data from a patient with T-ALL are shown in ►**Fig. 2**. Further, based on the expression of SmCD3 and the type of T-cell receptor, T-ALLs can be classified as $SmCD3^+/TCR\text{-}ab^+$ and $SmCD3^+/TCR\text{-}gd^+$ subgroups.

CD45, the pan-leukocyte gating marker, is often expressed brighter in T-ALLs than B-ALLs, and the expression level might significantly overlap with mature lymphocytes.[37] Other markers indicating an immature phenotype include CD34 and terminal deoxynucleotidyl transferase (TdT), which are positive in a proportion of T-ALL patients. CD10 (common acute lymphoblastic leukemia antigen [CALLA]) is positive in some patients (10% of all T-ALLs), but it seldom carries any diagnostic significance in classifying T-ALLs. CyCD79a, a marker associated with B-cell lineage, can be expressed at low levels in a fraction of T-ALLs, particularly the $TCRgd^+$ subgroup.[40] Expression of myeloid-associated markers such as CD13, CD33, and CD117 is found in some cases of T-ALL, especially in the recently described category of early T-cell precursor T-ALL (ETPALL).

ETPALL is a recently described category of immature T-ALLs (~15% of the cases) with low response rates to chemotherapy and dismal prognosis.[41] It is characterized by the absence of CD1a and CD8. CD5 is found weakly expressed (negative or dim, in <75% of the blast population) along with the expression of myeloid/stem cell–associated antigens (in at least >25% of the blast population) such as CD34, HLA-DR, CD13, CD33, CD11b, CD65, and CD117. Scoring systems incorporating a 6- or an 11-marker panel are used to identify patients of ETPALL. The expression of CD5 in patients with ETPALL is heterogeneous, and cases exhibiting a strong positivity for CD5 were further classified as near-ETPALL.[42]

The EuroFlow Consortium, in 2012,[8] designed an antibody panel for the diagnosis and immunophenotypic characterization of T-ALLs. This antibody panel was run when the ALOT tube suggested T-lineage blast proliferation. The panel was set with a combination of markers aimed at the identification of the blasts (cyCD3, CD45, and smCD3), markers for the differential diagnosis of T-ALL, and to specify the maturational stage of the blasts for subclassification of T-ALL with markers such as CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, TCR-ab, and TCR-gd. CD1a, CD34, CD99, and nuTdT were included to indicate the precursor nature of the blasts. Additional markers, including CD44, CD45RA, and CD123, were also used, which contribute to maturational staging.[8,43–45] Myeloid antigens such as CD13, CD33, and CD117 were added to the panel to identify cases with early precursor T-cell immunophenotype. ►**Table 6** shows the T-ALL panel designed by the EuroFlow Consortium.

T-ALL, especially the ETPALL, can be confused with MPAL (T/myeloid type). A combination of markers like CD3, CD7, and myeloperoxidase must be used for accurate lineage identification. The recent update on the classification of myeloid and histiocytic/dendritic cell neoplasm has redefined the lineage assignment criteria for the diagnosis of MPAL.[46] CD3

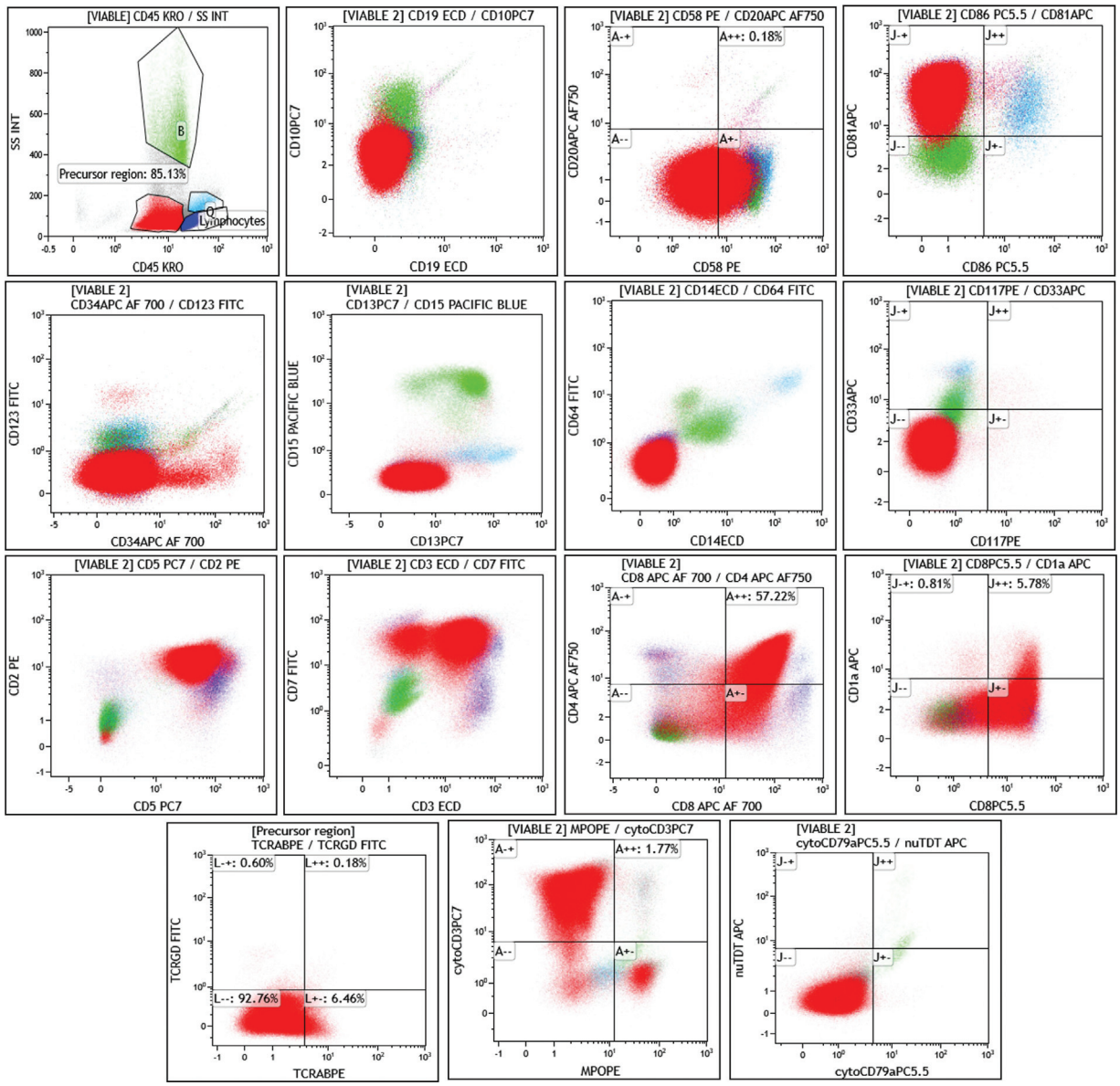


**Fig. 2** Flow cytometric dot plots of a case of T-ALL. The blasts (*red population*) are SSC low and CD45 dim, positive forcytoCD3, CD2, CD5, smCD3, CD7, CD4, CD8, and negative for CD1a, TCRab, TCRgd, and nuTdT. The blasts are negative for B-cell and myeloid markers.

expression (cytoplasmic or surface), with an expression intensity exceeding 50% of the mature T-lymphocytes on flow cytometry, is recommended for the assignment of T-lineage. Similarly, for myeloid lineage, myeloperoxidase expression intensity in part exceeds greater than 50% of the neutrophil level or expression of two or more WHO-defined markers of monocytic differentiation.[46]

Finally, information on the certain antigens expressed by the leukemic blasts can guide for targeted immunotherapies in T-ALL.[47] A lot of clinical trials have proved the efficiency of

**Table 6** EuroFlow consortium recommended eight color antibody panels for T-ALL

| T-ALL tubes | Fluorochromes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PacB | AmCyan | FITC | PE | PerCPCy5.5 | PECy7 | APC | AF700 |
| Tube 1 | CyCD3 | CD45 | NuTdT | CD99 | CD5 | CD10 | CD1a | SmCD3 |
| Tube 2 | CyCD3 | CD45 | CD2 | CD117 | CD4 | CD8 | CD7 | SmCD3 |
| Tube 3 | CyCD3 | CD45 | TCRgd | TCRab | CD33 | CD56 | CyTCRb | SmCD3 |
| Tube 4 | CyCD3 | CD45 | CD44 | CD13 | HLADR | CD45RA | CD123 | SmCD3 |

Abbreviation: T-ALL, T-acute lymphoblastic leukemia.

moAbs against specific antigens, particularly in relapsed/refractory T-ALLs. MoAbs to CD38 (daratumumab) and CD52 (alemtuzumab) have been tried in T-ALL patients and several new antigens such as CXCR4, IL7R, CD30, CD43, CD44, CD99, and CD194 are investigated as potential new targets for immunotherapy in T-ALL patients.[47,48] These antibodies can be utilized in the diagnostic panel of antibodies if clinical settings for immunotherapies are available.

## Conclusion

To summarize, immunophenotyping by flow cytometry is an essential component in the diagnosis of ALL and distinguishing it as B- or T-ALL and from other leukemias such as AML and MPAL. A suitable panel of antigens needs to be analyzed for improved sensitivity and specificity of the test. Flow cytometric immunophenotype at baseline is essential to identify the antigenic aberrancy in the leukemic blasts, which aids in minimal/measurable disease (MRD) assessment at follow-up and to differentiate residual leukemic blasts from their normal counterparts.

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Conflict of Interest
None declared.

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