# Clinical Flow Cytometry in Molecular Genetic Pathology

9

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## Contents



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L. Cheng, D.Y. Zhang, J.N. Eble (eds.), *Molecular Genetic Pathology*,
DOI 10.1007/978-1-4614-4800-6_10, 

## 9.1 Definition and Applications of Flow Cytometry

- Flow cytometry is the technique that measures the physical and antigenic properties of particles
- Any particle that can be suspended in a fluid, that is, cells, chromosomes, and individual molecules, can be detected and characterized by flow cytometry
- The most significant discovery that led to the advancement of flow cytometry and its subsequent widespread application to clinical practice was the development of monoclonal antibodies, for which Georges J.F. Köhler and César Milstein received a Nobel Prize in 1984
- Currently, immunophenotyping of hematolymphoid neoplasms using fluorochrome-conjugated monoclonal antibodies is the most common clinical application of flow cytometry
- In contrast to other applications of monoclonal antibodies, such as immunohistochemistry or Western blotting, flow cytometry examines antigens in their native (nonfixed) state
- Multicolor immunophenotyping is the current standard in clinical flow cytometry. Most commonly, 4–10 antibodies are analyzed at the same time. However, technical advances allow for the simultaneous detection of up to 17 antigens on an individual cell
- The most common applications of clinical flow cytometry include
  - Diagnosis and subclassification of malignant hematologic disorders such as leukemias and lymphomas
  - Detection of minimal residual disease in acute leukemia
  - Enumeration of T cell subsets for followup of HIV-positive patients
  - Determination of immunophenotypic/functional abnormalities in congenital immunodeficiencies
  - Enumeration of hematopoietic stem cells for bone marrow transplantation
  - Diagnosis of platelet disorders
  - Detection of fetal hemoglobin in fetomaternal hemorrhage
- The technique of flow cytometry can also be applied to
  - Cell sorting
  - Detection of chromosomal abnormalities based on in situ hybridization or polymerase chain reaction (PCR)
  - Functional assays
    - Proliferation
    - Apoptosis
    - Calcium efflux
    - Phosphorylation (cell signaling)

## 9.2 Technical Aspects of Flow Cytometry

### 9.2.1 Principle and Instrumentation

- Flow cytometry measures light scattering and fluorescence of individual particles as they are illuminated by a light (laser) source
  - In flow cytometer, individual particles are suspended in a fluid and pass one by one in front of a light source (Fig. 9.1)
  - As particles are illuminated, they scatter light and emit fluorescent signals
    - Light scattering
      - Forward scatter signal (FSC) measures cross-sectional area and is roughly proportional to size of a particle
      - Side scatter signal (SSC) reflects the internal complexity of a cell (cytoplasmic granules, vacuoles, and organelles). Cell size and refractive index may contribute to SSC characteristics
    - Fluorescence
      - The principle of fluorescence is illustrated in the simplified Jablonski diagram (Fig. 9.2): as an electron in its ground state absorbs light, it is raised to the excited state. The excess energy is emitted as nonradiative transition in a process of internal conversion and vibrational relaxation.

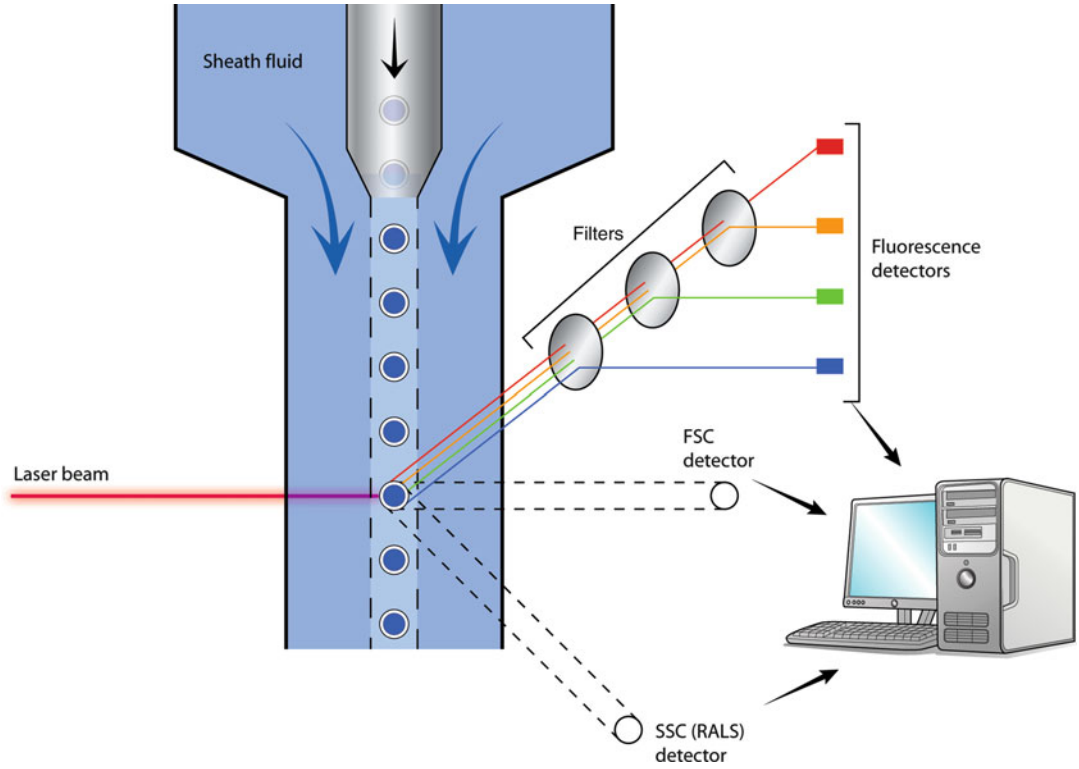


**Fig. 9.1** Diagram of flow cytometer. Cell suspension is injected into sheath fluid under pressure, which positions the cells in a single file in the center of the stream for interrogation by the laser. FSC and SSC signals and fluorescent signals of specific wavelength are recorded by separate detectors

Upon subsequent return to the ground state, the fluorescence is emitted. Since some energy is lost during nonradiative transitions, the energy content of emitted fluorescence is lower than the energy absorbed, resulting in the emission at a longer wavelength than the absorption (Stokes shift)

– Each fluorochrome is characterized by a distinct spectral pattern of absorption and emission (fluorescence). The fluorochromes must be specifically selected to absorb a certain wavelength of light emitted by the laser available in the instrument (some flow cytometers are equipped with more than one laser). Currently, a wide variety of monoclonal antibodies conjugated to various fluorochromes are available, which allows simultaneous detection of multiple antibodies bound to a single cell. However, great care should be taken to select fluorochromes with minimal overlap in the emission spectra to optimally resolve individual antibodies

- Flow cytometer consists of fluidics, a light source (laser), a detection system, and a computer (Fig. 9.1)
- Steps in the flow cytometric analysis of the sample:
  – Aspiration of the stained cell suspension into a stream of sheath fluid
  – Alignment of a single cell file centrally in the sheath fluid through the hydrodynamic focusing
  – Illumination of cells passing individually in front of the laser source

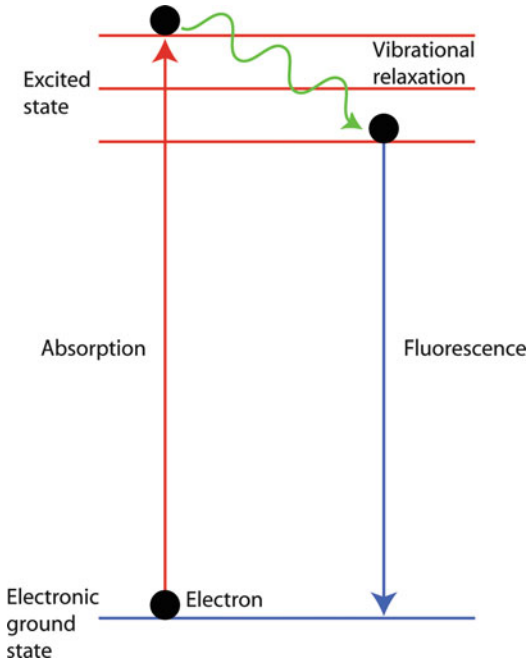


**Fig. 9.2** Jablonski diagram. As a result of energy absorption, electrons are raised to the excited state. The energy is emitted in a process of internal conversion or vibrational relaxation and subsequently as fluorescence when electrons return to their ground state

  - Registration of light scatter and fluorescence signals from individual cells by dedicated photodetectors (separate detectors for light scatter and each fluorochrome; partitioning into different wavelengths is achieved by a series of dichroic mirrors)
  - As the sample is run, the data is digitized and simultaneously displayed and stored for subsequent analysis

### 9.2.2 Sample Processing

- Any specimen in a form of single cell suspension is suitable for flow cytometric analysis
- Most common clinical samples analyzed by flow cytometry include
  - Bone marrow and peripheral blood (collected with an anticoagulant, i.e., sodium heparin, ethylenediamine tetra acetic acid, or acid citrate dextrose)
  - Solid tissues (lymph nodes and extranodal samples suspected to harbor hematologic malignancy should be submitted in culture media such as RPMI 1640 to maintain viability and subsequently mechanically dissociated usually by mincing with a scalpel and filtering to yield a single cell suspension)
  - Body cavity fluids
- Quality of the sample is critical for accurate analysis
  - Prolonged transport or transport in inappropriate conditions may render a sample unsuitable for analysis
  - Peripheral blood and bone marrow specimens should be processed within 24–48 h from the time of collection and, if transported, should be kept at room temperature
  - Certain samples, such as body cavity fluids or specimens with a high proliferation rate, may require even shorter time intervals between collection and processing
- Steps in sample processing:
  - Hypotonic lysis for specimens with an admixture of red blood cells
  - Determination of the cellularity and viability of all submitted samples
    - Cell count can be obtained using flow cytometry with standardized beads or using automated cell counters
    - Flow cytometry of a sample stained with DNA dyes (e.g., propidium iodide, 7-AAD) or a manual method utilizing trypan blue exclusion can be used to test viability
  - Preparation of a cytospin for the morphologic inspection of the cell suspension
  - Staining with a cocktail of fluorochrome-conjugated monoclonal antibodies (both surface, i.e., membrane bound, and intracellular antigens can be analyzed)

### 9.2.3 Selection of Antibody Panel

- Comprehensive antibody panels with multiple markers for myeloid and lymphoid lineage are recommended by 2006 Bethesda International

Consensus on the immunophenotypic analysis of hematolymphoid neoplasia

- Antibody panels are designed to identify multiple cell subpopulations expected to be present in the sample. Both terminally differentiated cell populations and successive developmental stages should be covered
- Numerous hematopoietic cell antigens and the corresponding antibodies have been cataloged by Workshops on Human Leukocyte Differentiation Antigens (HLDA) held regularly since 1982
- These workshops provide a forum for reporting new antigens/antibodies and defining a cluster of antibodies, which recognize the same antigen (cluster of differentiation [CD], Tables 9.1 and 9.2). Consecutive numbers are assigned to each new reported antigen. The recent, HLDA9 workshop, currently known as HCDM for Human Cell Differentiation Molecules, brought the number of characterized antigens to 350
- The selection of an antibody panel is based on the properties of the antibodies and fluorochromes
  - The selection of an antibody clone is often critical because antibodies can recognize different epitopes on the same antigen with different distributions in hematopoietic cells (e.g., antibodies against CD34 antigen recognize three different epitopes of this molecule). Additionally, clones may differ in binding capacity
  - The choice of the fluorochrome should be related to the density of a given antigen. For example, when only a few molecules of the antigen are expected, one should select the antibody coupled with a strong fluorochrome to enhance the detection
- Selected markers most commonly analyzed by flow cytometry in hematopathology are presented in Tables 9.1 and 9.2
- Dependent on the laboratory, a comprehensive antibody panel can be analyzed upfront. Alternatively, a limited screening panel is utilized initially with the subsequent addition of selected markers

**Table 9.1** Lineage-associated markers commonly analyzed in clinical flow cytometry

| Immature | Granulocytic/monocytic | Erythroid |
|---|---|---|
| CD34 | CD33 | CD71 |
| CD117 | CD13 | CD235a |
| TdT | CD15 | |
| | CD16 | |
| | CD14 | |
| Megakaryocytes | B cell | T cell |
| CD41 | CD19 | CD2 |
| CD42b | CD20 | CD3 |
| CD61 | CD22 | CD4 |
| | κ-light chain | CD5 |
| | λ-light chain | CD7 |
| | | CD8 |

## 9.3 Analysis and Interpretation of Flow Cytometric Data

- The evaluation of flow cytometric data is based upon analysis of the patterns of antigen expression presented graphically in the form of scattergrams and histograms and their comparison to normal expression (maturation and lineage specific)
- The detailed knowledge of immunophenotypic characteristics of normal hematopoietic differentiation, as well as normal variations (e.g., age-related), is critical for optimal interpretation of flow cytometric data
- Both qualitative (positive/negative, homogeneous vs heterogeneous expression in a given population) and semiquantitative information on antigen expression (low/moderate/high intensity) should be recorded since patterns of antigen expression are diagnostically significant
- The concept of gating:
  - Cells with similar physical properties (size, complexity/granularity, and the presence/absence of a specific antigen) form clusters on the displays of flow cytometric data
  - Gate is a borderline that identifies these clusters of cells
  - In the gating process, a population of interest is selected (outlined with a cursor) for further analysis (i.e., to determine antigen

**Table 9.2** Select hematolymphoid antigens commonly used in clinical flow cytometry

| Cluster of differentiation | Cellular expression |
|---|---|
| CD1a | Precursor T cells, Langerhans cells |
| CD2 | Precursor and mature T cells, NK cells, thymic B cells |
| CD3 | Precursor and mature T cells |
| CD4 | Precursor T cells, helper T cells, monocytes |
| CD5 | Precursor and mature T cells, subset of B cells (B1a cells) |
| CD7 | Precursor and mature T cells, NK cells |
| CD8 | Precursor T cells, suppressor/cytotoxic T cells, subset of NK cells |
| CD10 | Precursor B cells, germinal center B cells, granulocytes |
| CD11b | Granulocytic and monocytic lineage, NK cells, subset of T- and B cells |
| CD13 | Granulocytic and monocytic lineage |
| CD14 | Mature monocytes, neutrophils (dim expression) |
| CD15 | Granulocytic and monocytic lineage |
| CD16 | Granulocytic and monocytic lineage, NK cells |
| CD19 | Precursor and mature B cells |
| CD20 | Precursor and mature B cells |
| CD22 | Precursor and mature B cells |
| CD31 | Megakaryocytes, platelets, leukocytes |
| CD33 | Granulocytic and monocytic lineage |
| CD34 | Hematopoietic stem cells |
| CD36 | Megakaryocytes, platelets, erythroid precursors, monocytes |
| CD38 | Hematopoietic cells including activated lymphocytes and plasma cells |
| CD41 | Megakaryocytes, platelets |
| CD42b | Megakaryocytes, platelets |
| CD45 | Hematopoietic cells |
| CD56 | NK cells, subset of T cells |
| CD61 | Megakaryocytes, platelets |
| CD62P | Megakaryocytes, platelets |
| CD63 | Megakaryocytes, platelets |
| CD64 | Granulocytic and monocytic lineage |
| CD71 | High density on erythroid precursors, low density on other proliferating cells |
| CD79a | Precursor and mature B cells |
| CD117 | Hematopoietic stem cells and mast cells |

expression for the selected – "gated" population)

– Gating can also be applied at the time of data acquisition (so-called live-gating) to selectively collect high number of cells from a specific subpopulation, for example, CD19 positive cells, to facilitate the detection of a small number of monoclonal B cells
– For diagnostic purposes, the data is most commonly collected ungated, that is, all events detected by a flow cytometer are recorded, to comprehensively analyze the entire sample and retain internal positive and negative controls. A separate portion of the sample can be live-gated to better visualize specific cell populations (e.g., lymphocytes, blasts, or plasma cells)

- Steps in the analysis of flow cytometric data
  – Inspection of dot plots presenting cell size (FSC), internal complexity (SSC), and the expression of panhematopoietic antigen CD45
    - Specific cell populations can be identified based on their size and cytoplasmic complexity (granules/vacuoles) (Fig. 9.3a)
    - The identification is confirmed and further resolved on the display of CD45 antigen and SSC (Fig. 9.3b). This scattergram provides information on the relative proportion of specific cell populations in the flow cytometric sample and is of particular value when analyzing bone marrow/peripheral blood specimens
      – CD45 is a surface protein tyrosine phosphatase expressed at different levels on all hematopoietic cells
      – Lymphocytes show the highest density of CD45 expression with approximately 10% of the cell membrane occupied by this antigen
      – Granulocytic series including myeloid blasts, B cell precursors, and proerythroblasts show intermediate CD45 density
      – Late erythroid precursors along with megakaryocytes are negative for the CD45 antigen

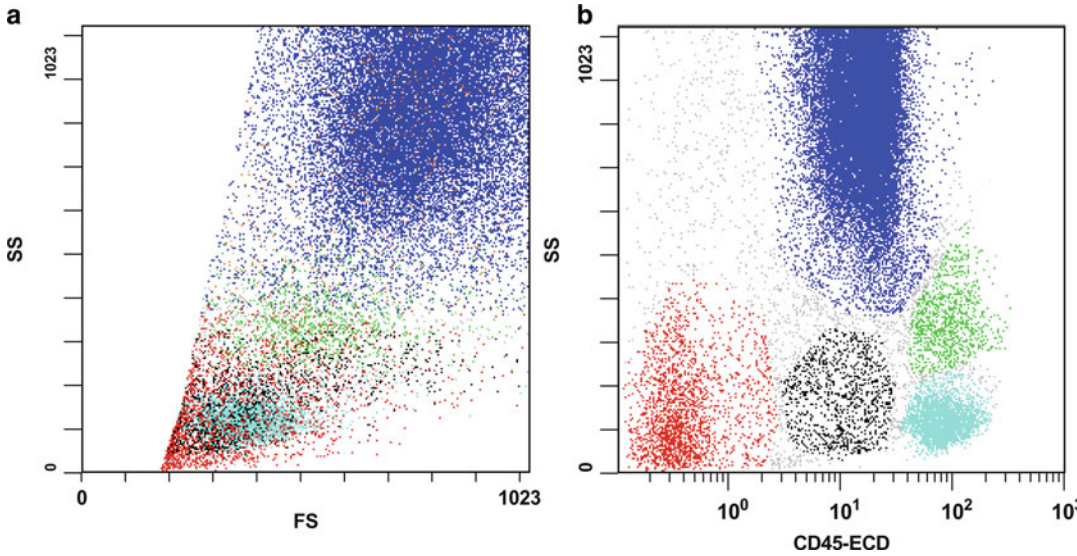


**Fig. 9.3** Main hematopoietic populations of normal bone marrow. (**a**) Scattergram of FSC (cell size) vs SSC (internal complexity) reflects the heterogeneity of bone marrow. Lymphocytes as smallest with negligible amount of cytoplasm are located closest to the origins of the axes (*shown in aqua*). Monocytes are slightly larger with occasional granules and vacuoles (*green*). Granulocytic series shows prominent granularity (*navy*). (**b**) Differential density of panhematopoietic marker CD45 on marrow leukocytes. Lymphocytes (*aqua*) and monocytes (*green*) show highest density of CD45 antigen. Intermediate expression of CD45 is seen in granulocytic population (*navy*) and blasts (*black*). Late erythroid precursors (*red*) are negative for CD45 antigen

- Focused analysis of the patterns of antigen expression including both qualitative data (antigen present/absent) and fluorescence intensity (on the logarithmic scale) as a relative measure of the antigen density on the cell surface
  - Residual normal cells present in a sample can be used as an internal negative and positive control and to gauge the intensity of staining
  - The intensity of staining is dependent on technical variables including antibody clone and type of fluorochrome. Thus, levels defining bright, moderate, and dim expression should be established by individual laboratories taking into account specific antibodies and fluorochromes used and previous experience
  - The autofluorescence and nonspecific background staining due to Fc receptors should be taken into account when evaluating antigen expression

## 9.4 Basic Cell Populations Identified by Flow Cytometry

- Genetically controlled differentiation program and bone marrow environment govern the expression of surface and cytoplasmic molecules that define hematopoietic cell populations
- Specific morphologic stages of development are accompanied by distinct changes in immunophenotypes. However, even though approximate morphologic–immunophenotypic correlates exist, transitions between immunophenotypes of various developmental phases are best described as a continuum
- All hematopoietic progeny are derived from pluripotent stem cells
  - These cells are morphologically unrecognizable and are defined by their functional and antigenic characteristics
  - They usually express a combination of intermediate density CD45, CD34,

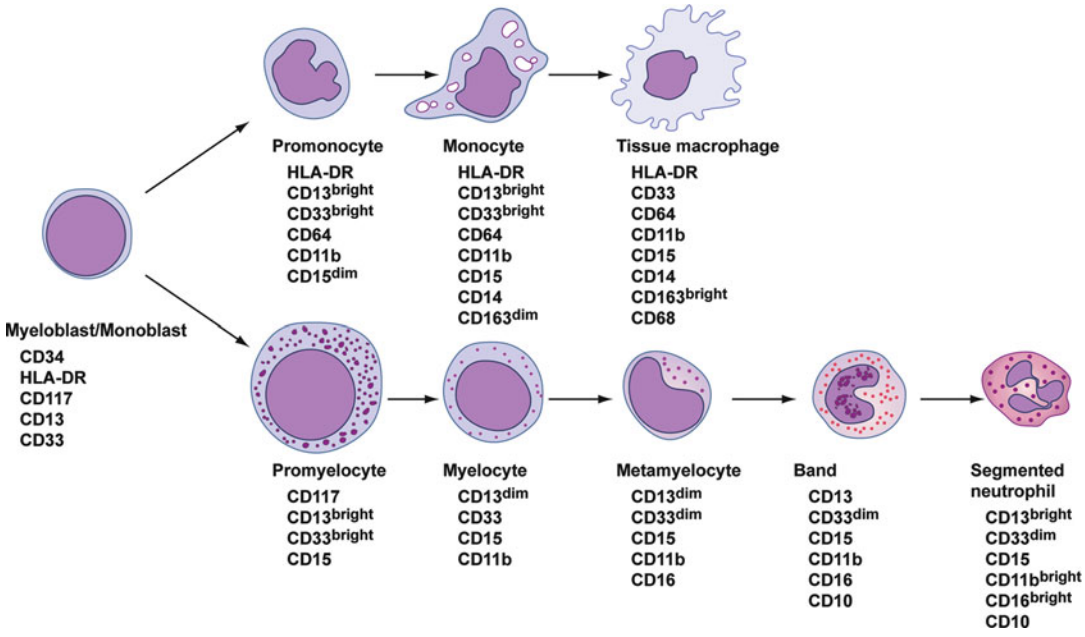


**Fig. 9.4** The maturation of myeloid series is a genetically driven developmental program characterized by the continuum of phenotypic and functional changes. Discrete morphologic stages correspond to specific immunophenotypes

CD133, CD117 (c-kit), variable density of CD38 and CD90, CD123, and HLA-DR

- As hematopoietic cells mature, they lose stem cell markers and acquire lineage-specific antigens
- Neoplastic hematopoietic cells to a certain extent mimic normal maturation stages; however, they frequently display aberrant antigen expression patterns

### 9.4.1 Granulocytic Lineage

- The differentiation of granulocytic lineage, as defined by the expression of specific antigens, corresponds closely to the morphologic maturation stages as depicted in Fig. 9.4
- Myeloblast is the first morphologically recognizable cell committed to the myeloid lineage and typically expresses immature cell markers CD34, CD38, HLA-DR, and stem cell factor receptor CD117, and panmyeloid markers, CD13 and CD33
- As the myeloblast matures to a promyelocyte, it loses CD34 and HLA-DR and gradually acquires the CD15 antigen
- Further maturation to myelocytes results in a complete loss of CD117 and downregulation of CD13 antigen. The decrease in the density of CD33 is also seen at this stage. CD15 and CD11b are positive
- Finally, as myeloid cells near the band stage, CD16 and CD10 are acquired and the density of CD13 increases. Further decrease of CD33 intensity is also noted
- The segmented neutrophil is characterized by high-density CD13, CD11b, and CD16 and dim CD33

### 9.4.2 Monocytic Lineage

- The immunophenotype of the earliest stage of monocytic development, a monoblast, overlaps with that of myeloblast and includes the expression of CD34, HLA-DR, CD117, and panmyeloid markers CD33 and CD13 (Fig. 9.4)

- Further monocytic maturation is marked with the increase in density of CD33 and CD13, appearance of CD64 and CD11b, and subsequently low density CD15 (promonocyte)
- Subsequent acquisition of CD14 and further increase in density of CD45 define the transition point to a mature monocyte
- The expression of CD163 and CD68 antigens is strongest on tissue macrophages

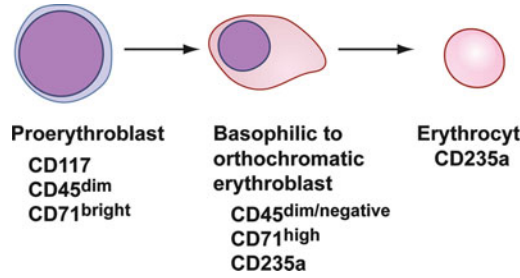


**Fig. 9.5** The development of erythroid series is defined by stepwise loss of CD45 along with acquisition of erythroid markers

### 9.4.3 Erythroid Lineage

- Erythroid precursors are characterized by a gradual decrease in the density of CD45 antigen to the undetectable level in reticulocytes. Thus, late erythroid precursors are one of the few cells in the bone marrow that express a negligible number of CD45 molecules
- The earliest marker of erythroid differentiation is the transferrin receptor, CD71 (Fig. 9.5). This marker increases in density starting from the proerythroblast stage and is rapidly downregulated in reticulocytes. Mature erythrocytes are negative for the CD71 antigen
- The decrease in CD45 intensity seen in basophilic erythroblasts is accompanied by the emergence of CD235a (glycophorin A). The latter marker persists through erythroid maturation and is also present in erythrocytes

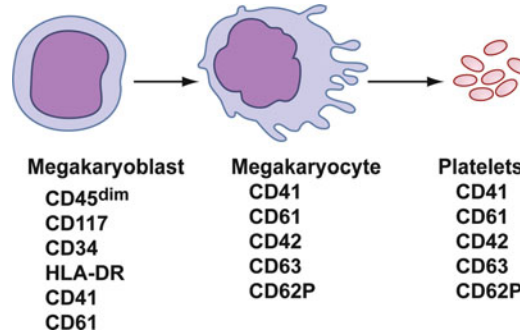


**Fig. 9.6** The sequence of immunophenotypic changes of megakaryocytic lineage is characterized by early appearance of CD41 and CD61, that is, gpIIb/IIIa complex

### 9.4.4 Megakaryocytic Lineage

- The identification of megakaryocyte population by flow cytometry is not done routinely
- CD41 and CD61 (gpIIb/IIIa complex) appear as the first markers of megakaryocytic differentiation and are present on a small subset of CD34 and CD117 positive cells believed to represent early megakaryoblasts
- CD31 and CD36, although not entirely specific for megakaryocytic lineage, are also present on megakaryoblasts
- As megakaryoblasts mature to megakaryocytes and platelets, additional antigens appear including CD42b, CD62P, and CD63
- CD41 and CD61 persist through the megakaryocyte differentiation (Fig. 9.6)
- The precise sequence of expression of megakaryocyte-associated antigens has not been well studied

### 9.4.5 Lymphoid Lineage

- The B and T lymphocytes are derived from lymphoid progenitors expressing CD34, terminal deoxynucleotidyl transferase (TdT), and HLA-DR

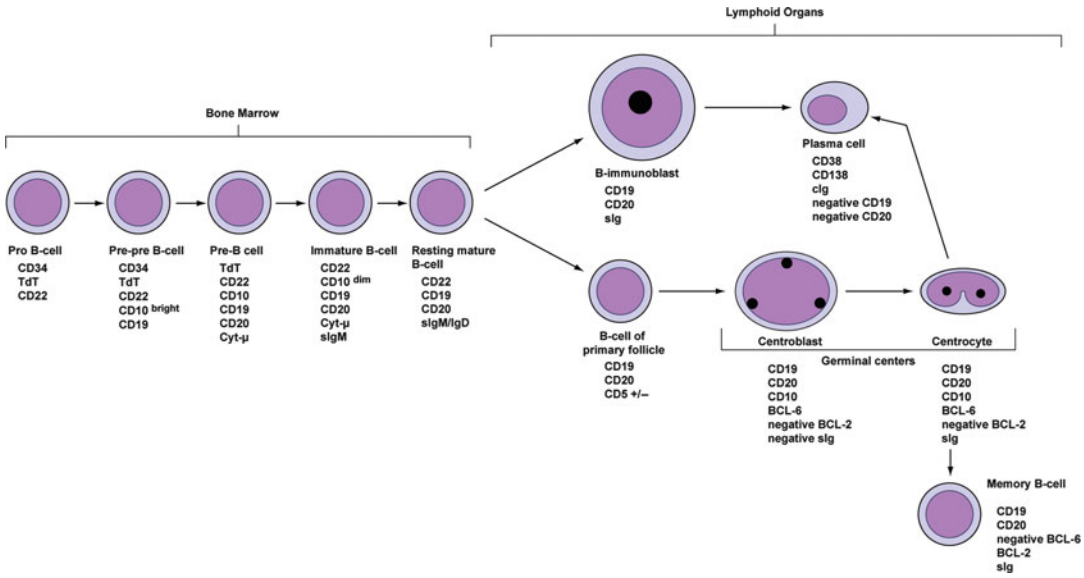


**Fig. 9.7** The early stages of B cell maturation are completed in the bone marrow. Subsequent maturation of B cell lineage occurs in lymph nodes and extranodal lymphoid tissues and results in the production of plasma cells and memory B cells

- The number of CD45 molecules steadily increases with B cell maturation and reaches characteristic high-density expression at the level of mature B cells. Early B cell precursors show low-density CD45
- The lymphoid differentiation represents a continuum of changes in the expression of surface and cytoplasmic antigens

### 9.4.5.1 B Cell Lineage

- The simplified schema of B cell differentiation is presented in Fig. 9.7
- The earliest B cell markers include cytoplasmic CD22, CD19, and cytoplasmic CD79a
- As B cell precursors proceed in their maturation, they acquire the CD10 antigen, which is initially expressed at high levels
- Subsequent appearance of the CD20 antigen is accompanied by the decrease in CD10 intensity and its subsequent loss
- The μ-heavy chain, a portion of immunoglobulin (Ig) molecule, is initially expressed in the cytoplasm and eventually transported to the cell surface where it forms a B cell receptor (BCR)
- The mature naïve B cell population expresses heterogeneous (polyclonal) surface light chains and, in this respect, differs from neoplastic B cells, which are restricted to only a single κ- or λ-light chain
- The mature B cells circulate to the secondary lymphoid organs including lymph nodes, spleen, and lymphoid tissues of extranodal sites, where they settle in the follicles and mantle zones
- Further differentiation of mature naïve B cells occurs upon antigen exposure and includes passage through germinal centers marked by the signature coexpression of CD10 and Bcl-6 antigens
- Plasma cells, the terminal stage of B cell differentiation, lose CD20 and surface Ig chains and can be identified by the high-density expression of CD38 and CD138 (syndecan-1)

### 9.4.5.2 T Cell Lineage

- Early stages of T cell development take place in the bone marrow (Fig. 9.8)
- The first committed T cell precursor (prothymocyte) expresses immature markers

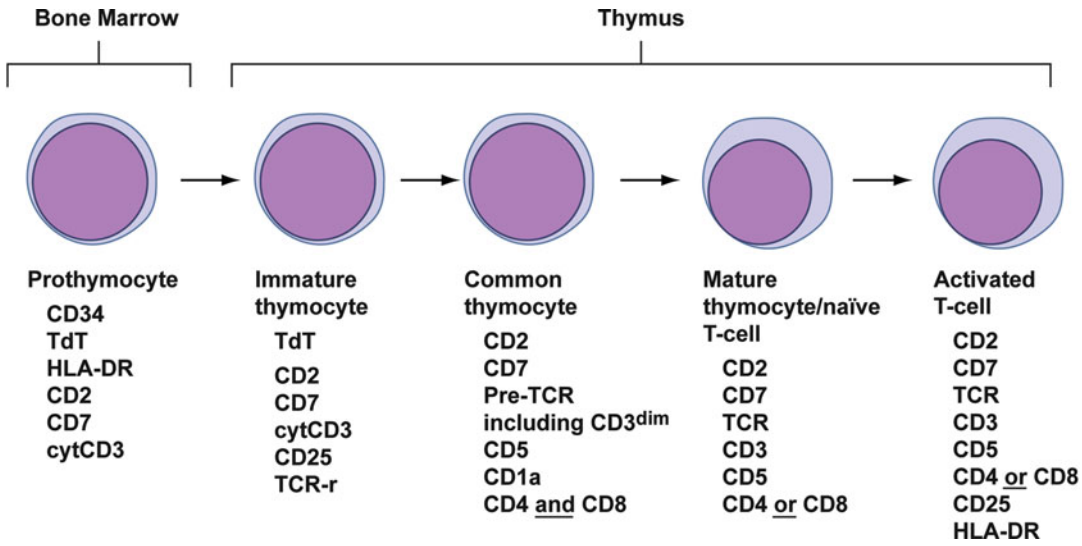


**Fig. 9.8** The early T cell precursors are generated in the bone marrow and migrate to thymus to complete their maturation

(CD34, TdT, and HLA-DR) and T cell-associated antigens including CD2, CD7, and cytoplasmic CD3
- Prothymocytes migrate to the thymus to complete T cell development
- Successive steps of T cell receptor (TCR) gene rearrangement with the production of TCR complex, expression of CD1a and CD5, and coexpression of CD4 and CD8 antigens define immature and common thymocyte stages
- As the double-positive (CD4+, CD8+) common thymocyte matures, the density of CD3 antigen increases and CD4 or CD8 is lost, giving rise to mature helper (CD4+) and suppressor (CD8+) T cells

#### 9.4.5.3 Natural Killer (NK) Cells

- NK cells are positive for CD2, CD7, CD56, and CD16
- Different densities of surface antigens allow for the separation of two functionally distinct NK subsets
  - Cytotoxic NK cells show expression of $CD56^{dim}$, $CD16^{bright}$, $KIR^{bright}$, and CD94/$NKG2A^{dim}$ (90% of NK population)
  - Immunoregulatory NK cells show expression of $CD56^{bright}$, $CD16^{dim/-}$, CD117, $KIR^{dim}$, and CD94/$NKG2A^{bright}$ (10% of overall NK population, however, represent the majority of the NK cells in lymph nodes)
- Published data suggests that the development of NK cells starts in the bone marrow from CD34+ progenitor cell
- It has been suggested that the later stages of development of immunoregulatory NK subset occur in the lymph nodes from $CD34^{dim}$ progenitor cells
- The site and exact sequence of the development of cytotoxic NK cells are unknown

## 9.5 Flow Cytometric Analysis of Myeloid Disorders

- In myeloid malignancies, flow cytometry can be used for the initial diagnosis, followup, and prognostication (specific immunophenotypes are associated with prognostically significant cytogenetic abnormalities) and less commonly for a determination of treatment targets (e.g., CD33 antigen for gemtuzumab ozogamicin therapy)

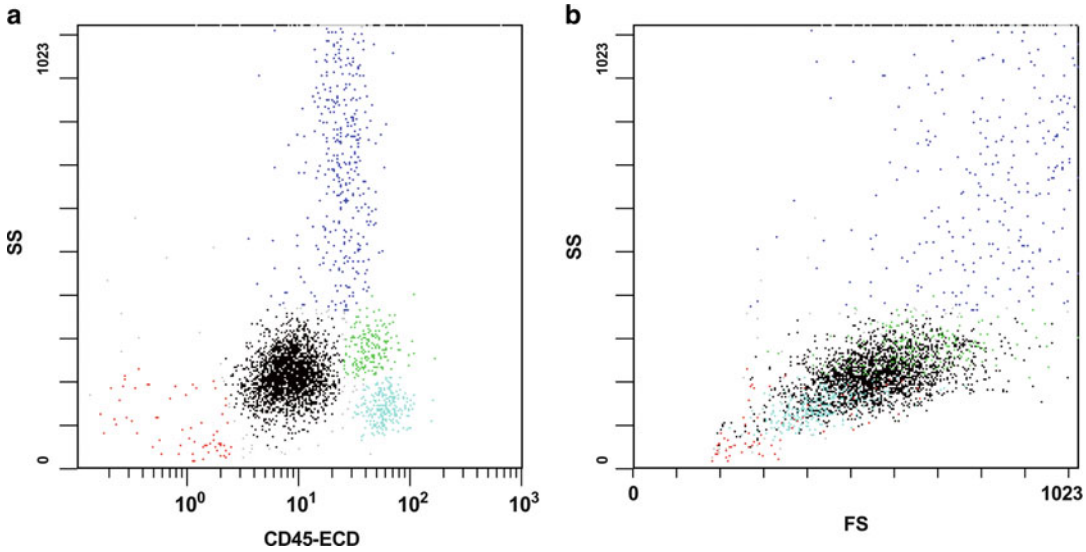


**Fig. 9.9** (**a**) The best resolution of blast population (shown in *black*) is achieved using scattergram of SSC vs CD45. The intermediate density of CD45 antigen together with low SSC results in clear delineation of blast population in the majority of cases of acute leukemia. The lymphoid cells (*aqua*), monocytes (*green*), and maturing granulocytic series (*navy*) localize outside the blast gate. (**b**) On the scattergram demonstrating cell size (FSC) and granularity (SSC), blasts usually overlap with other cell populations

- The majority of myeloid neoplasms are regarded as disorders of hematopoietic stem and progenitor cells
- In acute myeloid leukemias (AML), the maturation arrest leads to the accumulation of a homogeneous population of cells demonstrating an immature myeloid immunophenotype; thus, the blast region, best demonstrated on a CD45/SSC scattergram, is densely populated in most cases (Fig. 9.9a, compare with Fig. 9.3b). The increased number of blasts and the paucity of maturing marrow elements can also be visualized on SSC/FSC display (Fig. 9.9b)
- In myelodysplastic syndrome (MDS) and chronic myeloproliferative disorders, a myeloid maturation is at least partially preserved; thus, both evaluation of immature and maturing cells is considered essential for the diagnosis
- In this chapter, the immunophenotypic features of AML and chronic myeloid disorders are briefly discussed in the context of the 2008 World Health Organization (WHO) classification
- The WHO classification introduced new categories of AML defined by recurrent cytogenetic abnormalities. These leukemias often show specific immunophenotypes and will be presented separately in the following outline

## 9.5.1 Acute Myeloid Leukemias

### 9.5.1.1 AML with Recurrent Cytogenetic Abnormalities

**AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1***

- Majority of cases show an immature myeloid immunophenotype with a high density of CD34 and coexpression of low-density CD19 (Fig. 9.10)
- Numerous myeloid antigens, including CD33, CD13, and myeloperoxidase, are expressed
- Frequently, there is asynchronous coexpression of CD34 and CD15
- The presence of TdT is common
- The coexpression of CD56 has been reported to be associated with worse prognosis

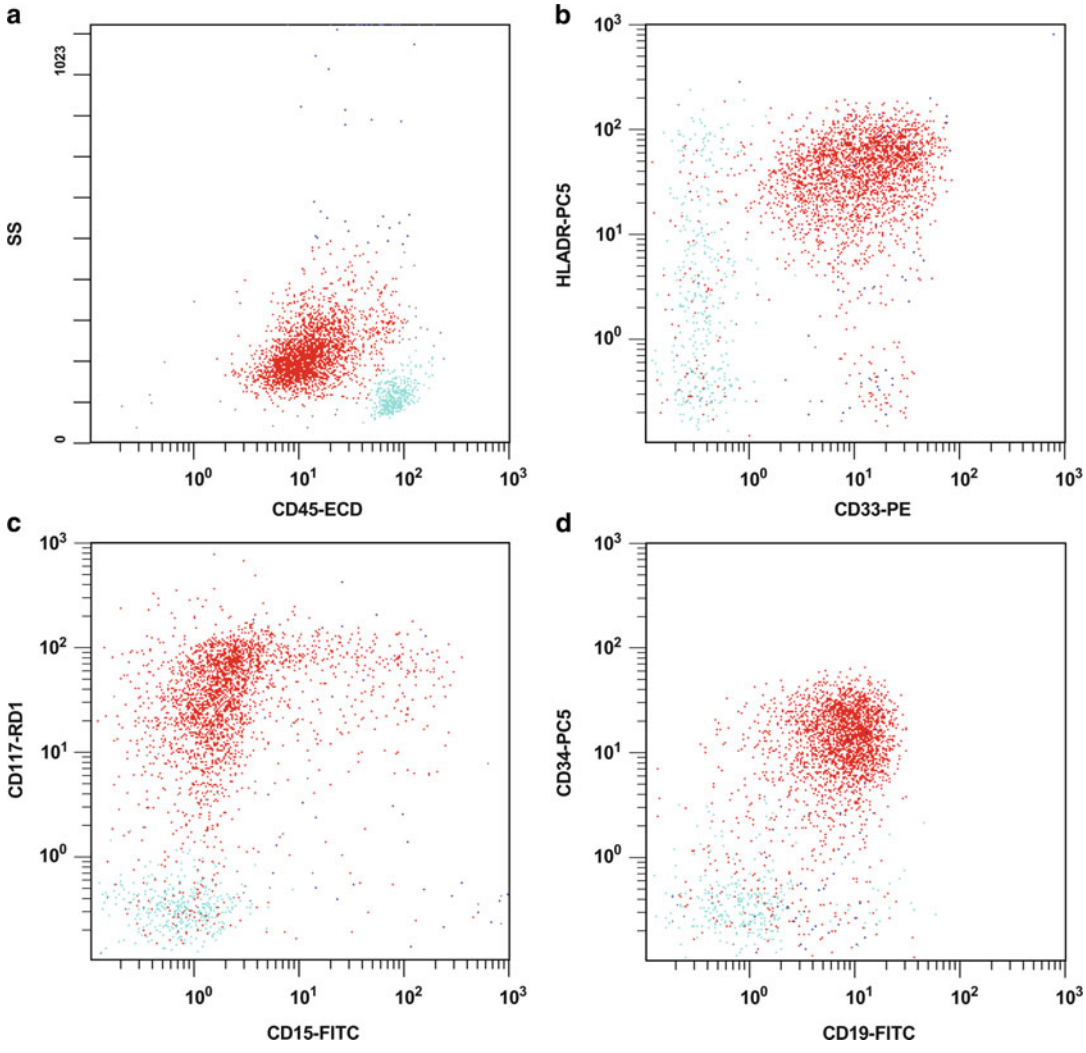


**Fig. 9.10** AML with t(8;21)(q22;q22); (*AML1*/*ETO*). Blasts are shown in *red* and residual lymphocytes in *aqua*. (**a**) CD45 vs SSC demonstrates a distinct blast population with marked decrease in other hematopoietic cells. The residual lymphocytes are present. (**b**–**d**) Blasts express immature markers frequently present in progenitor cells (CD34, HLA-DR, and CD117) and show characteristic coexpression of CD19

### AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*

- Immature cells show expression of CD34, CD117, and TdT
- Subpopulation of maturing cells expresses monocytic (CD14, CD11b, $CD4^{dim}$) and granulocytic (CD15) markers
- The coexpression of CD2, antigen normally seen on T and NK cells, is common (Fig. 9.11)

### Acute Promyelocytic Leukemia with t(15;17)(q22;q12); *PML-RARA* (APL)

- In contrast to most less differentiated myeloid leukemias, APL presents with high SSC reflecting the granular cytoplasm of leukemic cells (Fig. 9.12)
- A constellation of immunophenotypic features used to diagnose APL includes lack of CD34 and HLA-DR antigens

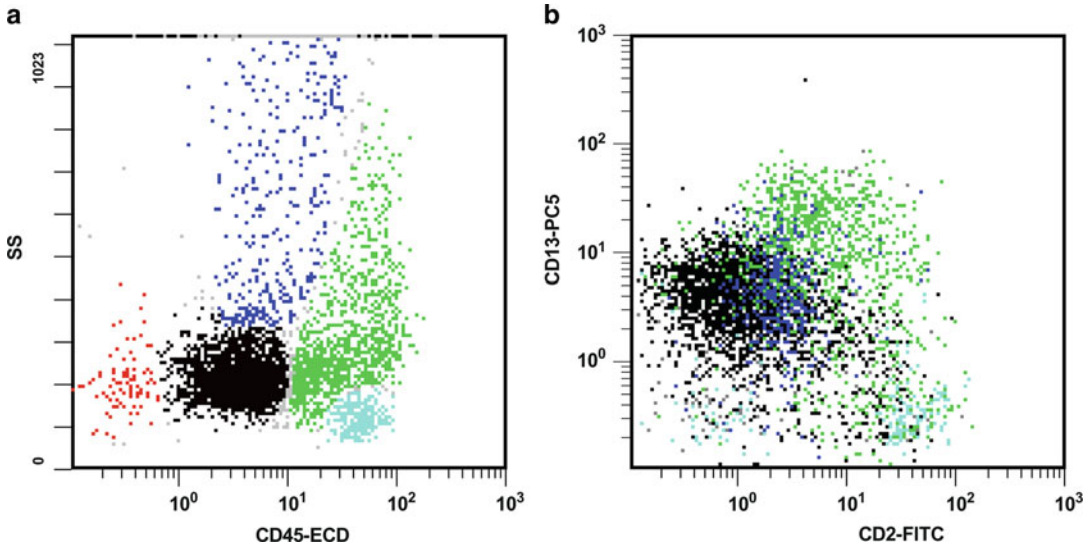


**Fig. 9.11** The AML with inv(16)(p13q22) or t(16;16)(p13;q22)/(*CBF-β/MYH11*) most commonly presents a myelomonocytic leukemia. (**a**) The CD45/SSC scattergram shows two merging populations of myeloid blasts (in *black*) and monocytic cells (in *green*). (**b**) The monocytic component with typical high-density CD13 coexpresses CD2 antigen

- Expression of homogeneous bright CD33 along with myeloperoxidase and variable expression of CD13 and CD15 are present
- CD2 and higher incidence of CD34 expression have been reported in APL with microgranular morphology

#### AML with t(9;11)(p22;q23); *MLLT3-MLL*

- Constitute a heterogeneous group most commonly presenting with monocytic differentiation
- The immunophenotypic features are not specific and can be seen in any acute myelomonocytic or monocytic leukemias
- Most commonly, these leukemias show variable expression of immature markers such as CD34 and CD117, and positive for CD33, CD13, CD14, $CD4^{dim}$, CD11b, and CD64

### 9.5.1.2 AML Not Otherwise Specified

#### AML with Minimal Differentiation and AML Without Maturation

- Blasts show low-density CD45 antigen expression and display low SSC reflecting their relatively agranular cytoplasm (Fig. 9.9a)
- The majority of even least differentiated AMLs express myeloid markers such as CD13, CD33, and/or CD117
- Primitive hematopoietic antigens, CD34 and HLA-DR, are often seen
- Myeloperoxidase is negative or only expressed in a minority of cells

#### AML with Maturation

- In addition to primitive hematopoietic and early myeloid antigens, more mature myeloid markers such as CD15 and myeloperoxidase are often expressed
- Occasionally, there is an asynchronous coexpression of antigens, which in normal hematopoietic cells are not expressed simultaneously (e.g., exclusive early and late myeloid markers such as CD34 and CD15 are expressed at the same time)
- Coexpression of markers associated with other lineages, for example, lymphoid, may be seen on the myeloid blasts. The most common example is the CD7 antigen

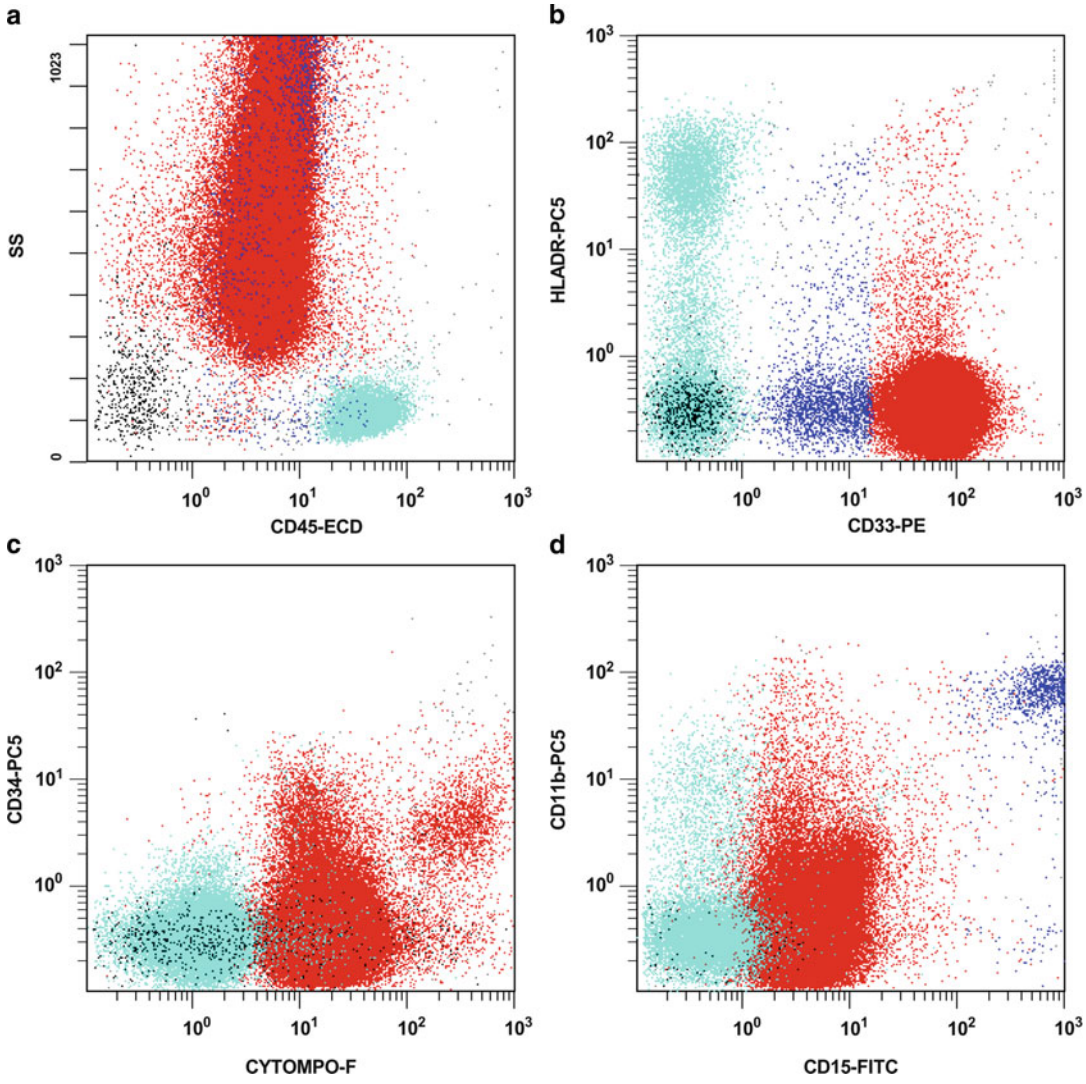


**Fig. 9.12** Acute promyelocytic leukemia. (**a**) High SSC of APL corresponds to prominent granularity of leukemic cells (in *red*). Residual lymphocytes are presented in *aqua*. (**b**) Leukemic promyelocytes are positive for CD33 (bright as compared with low-density CD33 of residual normal neutrophils, in *navy*) and negative for HLA-DR. (**c**) CD34 is negative and myeloperoxidase is expressed by the majority of cells. (**d**) Low-density CD15 antigen can be present

### Acute Leukemias with Monocytic Differentiation (Acute Myelomonocytic Leukemia and Acute Monoblastic/ Monocytic Leukemia)

- The SSC and CD45 expression in acute leukemias with monocytic differentiation is variable and dependent on the relative proportion of primitive myeloid blasts and the degree of differentiation of neoplastic monocytes. Patterns with a distinct and separate population of blasts and monocytes or a large merging cluster of cells, starting in the blast region and extending upward to monocyte region, can be seen (Fig. 9.13)
- In acute myelomonocytic leukemia, a population of primitive myeloid blasts is often distinct
- The expression of myeloid markers and antigens associated with monocytic lineage such as

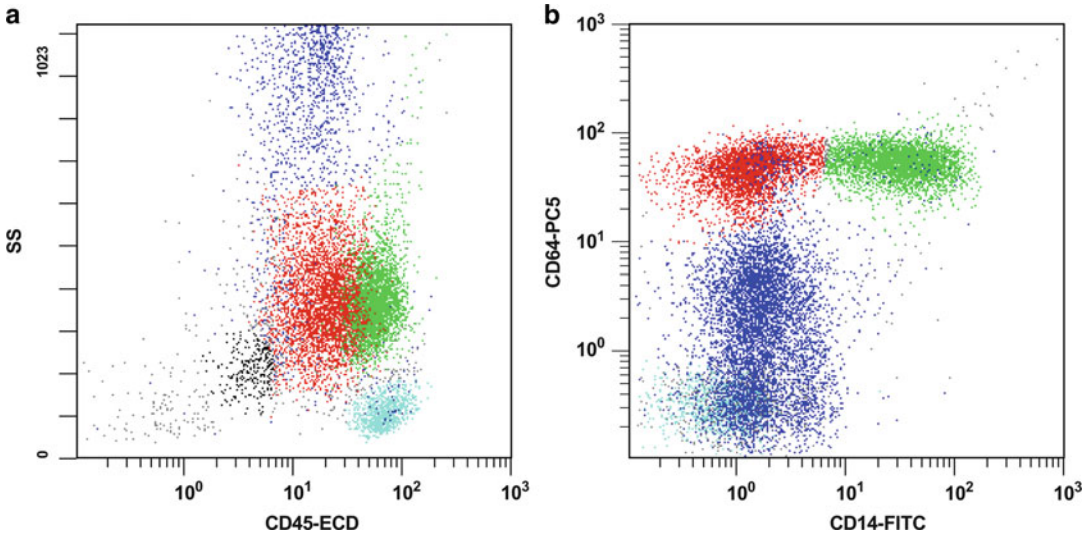


**Fig. 9.13** Acute leukemias with monocytic component frequently show a spectrum of monocytic maturation. (**a**) Both uncommitted myeloid blasts and different stages of monocytic maturation (in *red* and *green*) are present. (**b**) Immature monocytic cells, presented in *red*, show high-density CD64. More mature monocytic component expresses CD14 antigen (in *green*)

CD14, CD4, CD11b, and CD64 is commonly seen
- Despite the CD14 antigen being present on all mature monocytes, it can be negative in monocytic leukemias. Frequently, a heterogeneous pattern of CD14 expression is seen reflecting a maturation spectrum of neoplastic monocytes
- Markers ordinarily present on both immature and mature monocytes, such as high-density CD64, are more consistently expressed

#### 9.5.1.3 Acute Erythroid Leukemias

- Acute erythroid leukemias are categorized into two subtypes: pure erythroid leukemia and erythroid/myeloid leukemia (erythroleukemia)
- In erythroleukemia, both primitive myeloid blasts and erythroid precursors are present
- Erythroid markers CD71, glycophorin A (CD235a), and hemoglobin can be present
- When glycophorin A and hemoglobin are absent, the diagnosis is based on the absence of myeloid markers, the presence of bright CD71, and the scatter characteristics of leukemic cells

#### 9.5.1.4 Acute Megakaryoblastic Leukemia

- Usually shows low SSC and dim to absent CD45
- Early megakaryocytic markers, CD41 and CD61, are frequently expressed (Fig. 9.14)
- Occasionally, the late megakaryocytic marker, CD42b, is present
- There is variable expression of stem cell markers, CD34 and HLA-DR, on the population of leukemic megakaryoblasts

#### 9.5.1.5 Blastic Plasmacytoid Dendritic Cell Neoplasm

- Initially, this neoplasm was thought to have originated from NK cells and had been previously referred to as blastic NK cell lymphoma (agranular CD4+, CD56+ hematodermic neoplasm)
- The most current evidence suggests its origin from the plasmacytoid dendritic cells
- The immunophenotype reflects the cell of origin: CD56, CD4, HLA-DR, and CD123 antigens are positive. The latter, along with the expression of blood dendritic cell

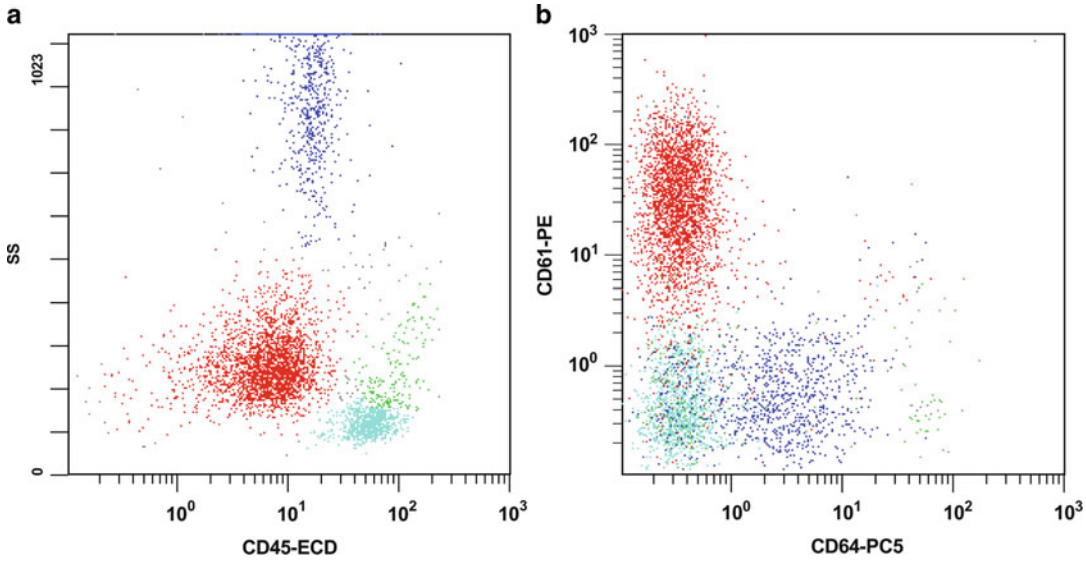


**Fig. 9.14** Acute megakaryocytic leukemias can be diagnostically challenging. (**a**) Leukemic cells usually show low SSC. (**b**) The expression of megakaryocytic markers, such as CD61, in otherwise undifferentiated acute leukemia proves the megakaryocytic lineage of leukemic cells (in *red*)

antigens 2 and 4, is highly specific for dendritic cells and their precursors
- Rare cases showing positivity for TdT and CD34 were reported
- The presence of T cell, B cell, and myelomonocytic lineage-associated markers should be excluded

## 9.5.2 Chronic Myeloproliferative Neoplasms and Myelodysplastic Syndrome (MDS)

- Multiparameter flow cytometry is helpful in diagnosis, prognostication, and prediction of a response to therapy in patients with chronic myeloid neoplasms, particularly in MDS. The contribution of flow cytometry in chronic myeloproliferative and myelodysplastic/myeloproliferative neoplasms is less well established
- The abnormalities detected by flow cytometry reflect abnormal morphologic features and abnormal maturation
- Flow cytometric abnormalities fall in two categories: quantitative (enumeration of marrow components, light scatter changes) and qualitative (altered antigen expression in maturation and lineage-specific hematopoietic compartments such as abnormal antigen density, asynchronous maturation, lineage "infidelity")
- A detailed review is beyond the scope of this text; however, a few examples are presented next to illustrate the contribution of flow cytometry to diagnosis of chronic myeloid neoplasms

### 9.5.2.1 Myelodysplastic Syndromes

- MDS is characterized by ineffective hematopoiesis with abnormalities in maturation and, often, decreased survival of hematopoietic progeny
- Diagnosis of MDS is based on the morphologic, immunophenotypic, and genetic features as well as clinical manifestations
- Flow cytometry contributes to the diagnosis of MDS through the identification of aberrant maturation and other immunophenotypic abnormalities, some of which are typical for MDS
- Aberrant immunophenotypes demonstrated by flow cytometry are seen in up to 98% of

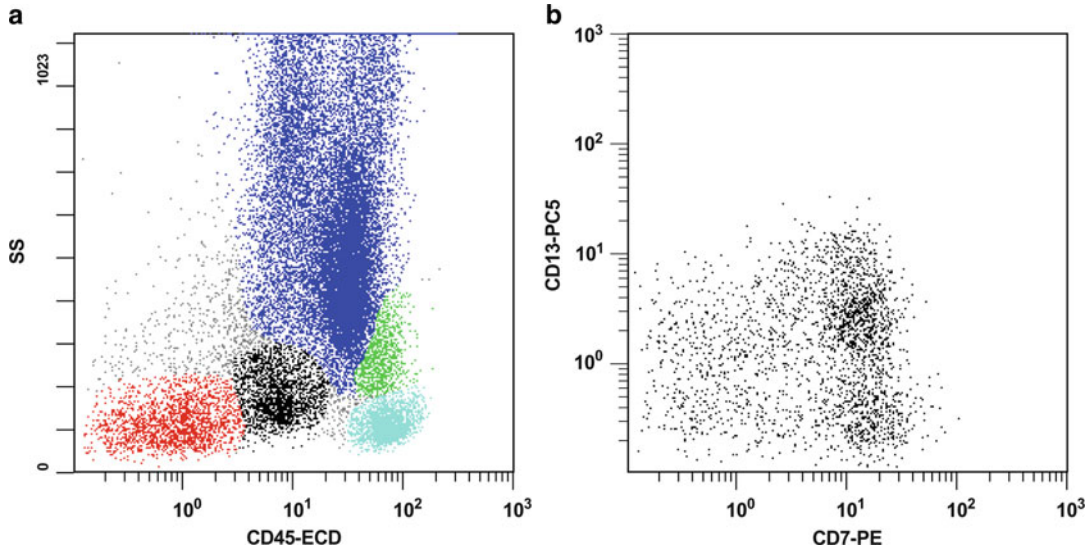


**Fig. 9.15** Flow cytometry demonstrates phenotypic abnormalities in the majority of MDS cases. (**a**) Hypogranulated neutrophils (in *navy*) can be visualized by their abnormally low SSC. Slight increase in the number of blasts is also seen in this case. (**b**) Coexpression of CD7 on myeloid blasts is frequent in MDS

MDS cases and can be routinely analyzed in granulocytic, monocytic, and erythroid lineages

- It has been previously reported that even in cases with minimal or no morphologic features indicative of MDS, flow cytometry was predictive of future cytogenetic abnormalities and diagnosis of myelodysplasia
- Recent studies underscored the prognostic significance of specific immunophenotypic abnormalities for the natural course of the disease or the outcome after bone marrow transplantation
- The abnormalities detected by flow cytometry in MDS include
  - Aberrant SSC reflecting morphologically identified dysplasia: hypogranulated neutrophils can be visualized by their abnormally low SSC in up to 70% of cases (Fig. 9.15a)
  - Changes in the relative proportion of cells at specific stages of myeloid maturation: the high-grade MDS usually demonstrates increased number of immature cells. A significant left shift in granulocytic maturation and an increase in blast percentage can be demonstrated by flow cytometry (Fig. 9.15a)
  - The disruption of normal maturation patterns as reflected by the asynchronous expression of myeloid markers
    - Appearance of late myeloid markers inappropriately early in the differentiation (i.e., CD15 on myeloblasts)
    - Persistent expression of immature markers in late granulocytic stages (e.g., retention of CD34 and HLA-DR on mature granulocytes)
    - Uncoupling of the normal sequence of CD13 and CD16 expression in granulocytic differentiation
  - In addition, blasts in MDS (and other myeloid neoplasms) can demonstrate
    - Detectable early progenitor population positive for CD34 and negative for CD38 antigen
    - Decreased or increased antigen density such as CD45
    - Aberrant expression of immature markers (CD34, CD117, HLA-DR)
    - Absence of myeloid markers CD13 or CD33

- Expression of markers associated with lymphoid lineage (CD7, CD56, CD4, CD2, CD5; Fig. 9.15b)

– The lymphoid populations in bone marrow of patients with MDS show the following changes
  - The paucity of B-lymphoid precursors, which can help to differentiate MDS from nonclonal cytopenias
  - The significant increase in CD8 positive T cells, expansion of CD4 positive cells, and reduced Th2 and regulatory T cells seen in low-risk MDS
  - V beta-restricted CD8 positive T cells in patients who respond well to immunosuppressive therapy
  - Increased number of CD3 + CD4 + IL-17 producing T cells (Th17) and regulatory T cells in high-risk MDS

#### 9.5.2.2 Chronic Myeloproliferative Neoplasms

- The utility of flow cytometry in chronic myeloproliferative neoplasms is less well established

**Chronic Myelogenous Leukemia, *BCR-ABL1* Positive**

- Application of flow cytometry as a diagnostic tool in chronic myelogenous leukemia is limited to the accelerated phase or blast crisis, in which the lineage of an expanding blast population needs to be determined
- In the chronic phase, the presence of the Philadelphia chromosome (as seen on conventional karyotyping or molecular analysis) remains the defining feature of this disorder

***BCR-ABL1* Negative Chronic Myeloproliferative Neoplasms**

- In general, flow cytometric abnormalities are seen in the majority of cases with cytogenetic abnormalities
- However, no consistent set of abnormalities to routinely subclassify neoplastic myeloproliferative states was described

## 9.6 Flow Cytometric Analysis of Lymphoid Neoplasms (Lymphoblastic Leukemia/Lymphoma and Mature Lymphoid Neoplasms)

- The diagnosis of lymphoid malignancies relies on the presence of lineage-associated markers corresponding to specific stages of lymphoid development
- No single marker can be used for a definite diagnosis; thus, the presence of several B cell or T cell-associated antigens is used for lineage assignment
- The sentinel feature of mature B cells and T cells is the presence of surface receptor complexes
- The immune system has to respond to a wide array of antigens. In consequence, in healthy individuals, B cells and T cells express a great diversity of surface receptor complexes (Ig and TCRs). This diversity defines reactive polyclonal lymphoid populations
- On the contrary, the neoplastic lymphoid cells are characterized by monoclonal B cell and T cell receptors. In the majority of cases, the presence of clonality is a definite confirmation of the malignant nature of lymphoid proliferation
- Lymphoid precursors often show an absence of surface receptor complexes. Thus, in precursor-derived neoplasms, the homogeneous expression of specific markers on lymphoblast population, rather than the presence of clonal surface receptors, is considered diagnostic of malignancy. The following paragraph presents the key immunophenotypic features of lymphoblastic and mature lymphoid malignancies

### 9.6.1 B Lymphoblastic Leukemia/Lymphoma (B-LL)

- B-LL shows expression of markers seen in normal B cell differentiation such as CD19, CD22 (cytoplasmic or membranous), CD79a, HLA-DR, CD34, and TdT (Fig. 9.16)

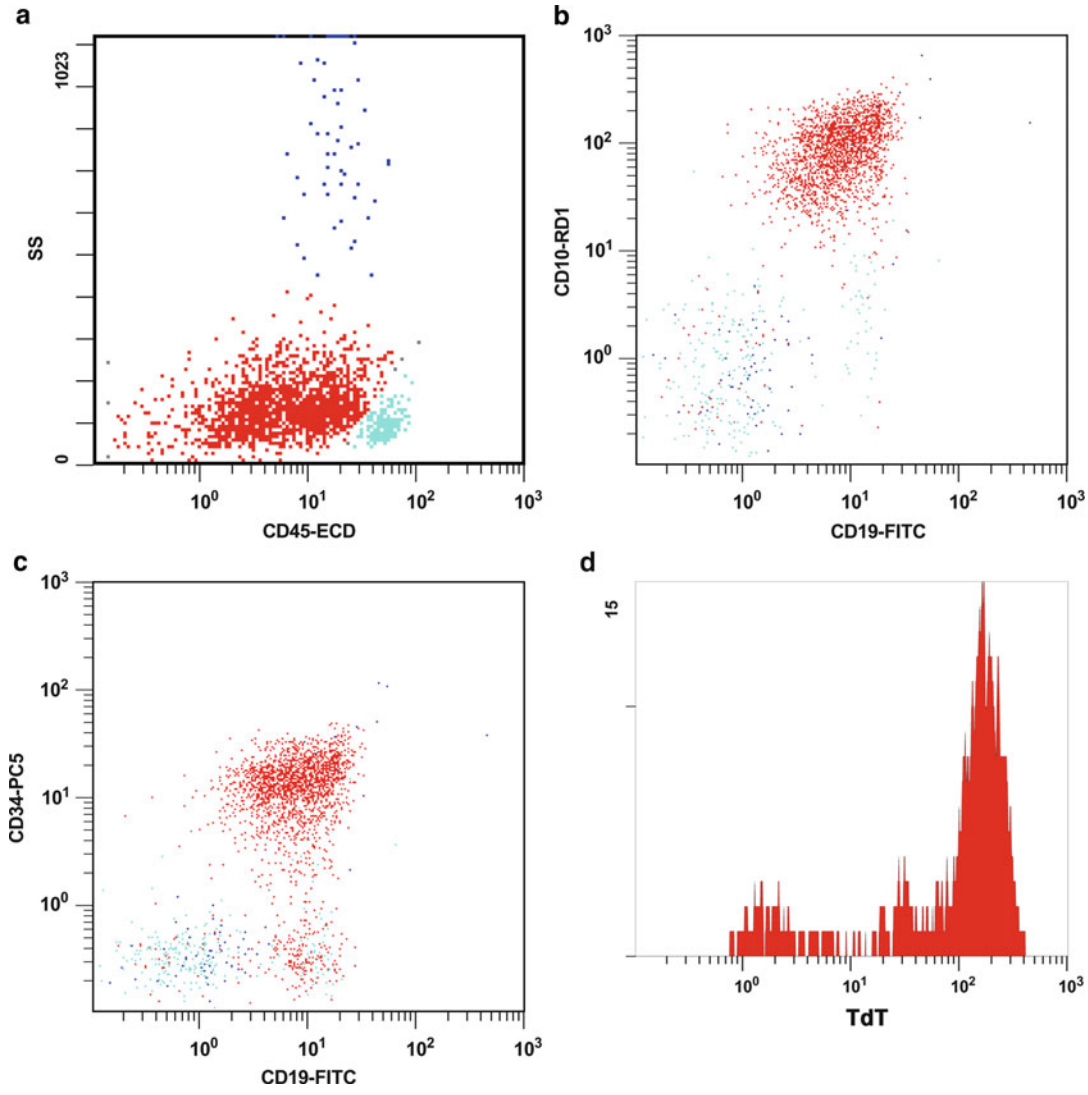


**Fig. 9.16** Pre-B ALL. (**a**) A distinct blast population is characterized by low SSC and low-density CD45 antigen expression. (**b**) The CD10 antigen tends to be expressed at higher levels than in normal B cell precursors. (**c**) The majority of leukemic cells are positive for CD34. (**d**) TdT is brightly positive

- High-density CD10 antigen is frequently seen (Fig. 9.16b)
- Surface Ig light chains are not present; however, cytoplasmic μ-chain or IgM may be detected
- Immunophenotypes of leukemic lymphoblasts resemble stages of normal B cell differentiation. However, even though maturation sequence is roughly reproduced, the majority of cases show aberrant expression of select markers. In addition, the categorization according to maturation stage is of limited practical value as clinical behavior is influenced mostly by clinical features and genetic abnormalities
- Specific immunophenotypes frequently correlate with cytogenetic and clinical features. However, in routine practice, the confirmation of cytogenetic abnormality with either conventional karyotyping and/or molecular techniques is necessary. Select examples of immunophenotypic-genotypic associations are presented below

#### 9.6.1.1 B-LL with t(v;11q23); *MLL* Rearranged

- This rearrangement commonly occurs in infant B-LL, while the frequency of *MLL* involvement in older children and adults is much lower (<5%)
- The most frequent fusion partner for *MLL* gene in ALL is *AF4* gene on chromosome 4q21 (t[4;11])
- Rarely other genes are involved including *ENL* (19p13.3) and *AF9* (9q21–22)
- CD19, CD34, and TdT are positive. The more mature B cell marker, CD20, is negative
- Contrary to the majority of B-LL cases, blasts in this leukemia are negative for the CD10 antigen, which indicates the early stage of B cell maturation
- Myeloid markers, CD15 and CD65, are frequently positive

#### 9.6.1.2 B-LL with t(9;22)(q34;q11.2); *BCR-ABL1*

- Philadelphia chromosome [t(9;22(q34;q11.2); *BCR-ABL1*] is a hallmark of chronic myelogenous leukemia. However, a *BCR-ABL1* translocation with a breakpoint in the minor breakpoint region (m-*BCR*) occurs in both pediatric and adult B-LL
- Cases of LL with *BCR-ABL1* translocation carry a particularly dismal prognosis; thus, their prompt identification is essential for effective treatment decision making
- Most *BCR-ABL1* cases have a classic intermediate (common) LL immunophenotype with the expression of CD19 and TdT
- The homogeneous CD10 and CD34 and dim/heterogeneous CD38 expressions are seen
- The expression of myeloid markers, especially CD13, is common
- The expression patterns of CD34 and CD38 allow differentiation between *BCR-ABL1-positive* and negative LL in multivariate analysis

#### 9.6.1.3 B-LL with t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)*

- *TEL-AML1* translocation occurs in 25% of childhood B-LL cases and is associated with a favorable prognosis
- The detection of t(12;21) by conventional karyotyping can be challenging; thus, the identification of this subset of B-LL by flow cytometry adds to a diagnostic accuracy
- The immunophenotype is that of a precursor B cell with expression of CD19, CD34, CD10, and TdT. CD20 antigen is negative
- The CD45 is more commonly positive and the aberrant coexpression of CD13 is frequent
- The most specific immunophenotypic features predictive of *TEL-AML1* fusion are negative or partially positive CD9 and negative CD20

### 9.6.2 T Lymphoblastic Leukemia/Lymphoma (T-LL)

- Both T lymphoblastic leukemia and T lymphoblastic lymphoma are derived from immature cells committed to T cell lineage
- Similar to B-LL, dependent on the primary site of involvement, that is, bone marrow or lymph node, the designation of leukemia or lymphoma is used
- The most specific marker of T cell differentiation is the CD3 antigen. Similar to normal T cells, in pre-T ALL, this antigen is initially seen in the cytoplasm before the transfer to the cell surface as a portion of the TCR complex
- Other T cell antigens include CD2, CD7, CD5, CD1a, CD4, and CD8
- T-LL mimics the normal maturation of T cells; however, aberrant antigen densities and/or antigen loss are frequent (Fig. 9.17)
- CD34 and CD10 may also be present; however, HLA-DR is typically absent
- In T-LL, the correlation of the immunophenotype with specific genetic lesions is not clear

### 9.6.3 Mature Lymphoid Neoplasms

- The flow cytometric diagnosis of lymphomas is based on the presence of clonal lymphoid population bearing numerous lymphoid markers
- Gating based on SSC/FSC characteristics is most commonly used

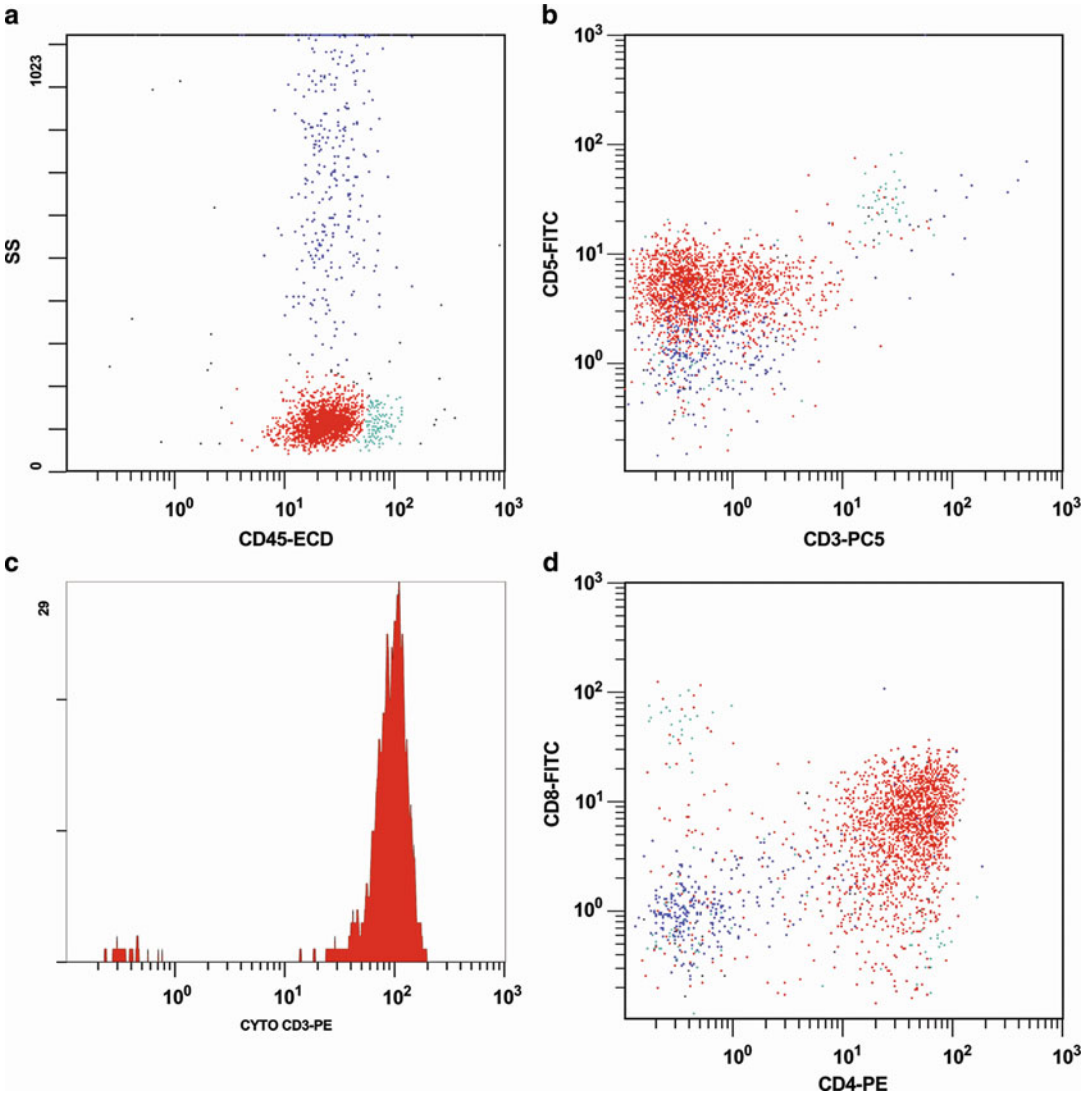


**Fig. 9.17** T-LL in the bone marrow. (**a**) Predominant cell population is seen in the blast gate. (**b**) CD3 antigen can be negative on the surface of leukemic cells. Note internal control, residual normal T cells (in *aqua*), positive for surface CD3 and CD5. (**c**) In surface CD3 negative cases, the CD3 antigen can be demonstrated in cytoplasm of leukemic cells. (**d**) The coexpression of CD4 and CD8 can be seen

- On FSC/SSC dot plots, neoplastic lymphoid cells are seen in the area of small or large lymphocytes. In large cell lymphomas or hairy cell leukemia, the neoplastic population can overlap with the monocyte region
- The display of SSC vs CD45 is not typically used for gating of lymphomas since most mature lymphoid malignancies display high-density CD45 antigen. Only rare cases of lymphoma show slightly dimmer CD45 or, even more infrequently, are negative for the CD45 antigen as in lymphomas with plasmablastic differentiation
- The designation "clonal" implicates that the entire lymphoma population is derived from a single lymphoid cell that underwent malignant transformation. Thus, all neoplastic cells

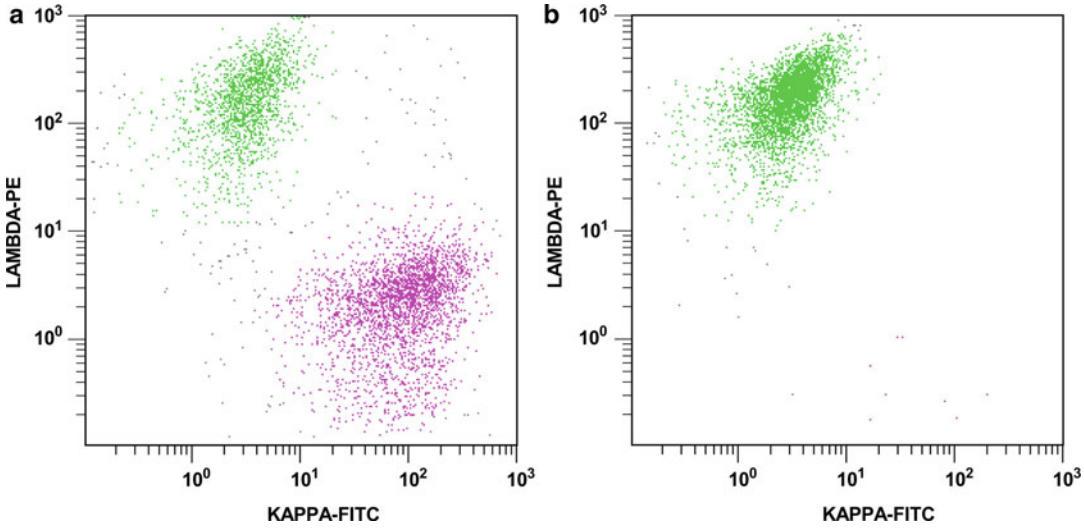


**Fig. 9.18** Surface Ig light chain expression in reactive and malignant B cells. (**a**) Reactive B cells show heterogeneous (polyclonal) expression of κ and λ. (**b**) Mature B cell neoplasms are monoclonal with the entire lymphoma population expressing only one type of Ig light chain

should demonstrate similar genetic and immunophenotypic features. The clonality is best represented as an expression of uniform (monoclonal) surface light chain or TCR. This stands in stark contrast to the highly variable, polyclonal immunophenotype of normal lymphocytes, which reflects a random receptor gene rearrangement as a response to a variety of antigenic stimuli

### 9.6.3.1 Mature B Cell Neoplasms

- Normal precursor B cells randomly rearrange Ig heavy and light chains. As a result, mature B cells show a polyclonal pattern of Ig heavy and light chains (Fig. 9.18a). In contrast, a monoclonal surface light chain expression (exclusively κ or λ) is seen in the majority of B cell lymphomas (Fig. 9.18b)
- The light chain monoclonality along with the expression of pan-B cell markers is in most instances diagnostic of B cell lymphoma. The lymphoma subclassification is based on the presence and density of specific lymphoid markers
- Rarely, mature lymphoid neoplasms lose their surface Igs, a feature not commonly seen in normal mature B cells
- On the contrary, neoplastic plasma cells typically lack surface Igs and show only cytoplasmic expression of κ or λ

#### Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL)

- CLL and SLL are derived from recirculating CD5+, IgM+, and IgD ± B cells normally present in the peripheral blood (Fig. 9.19)
- The WHO classification considers CLL and SLL as one entity with different presentations. The diagnosis is based on the predominant site of involvement (bone marrow/peripheral blood vs lymphoid organs)
- CLL/SLL is positive for pan-B cell antigens including CD19 and CD20 (dim CD20 expression, Fig. 9.19b and c)
- In addition, the expression of CD5, CD23, and weak surface monoclonal (κ or λ) light chain is seen
- The absence of FMC7 and cyclin D1 and presence of CD23 are features distinguishing CLL/SLL from mantle cell lymphoma (MCL)
- Currently, two groups of CLL/SLL are recognized

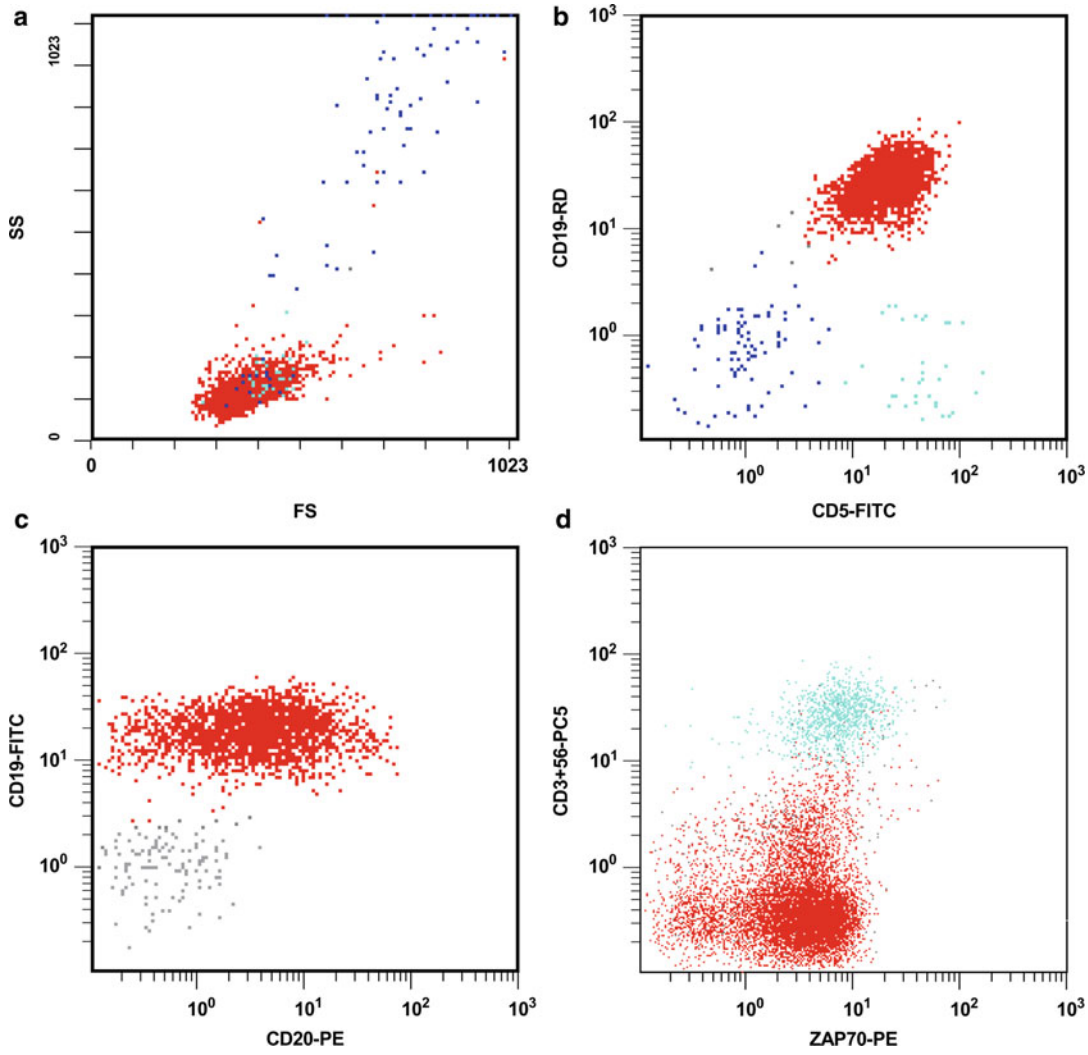


**Fig. 9.19** (**a**) CLL/SLL are composed of small lymphoid cells (in *red*). (**b**) Neoplastic cells are positive for CD19 and coexpress CD5. (**c**) The expression of CD20 antigen is dim. (**d**) ZAP-70, the expression which has been linked to the unmutated variable region of Ig heavy chain, is expressed in a proportion of cases. Note internal positive control in aqua (T and NK cells)

– One, corresponding to the pre-germinal center phenotype (naïve, showing no mutations in the variable region of Ig heavy chain [$V_H$] gene)
– The second type is derived from memory B cells (post-germinal center, mutated $V_H$ gene)
– The subclassification roughly corresponds to the expression of ZAP-70 (Fig. 9.19d) and CD38 molecules, which can be quantified by flow cytometry

### Monoclonal B Cell Lymphocytosis

- Occasionally, low numbers of monoclonal B cells are seen in peripheral blood of asymptomatic elderly individuals without any other site of involvement by lymphoma. This condition, named monoclonal B cell lymphocytosis, has to be distinguished from circulating lymphoma cells
- Monoclonal B cell lymphocytosis (MBL) is defined as monoclonal B cell population in

peripheral blood of a patient without signs and symptoms of B cell lymphoproliferative neoplasm, autoimmune disease, or infectious process
- These cells can demonstrate an immunophenotype similar to that seen in CLL/SLL and are present at the number below the threshold for the diagnosis of CLL/SLL (less than 5,000/ul)
- The incidence is variable; overall 3.5% of healthy individuals over 40 years of age are diagnosed with MBL
- The prevalence is highly dependent on the sensitivity of flow cytometric methodology
- In a proportion of patients, MBL can precede the overt CLL/SLL or other B cell lymphoproliferative disorder
- The level of MBL is linked to the risk of development of CLL/SLL

### Mantle Cell Lymphoma (MCL)
- MCL demonstrates expression of pan-B cell markers (CD19, CD20) and clonal surface light chains
- There is coexpression of CD5 antigen; however, CD23 is negative
- In contrast to CLL/SLL, the high-density CD20 and high-density light chains are seen, and there is coexpression of FMC7, the antigen that is invariably negative in typical cases of CLL/SLL
- The defining feature of MCL is the t(11;14), in which the *cyclin D1 (CCND1)* gene is translocated into the proximity of the Ig heavy chain gene promoter, resulting in the constitutive expression of this protein. The presence of cyclin D1 can be demonstrated by flow cytometry

### Follicular Lymphoma (FL)
- The immunophenotype reflects the follicle center cell origin of FL
- Pan-B cell markers (CD19, CD20) are present along with the coexpression of CD10 and clonal surface Ig
- The coexpression of CD10, similar in density to that seen in reactive follicular hyperplasia, and relative low-density CD19 are characteristic features of FL
- In contrast to reactive germinal centers, neoplastic follicular cells express BCL2, which results in decreased sensitivity to apoptosis and allows for the accumulation of neoplastic lymphocytes. The expression of BCL2 by FL cells is due to the t(14;18)(q32;q21), which places the *BCL2* gene under a promoter of the Ig heavy chain gene

### Marginal Zone Lymphomas
- Three subtypes of marginal zone lymphomas are recognized: nodal, extranodal (mucosa-associated lymphoid tissue lymphoma), and splenic. They all share similar immunophenotype
- Marginal zone lymphomas express the B cell markers (CD19, CD20, and CD22) and clonal surface light chains
- CD5 and CD10 are absent in the majority of cases
- No specific surface markers, routinely analyzed by flow cytometry, allow for subclassification of this lymphoma based on immunophenotype; therefore, the diagnosis is based on the absence of immunophenotypic features specific for other subtypes of lymphoma

### Lymphoplasmacytic Lymphoma/Waldenstrom Macroglobulinemia
- Lymphoplasmacytic lymphoma is a B cell lymphoproliferative disorder composed of a heterogeneous proliferation of small B cells, lymphoplasmacytoid lymphocytes, and plasma cells
- The defining feature is the demonstration of monoclonal IgM protein in the serum
- B cell-associated antigens, including CD19, CD20, and CD22, are consistently expressed
- In the majority of the cases, the B cell immunophenotype is nondescript
- Frequency of coexpression of other antigens seen in B cell lymphomas such as CD5, CD23, and FMC7 is variable. CD10 is most frequently negative

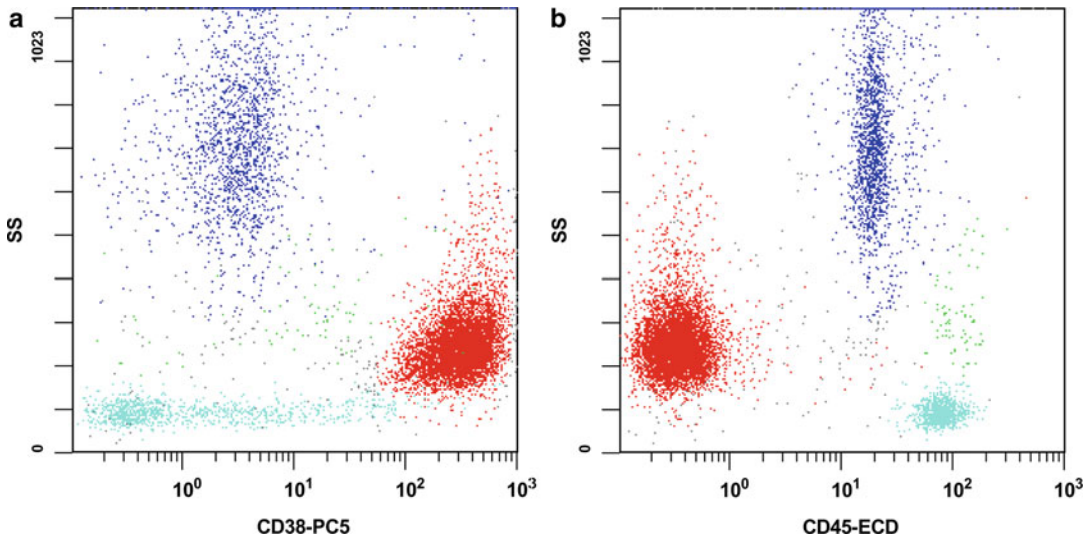


**Fig. 9.20** Plasma cells, both neoplastic and reactive, are best visualized using CD38 antigen. (**a**) The reactive plasma cells and the majority of their neoplastic counterparts express characteristic high-density CD38 (in *red*). (**b**) The neoplastic plasma cells are frequently negative or dim positive for CD45 antigen

- The surface IgM expression can be demonstrated in all cases
- The majority of cases show dim positivity for CD25 antigen
- In a proportion of cases a second subset of monoclonal B cells is identified, corresponding to lymphoplasmacytoid lymphocytes (intermediate FSC and SSC, positive for CD19, CD20, FMC7, and bright CD38 and negative for CD138)
- In addition, as reported previously, a minute monoclonal plasma cell component can be identified in the majority of cases

## Plasma Cell Neoplasms

- Plasma cell neoplasms are characterized by a monoclonal proliferation of terminally differentiated B cells, that is, plasma cells
- These disorders can present as a localized or disseminated process most commonly involving bone marrow, bone, and, more infrequently, extramedullary sites and peripheral blood
- Neoplastic plasma cells demonstrate a highly heterogeneous immunophenotype, different from that of their normal counterpart
- Due to overlap with various hematopoietic populations on SSC/FSC and SSC/CD45, the identification of plasma cells is best accomplished through their expression of CD138 and uniquely bright CD38 (Fig. 9.20a)
- The majority of neoplastic plasma cells, unlike their normal counterpart, show a decreased density of CD45 antigen (negative to weakly positive, Fig. 9.20b). Only about 20% of plasma cell myelomas demonstrate homogeneous bright to heterogeneous expression of CD45, similar to that of normal plasma cells
- Neoplastic plasma cells are most often negative for the pan-B cell markers, CD19 and CD20. The expression of CD20 is retained in approximately 20% of cases. CD19 is seen in $<5\%$ of neoplastic plasma cell proliferations
- Neoplastic plasma cells present with monoclonal cytoplasmic and occasionally surface Igs
- The CD56 antigen is seen in 70% of myeloma cases, and its absence has been associated with adverse clinical outcome
- The presence of myeloid markers including early antigens, such as CD117, is frequently reported

**Diffuse Large BCL (DLBCL)**

- The defining morphologic feature of DLBCL is a large cell size that can be appreciated on the displays of FSC vs SSC
- As in other B cell lymphomas, pan-B cell antigens such as CD19, CD20, and CD22 are positive
- DLBCL can originate from different stages in B cell development; hence, the coexpression of other markers is heterogeneous. The CD5, CD10, BCL-6, CD30, and CD138 can all be present

**Burkitt Lymphoma (BL)**

- This lymphoma is composed of medium-sized, highly proliferating lymphoid cells with basophilic vacuolated cytoplasm. The WHO classification distinguishes three variants of this lymphoma: endemic (occurring predominantly in Africa), sporadic, and immunodeficiency associated. All variants show similar immunophenotypic features
- The immunophenotype of BL reflects germinal center origin
  - CD19, CD20, CD10, and BCL-6 antigens are positive
  - BCL-2 is negative
- As in the majority of mature B cell neoplasms, there is surface expression of monoclonal Ig light chains
- Even though the immunophenotype of BL alone is not specific enough for the definitive subclassification of this lymphoma, the confirmation of high-proliferative activity associated with the expression of CD71 can be used to support the diagnosis. This feature is linked to the constitutive expression of *MYC* gene (cell cycle gate-keeping gene) due to its translocation under the promoter of Ig heavy or light chain genes (t(8;14), t(2;8), t(8;22)). The above translocations are pathognomonic of BL

### 9.6.3.2 Mature T and NK Cell Lymphomas

- Lymphomas derived from mature T and NK cells are much less common than the previously discussed mature B cell neoplasms and show greater geographic and ethnic variability
- The immunophenotypic features of T and NK cell malignancies are overlapping and frequently less specific than those seen in B cell lymphomas
- Expansion of a specific T cell subset (e.g., CD4 or CD8) with loss or altered intensity of T cell-associated markers (most commonly CD7, CD3, and CD5) is seen in the majority of T cell lymphomas (TCL)
- Aberrant immunophenotype is a reliable diagnostic feature only when the neoplastic population is significant since small numbers of T cells with unusual antigen makeup can also be seen in inflammatory conditions, autoimmune disorders, or viral infections. Thus, the aberrant immunophenotype alone cannot be considered pathognomonic of a T cell malignancy
- Considering these factors, an integration of morphologic, immunophenotypic, cytogenetic, molecular, and clinical information, as stressed by the WHO classification, is of particular importance in diagnosing T and NK cell malignancies
- Until recently, the demonstration of clonality in T cell proliferations was limited to molecular methods detecting *TCR* gene rearrangements (PCR or Southern blot analysis). The development of multiple Vβ-family antibodies directed against the variable region of the TCR β-chain allows for the determination of T cell clonality by flow cytometry. The determination of clonality is based on the preferential usage of a single Vβ-family and has close to 90% sensitivity and specificity in diagnosing T cell lymphoproliferative disorder. The results of this assay must be correlated with additional immunophenotypic and clinical data since rare cases with Vβ-family expansion have been reported in patients without a diagnosis of malignant lymphoma

**T Cell Prolymphocytic Leukemia**

- This is a mature TCL derived from helper T cells

- The majority of cases retain the mature T cell immunophenotype with expression of CD4 antigen
- Rare cases (~10%) can be double-positive or double-negative for CD4 and CD8
- CD25 can be coexpressed with the density similar to that of activated mature T cells

### T Cell Large Granular Lymphocytic Leukemia (T-LGL)

- T-LGL is an indolent lymphoproliferative disorder derived from cytotoxic T cells
- The demonstration of a monoclonal aberrant T cell population in bone marrow and peripheral blood in a patient with cytopenias is a hallmark of the disease
- Considering the variable morphology of T-LGL, flow cytometric immunophenotyping plays a key role in diagnosing this disease
- The immunophenotype is similar to CD8+ T cells. Varying degrees of loss or decreased density of CD7, CD2, and CD3 were reported
- CD57 is positive in the majority of cases. A small number of cases express CD56 and/or CD16
- NK receptors for class I major histocompatibility molecules (both of killer cell Ig-like receptor type, CD158 antigens; and C-type lectin type, CD94, and NKG2 molecules) showed aberrant expression in the majority of reported cases supporting the diagnosis of T-LGL

### Aggressive NK Cell Leukemia

- This is a rare neoplasm of NK cells typically presenting with systemic involvement and an aggressive clinical course
- As in normal NK cells, surface CD3 and CD5 are absent
- The CD2, CD56, and CD16 are present in the majority of cases. CD57 can be absent
- There is a varying degree of CD7 loss

### Extranodal NK/T Cell Lymphoma, Nasal Type

- Nasal type NK/T cell lymphomas are EBV-positive extranodal proliferations derived from NK or cytotoxic T cells. Both upper respiratory tract and other extranodal sites can be involved
- The immunophenotypic features include positivity for CD56, CD2, frequently CD7, and the cytoplasmic ϵ-chain of CD3 antigen
- CD8 and CD4, as well as other T cell-associated markers, are negative
- Rare cases demonstrate cytotoxic T cell immunophenotype

### Adult T Cell Leukemia/Lymphoma

- Adult T cell leukemia/lymphoma is a mature T cell neoplasm caused by human lymphotropic virus type 1 (HTLV-1)
- Even though classically it has been considered to be a neoplasm of helper T cells, subsequent studies demonstrated its immunophenotypic and functional similarity to regulatory T cells
- In addition to pan-T cell markers, CD3, CD5, and CD2, the expression of CD4, bright CD25, and regulatory T cell marker FoxP3 can be demonstrated by flow cytometry
- CD7 antigen and cytotoxic/NK cell markers are absent

### Hepatosplenic TCL

- Hepatosplenic TCL is a disseminated TCL originating from γδ T cells
- The neoplastic cells are positive for surface CD3 and associated γδ TCR. Only rare cases of αβ TCR type were also reported
- Typically, CD5, CD4, and CD8 antigens are negative
- CD56 and CD16 are expressed in some cases. CD57 is negative

### Angioimmunoblastic T Cell Lymphoma (AILT)

- AILT is most commonly diagnosed by the combination of morphologic, immunophenotypic, and clinical features. The origin of this lymphoma is a CD4+ T cell from the germinal center
- The pathologic diagnosis can be challenging due to morphologic heterogeneity and immunophenotypically mixed T cell

proliferation with a significant admixture of reactive component
- Even though the number of malignant T cells may be low, flow cytometry demonstrates the immunophenotypically aberrant T cell population in >90% of cases
- The immunophenotype is that of CD4+ mature T cells with a varying loss of the CD3 and CD7 antigens
- CD8 and CD56 antigens are absent
- Characteristic coexpression of CD10 is seen in 80% of cases

### Mycosis Fungoides (MF) and Sezary Syndrome (SS)

- MF is the most common cutaneous lymphoma. SS presents as a disseminated disease with widespread skin involvement, lymphadenopathy, and circulating lymphoma cells
- Flow cytometry is most commonly utilized to demonstrate circulating MF/SS cells with a specific T cell immunophenotype that includes the expression of pan-T cell markers CD3, CD5, and CD2 along with CD4 antigen
- An important feature is the absence of the CD7 and CD26 antigens. Other T cell-associated antigens such as CD2, CD3, and CD5 can also be negative (Fig. 9.21)

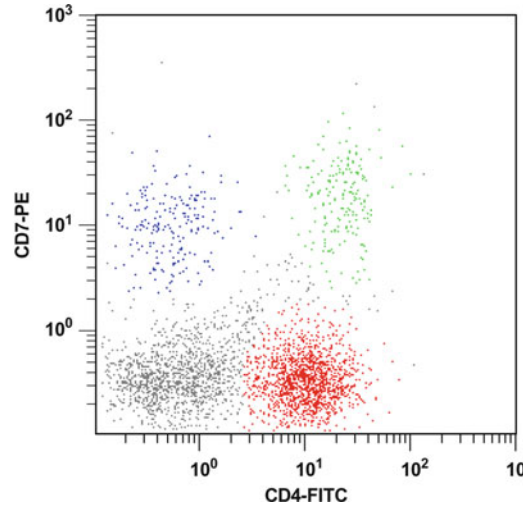


**Fig. 9.21** MF and SS are positive for CD4 and commonly lose CD7 antigen (in *red*; residual helper [T cell] cells are presented in *green* and cytotoxic T cells are in *navy*)

### Enteropathy-Associated TCL

- Enteropathy-associated TCL is derived from intraepithelial T cells and involves predominantly small bowel
- Flow cytometric immunophenotype was reported in rare cases and demonstrated neoplastic lymphoid cells positive for CD3, CD2, CD7, CD11c, and CD103 with variable loss of pan-T cell antigens, most commonly CD5
- Reported cases were positive for CD8 or double-negative for CD8 and CD4

### Peripheral T Cell Lymphoma, Not Otherwise Specified (PTCL)

- This is a morphologically heterogeneous group of lymphomas with mature T cell phenotype
- The majority of the cases are derived from CD4+ T cells and retain this immunophenotype. pproximately 10% of cases show CD8 expression. Double-negative cases have also been reported
- Variable loss of pan-T cell antigens is seen. Most frequently CD7 (in 50% of cases) and surface CD3 are lost

### Anaplastic Large Cell Lymphoma (ALCL)

- ALCL is composed of large pleomorphic cells, which, as other lymphomas with this morphology, often fall in the gate overlapping with large lymphoid cells and/or monocytes (Fig. 9.22)
- The presence of CD30 antigen and, in up to 70% of cases, ALK-1 protein is the defining immunophenotypic features of this lymphoma and can be demonstrated by flow cytometry. The overexpression of ALK-1 is most often due to t(2;5)(p23;35), between *ALK* gene and *nucleophosmin (NPM1)* gene. Alternative fusion partners for the *ALK* translocation have also been identified
- Various combinations of the CD30 antigen and T cell markers are seen including CD2, CD4, CD3, CD7, CD5, and CD8
- CD7 antigen is lost most frequently followed by CD5, CD3, and CD2
- CD25 antigen is positive in up to 90% of cases

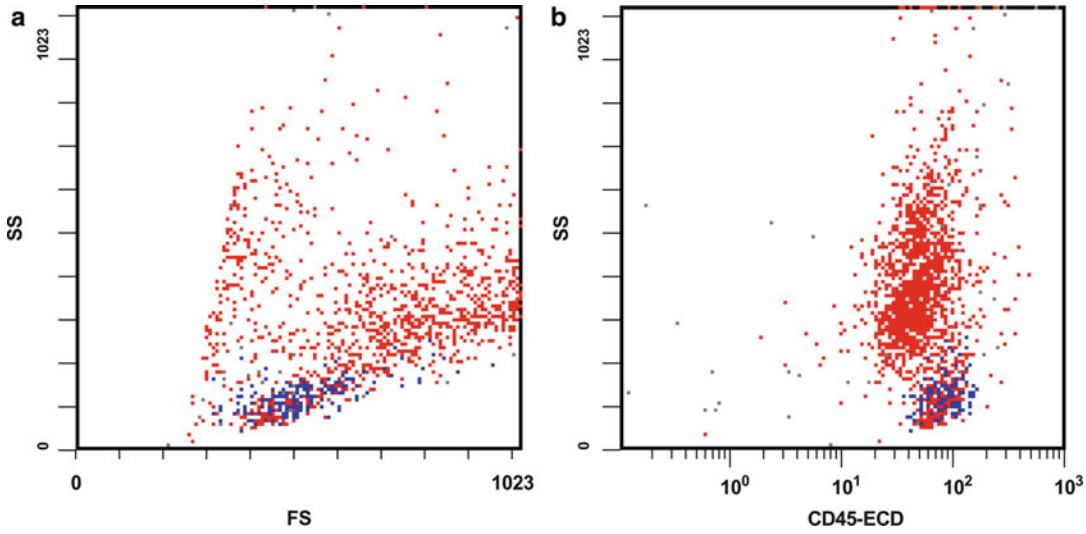


**Fig. 9.22** (**a**) Cells of ALCL show high FSC and frequently increased SSC. (**b**) SSC/CD45 shows an overlap with monocyte gate

- In ALK positive cases, ALK protein can be demonstrated by flow cytometry
- Interestingly, the expression of myeloid markers (CD13, CD33, and CD15) has also been reported, which, especially in the rare cases with leukemic involvement, can bring a myeloid neoplasm into the differential diagnosis

## 9.7 Other Clinical Applications of Flow Cytometry

### 9.7.1 Primary and Secondary Immunodeficiencies

- Flow cytometry is commonly used to diagnose and immunophenotype primary and secondary immunodeficiencies. The detailed discussion of this topic is beyond the scope of this text. The following examples are provided to illustrate the most common applications
  - Immunophenotyping: the loss of specific antigens, such as $\beta_2$ integrins, is easily demonstrated by flow cytometry and is used to diagnose leukocyte adhesion deficiencies
  - Functional defects: an absence or low levels of NADPH oxidase, an enzyme involved in oxidative burst, occurs in chronic granulomatous disease. The level of enzymatic activity can be assayed using flow cytometry and correlate with specific genetic lesions (Fig. 9.23)
    - In the presence of adequate levels of NADPH oxidase, the nonfluorescent compound, dihydrorhodamine 123, converts to fluorescent rhodamine. The phorbol 12-myristate-13-acetate-stimulated granulocytes from healthy volunteers serve as a positive control (Fig. 9.23b)
    - The X-linked recessive form of the disease most frequently results in the complete absence of the enzymatic activity (Fig. 9.23c), whereas the autosomal recessive-type such as defect of p47-phox enzyme subunit presents with markedly decreased level of fluorescence (Fig. 9.23d)
  - Enumeration of CD4+ helper T cells in human immunodeficiency virus infection is performed using flow cytometry and serves as an indicator of disease progression and response to treatment
    - The absolute number of helper T cells in peripheral blood correlates with the stage of the disease and patient prognosis

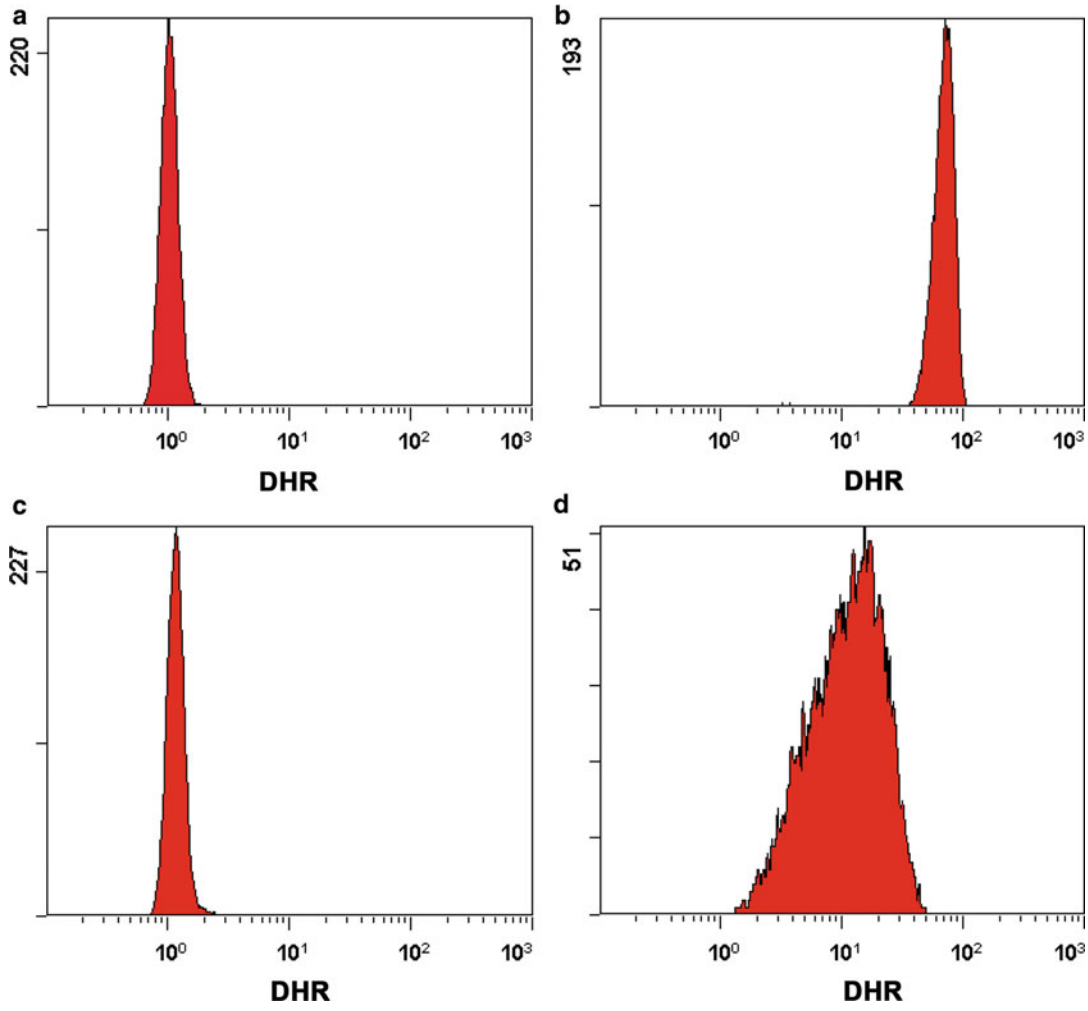


**Fig. 9.23** Chronic granulomatous disease. (**a**) Unstimulated neutrophils from a healthy volunteer show baseline level of dihydrorhodamine 123 fluorescence. (**b**) Normal granulocytes with adequate activity of NADPH oxidase show a distinct shift in fluorescence intensity upon phorbol 12-myristate-13-acetate stimulation. (**c**) X-linked recessive chronic granulomatous disease shows complete loss of enzyme activity (no fluorescence shift). (**d**) Low-level enzyme activity in a patient with p47-phox deficiency corresponds to decreased fluorescence intensity as compared to a normal control

- The enumeration of T cells and their subsets is easily accomplished by flow cytometry using a simple combination of antibodies against CD3, CD4, and CD8 antigens
- The absolute numbers are derived either from a routine white blood cell count of the concurrent peripheral blood specimen (dual platform) or from calibrating beads run simultaneously with the patient sample (single-platform method)

### 9.7.2 Paroxysmal Nocturnal Hemoglobinuria (PNH)

- PNH is caused by an absence or decreased numbers of membranous glycosylphosphatidylinositol (GPI) anchor, which results in a loss of GPI-linked proteins
- Diagnosis of PNH by flow cytometry relies on the demonstration of decreased expression of two GPI-anchored proteins on two cell populations. Various combinations of

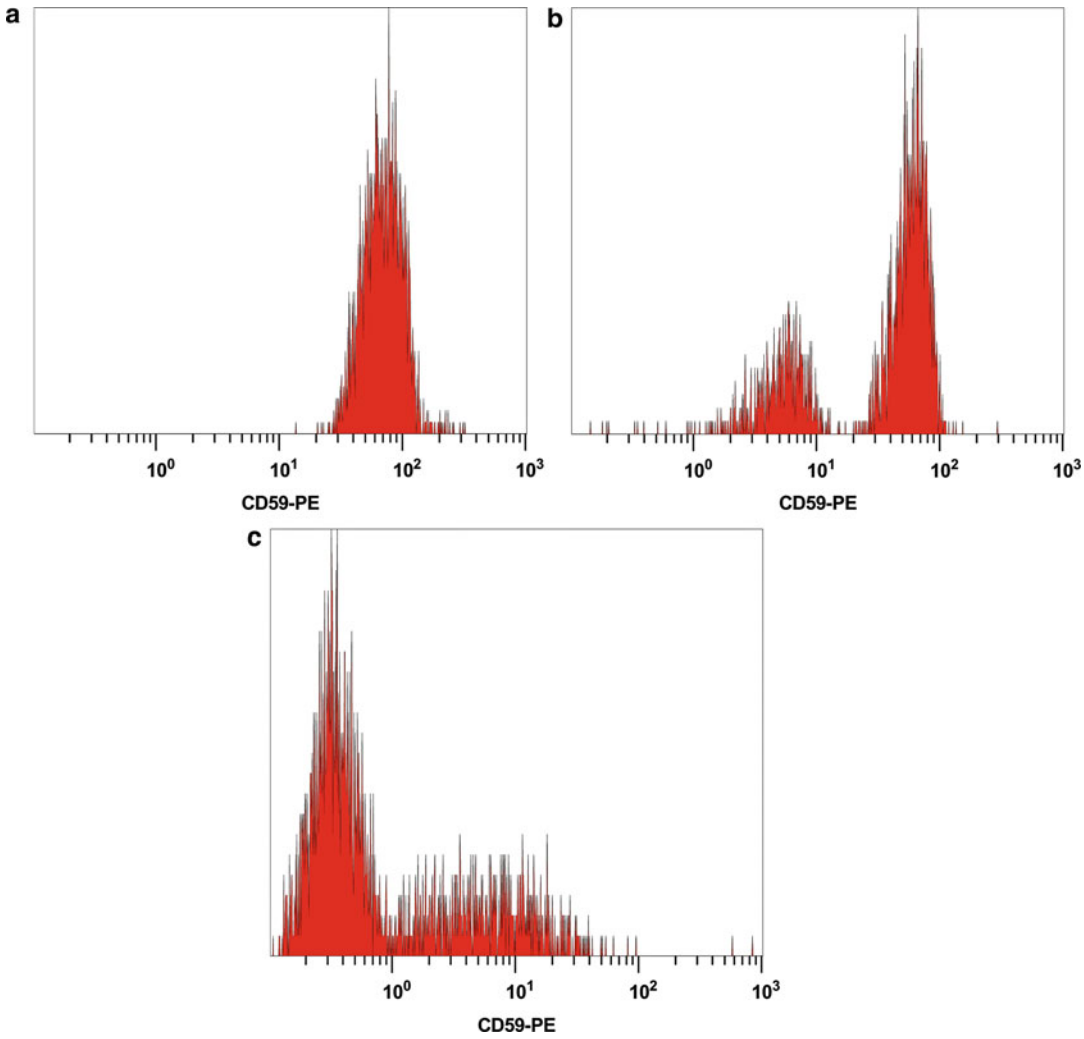


**Fig. 9.24** The diagnosis of PNH is based on the absence of GPI-anchored molecules. Type I (normal), II (partial deficiency), and III (completed loss) cells are best recognized among red blood cells using antibody against CD59 antigen. (**a**) Red blood cells from healthy volunteer, used as a positive control, show high-intensity CD59 (correspond to type I cells). (**b**) Type I and II cells (small peak with decreased expression) in a patient with PNH. (**c**) Complete loss of CD59 (type III cells) in a patient with PNH supported by red blood cell transfusion

antibodies are applied, most commonly against the following antigens: CD55, CD59, CD24, CD14, CD66b, CD16, CD48, and CD157
  - The analysis of CD59 expression on red blood cells provides the best discrimination between type I, II, and III cells (Fig. 9.24)
  - Granulocytes or monocytes are frequently analyzed in addition to red blood cells
- FLAER reagent, which binds directly to GPI-anchors, is also frequently used

### 9.7.3 Stem Cell Transplantation

- Flow cytometry is utilized for enumeration of CD34+ stem cells and cell sorting
  - CD34 population can be enriched using flow cytometric sorting. Using this approach, heterogeneous populations can be physically separated into cell subsets with different physical or immunophenotypic properties

– High-speed flow cytometric sorting is achieved through charging of droplets containing individual cells with a specific polarity. As a charged droplet passes through the electrostatic field, it is isolated from the remainder of the sample and collected into a separate container

### 9.7.4 Novel Applications of Flow Cytometry

- Flow cytometry-based molecular testing: detection of PCR target amplicons using liquid bead array systems
- Tissue typing
- Assaying response to medications, for example, monitoring platelet activation after antiplatelet therapy

## Further Reading


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