Standard immunophenotyping of leukemia cells in acute myeloid leukemia (AML)

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Abstract

Immunophenotyping of leukemia cells plays crucial role in identification of leukemia cell line, definition of maturation stage and finding possible aberrant antigens what in turn serves for individual treatment monitoring and detection of residual disease. The purpose of this study was to develop standard laboratory procedure for immunophenotyping of leukemia cells in acute myeloid leukemia (AML), to establish the kit of necessary antibodies and to work out strategy of typing in acute proliferations.

116 patients with AML type M1-M5 (according to French-American-British classification, FAB) were tested. Immunophenotype determination was carried out by direct antibody staining and analysis with FACSCanto Becton Dickinson flow cytometer.

Flow cytometry analysis revealed that for proper determination of leukemia cells' phenotype the first step is to separate leukemia cells with pan-leukocyte CD45 antigen. The next step is to determine line-specific antigens: CD13, CD14, CD15, CD33, CD64, CD117, CD36, MPO as well as antigens associated with maturation of hematopoietic cells: CD34, CD38, TdT and myeloid cells: CD16, CD66. We established useful screening kits for three- and four-color flow cytometry. Proposed three color kit is: surface: CD45/CD14, CD19/CD13/CD45, CD7/CD33/CD45, CD64/CD16/CD45 and cytoplasm: TdT/CD79a, MPO/cCD3. Proposed four color kit is: surface: CD14/CD64/CD45, CD19/CD13/CD45/CD3, CD7/CD33/CD45/CD117, CD66/CD16CD45 and cytoplasm: CD79a/MPO/CD3/TdT.

Auxiliary but important role plays determination of non-line specific antigens that are characteristic for myeloid and monocytic cells like CD11c, CD11b. Also, it is purposeful to look for lymphoid antigens like CD7, CD19, CD56 which, if co-expressed, are extremely useful to monitor residual disease.

Key words: *immunophenotyping, AML, leukemia cells, standardization.*

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Introduction

Acute myeloid leukemia is an uncontrolled proliferation of precursor myeloid cells with maturation stopped at various stages. As a consequence, those cells accumulate in the bone marrow, normal hematopoiesis is stopped and replaced by leukemic cells which finally appear in the blood and may infiltrate different organs.

Acute myeloid leukemia was initially classified according to FAB, based on morphology of the cells and cytochemistry, which was later completed with immunophenotyping [1]. In 2002 World Health Organization proposed a newer approach to AML classification based mainly on cytogenetic and molecular analysis [2]. However the latter has not been universally accepted and many centers still use either the former classification or both of them simultaneously.

The first classification of acute non-lymphoblastic leukemias was proposed in 1995 by EGIL (European Group Immunophenotyping Leukemia) [3] and in 1996 Rothe et al. presented a consensus for basic immunophenotyping [4]. Further improvement in immunophenotype detection and AML subtype classification was published by EWWGCA group and Orfao [5-7] who proposed modifications in basic diagnostic panels of antibodies.

Definition of cell immunophenotype in acute leukemias is commonly employed to identify malignant line, maturation stage and finally pattern of antigen expression

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in individual patients what permits better monitoring of treatment and residual disease.

Material and Methods

1. Laboratory preparation of diagnostic specimens before flow cytometry analysis

Sample type	Bone marrow aspirate or peripheral blood drawn on EDTA – minimal volume 1 mL
Initial preparation	1. Cell counting with automatic analyzer (optionally manual counting) 2. Washing with PBS twice and cell count correction to $10\text{-}20 \times 10^3$/mL
Sample storage	Storage at room temperature (18-22°C) with no preservatives added; samples should preferably be analyzed within 6 or 12 hours (bone marrow and peripheral blood respectively)

Method

Appliance	Flow cytometer with 488 nm laser (optionally with additional laser for greater number of simultaneous measurements) Minimal requirements: 5 simultaneous measurements: FSC, SSC, 3 fluorescent dyes (F1, F2, F3)
Type of staining	Direct staining with fluorochrome conjugated antibodies
Fluorescent dyes	Fluorochrome choice should depend on tested antigens: 1. PE for low antigen expression 2. PE-Cy5, PerCP-Cy5.5 or APC for intermediate antigen expression 3. FITC or PerCP for high antigen expression

Diagnostic test

Fluorescent dyes	At least 3 different dyes (4 dyes recommended)
Antibody producers	Choice of producer at laboratory discretion Restriction: comparative quantitative analyses (density of antigen on cell surface or fluorescence intensity) must be performed with antibodies from the same source
Lysing solution producer	Choice of producer at laboratory discretion We recommend PharmLyse BD as cell morphology is only minimally altered

Incubation with antibodies	If not otherwise recommended by antibody producer: 1. 15-20 min at room temperature in the dark 2. 30 min at 4°C

2. "Cornerstones" of immunophenotyping of acute leukemias at diagnosis

- Definition of leukemia cell line
- Definition of leukemia cell maturation stage (definition of AML subtype)
- Precise immunophenotype definition of leukemia cells. It is also recommended to determine density of analyzed antigens on cell surface or fluorescence intensity of antibodies bound to the antigens.
- Analysis of aberrant antigens – usually antigens typical for other cell lines or discordant with maturation stage – essential for detection of residual disease and early diagnosis of relapse

Results

1. Basis for flow cytometry analysis in AML

Mandatory antigens for flow cytometry analysis in AML:
- Pan-leukocyte antigen CD45 for separation ("visualization") of leukemia cells
- Myeloid line-specific antigens: CD13, CD14, CD15, CD33, CD64, CD117, GlyA, CD41,CD61, MPO (myeloperoxidase) as well as lymphoid line-specific antigens: CD19, cCD79a, cCD3 for determination of leukemia cell line
- Antigens for determination of maturation stage: CD34, CD38, HLA-DR, TdT (terminal deoxynucleotidyl transferase)
- Antigens for assessment of myeloid cells maturation: CD66, CD16

Supplementary antibody panel for precise characterization of leukemia cells should include non-line specific antibodies (antibodies for adhesion molecules and growth factors receptors): CD11c, CD11b, CD36, CD56, CD114, CD116, CD184 (chemokine SDF-1 receptor). It is recommended to use anti- CD71 (transferrin receptor) for determination of leukemia cell proliferation activity.

Employment of all above antibodies permits precise characterization of proliferating cells what is necessary for treatment monitoring and detection of residual disease. For the same purpose detection of aberrant antigens expression on myeloid cells is used (CD15/CD117, CD33/CD7, CD13/CD19 for example).

2. Steps of immunophenotyping

a. Initial step – leukemia cell separation ("gating")

The procedure of AML immunophenotyping starts with separation of malignant cells. The tool used for that purpose
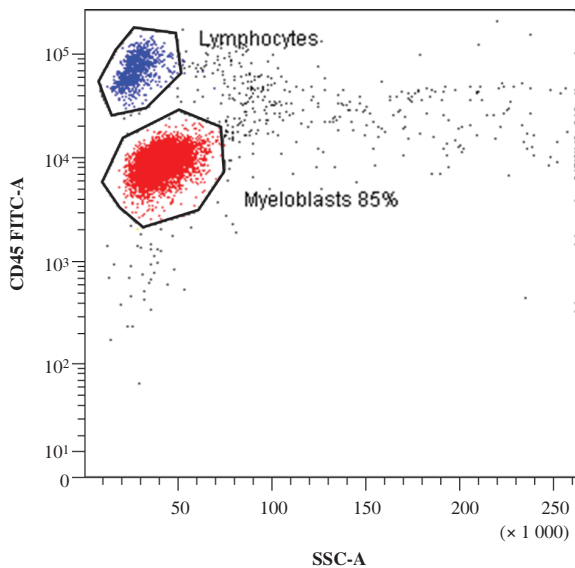
Fig. 1. CD45 expression as "gate marker" of leukemic cells, different from that on normal myeloid and monocytic cells. Lymphocytes expressing CD45 as reference for localization of examined leukemia cells on cytograms

is analysis of CD45, which shows diverse expression on leukemic cells different from that on normal myeloid and monocytic cells. CD45 is referred to in literature as "gate marker". Apart from screening it should be used for line identification and maturation stage (definition of AML subtypes) together with other antibodies. Simultaneously lymphocytes expressing CD45 are marked as reference for localization of examined leukemia cells on cytograms [8] (Figure 1).

b. First step – employment of screening panels of antibodies

In Europe a two-step approach to AML diagnosis is most frequently employed. The first step is definition of the main leukemia cell line (myelo- or monocytoid and exclusion of lymphoid proliferation). This goal is attained with screening panel of antibodies conjugated with three or four (as recently recommended) different fluorochromes. In either case there are two more parameters analyzed that reflect cell morphology: SSC – granularity and FSC – cell size.

Screening panel with 3 dyes includes 12 antibodies against (Figure 2):
• surface antigens 1) CD45/CD14, 2) CD19/CD13/CD45, 3) CD7/CD33/CD45, 4) CD64/CD16/CD45, 5) KFL1/KFL2/KFL3 (control – 3 fluorochromes FL);
• cytoplasmatic antigens: TdT/CD79a, MPO/CD3, KFL1/KFL2 (control – 2FL).

Expression of CD13, CD33, MPO with no expression of CD19, CD79a, cCD3, TdT indicates phenotype typical for acute myeloid leukemia.

Screening panel with 4 fluorochromes includes 15 antibodies against (Figure 3):
• surface antigens: 1) CD14/CD64/CD45, 2) CD19/CD13/CD45/CD34, 3) CD7/CD33/CD45/CD117, 4) CD66/CD16/CD45, 5) KFL1/KFL2/KFL3/KFL4 (control);
• cytoplasmmatic antigens: CD79a/MPO/CD3/TdT, K/FL1/KFL2/KFL3/KFL4 (control).

Expression of CD13, CD33, CD34, CD117, MPO (from high to weak) with no expression of CD19, CD7, cCD3, CD79a, CD14, CD64 is typical for acute myeloid leukemia from early progenitors (AML M1/M2) and excludes monocytoid or lymphoid proliferation.

c. Second step: definition of AML subtypes: M1 – M5

We recommend the following antibodies to precise AML subtype (after analysis with a three-color screening panel):
1) CD15/CD117/CD45, 2) CD38/CD34/CD45, 3) CD11c/CD11b/CD45, 4) CD36/CD56/CD45, 5) CD18/HLA-DR/CD45.

All AML cells show expression of CD13 or CD33 and additionally AML subtypes are characterized by:
1. AML M1/M2: CD117+, CD34+.
2. AML M3: CD34-/HLA-DR- (necessary confirmation of translocation t(15;17).
3. AML M4 – two cell subpopulation are detected with analysis of CD45 expression: monocytoid population with stronger expression of CD45 and myeloid population with weaker expression of CD45. Monocytoid and myeloid populations may also be distinguished with analysis of CD117 and CD64 expression. Typically myeloid cells are CD117+, CD64- while monocytoid cells are CD117-, CD64+. Discrimination of two cell populations at diagnosis is later useful in treatment monitoring of patients (Figure 4).
4. AML M5 CD14-: CD64+ CD36-/+,CD11c+CD15++CD117+/-CD34-/+.
5. AML M5 CD14+: CD64+ CD36+,CD11c+CD15++CD117-CD34-.

Detailed antigen expression in particular AML subtypes is shown in Table 1.

3. Aberrant antigen expression on acute myeloid leukemia cells

One of very important tasks for immunophenotyping of AML at diagnosis is detection of aberrant expression of antigens. Those aberrant expressions are divided into four groups: a/ asynchronic expressions, b/ expression of antigens specific for other cell lines, c/ lack of expression of line-specific antigens, d/ over-expression of antigens [9].

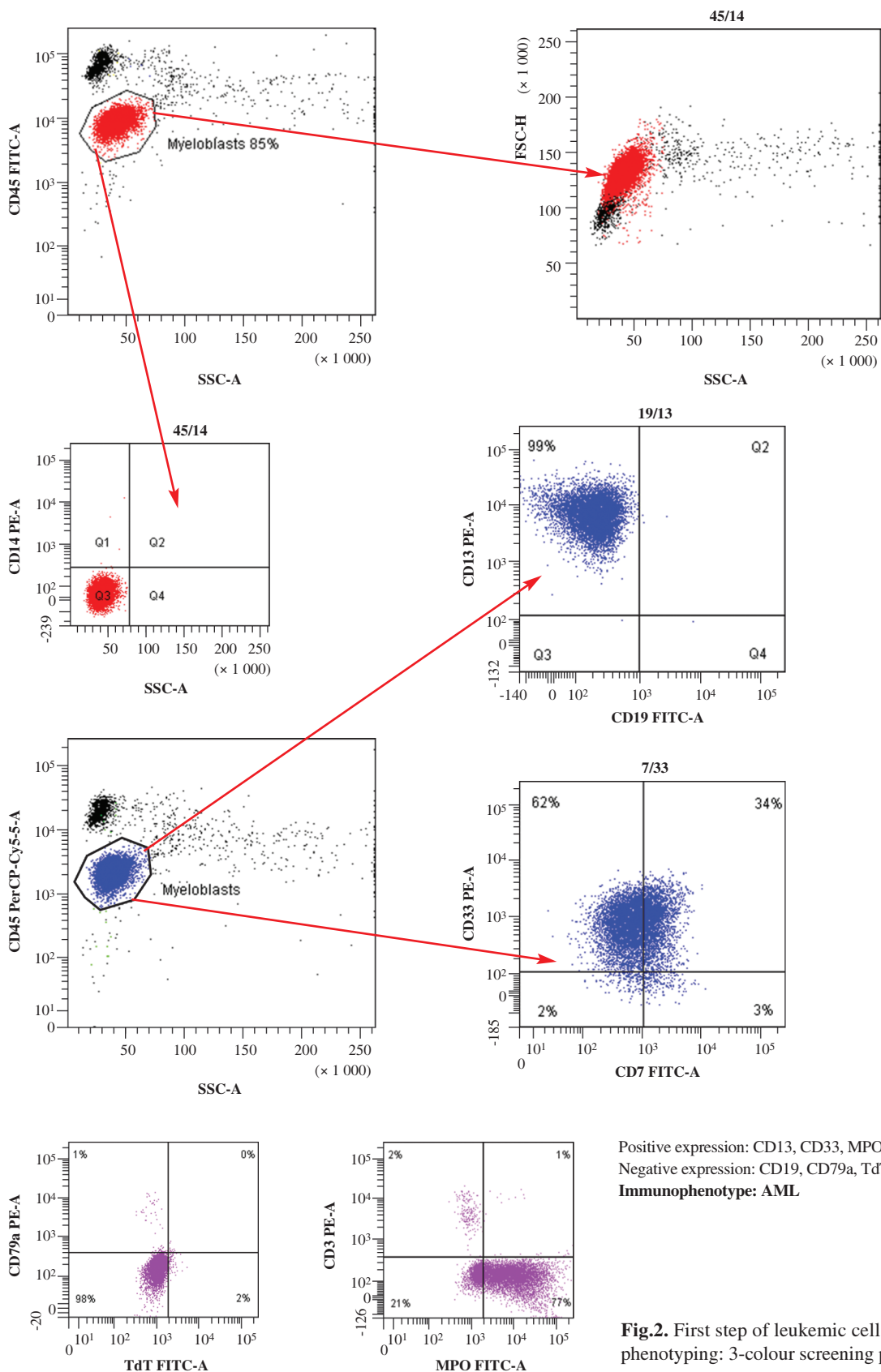Most often in AML aberrations include asynchronic expression of antigens (for example CD15+/CD117+

Positive expression: CD13, CD33, MPO, CD7low
Negative expression: CD19, CD79a, TdT
Immunophenotype: AML

Fig.2. First step of leukemic cell immuno-phenotyping: 3-colour screening panel
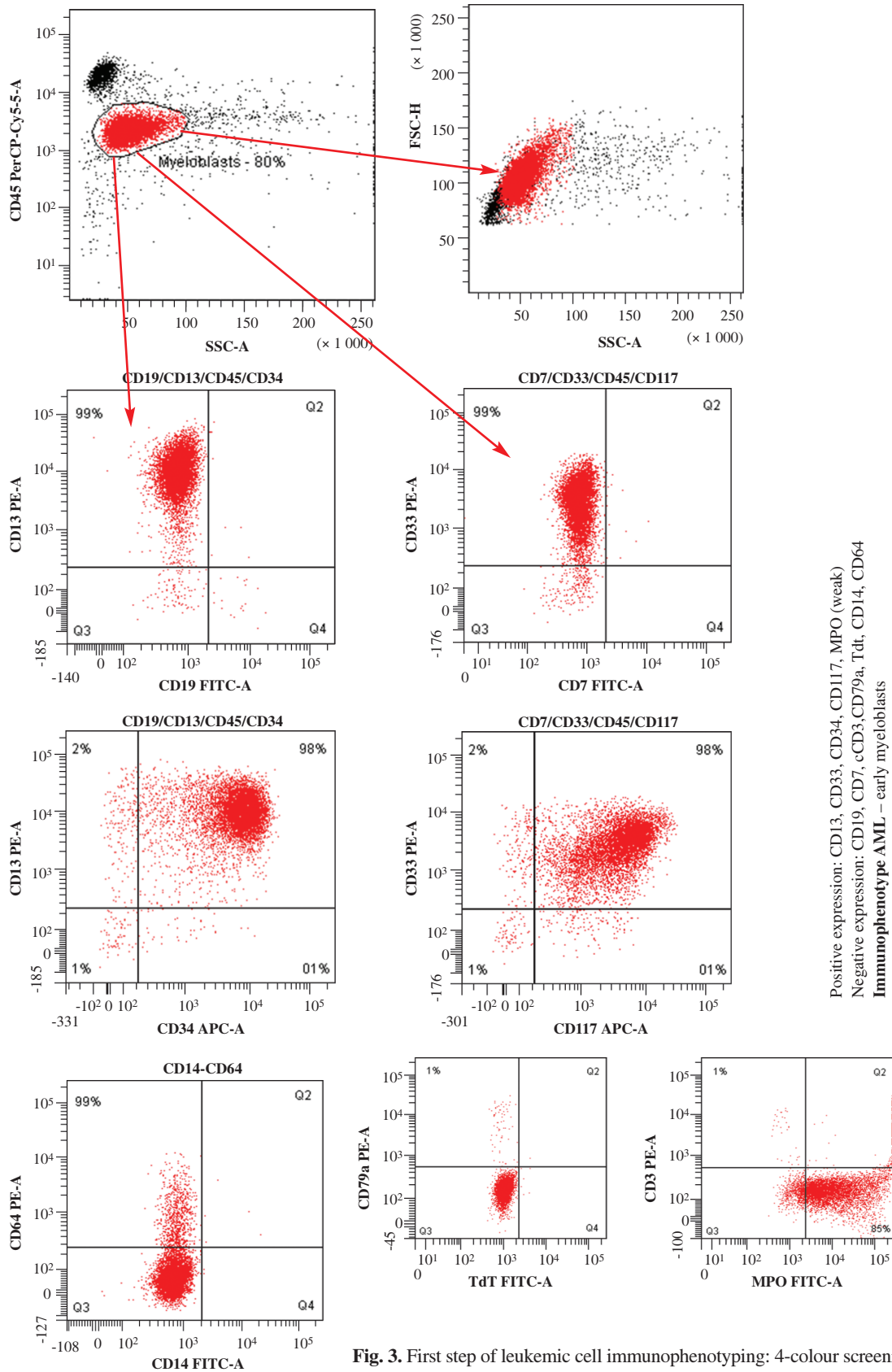
Fig. 3. First step of leukemic cell immunophenotyping: 4-colour screening panel

Positive expression: CD13, CD33, CD34, CD117, MPO (weak)
Negative expression: CD19, CD7, cCD3,CD79a, Tdt, CD14, CD64
Immunophenotype AML – early myeloblasts

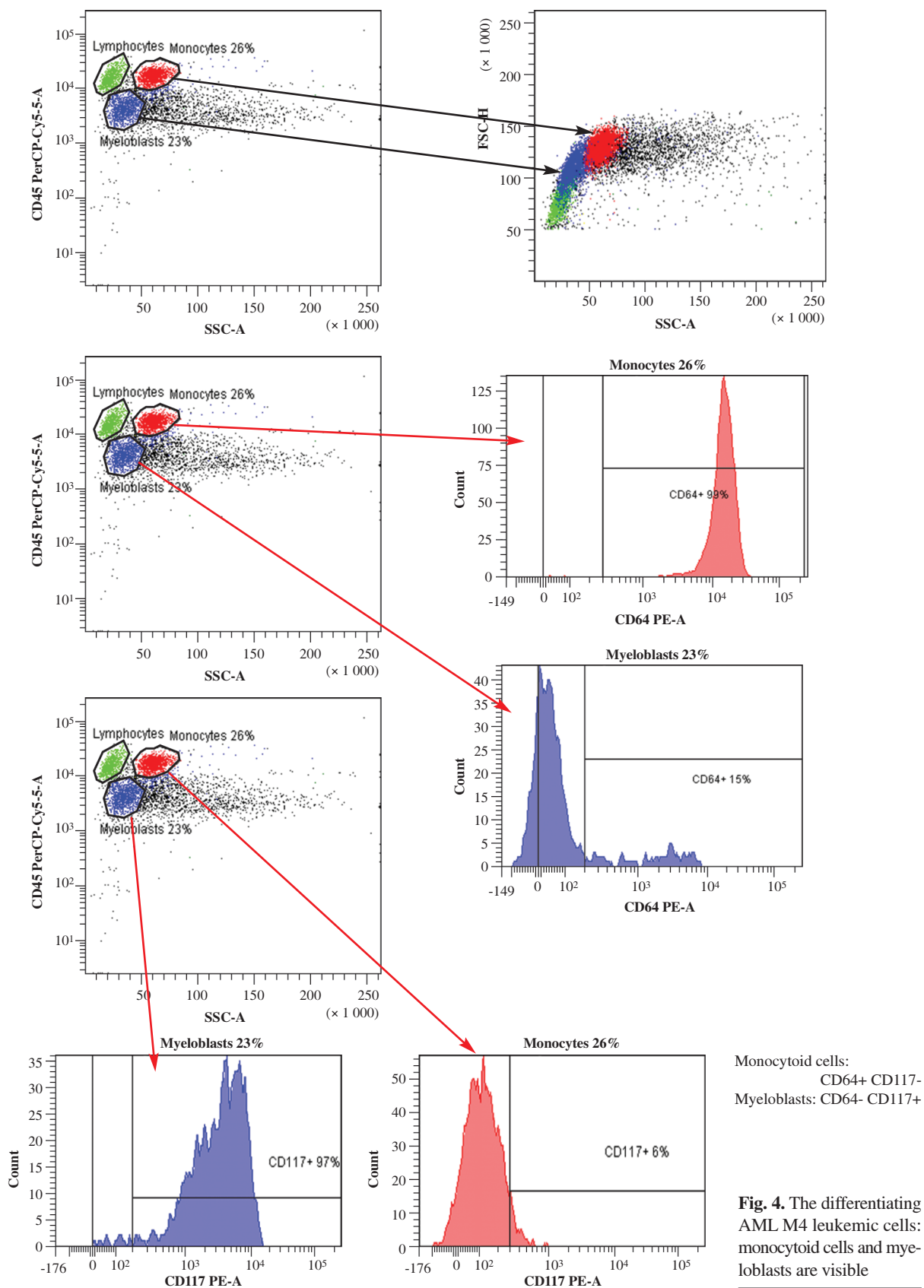Monocytoid cells:
CD64+ CD117-
Myeloblasts: CD64- CD117+

Fig. 4. The differentiating AML M4 leukemic cells: monocytoid cells and myeloblasts are visible

Table 1. Patients (%) with 20% or more leukemic cells presented CD antigen expression

		cell line CD									cytoplasmic CD	
		CD13[1]	CD33[1]	CD15[1]	CD117[1]	CD34[1]	HLA-DR[1]	CD14[3]	CD64[3]	CD36[3]	MPO[2]	TdT[3]
	M0/M1	CD13[1]	CD33[1]	CD15[1]	CD117[1]	CD34[1]	HLA-DR[1]	CD14[3]	CD64[3]	CD36[3]	MPO[2]	TdT[3]
	M2 t(8;21)	CD13[1]	CD33[1]	CD15[3]	CD117[1]	CD34[1]	HLA-DR[1]	CD14[3]	CD64[3]	CD36[3]	MPO[1]	TdT[3]
	M2	CD13[1]	CD33[1]	CD15[2]	CD117[1]	CD34[1]	HLA-DR[1]	CD14[3]	CD64[2]	CD36[3]	MPO[1]	TdT[3]
AML	M3 t(15;17)	CD13[1]	CD33[1]	CD15[2]	CD117[1]	CD34[3]	HLA-DR[3]	CD14[3]	CD64[1]	CD36[3]	MPO[1]	TdT[3]
	M4 myeloblasts	CD13[1]	CD33[1]	CD15[2]	CD117[1]	CD34[2]	HLA-DR[1]	CD14[3]	CD64[1]	CD36[2]	MPO[3]	TdT[3]
	M4 monocytoid cells	CD13[1]	CD33[1]	CD15[1]	CD117[3]	CD34[3]	HLA-DR[1]	CD14[1]	CD64[1]	CD36[1]	MPO[3]	TdT[3]
	M5 CD14-	CD13[2]	CD33[1]	CD15[1]	CD117[2]	CD34[2]	HLA-DR[1]	CD14[3]	CD64[1]	CD36[1]	MPO[2]	TdT[3]
	M5 CD14+	CD13[1]	CD33[1]	CD15[1]	CD117[3]	CD34[3]	HLA-DR[1]	CD14[1]	CD64[1]	CD36[1]	MPO[2]	TdT[3]

		cytokin receptors			aberrant phenotypes					
		G-CSF	**GM-CSF**	*CD184 (CXCR4)*	adhesion molecules			limfoid cell antigens		*asynchronic expression*
	M0/M1	CD114[2]	CD116[2]	CD184[1]	CD11b[3]	CD11c[3]	CD56[2]	CD7[1]	CD19[3]	CD15+117+[2]
	M2 t(8;21)	CD114[1]	CD116[3]	CD184[3]	CD11b[3]	CD11c[3]	CD56[2]	CD7[3]	CD19[1]	CD15+117+[3]
	M2	CD114[1]	CD116[2]	CD184[1]	CD11b[2]	CD11c[2]	CD56[3]	CD7[2]	CD19[3]	CD15+117+[3]
AML	M3 t(15;17)	CD114[1]	CD116[2]	CD184[2]	CD11b[3]	CD11c[2]	CD56[3]	CD7[3]	CD19[3]	CD15+117+[3]
	M4 myeloblasts	CD114[1]	CD116[1]	CD184[1]	CD11b[1]	CD11c[1]	CD56[3]	CD7[2]	CD19[3]	CD15+117+[3]
	M4 monocytoid cells	CD114[2]	CD116[1]	CD184[1]	CD11b[1]	CD11c[1]	CD56[3]	CD7[3]	CD19[3]	CD15+117+[3]
	M5 CD14-	CD114[2]	CD116[1]	CD184[1]	CD11b[1]	CD11c[1]	CD56[3]	CD7[3]	CD19[3]	CD15+117+[3]
	M5 CD14+	CD114[2]	CD116[1]	CD184[1]	CD11b[1]	CD11c[1]	CD56[1]	CD7[3]	CD19[3]	CD15+117+[3]

[1] – 100-50%, [2] – 49-21% patients, [3] – 20-0% patients

myeloblasts that show simultaneous expression of early CD117 and late CD15 antigens) or expression of antigens typical for other cell lines (for example lymphoid: CD19, CD7 or CD56).

In our study nearly all AML patients with monocytoid features showed expression of CD184; half of AML patients with myeloid features had CD184 expression; the lowest CD184 expression was seen in AML M3 patients (40%) while no CD184 expression was detected in AML with translocation t(8;21). The percentage of CD184 positive myeloblasts and promyelocytes was significantly lower than monocytoid leukemia cells. Also density of CD184 was significantly lower on myeloblasts and promyelocytes in comparison to monocytoid cells.

4. Results presentation

The results are usually given as percentage of positive cells within blast cell population discriminated with SSC/CD45 or FSC/SSC gates. The population is regarded positive if at least 20% of cells within the gate show expression of a particular antigen. Blast cell count within the gate on dot plot SSC/CD45 is expressed as percentage of all CD45+ bone marrow cells. It is consistent with recommendations from Vecchio et al. [10].

Leukemia cell phenotype may be presented in a descriptive form if for example it is impossible to separate leukemia cell gate. Intensity of antigen expression may serve as additional characteristics of leukemia cells. It is presented as fluorescence intensity (Mean Channel Fluorescence MFI) or as antibody molecules bound by cells (MESF, ABC).

Discussion

It is commonly accepted that multiparameter flow cytometry immunophenotyping is an important and sensitive diagnostic tool in hematological malignancies. Flow cytometry is employed for analysis of peripheral blood, bone marrow as well as "non-hematological" samples such as cerebrospinal fluid and permits precise classification of AML subtype.

The procedure that we propose includes general recommendations for immunophenotyping in AML [6-8, 11] and additionally suggestions based on our own experience. Proposed antibody panels include antibodies employed for analysis of normal myelo- and monocytoid cells in the bone marrow as well as expected abnormal antigen expression on myeloid leukemia cells or aberrant coexpressions [6,12].

Our analysis of acute myeloid leukemia phenotypes, based on latest literature [13-16] and on our own experience

proved that employed antibody panels are adequate for diagnosis and precise subtype classification in AML.

Antibodies against CD13, CD33 and CD117 are the basic ones to identify acute myeloid proliferation, additionally with anti CD34 and CD15 for proliferations of early progenitors (AML M0/M1, AML M2 regardless of translocation t(8;21) and M3 t(15;17). Comparison of phenotypes of AML with translocation t(8;21) and AML M2 in our material suggests a possibility of discrimination between the two. AML M2 with t(8;21) is characterized by lack of expression of CD116, CD 184, TdT, more frequent expression of MPO and more frequent coexpressions of CD19 and CD56. However those observations must be confirmed in a larger study population.

Some authors (for example Orfao et al.) suggest correlations between expression of certain molecules and cytogenetic findings: AML M3 is supposedly characterized by cMPO+, CD33++, CD13+, HLA-DR -/dim, and differential expression CD11b-, CD117-/+ [6].

In AML M4 two cell populations must be distinguished: myeloblasts and monocytoid cells, which are usually more differentiated than monocytoid cells in AML M5 [17]. It seems that analysis of CD64 and CD36 is very helpful and more sensitive than CD14 [18].

In AML M5, apart from well described expression of CD64 and CD36, presence of CD184 (CXCR4 – chemokine SDF-1 receptor) is a characteristic feature in nearly all patients [19, 20] as well as expression of CD36 and co-expression of CD56 (50-70%) [21, 22].

Analysis of AML M5 cases revealed two phenotypes of leukemia: CD14- and CD14+ which do not completely correlate with AML M5a and AML M5b subtypes according to FAB. In CD14- form, detected monocytoid cells were on various maturation stages from monoblasts to mature monocytes. However on most instances those cases could not be classified as AML M5a as the percentage of monoblasts was less than 80%. Only one case in a two year period fulfilled criteria of a "classical" AML M5a with all leukemic cells being monoblasts. Patients with AML M5 CD14- showed more often expression of CD34 and less often expression of CD13, CD36, CD11b and CD56 than patients with AML M5 CD14+.

Our experience, as well as other publications [19, 20], indicates that lack of CD184 expression on myeloblasts and promyelocytes may serve as a good prognostic factor. On the other hand expression of CD184 on monocytic cells could be used as additional marker discriminating AML subtypes. Therefore it may be recommended to analyze CD184 expression on proliferating cells, both as supplementary differentiating marker in AML and as additional prognostic factor, corresponding to leukemia phenotype. Expression of CD56 was found to be linked to hyperleukocytosis and central nervous system infiltration [22] and as a consequence determination of CD56 expression may be an important prognostic factor in AML. Of other adverse prognostic factors in AML, expression of CD7 in AML M0 was found to bear importance [23].

Flow cytometric immunophenotyping of leukemia cells has dramatically evolved and improved over the last years. It is one of basic and most important tools in diagnosing acute leukemia. As no other method it permits definition of individual profile of malignancy at diagnosis what permits monitoring of treatment and detection of residual disease or early relapse in particular patients. It must however be strongly emphasized that only application of standard and up-to-date procedures allows achievement of optimal results.

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