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New methodologic approaches for immunophenotyping acute leukemias

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Background and Objectives. Flow cytometry is nowadays the preferred method for immunophenotypic identification, enumeration and characterization of blast cells at diagnosis. Despite widespread application of standardized protocols, inter-laboratory reproducibility has still not been achieved. The complexity of diagnosis and evaluation of minimal residual disease, in immunophenotyping acute leukemia, demands the use of a test that provides all the necessary information.

Data Sources and Methods. The information given here is derived from the experience of the authors and from literature files. The most relevant studies with adequate conclusions were considered. We report on the current status of multiparametric immunophenotyping using simultaneous three and four-color staining and the applications of this technique.

Results. Multiparametric immunophenotyping is a powerful method for achieving a clear discrimination between normal and pathologic cells. The specific identification of leukemic cells by immunologic gating forms the basis for immunophenotypic diagnosis, classification as well as prognostic evaluation of patients with acute leukemias. The performance of the procedure with regards to the panels of reagents and the analytic processes, is necessarily different in lymphoblastic and myeloblastic leukemias, since the diagnostic questions are different. Phenotypic information should be specifically provided for the blast cells and antigen expression should preferably be reported in quantitative units and CV. This would allow a standardized cross evaluation of immunophenotypic results between different investigators and laboratories.

Interpretation and Conclusions. Recent reports indicate that phenotypic aberrations reflect genetic abnormalities of leukemic cells and therefore their definition and identification is of clinical relevance not only for minimal residual disease monitoring but also for subclassifying acute myeloid and lymphocytic leukemias.
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Key words: acute leukemia, diagnosis, immunophenotyping, protocols

An adequate diagnosis is the first goal towards tailoring correct treatment for patients with acute leukemias. For a long time morphologic and cytochemical evaluations have been considered the principal diagnostic criteria and the FAB classification has been generally accepted, despite difficulties remaining in achieving inter-laboratory reproducibility.[1-8]

Immunophenotyping is considered to be very useful for lineage assignment of immature and mature leukemic cells.[9-13] The commercial availability of large panels of high quality reagents facilitates the applications of this analytic method in diagnosis, classification, prognostic evaluation, and minimal residual disease evaluation.[10-32] At present flow cytometric analysis is the preferred method for immunophenotyping while the role of microscopic evaluation of antibody-stained cells is reserved for exceptional situations. Flow cytometry allows the analysis of a large number of cells (usually between ten thousand cells per sample, and more than one million in minimal residual disease studies) providing high statistical accuracy. The simultaneous analysis of several different parameters (typically 5 or 6: two related to physical properties of the cells and 3 or 4 to the immunophenotype) contributes to increasing both the specificity and sensitivity of the test. Furthermore, the results can be stored in list-mode files and interpreted by other observers to facilitate objective interpretation. At present the two major sources of variability related to flow cytometric

immunophenotyping of leukemic samples are: the methods used for antigen expression assessment and the criteria employed for the interpretation of the results.

The first goal of immunophenotyping is the identification of pathologic cells; the second aim is phenotypic characterization.

Test principle

Immunophenotyping is based on the identification of specific patterns of both surface and intracellular antigen expression in unique populations of cells.[11-13,18,22] Accordingly the phenotypic expression patterns of normal cells present in a sample analyzed for diagnostic purposes must have been established in advance.

This principle should be applied even when normal patterns are *dynamic*, such as in bone marrow, where the normal regenerating cells are present at various stages of maturation.[33]

Classically, leukemic blast cells have been considered to represent the neoplastic counterpart of normal immature cells blocked at a specific differentiation stage. At present this opinion needs to be reconsidered. The multiparametric, quantitative approaches have shown that expression in leukemic blasts is frequently aberrant in terms of both the presence of cross-lineage antigens (i.e. myeloid antigens in lymphoid cells and *vice versa*) and the presence of abnormal expression of *normal* antigens. This anomalous antigen expression is not directly related to an abnormal differentiation pattern but instead seems to reflects an underlying abnormal *genetic pattern* of these leukemic cells.[13,22,25,32-40] Such phenotypic aberrations are essential for the identification of pathologic cells among normal cells.[12,13] Initially light scattering properties of the cells were used for the specific identification of abnormal cells, but it is now well-established that this is not sufficient (Figure 1A).[41] When associated with other immunologic markers, light scattering parameters are quite helpful in obtaining specific and sensitive identification of blast cells, especially when the percentage of the pathologic cells is low. At present, the association of angular light scatter (side scatter, SS) with an immunologic marker is the preferred method for blast cell identification.[12,13,42] The single immunologic marker best suited for this purpose is CD45. CD45 is a pan-leukocytic antigen which displays different patterns of expression with unique densities in the different normal peripheral major leukocyte subpopulations (Figure 1) being more intense in lymphocytes and monocytes than in granulocytes.

CD45 expression in blast cells is typically lower than in lymphocytes[22,42] (Figure 1B). In our experience, this approach can be further strengthened by the use of additional lineage-associated antigens since the lineage-specific antigen will contribute to improving the SSC/CD45 gating strategy especially in bone marrow samples which may contain normal residual precursors.

Selection of reagents

In almost all cases assessment of antigen expression is based on the use of a combination of fluorochrome conjugated monoclonal antibodies (MoAb) and direct immunofluorescence techniques. At present clinical flow-cytometers are usually equipped with three or four fluorescence detectors in order to allow for the simultaneous analysis of up to five or six parameters; two light scatter (forward and side) and three or four immunofluorescence colors. Instruments equipped with four photo-multipliers can increase the light emission of up to 4 different fluorochromes either using two fluorochrome tandems [(R-phycoerythrin-Texas red (ECD) + R-phycoerythrin-cyanin 5 (R-PE/Cy5)] and a single blue (488 nm) laser (Beckmann Coulter, Inc., Miami, FL, USA) or allophycocyanin (APC) and either peridinin-chlorophyll protein (PerCP) or the PerCP/Cy5 tandem using both a blue laser (488 nm) and a red diode laser (635 nm) (Becton Dickinson Biosciences, San Josè, CA, USA).

In order to select for the most adequate combinations of reagents, the investigator must know the characteristics of both the antigens and the fluorochromes. Accordingly, MoAb clones must be carefully selected since several clones recognizing the same CD may differ in their reactivity, as proven by the CD15 (glycosylated or not glycosylated form) and CD34 (class I, class II, class III) reagents.[42,43]

Variability between different batches of the reagents is not a major problem. However, it is recommended that every new vial is checked. Recent studies have demonstrated that intracellular antigens can currently be easily detected with different commercially available fixation/permeabilization kits. However, when selecting a particular fixation/permeabilization reagent its combination with particular antibody-conjugates should be tested.[44,45]

In general PE-conjugates are more sensitive than FITC ones while PE/Cy5 and APC reagents show an intermediate performance. Accordingly, the brightest fluorochrome, PE should be used for the weakest antigens. The choice for a third and fourth flu-

orochrome combination depends on the instrument that will be used. Of the fluorochromes available, PerCP-conjugated MoAb have some limitations related to their low sensitivity and the low number of commercially available conjugated MoAb reagents. In contrast, the PE/Cy5 tandem is available for a very large number of MoAbs, although it shows non-specific binding especially to monocytic cells.[11] Recently new fluorochromes or tandems have been proposed by various companies to increase the possible number of simultaneously measured antibody combinations (5/6 color analysis) but only APC/Cy5, PerCP/Cy5, APC/Cy7 and PerCP/Cy7 conjugates have been produced on a relatively large scale. The APC reagents are quite sensitive, and their combined use with PerCP/Cy5-Cy7 conjugates avoids the need for both fluorescence compensation and non-specific binding.

At present, the use of isotypic controls is controversial. Agreement exists on the evaluation of the baseline autofluorescence levels of the specific cell populations under study.

Sample preparation

At present there is no rationale for using either peripheral blood or bone marrow (BM) samples which have been separated for analysis by gradient centrifugation (Ficoll hypaque solution). There are many reasons that support the use of either whole blood or whole BM immunophenotyping techniques. These procedures avoid selection or arbitrary loss of specific cell populations and allow reliable enumeration of the cell populations present in the sample. The direct procedures minimize the chances of modifications in antigen expression, and reduce the work time and work load.[12,13]

Whole blood sample preparation procedures usually consist in a short period of incubation (typically 15-20' in the dark at room temperature) of the sample with appropriate amounts of high-affinity fluorochrome-conjugated MoAb followed by erythrocyte lysis.

Analyses must be performed on the total sample, only excluding debris/platelets, by light scatter and should be based on at least 10,000 cells per tube and ideally more than 15,000 viable cells.

Leukemia diagnosis

The diagnosis of acute leukemia is currently a multi-step procedure. Typically one of the first steps consists of microscopy examination of blood or bone marrow smears. The morphologic/cytochemical (FAB classification) examination of these smears still plays a relevant role. The presence of myeloperoxidase (MPO) and/or Sudan black or a strong/diffuse positivity for non-specific esterases (ANAE) in the blast cells represents an unequivocal sign of non-lymphoblastic origin of leukemia.[1,2]

Multiparametric (three or four color) flow cytometry immunophenotyping must be used for characterization of the cells present in the sample.

The major difference between two color immunophenotyping and the herein proposed analysis is related to the gating strategy. In two color analysis, gating is typically based on morphology-related parameters (i.e. light scattering parameters, FSC, SSC).[11,12,22] In three/four color immunophenotyping one marker is usually used in association with orthogonal scatter (SS) for immunologic gating of the cells of interest. With the last method, the sensitivity and specificity achieved in the identification of blast cells is clearly higher.

Enumeration and characterization of blast cells

The number of pathologic cells in bone marrow or in peripheral blood is critical for diagnosis under various conditions. For example, according to the FAB and WHO classifications, the differential diagnoses of non-Hodgkin's lymphomas and ALL or myelodysplastic syndromes (MDS) and acute myeloblastic leukemias (AML) are based on the percentage of blast cells in the bone marrow.[1,2,14]

Immunophenotypic characterization of blast cells has several goals: 1) lineage assignment; 2) evaluation of cell maturation; 3) assessment of phenotypic aberrations. At the same time, it provides information on the degree of heterogeneity of a leukemic cell population.

Adequate instrument set-up and calibration are essential for all these goals and especially for day-to-day comparison of results.

Lineage assignment of blast cells by immunophenotype still represents a major challenge in some acute leukemias. This is mainly due to the existence of cross-lineage antigen expression. Several antigens which were claimed, in normal samples, to be specific to or associated with lymphocytes and NK cells (CD2, 7, 19, 56), and myeloid cells (CD13, 14, 15, 33, 65) have now been reported as positive in a proportion of AML and ALL cases.[18,23,26,29,35-40,46] These observations emphasize the need to use combinations of several lineage-associated markers in order to establish the lineage of blast cells. The choice must include antigens with high sensitivity that are fully present in a certain lineage (e.g. CD7 in T-cells or CD19 in B-cells) together with more specific markers (e.g. CD3 for T-cells, or MPO for AML). Additional progenitor cell

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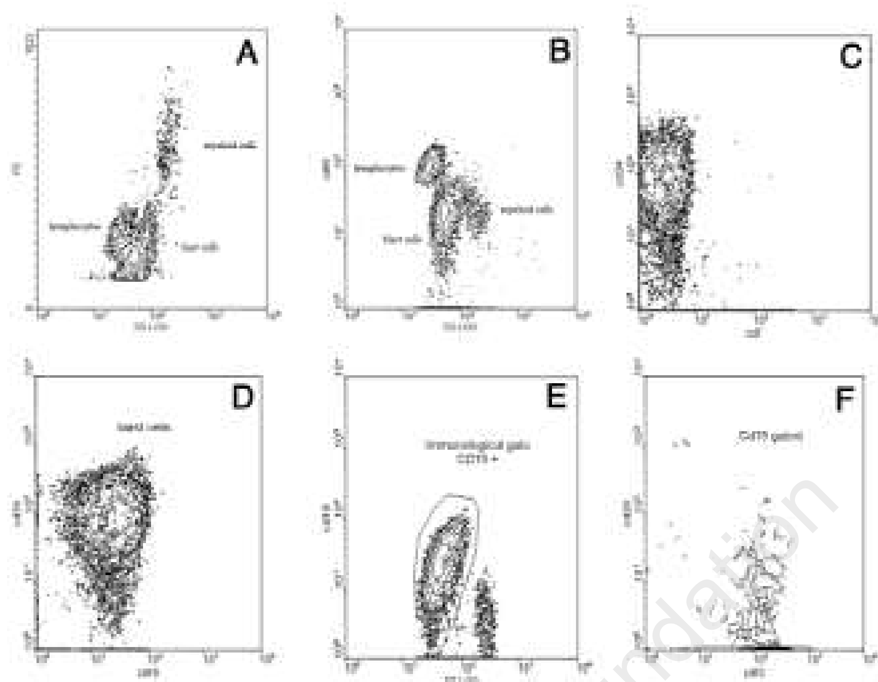

Figure 1. The four color combination CD7/CD34/CD19/CD45. Blast cell identification is obtained using the combination based on light scatter parameters and 2 antigens with high sensitivity (CD7 ,CD19) and 2 with high specificity (CD34,CD45). The A and B histograms demonstrate the different ability of either light scattered parameters (FS and SS log) or CD45 and light scatter (SS log) in identifying various bone marrow populations (lymphocytes, monocytes and blast cells). The combinations with CD7 and CD34 (C), CD19 and CD34 (D), permit an adequate identification of blast cells. The CD19 immunological gate (E) and combination CD10/CD20 (F) in CD19 gated cells.

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markers such as CD34 are used to confirm the immaturity of the pathologic cells (Table 1).

From the practical point of view, lineage assignment should typically be based on the combined use of markers for immaturity and for the different lymphoid (B, T) and myeloid lineages together with markers that allow the identification of blast cells. The CD7/CD34/CD19/CD45, cCD3/cyCD79a/CD34/CD45 and cMPO/CD33/CD34/CD45 combinations represent practical examples. Using the combination CD7/CD34/CD19/CD45, precursor B-ALLs result as being CD19 and CD34 positive. In the few CD34$^-$ cases the altered expression of CD45 (lower expression than normal B-lymphocytes) allows differentiation between the normal immature and the neoplastic B-cells (Figure 1 C,D,E,F).[22] The precursor T-ALLs are CD7 positive and CD19 negative. The simultaneous absence or anomalous reactivity for CD19 and CD7 in the presence of positivity for other myeloid-associated markers supports a non-lymphoid origin of the leukemia even in the absence of MPO.[46-49]

Specific combinations could be used to monitor therapy efficacy and minimal residual disease.[13, 22-25]

Table 2 gives a list of the most useful antigens for immunophenotypic diagnosis of acute leukemias. Once lineage and maturation stage of the blast cells have been identified the role of immunophenotyping focuses on their subclassification through the identification of homogeneous clinically relevant subgroups.

Acute lymphoblastic leukemias

Specific phenotypic characterization of ALL blast cells is usually performed using lineage associated markers such as CD19 for B-cells and CD7 for T-blasts in all tube combinations. Other markers are used to identify the maturation level of the blast cells and eventually establish atypical or aberrant

phenotypes indicative of specific underlying genetic lesions.

Assessment of the blast cell maturation stage has proven to be of unequivocal clinical value. As an example differentiation of mature B-ALL cases from early B-cell ones is of great clinical relevance. B-ALL cases are characterized by translocations involving the c-Myc gene located on chromosome 8 q24 with three different partner chromosomes: t(8;14); t(2;8) and t(8;22). These genetic events are translated into a high proliferative rate (more than 30% S-phase) of tumor cells which, from the therapeutic point of view, benefit from more intense and shorter therapeutic protocols.

In various large studies the clinical relevance of myeloid-related antigens in ALL has been recently denied, at least in children with ALL, putting an end to the enormous amount of controversy in the literature.[50-53]

The three/four color panel of reagents recently utilized in the AIEOP central laboratory for the phenotypic characterization of B-cell ALL is reported in Table 3. This panel is able to identify the major subtypes of B-cell ALL and prognostically relevant genotypes:

major subtypes of B origin ALL

BI/ProB/Early B: (cyCD79a$^+$), CD19$^+$, HLA-DR$^+$, Tdt$^+$, CD10$^-$ CD20$^-$, cyIgm$^-$, Sig$^-$

BII/common/EarlyB: (cyCD79a$^+$), CD19$^+$, HLA-DR$^+$, Tdt$^+$, CD10$^+$, CD20$^{+/-}$,cyIgm$^-$, Sig$^-$

BIII/Pre B: (cyCD79a$^+$), CD19$^+$, HLA-DR$^+$, Tdt$^+$, CD10$^+$, CD20$^{+/-}$, cyIgm$^+$, Sig$^-$

BIII/Pre B/B: (cyCD79a$^+$), CD19$^+$, HLA-DR$^+$, Tdt$^+$, CD10$^+$, CD20$^{+/-}$, cyIgm$^+$, Sig$^+$ (κ or λ^-)

BIV/B mature: (cyCD79a$^+$), CD19$^+$, HLA-DR$^+$, Tdt$^{+/-}$, CD10$^{+/-}$, CD20$^+$, cyIgm$^-$, Sig$^+$ (κ or λ^-)

Finally, specific antibody combinations could be used in order to identify prognostically relevant genetic translocations. The identification of the genetic translocations is relevant for the correct stratification of patients into risk groups receiving different treatment protocols and also provides a sensitive and specific tool for minimal residual disease (MRD) follow-up.[55] In acute lymphoblastic leukemia of B-cell origin it is possible to define homogeneous subgroups with different prognoses related to associated chromosomal abnormalities.

Table 1. Antigen utilization in the immunophenotyping of acute leukemias.

Antigens with broad expression	
Pan-myeloid	CD13,CD33, CD64, MPO
Pan-B-cell	cyCD22, CD19, CD24, cyCD79α–β
Pan-T-cell	cyCD3, CD5, CD7
Antigens associated with immaturity	
Pan-lymphoid and myeloid	Tdt, CD34,CD133, CD135
Pan-myeloid	CD117
Antigens with lineage-specific and maturation-dependent expression	
Myeloid cells	CD14, CD15, CD65, Lactoferrin
Erytroid cells	Glycophorin A
Platelets	CD41a, CD61
B-cells	CD20, cyIgμ, sIg, κ–λ
T-cells	CD1a, CD2, sCD3, CD4, CD8
NK-cells	CD16, CD56

Cy: cytoplasmic. s: surface membrane.

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A very good prognosis has been associated with *t(12;21) (q12;q22)- (TEL/AML1)*[55-57, 96,99] and *hyperdiploid DNA* content.[36,57-60] It has been demonstrated that this translocation occurs in a substantial subgroup of childhood leukemia whereas it is uncommon in adults.[95,95,98] The immunophenotype is characterized by low or absent CD45 and CD20, high CD10, and bimodal CD34 expression.[96,99]

In contrast, poor prognosis has been associated with *BCR/ABL* and *MLL/AF4* fusion genes – resulting from *t(9;22) (q34;q11)* and *t(4;11) (q21;q23)* translocations, respectively. Immunophenotyping shows specific CD34 and CD38 expression in t(9;22) translocations.[62] The 11q23 translocations are characterized by positive antibody staining for NG2 and CD15 and negative for CD10.[55,60,61,81,82]

T-cell ALL

The CD7 marker shows a high specificity in T-ALL, in fact it is present in about 100% of patients. However the sensitivity is not absolute since this antigen can also be detected in around 20% of AML[63] cases. This renders this marker useful for blast cell gating strategies in combination with CD45 in T-ALL. Further characterization of the phenotype of T-ALL blast cells is aimed at subclassification into early T versus more mature T-cell ALL.

T-lineage ALL has been previously divided into three stages of immunophenotypic differentiation: early (CD7$^+$, cCD3$^+$, surface CD3$^-$, CD4$^-$ and CD8$^-$), intermediate (cCD3, surface CD3$^-$, CD4$^+$, CD8$^+$ and

Table 2. Relevant antigens in immunophenotyping acute leukemias.

Designation	*Cell type*	*Antigen*
CD1a	Cortical thymocytes, subpopulation of B-cells, dendritic cells	MHC-like protein, can associate with β2-microglobulin
CD2	T-cells, most NK cells	Erythrocyte-rosette receptor
CD3	Surface expression on mature T-cells, cytoplasmic	Associated with the T cell receptor, mediates signal expression in immature T cells transduction
CD4	Helper/'inducer' T-lymphocytes, monocytes	Receptor for MHC II molecules
CD5	Thymocytes, mature T cells, subpopulation of B cells	Linked to T cell proliferation
CD7	T-cells, NK-cells, subpopulation of immature myeloid cells	40 κDa protein
CD8	Cytotoxic/suppressor T-cells, subpopulation of NK-cells	Receptor for MHC I molecules
CD10	c-ALL, lymphatic precursor cells, neutrophils subset of mature B cells	Common acute leukemia antigen (CALLA), neutral endopeptidase
CD11b	Monocytes, macrophages, neutrophils, NK-cells	Adhesion molecule, C3bi receptor
CD11c	Monocytes, neutrophils, NK-cells, subpopulation of B-cells	Adhesion molecule, gp 150/95
CD13	Myeloid cells	Aminopeptidase N
CD14	Monocytes and neutrophils	LPS receptor
CD15	Neutrophils and monocytes	X-hapten, 3-fucosyl-N-acetyl-lactosamine
CD16	NK-cells, neutrophils, subpopulation of monocytes	Low-affinity Fc-receptor for IgG
CD19	Precursor B-cells, B-cells	Bridge for surface immunoglobulin signal
CD20	Subpopulation of precursor B-cells, B-cells	Ion channel, protein kinase C substrate
CD22	Surface expression on B-cells, cytoplasmic expression in precursor B-cells	Related to neutral cell adhesion molecule, bridge for surface immunoglobulin signal
CD24	B-lymphocytes, activated T lymphocytes, neutrophils	35-45 κDa glycosylphosphatidylinositol-linked glycoprotein
CD33	Monocytes, myeloid precursor cells, weak expression on	67 κDa glycoprotein neutrophils
CD34	Myeloid and lymphoid precursor cells	105-120 κDa glycoprotein
CD38	Activated lymphocytes, subpopulation of B-cells, plasma	45 κDa glycoprotein cells
Cd41a	Platelets, megakaryocytes	Glycoprotein Iib/IIIa, fibrinogen receptor
CD42b	Platelets, megakaryocytes	Glycoprotein Ib, part of the Willebrand factor receptor
CD45	All leukocytes	T200 antigen, protein-tyrosine-phosphatase
CD56	NK-cells	N-CAM
CD61	Platelets, megakaryocytes	Glycoprotein IIIa, vitronectin receptor β-chain
CD64	Monocytes, macrophages	High-affinity receptor for IgG
CD65	Neutrophils, weak expression on monocytes	Ceramide dodecasaccharide 40
CD66c	Neutrophils	Glycosyl-phosphatidylinositol (GPI) linked
CD68	Monocytes	GP 110
CD71	Erythroid cells, activated T- and B-lymphocytes,	Transferrin receptor macrophages
CD79a	B-lymphocytes, including immature B-cells	Ig-α/mb1, part of the B-cell antigen receptor
CD80	B-activated lymphocytes and monocytes	B7 antigen , glycosylated single-chain protein
CD83	Dendritic cells	43kDa glycoprotein
CD95	Mature and immature cells	APO1
CD116	Myeloid cells	GM-CSF receptor α chain
CD117	Myeloid precursors cells	C-kit, stem cell factor receptor
CD133	Precursor myeloid and lymphoid cells	?
CD135	Precursor myeloid and lymphoid cells	FLK2/ FLT3
Glycophorin A	Erythrocytes, erythroblasts and erythroid precursor cells	Sialinic acid-rich polypeptide
HLA-DR	B-lymphocytes, activated T-lymphocytes, monocytes,	Part of the MHC II complex precursor cells
MPO	Lysosomal expression in neutrophils and monocytes,	Myeloperoxidase including immature myeloid cells
Lactoferrin	Neutrophils and monocytes	Lactoferrin
TdT	Nuclear expression in lymphoid precursor cells	Terminal deoxynucleotidyl transferase
TcR $\alpha\beta$	Majority of T-lymphocytes	α/β chains of T-cell receptor
TcR $\gamma\delta$	Subset of T-lymphocytes	γ/δ chains of T-cell receptor
K	Surface expression on B-lymphocytes	Immunoglobulin-light chain type κ
λ	Surface expression on B-lymphocytes	Immunoglobulin-light chain type λ
Ig μ-chain	PreB and B-lymphocytes	Immunoglobulin M-heavy chain

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CD1a$^+$) and late (surface CD3$^+$, CD1a$^-$, and either CD4$^+$ or CD8$^+$).[64]

There is no general consensus about the clinical relevance of maturation levels for T-ALLs. Some groups have reported a better outcome for the intermediate CD1a positive subgroup[64-67] while these data are not fully confirmed by other series in which the subclassification of T-ALL did not show clinical relevance.[68-71] Furthermore controversial data have been reported about the prognostic significance of some antigens in T-ALL. For example a favorable significance has been associated with CD2,[72-74] CD3, CD4, CD8, and CD10.[75,76] These results (like the ones related to myeloid anti-

Table 3. Possible antibody combinations for B-ALL characterization.

CD19 POSITIVE BLASTS

A

FITC	R-PE	Third color
CD2	HLA-DR	CD19
CD24	CD33	CD19
CD65	CD13	CD19
CD38	CD22	CD19
CD34	CD135	CD19
CD16	CD56	CD19
CD15	CD14	CD19
CD61	GlycoA	CD19
cyCD79α	CD79β	CD19
TdT	cyIgμ	CD19
SmIg	CD20	CD19
κ	λ	CD19

Beckmann-Coulter instruments: third color PE-Cy5 or ECD.
Becton-Dickinson instruments: third color PerCP or PE-Cy5.

B

FITC	R-PE	Third color	Fourth color
CD66c	NG2	CD45	CD34
CD34	CD133	CD19	CD38
CD15	CD14	CD19	HLA-DR
CD65	CD33	CD19	CD13
CD2	CD22	CD19	CD38
SmIg	cyIgμ	CD19	CD20
CD61	CD135	CD19	CD45
TdT	cyIgμ	CD19	CD20
CD10	CD34	CD19	CD20
CD58	CD10	CD19	CD45
cyCD79α	CD79b	CD19	CD20
CD16	CD56	CD19	CD24

Beckmann-Coulter instruments: third color ECD, fourth color PE-Cy5.
Becton-Dickinson instruments: third color PerC , fourth color APC.

Table 4. Possible antibody combinations for T-ALL characterization.

CD7 POSITIVE BLASTS

A

FITC	R-PE	Third color
CD3	CD5	CD7
CD10	CD2	CD7
CD1a	HLA-DR	CD7
CD65	CD13	CD7
CD34	CD38	CD7
CD33	CD117	CD7
CD16	CD56	CD7
CD15	CD14	CD7
CD61	GlycoA	CD7
TdT	cyCD3	CD7
TCRαβ	TCRγδ	CD3
CD45RA	CD45RO	CD7

Beckmann-Coulter instruments: third color PE-Cy5 or ECD.
Becton-Dickinson instruments: third color PerCP or PE-Cy5.

B

FITC	R-PE	Third color	Fourth color
CD66c	NG2	CD45	CD34
CD10	CD5	CD3	CD7
CD1a	CD38	CD3	CD7
CD65	CD33	CD45	CD7
CD15	CD2	CD14	CD7
CD16	CD56	CD8	CD7
CD13	CD33	CD45	CD7
CD61	GlycoA	CD45	CD7
CD45RA	CD45RO	CD3	CD7
CD13	CD117	CD34	CD7
TdT	cyCD3	CD3	CD7
TCRαβ	TCRγδ	CD3	CD7

Beckmann-Coulter instruments: third color ECD, fourth color PE-Cy5.
Becton-Dickinson instruments: third color PerCP, fourth color APC.

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gen expression) have not been unequivocally confirmed.[67, 70,74,76] These results are not clinically useful, and indeed they are not included in stratification for treatment. Some groups, including AIEOP, BFM and CCSG[70,76,77] are putting major emphasis on early good response to therapy as the best predictor of outcome in T-ALL.

A possible potentially useful panel of monoclonal antibody combinations for the characterization of T-ALL is shown in Table 4.

Acute myeloid leukemia

From both diagnostic and clinical points of view, three different major groups should be established among non-lymphoblastic acute leukemias: *minimally differentiated (MPO-)AML*, *acute promyelocytic leukemia (APL)* and the *other AML cases.*

A rare AML subtype is constituted by the *minimally differentiated MPO negative AML*. This subgroup is characterized by being of myeloid origin without evidence (less than 3%) of MPO in flow cytometry or in cytochemistry and negativity for ANAE. The immunophenotype of these cases is characterized by the absence of expression of highly specific lymphoid markers (i.e. CD3, cyCD79a) and by reactivity for myeloid related antigens (CD13, CD33 and/or CD117) and for CD7 and CD34 (Figure 2 D,E,F).[11,47-49] These leukemias are usually heterogeneous both from the clinical/cytogenetic

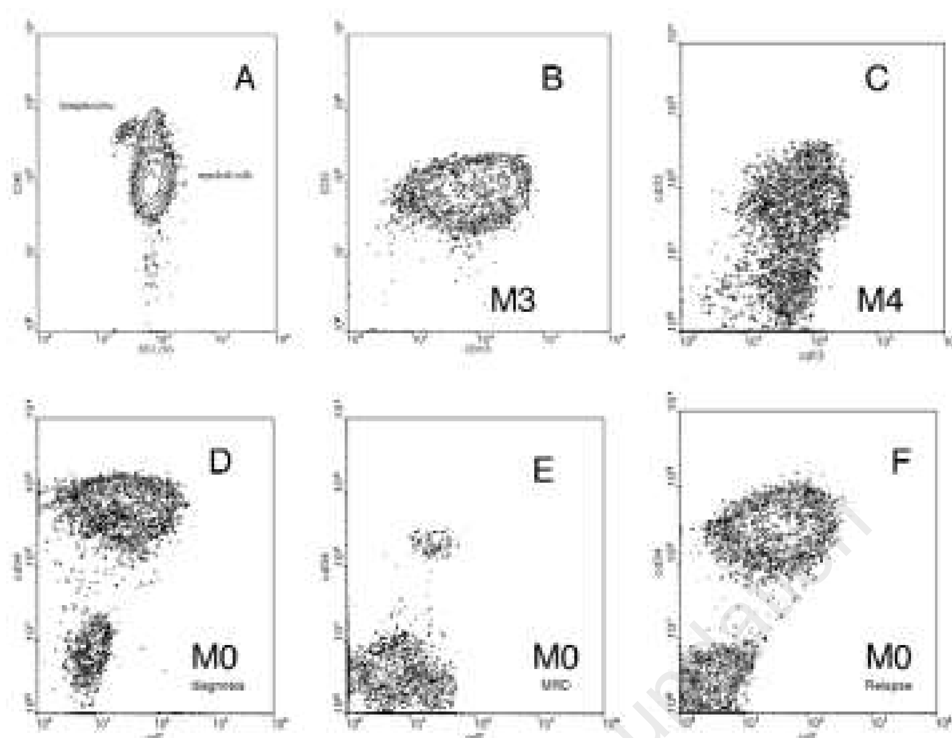

Figure 2. Myeloid cell identification with light scatter and CD45 (A). High and homogeneous CD13 and CD33 in M3 classic (B), heterogeneous expression of CD33 and CD13 in M4 (C). M0 diagnosis and follow-up (D,E,F). The CD34/CD7 pattern at diagnosis (D), MRD detection after three months (10^{-3}–10^{-4}) (E), relapse after 1 year (F).

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and prognostic points of view as well as with respect to their light scattering and phenotypic properties.

More differentiated AML cases

For a long time the FAB classification has been the most widely accepted set of criteria for classifying acute non-lymphoid leukemias.[2] The correspondence between FAB subclasses and some specific genetic aberrations reinforces the relevance of conventional morphologic criteria. The role of immunophenotyping in the subclassification of AML is still under discussion although general agreement exists on its relevance to the diagnosis of M0, M6 and M7 subtypes.[11,13,47-49]

In spite of this, in recent years multiparametric immunophenotyping has shown that some specific genetic aberrations are associated with unique patterns of antigen expression. Furthermore, in some instances flow cytometry provides a more accurate enumeration of the blast cells than do morphological criteria. Immunophenotyping may be of help in areas such as the differential diagnosis between AML and myelodysplastic syndromes (MDS) and the subclassification of these latter.[2] As opposed to what happens in precursor-B or T-ALL, the blast cells in AML are usually more heterogeneous and more than one cell lineage can be involved.[11,17,20,22]

AML represents a group of leukemias composed by less (M0, M1, M5a) or more (M2, M3, M5b) differentiated diseases and by single (M1, M3, M5) or mixed (M4, M6, M7) cell lineage leukemic blasts. The greater heterogeneity of non-lymphoid blast cells in the bone marrow may have an impact on the ability to identify blast cells precisely. At the same time, exclusive evaluation of typical blast cells could be misleading since leukemic myeloid cells frequently retain a certain ability to mature. The identification and interpretation of the differentiation pathways of myeloblasts represent major challenges for the classification of AML.

This supports the relevance of minimizing manipulation of samples during sample preparation or data acquisition/analysis.

Based on this, flow cytometric multiparametric

analysis of AML should deal first with the identification of the pathologic myeloid cells as opposed to the normal cells present in the sample. Assessment of the potential involvement of mature cell populations should be done in a second step. Finally, the maturation pathways of the pathologic cells should be identified based on tumor heterogeneity for antigen expression.

At present consensus exists on the choice of the CD45 immunologic marker for gating purposes in AML, although it is well-known that a single marker for all possible myeloid leukemic populations does not exist. CD45 should be used in association with light scatter; we have found this to be adequate in gating the majority of AMLs studied. In some cases CD45 is not suitable for gating purposes because of the marked heterogeneity of bone marrow cells or the limited number of blasts present in the sample. In such cases CD13, CD33 or CD34 may be of help.

Some antigen combinations which might be of great utility for the follow-up of monocytic and granulocytic differentiation include SSC/CD14/CD64/CD45/CD34 and SSC/CD15/CD16/CD45/CD65, respectively. In spite of the similarities between leukemic and normal myeloid cells, the existence of atypical antigen expression is currently considered a useful finding for specific identification of leukemic cells.[35,37–40,46] In recent years it has been shown that, similarly to ALL, the patterns of leukemic-associated antigen expression correlate with specific translocations. These observations together with the production of a new set of reagents against fusion proteins or proteins expressed in cells carrying a specific translocation have provided new insights into the importance of immunophenotyping in the classification of AML.[35,37-39,79-87] The identification of three subtypes of AML has been critical for prognosis-based treatment. A diagnosis of M3 t(15;17) implies a specific therapeutic program[89] while identification of M2 t(8;21) and M4 Eo (inv 16, t(16;16)) is relevant because of their very good response to therapy.[101]

Acute promyelocytic leukemia (APL) with t(15;17)

Acute promyelocytic leukemia (APL) is characterized by a translocation [t(15 ;17)] involving the PML gene in chromosome 15 and the retinoic acid receptor gene in chromosome 17. APL is associated with a unique pattern of CD34, CD15 expression and a heterogeneous reactivity for CD13. In addition t(15;17) blast cells are usually homogenously CD33+ (Figure 2B) and they lack both CD34 and HLA-DR expression.[87,89,90] Several groups have suggested that CD2 expression found in some cases might be associated with M3v blasts.[37,87-91] Recently a PG-M3 MoAb has been produced against the PML protein. This reagent shows a distinctive immuno-cytochemical staining pattern between normal and leukemic promyelocytes and is of great clinical utility for the diagnosis of t(15;17).[79] Overall the sensitivity and specificity of immunophenotyping in the diagnosis of t(15;17) is higher than 90%.[90] The clinical impact of such diagnosis is related to the sensitivity of APL with t(15;17) to treatment with all trans retinoic acid (ATRA).

Acute myeloblastic leukemia with granulocytic differentiation and t(8;21)

The M2 leukemia with t(8;21) is a unique subgroup of M2 cases characterized by a balanced translocation involving the AML1 and ETO genes in chromosomes 21 and 8, respectively. From the clinical point of view it has been suggested that in AML this genotype is associated with a higher sensitivity to therapy and a better outcome.

The classical myeloid immunophenotyping of the latter is reflected by a strong positivity for MPO, CD13 and CD33, associated with CD15, CD34, CD65, CD117, HLA-DR expression in a high proportion of cases. The CD19 and/or CD56 lymphoid-related markers are more frequently expressed and furthermore the high CD56 expression has been demonstrated to be prognostically relevant.[35]

Acute myelomonocytic leukemia with eosinophilia (M4Eo)

Morphologically this myelomonocytic AML subtype is characterized by coarse, densely-staining granules in the eosinophils, with a high percentage of these cells in the smear. From the genetic point of view inversion or translocation of chromosome 16 [inv16 or t(16;16)] involving the CBFβ-MyH11 genes is typical of these cases. Clinically this chromosomal abnormality has been associated with a more favorable prognosis. In a high percentage of cases, immunophenotypic studies show co-expression of CD2, CD4, CD7,CD13, CD14, CD15, CD33, CD34, CD64, CD65, CD117 and HLA-DR in the blast cells.[38,39,92]

Immunophenotyping of other AML cases not genetically classifiable

The most important point regarding the immunophenotypic characterization of these AML cases is the evaluation of the degree of differentiation and the identification of aberrant phenotypes. Within this subgroup, the identification of AML cases displaying similar patterns of antigen expression will in the near future certainly contribute to a clinically relevant subclassification of these patients, especially among M2, M4 and M5 (clini-

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cally relevant) leukemias.

Assessment of megakaryocytic or erythroid lineage involvement through the expression of megakaryocytic [CD41a, CD42b, and CD61] and erythroid [glycophorin A, CD36, CD71++] related markers is of great utility for the identification of these subtypes of AML. However, reactivity for these antigens must be examined with care since the possible non-specific coincidence of platelets or red cell membrane fragments with the blast cells during analysis may lead to misclassification.

Although expression of some antigens has been correlated with prognosis (i.e. CD7 and CD34, CD56), controversial results exist in the literature concerning this subject.[63] Recently in adult *de novo* AML, pan-myeloid phenotype (defined by the full expression of MPO, CD13, CD33, Cdw65 and CD117), performance status, and permeability glycoprotein activity have been demonstrated to influence treatment outcome with a high prognostic impact in a multivariate analysis.[100] These results are very interesting even if not confirmed by following series and despite the employed analysis criteria not having been adequate. The back-bone of the analysis, that a pattern of various antigens and not single antibody positivity could be relevant for the prognosis, is shared. Extensive studies with a multiparametric quantitative approach may increase the importance of some myeloid antigens in identifying homogeneous AML subtypes with prognostic significance. This approach overlaps the results obtained using traditional studies with 20% criteria of positivity

A possible potentially useful panel of monoclonal antibody combinations for the characterization of AML is shown in Table 5.

Table 5. Possible antibody combination for myeloid ALM characterization.

CD7 POSITIVE BLASTS

A

FITC	R-PE	Third color
CD65	HLA-DR	CD45
CD5	CD7	CD45
CD15	CD14	CD45
CD16	CD56	CD45
CD2	CD38	CD45
CD64	CD19	CD45
CD66b	CD11b	CD45
CD68	CD33	CD45
CD66b	CD15	CD45
CD16	CD11b	CD45
CD10	CD20	CD45
CD4	CD7	CD45
CD34	CD117	CD45
CD34	CD135	CD45
MPO	LACTOFER.	CD45

Beckmann-Coulter instruments: third color PE-Cy5 or ECD. Becton-Dickinson instruments: third color PerCP or PE-Cy5.

B

FITC	R-PE	Third color	Fourth color
CD65	CD15	CD45	HLA-DR
CD16	CD11b	CD45	CD34
CD34	CD133	CD45	CD38
CD19	CD56	CD45	CD34
CD71	CD135	CD45	CD33
CD15	CD14	CD45	CD34
CD2	CD33	CD45	CD13
CD64	CD11a	CD45	HLA-DR
CD61	GlycoA	CD45	CD20
CD7	CD117	CD45	HLA-DR
MPO	Lactof:	CD45	CD34
CD64	CD14	CD45	CD34
CD65	CD15	CD45	CD16

Beckmann-Coulter instruments: third color ECD, fourth color PE-Cy5. Becton-Dickinson instruments: third color PerCP, fourth APC.

Acute biphenotypic leukemia

A certain number of acute leukemias have blast cells that simultaneously demonstrate features characteristic of both the myeloid and lymphoid lineages and for this reason are designated *mixed-lineage, hybrid or biphenotypic acute leukemias (BAL)*.[102,103] The real incidence of this form of leukemia varies considerably between different studies probably because of various technical aspects (antigen studied and gating strategy criteria).[12]

There are no widely accepted criteria for defining such leukemias and there are difficulties in establishing whether they represent a distinct clinical and biological entity. Probably the real biphenotypic leukemias are rare entities originating from a primitive stem cell with the potential to differentiate along the lymphoid or myeloid lineage.[104] The scoring system devised by the *European Group for the Immunological Classification of Leukemia* (EGIL) based on the number and degree of specificity of the markers (lymphoid and myeloid) expressed by the blasts[105] is not fully adequate for identification of these forms. We considered as hybrid a leukemia characterized by positive myeloperoxidase (more than 3% in the blast cells) and a complete lymphoid immunophenotype with or without myeloid antigen expression.

Positive myeloid antigens in ALL is not a criterion for diagnosing hybrid leukemia. The high frequency of myeloid antigen positivity in t(12;21)

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and in t(9;22) ALL confirms this. Myeloid-associated antigens have no prognostic significance in childhood ALL subtypes.[51,52,106,107] Different results have been reported in adult myeloid antigen positive ALL, in which a poorer response to standard ALL therapy has been reported.[107] Since these phenotypes are frequently associated with molecular genetic abnormalities, the data related to poor prognosis could be explained by frequencies of prognostically relevant chromosome abnormalities in childhood [t(12;21)] and in adults [t(9;22)].

Acute leukemias with other phenotypes

The disease spectrum of rare forms of leukemias such as natural killer (NK) cell leukemias has been continuously expanding. Acute leukemia of NK-cell lineage is a very rare disease.

Suzuki *et al.*[108] described seven cases of *acute leukemia of myeloid and NK cell precursor phenotype* with strikingly extramedullary involvement at presentation. The expression of CD7, CD33, CD34, CD56 and frequently HLA-DR, but not MPO nor other NK, T-cell and B-cell markers was observed.[109,110]

Most reports suggest that this form of acute leukemia, most common in adults but described also in childhood,[107] may arise from the transformation of a precursor cell common to the NK-cell lineage and myeloid or T-lineage.[109,111-113]

Its recognition appears to be particularly important for the clinical pathologic evaluation of CD56$^+$ hematolymphoid malignancies and for the development of therapeutic approaches to such diseases with an aggressive outcome.[108] The T-cell receptor DNA rearrangement demonstrated in these forms makes the NK cell origin of these leukemias doubtful. Furthermore CD56 is positive in a few CD19$^+$ ALLs and these leukemias are not classified as NK leukemias.

A dendritic cell leukemia has been included in the WHO classification.[14] No cases with principal and exclusive bone marrow involvement have yet been described in the literature. In our experience only one case has been classified as acute dendritic leukemia. The diagnosis was made by characteristic morphology and by immunophenotyping showing positivity for CD83 and CD86.

Criteria for positivity and quantitative evaluation of antigen expression

Flow cytometry immunophenotyping allows qualitative and quantitative evaluation of antigen expression by individual cells. The analysis of high numbers of events provides patterns of antigen expression for leukemic cell populations. Accordingly, apart from the assessment of the presence/absence of an antigen, information is provided on the amount of antigen expressed/cell and the pattern -homogeneous versus heterogeneous- of antigen reactivity. Thus, quantitative analysis of antigen expression is essential and is very useful for the differentiation between normal and leukemic cells as well as for the identification of different subtypes of AL with a similar genetic background.[94-98] Antigen expression should therefore be evaluated by the mean fluorescence intensity and coefficient of variation found for the leukemic blasts as opposed to the frequently used cut-off values of positive cells (typically more than 20% positive cells). We consider a leukemic cell population to be positive for a specific antigen when the median fluorescence intensity (MFI) found for this antigen is higher than that obtained for the negative cells (unstained cells) plus 2 standard deviations (SD).

Antigen intensity (MFI) can be expressed in several different units including MESF or ABC (antibody binding capacity) depending on the operative system used. The use of standardized units is usually preferred for obtaining reproducible results at either different time points in the same laboratory and/or in different laboratories. Fluorescence quantification in standardized units such as MESF, is typically obtained through instrument calibration with a set of fluorescent standard beads with different well-known amounts of fluorochrome. The peak channel distribution for each of the different beads (1=negative) obtained in the fluorescence histogram is plotted against the known value of molecules of soluble fluorochrome (MESF) for each population of beads in order to provide a standard curve.

As mentioned earlier, antigen quantification has proven of use in the identification of specific prognostic subtypes of both ALL [i.e. t(12,21)$^+$, t(9;22)$^+$] and AML [i.e. t(15;17)]. Accordingly, the use of quantitative multiparametric immunophenotyping techniques for a more profound analysis of the patterns of expression of antigens on blast cells has been reported. The t(12;21) in precursor B-ALL is identifiable with high sensitivity (87%) and specificity (100%) by high expression of CD10, HLA-DR, and by low expression or absence of CD20 and CD135 and by bimodal expression of CD34.[99] A unique pattern of CD38, CD34, CD13, and CD10 has been demonstrated in adults with t(9;22)$^+$ precursor B-ALL.[62] The APL with t(15;17) shows a typical phenotype with regard to CD13, CD34 and CD15 expression which allows highly specific and sensitive identification of this AML subgroup.[90] The t(4;11) ALLs characteristically show CD45, CD15, CD19, NG2 positivity. and are usually CD10 negative.

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Minimal residual disease

Common therapeutic protocols have considered 5% of blasts cells in the BM as the morphologic limit for considering the patient in complete remission (CR). More recent studies using molecular and immunophenotypic methods have demonstrated the presence of quantifiable disease in patients considered in morphologic CR. The studies of minimal residual disease (MRD) should improve the estimate of the number of blast cells present in the bone marrow in CR during and after therapy thus improving the clinical management of patients with acute leukemia. MRD studies involve early detection of relapse and evaluation of drug efficacy by estimating the *in vivo* clearance of blast cells. MRD studies are also of potential utility in the setting of autologous stem cell transplantation in order to evaluate the possible presence and levels of blast cells in the harvested sample.

Numerous methods including cytogenetic, molecular genetic and immunologic approaches have been developed over the last decade aimed at the sensitive detection of MRD. The cytogenetic methods use karyotypic abnormalities, defined at diagnosis, during follow-up either after cell culture or by interphase fluorescent *in situ* hybridization (FISH) to identify the blast cells. This approach has a high specificity but its sensitivity is relatively low. In fact, in the best conditions, 1/1000 cells have been identified.[114] Molecular approaches are usually based on PCR technology to identify, with high specificity and sensitivity (10^{-5}/10^{-6} cells), either fusion products of translocations (BCR-ABL, E2A-PBX1, MLL-AF4, TEL-AML1, AML1-ETO, PML-RARα, ...) or specific B- and T-cell antigen receptor-gene rearrangements.[115,116] Identification of fusion products of translocations is a simple, fast and cheap but only about 30 % of ALL and 25-30% of AML can be monitored in this way.[22–24,26,29,115,117] The B and T cell antigen rearrangements are not easy, not fast and although able to monitor more than 90% of ALL, are not useful for AML. The immunophenotyping approach in minimal residual disease is fast, cheap and can be applied in about 90% of cases of ALL and 80% of AML. Current strategies for MRD detection by immunophenotyping are based on two different approaches. The first exploits the identification of aberrant phenotypes in leukemic cells at diagnosis which are not detectable in normal bone marrow cells of the same lineage. This approach includes the positive myeloid antigens in ALL and lymphoid antigens in AML.[23,24,27,28,34,54] The second approach is based on the existence of abnormal patterns of antigen expressions in the blast cells with respect to the pattern of the same antigens in normal bone marrow cells (Figure 3).[33] The latter approach utilizes either the abnormal higher expression of CD10, CD38 and CD58[118] or the lower expression of others such as CD45 and CD11a in the ALL of B-cell origin. In the last methodology the empty space approach has been suggested by Biomed concerted action[119] and by Borowitz.[120] According to this methodology, a series of three color[119] or four color combinations[120] have been employed to design a map of the normal pattern of B-cell differentiation. The blast cells are identified by their aberrant position in the dot plot. This methodology is very interesting but the MRD studies are performed in treated patients in whom bone marrow is not normal but regenerating or hypoplastic. The pattern of regenerating B-cells is therapy-dependent as demonstrated by the Dutch group.[121] For these reasons the comparative phenotype mapping based on identifying the specific dot plot of blast cells at diagnosis and in the follow-up could make an interesting, strong methodology to increase sensitivity and specificity during the follow-up. In any case the strategy to increase the sensitivity of the immunologic detection of MRD consists in acquiring only cells from a specific cell lineage — that of the blasts. This is achieved by using a specific immunologic gate in the acquisition or in the analysis. Thus, a high level of sensitivity can be obtained by screening more than 10^6 or even 10^7 cells without problems with the computer hardware. The presence of residual blast cells is confirmed once a homogeneous (for

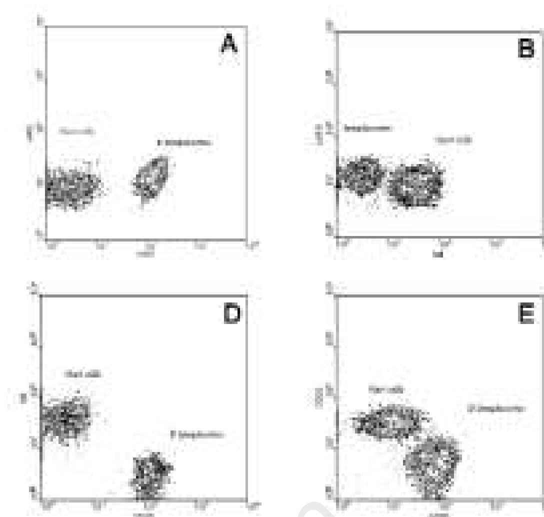

Figure 3. Different antigens expression of B lymphocytes and blast cells of B origin in an ALL bone marrow using CD19 gating strategy; the blast cells are characterized by lower CD19 and negative CD20(A,C), positive Tdt (B,C), lower CD22 and higher CD38.

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antigen expression and light scatter characteristics) group of cells (a cluster) with a leukemic phenotype is identified (at least 10^{-15} events would be necessary). Usually, detection of minimal residual disease in T-ALL by flow cytometry is relatively simple. In fact, the association of terminal deoxynucleotidyl transferase (TdT) and CD3 or CD5 represented in more than 90% of T-ALL is not present in normal bone marrow. The test sensitivity is high, around 10^{-5}. In the precursor B-ALL a more convenient approach consists in using a back-bone combination, such as CD10 /CD19 /CD34 (useful in 80% of ALL), to which a fourth antigen is associated with anomalous expression at diagnosis. Useful antigens were CD20 or CD45 or CD58 or CD66c or one positive myeloid antigen at diagnosis.[13,23,24,29-32,42,99,115,117,118] Immunophenotyping is able to monitor MRD in about 80% of all AML, which compares with the 30% using molecular techniques. Various combinations have been reported in AML monitoring using the identical ALL approach. The great advantage consists in a high number of atypical expression antigens. The backbone CD34/CD45 combination (utilized as the gate) associated with two positive antigens offers a high sensitivity and specificity in MRD (Figure 2 D,E,F).

Previous reports have confirmed the clinical value of MRD monitoring of both ALL and AML patients as far as regards relapse prediction.[23,24,122] An additional clinical value of the MRD approach is the confirmation of lymphocyte regeneration in the bone marrow after the therapy.[121]

Apoptosis and multi-drug resistence

Some new apoptosis-related molecules such as CD95 (Fas/APO-1)[123] and Bcl-2[124,125] are important factors for tumor cell survival and resistance to chemotherapy in hematologic malignancies.[126] The role of the expression of these molecules is controversial in AL. The role of these molecules appears to be more relevant in AML than in ALL. Several studies have demonstrated a correlation between constitutive Bcl-2 expression and response to chemotherapy in AML.[127-131] Only one study revealed a correlation between Bcl-2 expression levels and response to chemotherapy in ALL.[128] Other studies could not confirm this finding.[132-135] Constitutive CD95 expression and function in AML have been correlated with response to induction chemotherapy;[131,136,137] these data are not confirmed in ALL.[141]

One of the best-characterized resistance mechanisms is drug expulsion by P-glycoprotein (Pgp), the product of multidrug-resistance-1 (MDR1) gene. This mechanism contributes to chemotherapy failure in acute leukemia.[138] Its expression level and its functional activity have been demonstrated to have a prognostic impact in adult AML but not in childhood ALL.[139,140,141] Quantification of resistance will yield new, relevant information about this mechanism.

Conclusions

In summary the immunophenotypic analysis of leukemic cells provides essential information for the diagnosis and the follow-up of AL and this information should be combined with that of morphology and genetic investigations. Furthermore, the introduction and diffusion of new reagents specific for abnormal fusion products[79-86] and antigen quantification[41,90,98] will provide us with new tools for such investigations and the management of AL.

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GB and AO designed the study, LDZ and BB did the experiments and contributed to the writing of the paper.

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