



Methodological approach to minimal residual disease detection by flow cytometry in adult B-lineage acute lymphoblastic leukemia

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A flow cytometric approach to minimal residual disease (MRD) monitoring useful in childhood B-lineage acute lymphoblastic leukemia (ALL) is discussed here in the context of ALL in adults. Of 64 leukemia samples analyzed, 95.3% had at least one abnormal phenotype (57.3% had two or more) as compared to physiologic B-cell precursors in adult bone marrow. The method was sensitive enough to detect one leukemic cell among 10,000 normal cells in 16/19 experiments (84.2%). Blast phenotypes were stable in culture and at relapse, and were useful for MRD monitoring in patients. Marker combinations for childhood ALL are also applicable to adult cases.

Key words: MRD, flow-cytometry, B-lineage ALL, normal B-cell precursors.

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A number of patients with adult acute lymphoblastic leukemia (ALL) who achieve hematologic complete remission subsequently have a relapse. Early assessment of treatment response is useful to design risk-adapted treatments.¹⁻⁴ In childhood ALL, the presence of minimal residual disease (MRD) during treatment is associated with a significantly higher relapse risk, and is currently used to tailor treatment intensity.³⁻⁴

Flow cytometry is widely used for the diagnosis of leukemia and is suitable for routine MRD assays.^{3,5-8} In childhood ALL results of flow cytometry and polymerase chain reaction (PCR) analysis can be highly concordant.⁹ MRD detection by flow cytometry is based on the identification of leukemia-associated immunophenotypes (LAIP), which are not expressed by normal bone marrow (BM) or peripheral blood cells.³⁻¹⁷ While T-ALL blasts normally express nuclear TdT/cytoplasmic CD3, a thymus-restricted marker combination, in more than 90% of cases, B-lineage ALL blasts usually share most phenotypes of normal BM B-cell precursors. Thus, the identification of LAIP requires first that the phenotypic profiles of normal cells are fully characterized.^{7-8,10-17} LAIP can be currently identified in 95% of childhood ALL cases.^{7-8,10-16} Fewer studies have addressed the feasibility, sensitivity, reproducibility, and prognostic significance of flow cytometric MRD detection in adult ALL.^{7,11,17-18} We characterized adult normal BM B-cell precursors and compared the marker combinations to those expressed by adult B-lineage ALL blasts. We then assessed the expression stability of the identified phenotypes, and the potential prognostic significance of MRD monitoring using these phenotypes.

Design and Methods

Cell analysis

We analyzed 20 normal BM samples (12 from BM donors and 8 from patients treated with chemotherapy for non-B-cell neoplasia). The study was conducted according to institutional ethical committee requirements and informed consent was obtained from donors and patients. Mononuclear cells were immediately processed or cryopreserved in a few hours. Similarly, we studied peripheral blood or BM mononuclear cells from 64 adult B-lineage ALL samples at diagnosis (20 freshly analyzed, and 44 after thawing).

Mononuclear cells were characterized by four-color flow-cytometry using the following monoclonal antibodies: (i) fluorescein isothiocyanate (FITC)-conjugated anti-CD13, CD33, CD10, TdT (DakoCytomation, Copenhagen, Denmark), CD38, CD58, CD21, CD66c (Beckman Coulter, Fullerton, CA, USA), CD22, CD45, CD15 (Becton Dickinson, [BD], San José, CA, USA), CD65 (Caltag Laboratories, Burlingame, CA, USA); (ii) phycoerythrin (PE)-conjugated anti-CD10 (DakoCytomation), NG2 (Beckman Coulter), CD56 (BD); (iii) peridinin-chlorophyll-protein (PerCP)-conjugated anti-CD34; (iv) allophycocyanin (APC)-conjugated anti-CD19 (BD). Mononuclear cells were diluted to a final concentration of 2×10^7 /mL in phosphate-buffered saline, with 1% bovine serum albumin and 0.1% sodium azide (Sigma-Aldrich, Milan, Italy), and pre-incubated with 50 μ L of rabbit normal immunoglobulins (DakoCytomation) to lower non-specific antibody binding. For TdT intracellular staining, cells were permeabilized with 8E (modified ORTHO-

Permeafix). Data were acquired and analyzed with a FACScalibur flow-cytometer (BD) and CellQuest software. In the entire cell population (10,000 events), we defined two gates of analysis, one surrounding mononuclear cells (R1, forward scatter versus side scatter), the second delimiting the B-cell population (R2, side scatter versus CD19 or CD34 versus CD19). Within both gates (R1+R2) we acquired at least 500,000 events for normal BM and B-lineage ALL samples in complete remission, and at least 30,000 events for samples at diagnosis. In normal B-cell precursors we analyzed CD19⁺/CD34⁺ and CD19⁺/CD34⁻ cell populations for the expression of the other antigens. We then compared normal and leukemia cell patterns. Nineteen dilution tests of leukemia cells from nine samples (with at least three LAIP each) were carried out with mononuclear cells from normal BM samples (1/10²- 1/10⁵) to test the sensitivity of the method with the most frequent LAIP. By definition, no less than 10 clustered cells/100,000 total events in the LAIP-positive gate were considered MRD.

Co-culture experiments

To assess LAIP expression by blasts following contact with BM microenvironmental cells, we used adherent mesenchymal stem cells (MSC) as a feeder layer. These cells were isolated from normal BM samples as previously described.¹⁹⁻²⁰ On a MSC monolayer we co-cultured for 3 weeks different dilutions of either blasts from five patients with suitable LAIP (CD38: 5 cases, CD45: 4 cases, NG2: 3 cases, CD66c: 4 cases, CD13: 1 case, CD33: 1 case), or normal BM cells, or blasts with normal BM cells (1:10 ratio, to maintain the same mononuclear cell-MSC ratio). The blasts and normal cells were discriminated using the same method of analysis carried out with blasts at diagnosis. At days +14 and +21 of co-culture cell recovery was >80%, and blasts were analyzed to assess any change in the expression of the LAIP identified at diagnosis.

Detection of MRD in B-lineage ALL patients

From January 2003 to October 2005, we monitored MRD by flow cytometry in 14 patients with adult B-lineage ALL. These patients were aged 16-68 years (median 37), six were male and four had a WBC >30×10⁹/L. The EGIL subtypes were B-I (pro-B) (n=2), B-II (common) (n=10) and B-III (pre-B) (n=2). Seven cases were Philadelphia-positive and one case each was t(4;11)-positive or hyperploidy-positive. All patients had one to three suitable LAIP (median: two) that were monitored at the end of induction, consolidation, and maintenance therapy. Seven patients were treated with the *Linee Guida GIMEMA LAL 2000* protocol (induction and consolidation for 3 months, then cranial chemio/radioprophylaxis, and maintenance/re-induction cycles for up to 3.5 years from diagnosis). Philadelphia-positive patients were treated according to the *GIMEMA STI 571-0201* protocol, which is similar to the *Linee Guida GIMEMA LAL 2000* protocol for

patients <60 year old, but with more intensive consolidation, and maintenance based on STI 571 alone. The protocol for patients >60 years old was prednisone plus STI 571, followed by maintenance with STI 571 alone.

Statistical analysis

The probability of relapse was evaluated using the cumulative incidence procedure (follow-up time until the end of October 2005). The survival rate was calculated with the Kaplan-Meier method.

Results and Discussion

Antigen expression in normal or regenerating BM cells

The normal distribution of B-cell precursors was used to draw the *normality templates* for each combination and to identify *empty spaces* with <0.01% normal B-cell precursors (Figure 1 A and B). No significant differences were found between normal and regenerating BM samples (CD19⁺ cells: 14.4±3.5% and 10.5±4.4%, respectively; CD19⁺/CD10⁺/CD34⁺ cells: 1.1±0.5% and 0.89±0.45%, respectively). The most immature B-cell precursors (CD19⁺/CD34⁺/CD10⁻) expressed medium-high levels of CD38. Along maturation, CD34 was down-regulated first, followed by CD38 and CD10. The percentages of CD34⁺/CD38⁻ and CD10⁺/CD38⁻ normal B-cell precursors in any of the normal or regenerating BM samples analyzed were always under the detection threshold of the method (<0.01%), and cells were not clustered. CD19⁺/CD34⁺/CD10⁺ cells also expressed low levels of CD45, whose intensity increased during B-cell maturation. As for CD38⁻ cells, there were always less than 0.01% of CD34⁺/CD45⁻ and CD10⁺/CD45⁻ normal B-lineage precursors, with no evidence of cell clustering.

CD19⁺/CD34⁺/CD10⁺ cells did not express CD21 and weakly expressed CD22; these markers were both up-regulated during maturation, in parallel with CD10 down-modulation by CD19⁺/CD34⁺ cells. We considered the hyper-expression of CD21 and CD22 abnormal, particularly in CD34⁺ B-cell precursors. All CD19⁺ (both CD34⁺ and CD34⁻) B-cell precursors expressed low levels of CD58. TdT, CD34 and CD10 were highly expressed by immature B-cell precursors. TdT was down-regulated along B-cell maturation together with CD34. TdT-positive normal B-cell precursors did not express either myeloid markers (CD13, CD15, CD33, CD65, CD66c) or CD56 or NG2 (Figure 2). For all the markers mentioned above the background levels in normal and regenerating BM samples were always lower than 0.01%.

Prevalence of LAIP

Sixty-one of the 64 cases studied (95.3%) had at least one LAIP. Of these, 26 (42.6%) had only one LAIP and 35 cases (57.3%) had two or more LAIP. In 11/64 cases (17.1%) not all 13 marker combinations could be studied because of low cell count. LAIP frequencies are shown in Table 1.

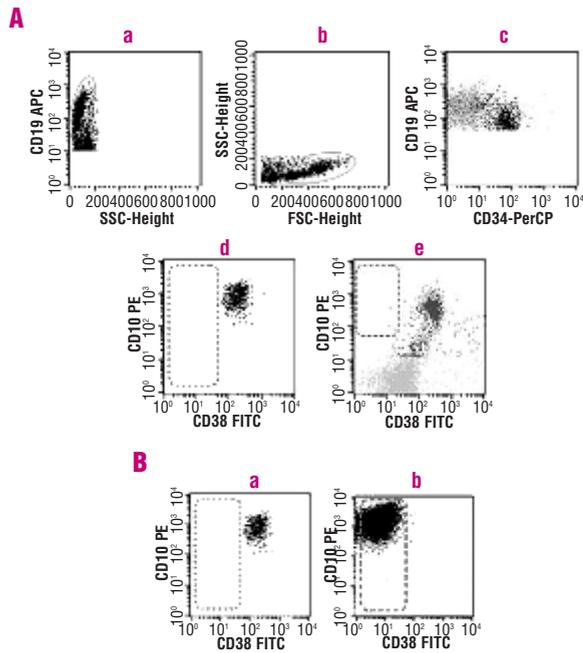


Figure 1 (left). **A.** Analysis of normal BM CD19⁺ cells. Two gates (gray lines) were drawn, the first surrounding CD19⁺ cells (a) and the second mononuclear cells (b). Then, CD19⁺ B cells (identified by both gates) were distinguished, according to surface CD34 expression, into CD34⁺ (black) and CD34⁻ cells (gray) (c). The separate analysis of CD34⁺ and CD34⁻ B cells enabled us to study the relative expression of two other antigens (i.e. CD38 and CD10) (d,e). Two empty spaces (broken line) can be identified, where normal BM cells are never detected (d,e). The figure shows a representative case. **B.** Analysis of LAIP in adult B-lineage ALL blasts in comparison with the immunophenotypic patterns of normal B-cell precursors. Normal BM CD19⁺/CD34⁺ cells (a) express both CD10 and CD38, while in this case of adult common B-lineage ALL (b) blasts are CD10⁺ but do not express CD38, thus falling in the empty space of abnormality (broken line). The figure shows a representative case.

Table 1. Useful antibody combinations for immunophenotypic MRD detection.

Antibody combinations*	Suitable cases (n)	(%)	LAIP frequency in childhood ALL ^o
CD38/CD10/CD34/CD19	36/63	57.1%	30-50%
CD45/CD10/CD34/CD19	17/64	26.6%	30-50%
CD21/CD10/CD34/CD19	0/57	0	5-10%
CD22/CD10/CD34/CD19	0/55	0	20-30%
CD58/CD10/CD34/CD19	11/62	17.7%	40-60%
TdT/CD10/CD34/CD19	22/61	36.1%	30-50%
CD13/CD10/CD34/CD19	8/62	12.9%	10-20%
CD15/CD10/CD34/CD19	3/58	5.2%	5-10%
CD33/CD10/CD34/CD19	2/59	3.4%	5-10%
CD65/CD10/CD34/CD19	0/59	0	5-10%
CD66c/CD10/CD34/CD19	15/62	24.2%	10-20%
CD10/NG2/CD34/CD19	9/60	15.0%	3-5%
CD10/CD56/CD34/CD19	3/57	5.3%	5-10%

*FITC/PE/PerCP/APC; ^oRefs. 2,3,5,8.

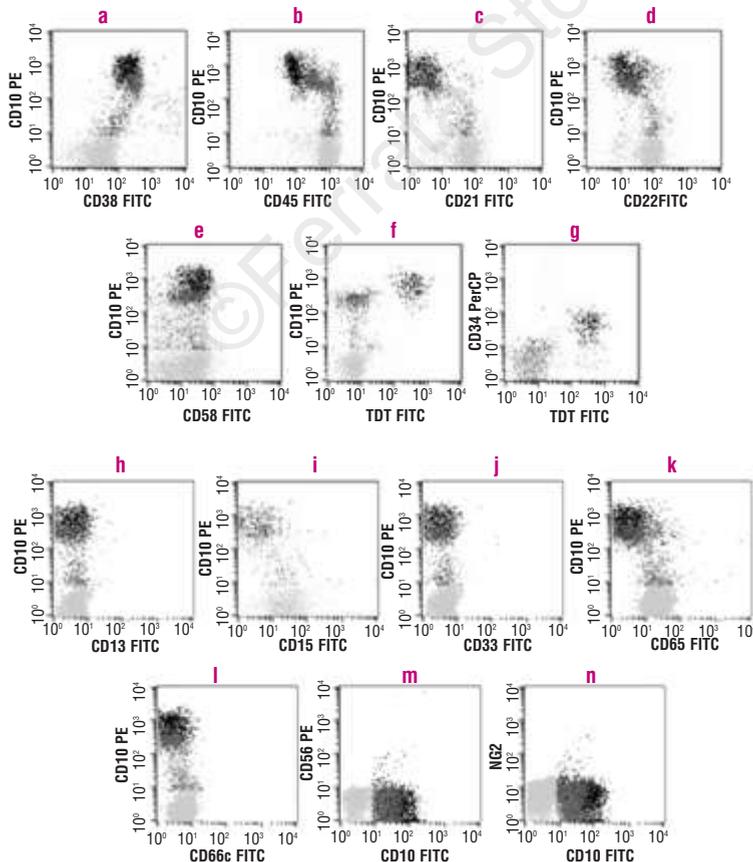


Figure 2. Expression pattern of CD38 (a), CD45 (b), CD21 (c), CD22 (d), CD58 (e), TdT (f and g), myeloid markers (h-l), CD56 (m), and NG2 (n) by normal BM CD19⁺ cells. Black dots: CD19⁺/CD34⁺/CD10⁺ normal BM cells; dark gray dots: CD19⁺/CD34⁻/CD10⁺ normal BM cells; light gray dots: CD19⁺/CD34⁻/CD10⁻ normal BM cells. The figure shows a representative case.

Sensitivity assessment of MRD detection by flow cytometry

We could identify one blast in 1,000 normal cells (a sensitivity of 10^{-3}) in all cases, and in most cases (84.2%) up to one blast in 10,000 normal cells (a sensitivity of 10^{-4}). The sensitivity of detection was 10^{-4} in all cases displaying LAIP with abnormal intensity expression, but in only 66.7% of cases with cross-lineage marker expression.

LAIP persistence after blast-MSL co-culture

At the end of co-culture, blasts expressed their original phenotype in all cases, without any significant immunophenotypic change. We observed a slight increase of marker expression intensity following the interaction with MSL, but this not jeopardize LAIP recognition.

Detection of MRD in patients

All patients obtained morphological complete remission. The median follow-up was 8 months (range 3-28 months). Eight of 14 patients relapsed, and cells expressed the same LAIP observed at diagnosis. All but one patients who relapsed had persistent MRD at two time points during complete remission (median \pm SD: 1.02 \pm 0.9%, range 0.06-2.6%), and six out of eight were high-risk according to karyotype at diagnosis. The other six patients remained in complete remission at the end of follow-up and were

consistently negative for MRD with the exception of one, who had detectable MRD after induction (0.8%), but converted to MRD-negativity during maintenance therapy with STI 571. Of the five patients in complete remission who were MRD-negative, only one was high-risk according to karyotype at diagnosis. All (n=13) samples from Philadelphia-positive patients (n=7) who were MRD-positive by flow-cytometry (6/7 patients) resulted bcr-abl positive by PCR. Conversely, according to the differences in sensitivity of either method, 1/7 Philadelphia-positive patients was persistently bcr-abl positive but MRD negative by flow-cytometry in 1/2 samples.

Our clinical studies are preliminary because of the small number of patients. However, MRD detection was associated with a worse outcome, whereas most MRD-negative patients had durable remissions. For this reason, we are currently testing this methodological approach in larger cohorts of patients enrolled in Italian GIMEMA trials.

MK and OP designed the study, analyzed the data and wrote the paper; CV and FZ prepared and acquired the samples; AP and MTS contributed to co-culture experiments; AG, SDP, ECS, and DC contributed to data analysis; AV contributed to sample collection; RF and GP contributed to the paper writing. Funding: This work was supported by grants from the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR), Consiglio Nazionale delle Ricerche (CNR) and Fondazione Cariverona, Verona, Italy.

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