



## Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: implications for the detection of minimal residual disease in acute lymphocytic leukemia

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**Background and Objectives.** CD58, a member of the Ig superfamily, is expressed by hematopoietic and non-hematopoietic cells. It has been demonstrated to be over-expressed in precursor-B acute lymphoblastic leukemia (ALL) blasts when compared to in their normal counterparts, suggesting its potential use in the detection of minimal residual disease (MRD) by flow cytometry (FC). To assess the reliability and accuracy of CD58 for this purpose, we studied its expression in a large series of normal and ALL bone marrow (BM) samples using quantitative FC.

**Design and Methods.** We studied 180 precursor-B ALL BM samples at diagnosis (8 pro-B, 164 early-B, 8 mature-B ALL) and 123 follow-up BM samples (n=54 at day +15 and n=69 at day +78), as well as 51 normal BM samples and 7 regenerating BM samples from patients with T-ALL at week 12. We used four-color quantitative FC, focusing analysis on CD58 expression. In follow-up samples from day +78, the MRD level was simultaneously evaluated by real time quantitative polymerase chain reaction (RQ-PCR) amplification of antigen receptor genes.

**Results.** CD58 expression was significantly higher in ALL blasts than in normal B lymphocytes, while no significant differences between regenerating and normal B lymphocytes were observed. CD58 was expressed in 99.4% of the precursor-B ALL cases and 93.5% of these showed over-expression compared to normal. No significant modulation of CD58 expression during remission induction therapy was noted. Finally, 66 (95.6%) of 69 BM samples simultaneously analyzed using both FC and RQ-PCR at day +78 showed concordant results regarding MRD.

**Interpretation and Conclusions.** Our results confirm and further evidence the role of CD58 in the diagnosis and monitoring of precursor-B ALL. In particular, we demonstrated its stability and accuracy in MRD detection at clinically relevant time points. These findings indicate that CD58 is a powerful tool for MRD detection in precursor-B ALL.

**Key words:** flow cytometry, CD58, residual disease, acute lymphoblastic leukemia.

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The level of minimal residual disease (MRD) in childhood acute lymphoblastic leukemia (ALL) measured during different phases of therapy represents an independent prognostic indicator with clinical relevance.<sup>1-3</sup> It has been demonstrated that the measurement of MRD by multi-parametric flow cytometry (FC), based on leukemia-associated immunophenotypes, also provides independent prognostic information in children with ALL.<sup>3-5</sup> One of the major goals of flow cytometric MRD studies is the identification of new immunophenotypic markers able to detect blast cells with high sensitivity and specificity among their normal counterparts.<sup>6</sup> Recently, it has been demonstrated that multiparametric FC detection of antibody directed to CD58 (lymphocyte function-associated antigen-3, LFA-3, a member of the Ig superfamily expressed by hematopoietic and non-hematopoietic cells) is an attractive tool for diagnosing and monitoring MRD in children with B-lineage ALL. CD58 has been shown to be over-expressed in leukemic blasts when compared to in their normal counterparts, CD19<sup>+</sup>CD10<sup>+</sup> B-cell progenitors.<sup>6-8</sup> This feature allowed for the detection of MRD in bone marrow (BM) samples from children affected by ALL in clinical remission.<sup>6</sup>

In order to assess the feasibility of using CD58 to monitor MRD in a co-operative study, we applied a well standardized multi-parametric quantitative immunophenotyping approach<sup>9-11</sup> to evaluate the expression of CD58 in blast cells from a large cohort of childhood B-lineage ALL patients and in non-leukemic B cells obtained from normal and regenerating BM.

Furthermore, the stability of CD58 staining was tested in ALL samples collected during the early phase of remission induction chemotherapy (day+15) and the sensitivity was evaluated in the regenerating BM phase (day +78), by comparing FC and molecular evaluation of MRD levels.

### Design and Methods

#### Samples

(i) *Leukemic bone marrow (at diagnosis).* Samples were taken at diagnosis from 180 patients with B-lineage ALL (8 pro-B, 164 early-B, 8 mature-B ALLs) enrolled in the ongoing I-BFM-2000 ALL protocol AIEOP ALL 2000, of the *Associazione Italiana Ematologia Oncologia Pediatrica* (AIEOP). The diagnosis of B-lineage ALL was established according to conventional FAB and immunological criteria.<sup>12,13</sup> Of the 180 patients, 112

were male and 68 were female, with a median age of 5.72 years (range: 0.72-17.64 years). This study was approved by the institutional ethical committees and was done with the informed consent of the patients' parents or guardians.

(ii) *Leukemic bone marrow (day +15)*. In order to verify the stability of CD58 expression during the first phase of chemotherapy induction, 54 patients with B-lineage ALL were investigated. To analyze statistically relevant cell populations, we included patients in whom a cluster of at least 150 leukemic cells out of 300,000 cells were detected by flow cytometry.

(iii) *Leukemic bone marrow (day +78)*. MRD was investigated on day +78 (week 12) of remission-induction chemotherapy in 69 patients using both FC and real-time quantitative polymerase chain reaction (RQ-PCR) techniques.

(iv) *Normal bone marrow*. Fifty-one samples, collected from children undergoing bone marrow aspiration for non-lymphoproliferative disorders or as donors for transplantation, were evaluated. The median age of this category of patients was 8 years (range: 1.24-19.24 years).

(v) *Regenerating bone marrow*. Seven samples, collected from T-ALL cases at day +78 (week 12) and characterized by massive bone marrow expansion of precursor-B cells after remission-induction chemotherapy, were studied.

#### Flow cytometric studies

Analyses were performed as previously described.<sup>10</sup> Briefly, whole blood (at least 500,000 cells/tube) was incubated for 15-20 minutes in the dark at room temperature together with a cocktail of monoclonal antibodies (MoAbs) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Texas-red (ECD) and phycoerythrin-cyanin 5 (PE-Cy5) fluorochromes. Samples were lysed using  $\text{NH}_4\text{Cl}$  solution, then washed twice in phosphate-buffered saline (PBS), re-suspended in 1 mL of PBS and analyzed by flow cytometry. All samples were processed within 24 hours after collection. Antibody labeling was measured by multi-parameter four-color FC using an Epics XL cytometer (from Beckmann Coulter, Inc., Miami, FL, USA) equipped with an argon laser tuned to 488 nm. Data were acquired with the EXPO32 software (Beckman-Coulter). We analyzed at least 15,000 and 300,000 viable cells per tube at diagnosis and for MRD measurements, respectively. The instrument set-up was optimized daily by analyzing the expression of peripheral blood lymphocytes labeled with the anti-CD4 FITC/CD8 PE/CD3 ECD/CD45Pe-Cy5 four color combination.<sup>9</sup> The following MoAbs were used: anti-CD58 FITC, CD3 ECD, CD10 PE, CD19 ECD, CD20 PeCy5 (from Beckmann Coulter/Immunotech, Miami, FL, USA),

CD4 FITC, CD8 PE, CD34 PE (from Becton Dickinson Biosciences, San José, CA, USA), and CD45 PeCy5 (from Caltag Laboratories, San Francisco, CA, USA). The leukemic and normal B cells were identified using an immunological gate including all CD19 positive cells associated with a physical parameter ( $90^\circ$ -scatter, SSC). Minimal residual disease was defined as an accumulation of at least 10 clustered events displaying the leukemia-associated immunophenotypic characteristics identified at diagnosis. To guarantee quantitative and reproducible results of CD58 expression, we transformed the mean fluorescence intensity (MFI) values (channel numbers, scaled from 0 to 1024) into molecules of equivalent soluble fluorochrome (MESF) using DAKO Fluorospheres with assigned values of MESF (Dakopatts, Glostrup, Denmark), according to the manufacturer's instructions. The standard calibration curve was obtained by using the TallyCal™ software (DAKO). Background fluorescence was calculated for FITC-conjugated CD58, and a sample was considered positive when the MESF value was higher than 974 (mean $\pm$ 2 SD of the negative controls). CD58 was considered over-expressed in leukemic cells when the MESF value resulted higher than 3043 (mean $\pm$ 2 SD of the value for normal BM).<sup>11</sup>

#### PCR amplification of antigen receptor genes

Diagnostic bone marrow samples were analyzed for incomplete (D-J) and complete (V-D-J) rearrangements of heavy-chain immunoglobulin gene (IGH), light-chain  $\kappa$  (IGK), T-cell receptor  $\delta$  (TCRD), and T-cell receptor  $\gamma$  (TCRG) gene rearrangements. PCR amplification, heteroduplex analysis, and sequencing were performed as previously described.<sup>14,15</sup> The involved segments of IGH, IGK, TCRD and TCRG gene rearrangements were identified by comparison with published germline sequences using the sequence alignment software (V-QUEST) from the IMGT database. All primers and probes for RQ-PCR were designed as reviewed,<sup>16</sup> using the Primer Express 1.0 software (Applied Biosystems). An allele-specific oligonucleotide (ASO) primer approach was applied, by designing a germline probe and germline reverse primer; ASO forward primers were complementary to the junctional region. The ABI PRISM 7700 Sequence Detection System, containing a 96-well thermal cycler, was used (Applied Biosystems). A standard annealing temperature of 60°C was used, unless indicated otherwise. To determine the efficiency and sensitivity of the target-specific PCR amplification, diagnostic DNA was serially diluted 10-fold into DNA from mononuclear cells (MNC) of a pool of 5 different healthy donors, from a dilution of  $10^{-1}$  down to  $10^{-6}$ . Serial dilutions of diagnostic samples and fol-

**Table 1. Expression of CD58 in normal, regenerating and leukemic bone marrow.**

	CD58 MESF		
	Regenerating BM (N = 7)	Normal BM (N = 51)	Leukemic BM (N = 180)
Mean	1649	1705	11245
Median	1282	1577	9857
SD	837	669	7672
Min	641	497	962
Max	3206	3752	41028
		<i>p = ns</i>	<i>p &lt; 0.001</i>

BM: bone marrow; ns; not significant.

low-up samples were analyzed in triplicate. To correct for the quantity and quality (*amplifiability*) of DNA, the albumin gene was amplified in parallel.<sup>16,17</sup>

#### Statistical analysis

We calculated the mean, median and standard deviation (SD) of each MESF and CV value. The statistical significance of any differences reported was determined using the paired Student's t-test ( $p < 0.05$ ).

#### Results

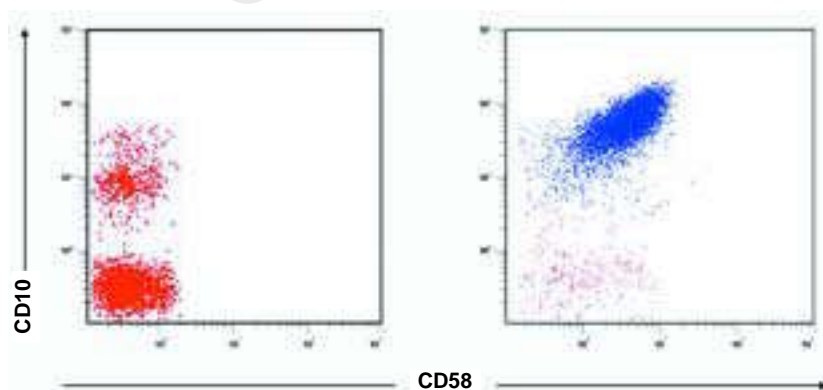
##### Quantitative expression of CD58 in normal and leukemic B-cells

To evaluate the expression of CD58 in B-lineage ALL and to compare it with that of normal precursor-B cells, we tested this antibody in 180 children with B-lineage ALL at diagnosis, in 51 samples of

normal BM and in 7 regenerating BM samples. As shown in Table 1, CD58 expression was higher in leukemic lymphoblasts than in normal B lymphocytes [median MESF value 9857 (range 962-41028) vs 1577 (range 497-3752) MESF,  $p < 0.0001$ ]. By contrast, we did not observe any significant differences between regenerating and non-regenerating normal B lymphocytes. Figure 1 illustrates a representative example of the different CD58 expression in normal and leukemic BM samples. The CD58 resulted positive (MESF value  $> 974$ ) in 171/172 (99.4%) cases of precursor-B ALL (pro-B and early-B), of which 160 (93.5%) showed overexpression of this antigen compared to the level of expression in normal B lymphocytes (MESF value  $> 3043$ ). One hundred and fifty out of 171 CD58 positive cases were also CD34 positive, while 21/171 were CD34 negative. We did not find any correlation between overexpression of CD58 and expression of CD34. The case of CD58-negative precursor-B-ALL expressed the CD34 antigen. Five out of 8 cases of mature B-ALL (Smig<sup>+</sup>) were positive for CD58 with overexpression in just two cases and none of them (0/8) expressed the CD34 antigen. The detailed results of MESF measurements in individual cases are reported in Figure 2.

##### Stability of CD58 expression during treatment

One of the potential sources of false negative results in immunologic MRD studies is immunophenotypic changes of the leukemic clone occurring during or after treatment.<sup>18</sup> Of note, we have recently observed antigen modulation in ALL during the first week of treatment, when patients received steroids and IT MTX, in the context of a *Berlin-Frankfurt-München* protocol (Gaipa G *et al*, unpublished data). In order to test the stability of CD58 expression in this phase of treatment, we studied 54



**Figure 1. Flow cytometric analysis of CD58 and CD10 expression in normal and leukemic bone marrow. Expression of CD58 (x-axis) and CD10 (y-axis) on CD19 positive-gated cells as measured in normal bone marrow (left cytogram), and in a representative case of B-lineage ALL (right cytogram). The red dots and blue dots represent normal and leukemic cells, respectively.**

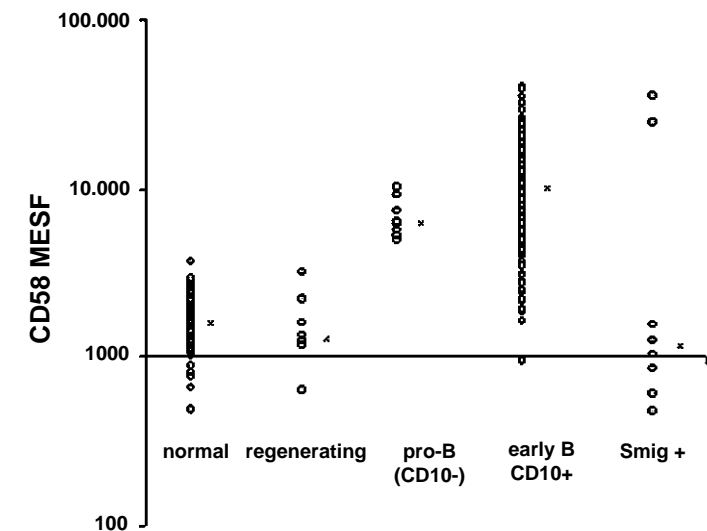


Figure 2. Quantitative expression of CD58 antigen in normal and leukemic bone marrow. MESF value (y-axis) of CD58 according to immunophenotype in 180 ALL patients, in normal and in regenerating precursor-B cells. The median values are indicated by a cross. The horizontal line (974 MESF) indicates the threshold of positivity.

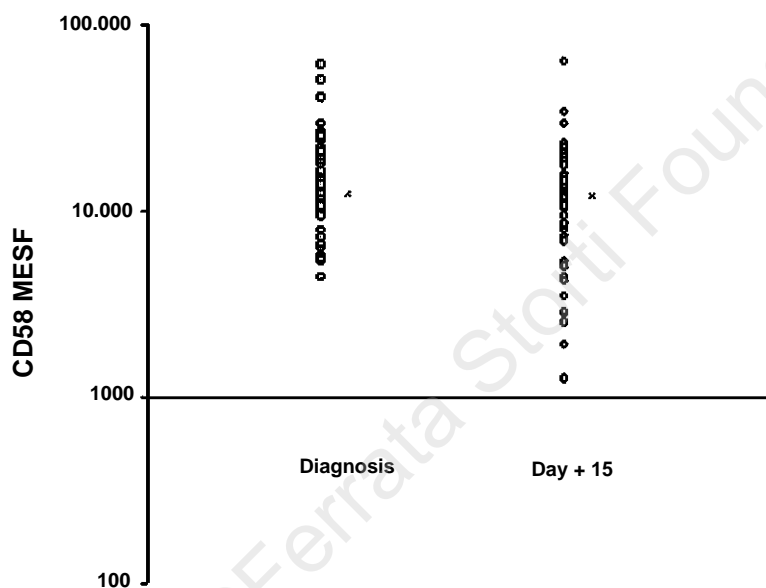


Figure 3. Quantitative expression of CD58 antigen in leukemic bone marrow at diagnosis and at day+15. MESF value (y-axis) of CD58 in 54 cases of B-lineage ALL at diagnosis and day +15. The difference between median values (indicated by a cross) was not significant by the t test. The horizontal line (974 MESF) indicates the threshold of positivity.

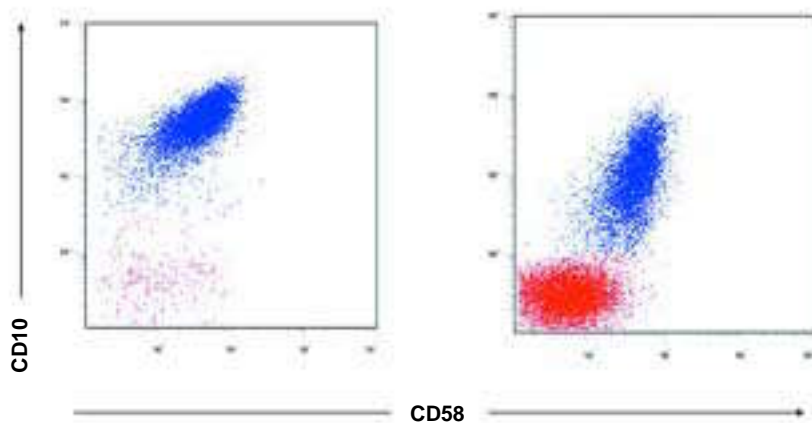
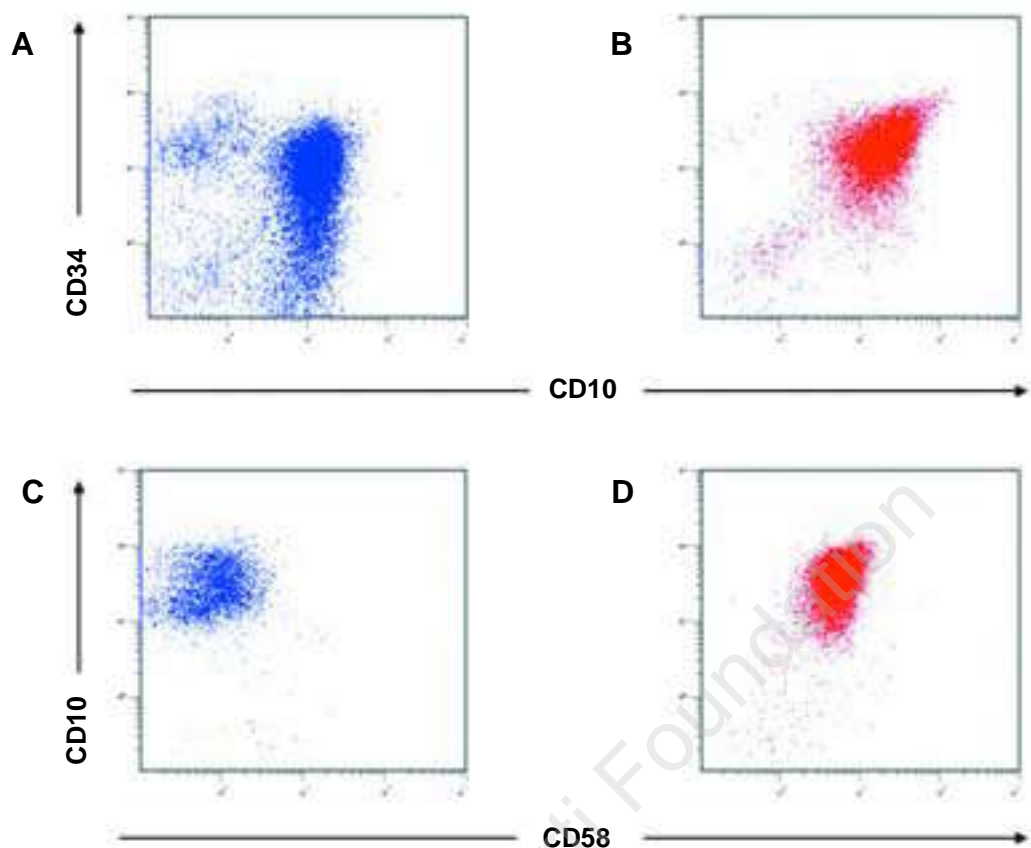


Figure 4. Flow cytometric analysis of CD58 and CD10 expression in leukemic bone marrow. Expression of CD58 (x axes) and CD10 (y axes) in CD19 positive gated cells, as measured at diagnosis (left cytogram) and at day +15 (right cytogram) in a representative case of B-lineage ALL. The blue dots and red dots represent blast cells and normal cells, respectively.



**Figure 5.** Flow cytometric analysis of leukemic cells and regenerating bone marrow. CD19 gated cells. The expression of CD10 and CD34 in regenerating BM (A) shows a very similar distribution to that observed in leukemic cells (B); by contrast, when CD58 is used in addition to CD10, leukemic (C) and regenerating (D) cells can be discriminated easily.

B-lineage ALLs at diagnosis and again at day +15. As shown in Figure 3, we did not observe significant differences overall [median MESF 12332 (range 4488-61873) vs 11978 (range 1282-65301) at diagnosis and at day +15, respectively,  $p=NS$ ]. However, in some cases relevant down-modulation of CD10 expression was observed (Figure 4).

#### *CD58: a marker for monitoring MRD*

By 12 weeks after the start of treatment, the BM is in a regenerating phase dominated by very immature B-cell precursors with an antigen expression pattern overlapping that of leukemia (Figure 5). In an attempt to assess whether the detection of anti-CD58 could be useful for demonstrating MRD at this particular time point, we analyzed 69 ALL patients using both multi-parametric FC and PCR amplification of antigen receptor genes. The flow cytometric studies used four-col-

or antibody combinations containing anti-CD58. The leukemic cells in MRD positive samples were quantified according to the criteria described previously.<sup>5,10</sup> The analysis of CD58 positive cells also included MFI calculations (*data not shown*). All patients were considered, by an expert hematologist, to be in complete morphologic remission at the time of sampling. Ten out of 69 specimens were MRD positive according to both techniques. The proportion of leukemic cells in these cases ranged from 0.28% to 0.01% of nucleated cells by FC and from 0.46% to 0.001% by PCR. In fifty-six samples residual disease was undetectable by either technique. In three samples MRD was detected by PCR, but not by FC; the proportion of MRD in these three cases ranged from 0.01 to 0.001%. Of note in two of these three cases, MRD was detected by only one of the two PCR markers studied (Table 2).



**Table 2. MRD detection in BM samples from ALL patients at day+78, analyzed by FC and PCR.**

	PCR+	PCR-	
FC+	10	0	10
FC-	3	56	59
	13	56	69

## Discussion

The classical concept that acute lymphoblastic leukemia develops from normal hematopoietic cells that are blocked at a particular stage of differentiation has recently been overturned by the demonstration that leukemic cells often display immunophenotypic features that are usually undetectable in normal precursor cells, the so-called aberrant phenotype.<sup>9,20-23</sup> With this in mind, emphasis is being increasingly placed on the identification of new monoclonal antibodies capable of optimizing the discrimination between leukemic and normal cells.<sup>6,8</sup> CD58 has been reported to be a novel marker suitable for diagnosing and quantifying MRD in childhood ALL, due to the over-expression displayed by leukemic lymphoblasts when compared to normal B-cell progenitors.<sup>6,7</sup> On this background, we screened 180 B-lineage childhood ALL at diagnosis for quantitative expression of CD58 antigen, as extensive data regarding the use of this marker were not present in the literature. Among the cases of early B-ALL, we observed expression of the antigen in 99.4% of tested cases and over-expression in 93.5% of positive cases. Of the 8 mature B-ALL cases investigated, 5 expressed CD58, and only 2 showed over-expression compared with the normal counterpart. We did not find any correlation between CD58 intensity and CD34 expression.

The occurrence of either lineage switch or phenotypic shift (disappearance of one or more antigens) between diagnosis and relapse has been described in acute leukemias by several authors and its potential impact on MRD detection has been extensively reviewed by Campana *et al.*<sup>18</sup> Partial or total antigen modulation can also occur in ALL during the early phase of treatment, in particular during steroid administration (Gaipa G *et al.*, unpublished data). To investigate whether CD58

expression on leukemic blasts is altered by steroid treatment, we compared 54 leukemic bone marrow samples at diagnosis and at day +15 of remission-induction chemotherapy. We did not observe any significant differences in CD58 expression between diagnostic and follow-up samples, suggesting that CD58 is a very stable immunophenotypic marker for use in MRD detection.

The overexpression of CD58 observed in a high proportion of ALL cases (>90 %) included in the present study prompted us to test the feasibility of measuring this antigen in regenerating bone marrow. It is known that normal precursor-B cells bear a close resemblance to leukemic lymphoblasts, sharing morphologic and immunophenotypic features, in particular the expression of CD10, CD34 and TdT,<sup>22,24-26</sup> which could hamper MRD assessment in the regenerating phase occurring during chemotherapy (week 12, and week 22-24) and after treatment withdrawal, when normal precursors often expand.<sup>5,24-26</sup> In an attempt to evaluate the interference of these cells with the detection of leukemic lymphoblasts, we investigated CD58 expression in normal B cells from 7 T-ALL patients on day +78 (week 12). Interestingly, the intensity of CD58 expression was similar in normal and regenerating bone marrow samples when assessed by measuring the MESF value in the two groups. In contrast, the expression of CD58 in precursor-B ALL (but not in mature-B ALL) was significantly higher than that observed in normal regenerating precursor-B cells. In spite of the small number of regenerating cases studied, we can speculate that CD58 could be a great advantage during MRD detection in this particular setting. To verify this hypothesis, we simultaneously used FC, with a CD58-based combination of antibodies, and RQ-PCR of antigen receptor genes, to investigate BM samples from 69 patients at week +12 of treatment. The two methods yielded concordant results in 66 of the 69 patients (95.6%), while in the remaining three samples MRD was detected by PCR at a proportion ranging from 0.28% to 0.01%, but not by FC. Notably in 2 of these 3 cases, one of the two molecular markers failed to detect MRD (Vkl-1kde and Vd2Dd3, respectively). These discrepancies between FC and RQ-PCR results may be explained by the different concentrations of lymphocytes in the tested samples, since the DNA used for RQ-PCR was obtained from mononuclear cells enriched by Ficoll centrifugation, while the FC analysis was performed on whole blood. Very similar results were obtained by Chen *et al.*;<sup>6</sup> these authors compared FC and the PCR technique for detecting MRD in BM from children with ALL in morphologic remission. They obtained three discordant samples out of 55 tested using CD58 (one FC-/PCR+ and two FC+/PCR-). This study examined samples collected at the end of remission induction

during continuation therapy (approximately six weeks after diagnosis). While differences in the therapeutic regimen should be considered, our results obtained from samples at week 12 suggest that MRD detection using CD58 performs very well even in the presence of a high proportion of immature B cells.

In summary, our results, obtained from a large series of normal and leukemic samples using quantitative FC, not only provide further evidence on the role of CD58 in the diagnosis and monitoring of precursor-B ALL, but also demonstrate the stability and accuracy of this marker in MRD detection at critical time points. In conclusion, CD58 is a powerful tool for MRD detection in precursor-B ALL and should be used to complement current FC strategies.

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## Pre-publication Report & Outcomes of Peer Review

### Contributions

MV: analysis and interpretation of data; drafting the article; LDZ: analysis and interpretation of data; MCS: analysis and interpretation of data; OM: analysis and interpretation of data; MND: revising the article critically for important intellectual content; RR: revising the article critically for important intellectual content; AB: final approval of the version to be published; GB: conception and design; revising the article critically for important intellectual content; final approval of the version to be published; GG: conception and design; revising the article critically for important intellectual content.

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### Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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### Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Dario Campana, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Campana and the Editors. Manuscript received June 19, 2003; accepted September 30, 2003.

In the following paragraphs, Dr. Campana summarizes the peer-review process and its outcomes.

### What is already known on this topic

Minimal residual disease (MRD) is a key prognostic factor in acute leukemia. Flow cytometry is the most widely usable method for MRD detection; its clinical utility has been convincingly demonstrated. However, there is room for improvement in the current antibody panels, which must be extensive (and expensive) to be reliable.

### What this study adds

In this careful and extensive study, Veltroni *et al.* tested the usefulness of CD58, a marker previously shown to be overexpressed in acute lymphoblastic leukemia (ALL) cells, to monitor MRD at different time points during treatment. The results are important because they conclusively demonstrate that CD58 is overexpressed in ALL and is a robust marker for MRD studies; they sanction its use in a large multicenter trial of childhood ALL. Unfortunately, CD58 does not achieve the status of *universal marker* of ALL; up to half of the cases have levels of expression that may not be sufficient to distinguish ALL from normal cells with confidence. Nevertheless, CD58 sets an important precedent; it seems likely that modern methods to probe gene and protein expression will identify other similar or more powerful markers in the near future.