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Drug-induced immunophenotypic modulation in childhood ALL: implications for minimal residual disease detection

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Assessment of minimal residual disease (MRD) by flow cytometry is considered to be based on the reproducibility of the leukemic immunophenotype detected at diagnosis. However, we previously noticed modulation of surface antigen expression in acute lymphoblastic leukemia (ALL) during the early treatment. Hence, we investigated this in 30 children with B-cell precursor ALL consecutively enrolled in the AIEOP-BFM ALL 2000 protocol. Quantitative expression of seven antigens useful in MRD monitoring was studied at diagnosis and compared to that measured at different time points of remission induction therapy. Downmodulation in the expression of CD10 and CD34 occurred at follow-up. By contrast, upmodulation of CD19, CD20, CD45RA, and CD11a was observed, while the expression of CD58 remained stable. Despite this, we could unambiguously discriminate leukemic cells from normal residual B cells. This holds true when bone marrow (BM) samples from similarly treated T-ALL patients, but not from healthy donors, were used as reference. Our results indicate that immunophenotypic modulation occurs in ALL during the early phases of BFM-type protocols. However, the accuracy of MRD detection by flow cytometry seems not negatively affected if adequate analysis protocols are employed. Investigators should take this phenomenon into account in order to avoid pitfalls in flow cytometric MRD studies.
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Introduction

Studies of minimal residual disease (MRD) have a powerful clinical application in the management of acute leukemia patients.[1–2] The prognostic value of MRD detection in acute lymphoblastic leukemia (ALL) during remission induction therapy has been demonstrated by several studies.[3–8] Strategies for MRD detection include multiparametric flow cytometry, which differentiates leukemic cells from their normal counterparts based on the expression of aberrant phenotypes.[9] The sensitivity and specificity of MRD studies by flow cytometry depend on various technical issues[10] including the stability of the leukemia-associated immunophenotypes during therapy. Immunophenotypic changes between diagnosis and relapse have been extensively described in acute leukemias. These changes include either acquisition/loss of lineage-associated antigens[5,11–18] or lineage switch.[14,16] Implications of leukemic clone evolutions by means of immunophenotyping in MRD studies are mainly related to false negative results,[5,18] which can be prevented by the simultaneous use of various immunophenotypic markers.[10] However, little is known as to whether such changes also occur during therapy. In this report, we describe a characteristic type of immunophenotypic modulation that occurs within the first 5 weeks of treatment. We studied this by investigating 30 consecutive children with B-cell precursor ALL. We analyzed the expression of the antigens mostly involved in the recognition of leukemia-associated immunophenotypes[6,19] by quantitative flow cytometry and we investigated the possible implications for MRD detection.

Patients and methods

Patients and treatment protocol

From January to October 2002, a series of 37 children with ALL were consecutively diagnosed and treated at the Pediatric Clinic of Milan-Bicocca University, Monza (Italy). A total of 30 children from this cohort were included in the present study according to the following criteria: they had to (1) be B-cell precursor ALL cases; (2) present leukemia-associated immunophenotypes suitable for MRD investigation; and (3) have a level of MRD of at least 150 events in peripheral blood (PB) or bone marrow (BM) follow-up specimens, as assessed by flow cytometry. This study was approved by the institutional ethics committee, and was carried out with the informed consent of the patients' guardians. The diagnosis of B-cell precursor ALL was established according to conventional FAB and immunological criteria.[20,21] From these 30 patients, 16 were common ALL and 14 were pre-B ALL; 18 were female and 12 male subjects with a median age of 3 years (range 1–16 years of age). A normal leukemic karyotype was found in 12 of 22 patients with cytogenetic results; seven patients had hyperdiploid leukemia and one patient showed pseudodiploidy. Translocation t(1;19) (q23;p13) was found in one case while another showed various aberrations other than t(9;22) and t(4;11). Translocation t(12;21) was found in eight out of 26 analyzed patients as assessed by molecular screening.

All these patients were enrolled and treated according to the AIEOP-BFM ALL 2000 protocol. The remission induction therapy, relevant for the findings here reported, was scheduled over 9 weeks, and included a 7-day prephase with daily oral prednisone (60 mg/m² of body surface area daily) and a single dose of intrathecal methotrexate (age-adjusted) on day 1, followed by a randomized regimen with either prednisone (60 mg/m² daily) or dexamethasone (10 mg/m² daily) given

from day 8 to day 28 and tapered thereafter over 9 days, plus L-asparaginase (5000 U/m² q 3 days ×8 from day 12), daunorubicin (30 mg/m² ×4 on day 8, 15, 22, and 29), vincristine (1.5 mg/m² on day 8, 15, 22, and 29) and two doses of intrathecal methotrexate (age-adjusted) on days 15 and 29. From day 36 to day 64, the regimen included: two doses of intrathecal methotrexate (age-adjusted) on days 38 and 52, cyclophosphamide (1 g/m² on days 36 and 64), cytarabine (75 mg/m² daily on days 38–41, 45–48, 52–55, and 59–62), and 6-mercaptopurine (60 mg/m² daily from day 36 to day 62). All patients were good prednisone responders according to protocol criteria (less than 1000 blasts/μl PB on day 8, as assessed by morphological evaluation). BM samples from healthy transplantation donors (*n* = 10) and from pediatric T-ALL patients (*n* = 3) undergoing the same treatment were used for comparisons.

Flow cytometric studies

Samples were collected at diagnosis from both PB and BM. Follow-up samples for MRD study were collected from PB at day 8 and from BM at days 15, 33, and 78. Leukemia-associated immunophenotypes were assessed by multiparametric flow cytometry using three-colour monoclonal antibody combinations, conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cyanin 5 (PE-Cy5) fluorochromes: CD20/CD10/CD19; CD58/CD10/CD19; CD10/CD34/CD19; CD10/CD45RA/CD19; CD10/CD11a/CD19. Terminal deoxynucleotidyl-transferase (TdT), in combination with CD10 and CD19, was employed in three patients. Antibodies to CD58, CD10 (FITC), CD19, and CD45RA were purchased from Coulter/Immunotech (Coulter/Immunotech, Miami, FL, USA); CD20, CD34, and CD11a from Becton Dickinson (BD; Becton Dickinson Biosciences, San José, CA, USA); CD10 PE and TdT (HT-6-FITC) from DAKO (Dakopatts, Glostrup, Denmark). For each patient, at least two marker combinations, allowing for the identification of leukemia-associated immunophenotypes, were selected at diagnosis and then applied during follow-up for MRD detection.

The engaged stain-lyse procedure has been previously reported.[6] Briefly, whole blood was incubated for 25 min at +4°c, samples were lysed using a commercially available red cell lysing solution (BD), then washed in phosphate-buffered saline (PBS) and resuspended in 0.5 ml of PBS. Stainings with the cell-permeant, live-cell nucleic acid fluorochrome SYTO 16 (Molecular Probes, Leiden, The Netherlands) combined with CD19 PE (from Coulter/Immunotech) and CD45 peridinin-chlorophyll protein (PerCP; BD) were used in follow-up analyses to exclude residual non-nucleated erythroid cells, thrombocytes, or debris. Intracellular staining was performed by a two-step fixation and permeabilization procedure using a commercial kit (Fix & Perm™, Caltag Laboratories, Hamburg, Germany) according to the manufacturer's instructions. All samples were processed within 24 h after collection. The immunophenotypic diagnosis and the SYTO16 evaluation were performed collecting 30 000 events, while for MRD measurements 300 000 ungated events were collected. Cell acquisition was performed using a FACScan™ flow cytometer equipped with a 488 nm argon laser, and the Cell-Quest™ software program (all from BD).

Instrument set-up was optimized daily by analyzing the expression of normal peripheral blood T lymphocytes labelled with CD4 FITC/CD8 PE/CD3 PerCP (all from BD), as previously reported.[22] For analysis, the PAINT-A-GATE™ software program (BD) was used. Leukemic cells were identified using an immunological gate based on CD19 expression associated with a physical parameter (90°-scatter, SSC) and they were discriminated from normal B lymphocytes on the basis of leukemia-associated immunophenotypes (ie overexpression of antigens such as CD10, CD34, CD58 and/or underexpression of CD45RA and CD11a). Antigen expression was quantified on the basis of mean fluorescence intensity (MFI) values (channel numbers, scaled from 0 to 1024), which were subsequently transformed into values of molecules of equivalent soluble fluorochrome (MESF) to allow quantitative comparisons over time. MESF values were calculated by using simultaneous measurements of DAKO Fluorospheres™ with assigned MESF-values as standards. The calibration curve was obtained by using the TallyCal™ software (DAKO).[27]

PCR amplification of antigen receptor genes

Diagnostic BM 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.[23,24] Real-time-Quantitative PCR (RQ-PCR) was performed by using patient-specific primers on IG/TCR junctional regions (reviewed in van der Valden *et al*[25]). To correct for the quantity and quality of DNA, the albumin gene was amplified in parallel.[25,26]

Statistical analysis

The paired Wilcoxon signed rank test was used to compare the median values of MESF at diagnosis and at follow-up time points, while the paired *t*-test was used to compare the mean values of FSC and SSC parameters. The adopted significance level was α = 0.05. Box and whisker plots were used for descriptive purposes.

Results

Antigen modulation in leukemic cells

We investigated paired samples from diagnosis and early follow-up from 30 consecutive B-cell precursor ALL cases (PB: day 0–day 8; BM: day 0–day 15) for the expression levels of seven antigens, highly relevant for MRD studies, on leukemic cells. We observed a downmodulation of CD10 and CD34 expression; by contrast, CD19, CD20, CD45RA, and CD11a were upmodulated, while expression of CD58 was not significantly affected (see Figure 1 for four representative examples). Immunophenotypic modulation occurred, to different extents, in all analyzed day 15 BM samples compared to diagnosis. As reported in panel a of Figure 2, downmodulation was statistically highly significant for CD10 and CD34 (*P* < 0.001 for both markers); by contrast, both CD19 and CD20 were significantly upmodulated (*P* = 0.02 and < 0.001, respectively). Although similar statistical evaluations were not performed for CD45RA and CD11a due to the low number of patients studied with these markers (*n* = 7 and *n* = 11, respectively), their expression was generally increased at variable rates in all analyzed samples; median MESF 996 (range 389–22 064) *vs* 11 683 (range 1396–29 782) and 995 (range 390–33 207) *vs* 2626 (range 404–47 610), respectively. Expression of CD58 was not significantly changed (*P* = 0.085). Very similar results were obtained for the same antigens by comparing PB samples at
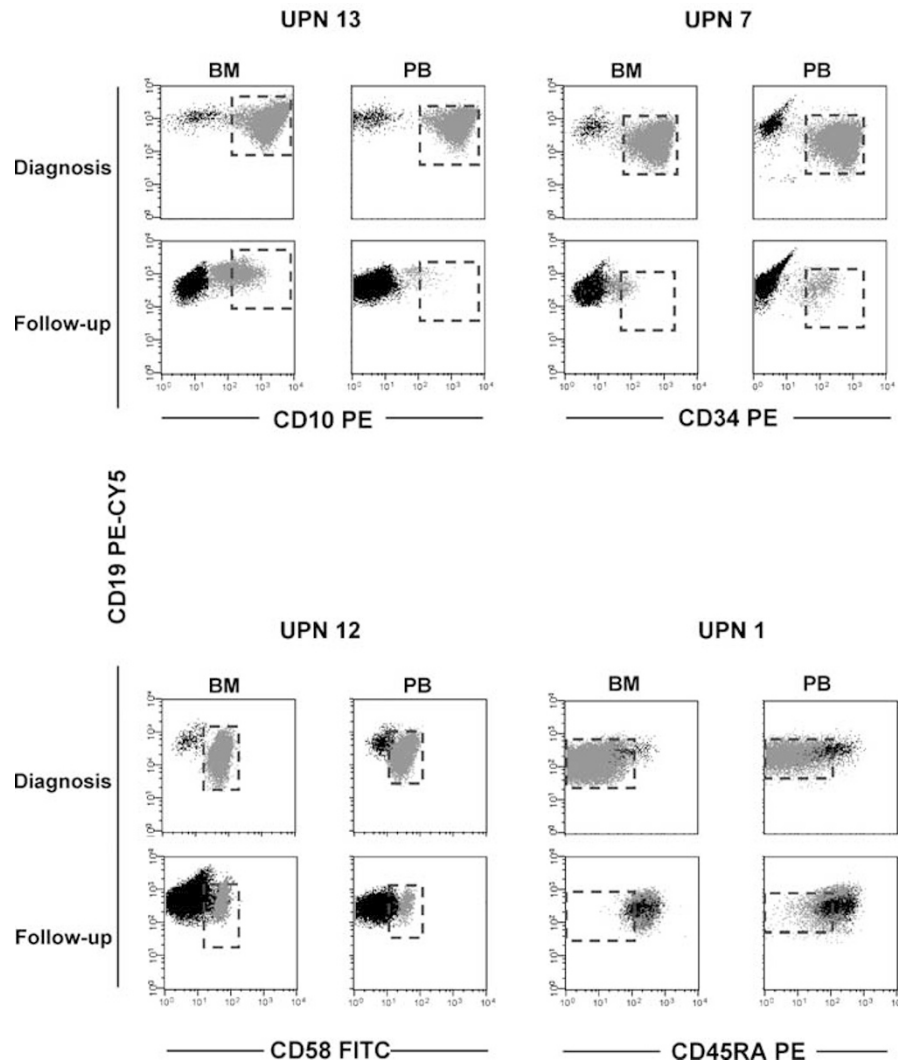
Figure 1 Immunophenotypic modulation in four representative B-cell precursor ALL representative cases. Only CD19-positive cells are represented. Fluorescence biparametric cytograms of CD10, CD34, CD58, and CD45RA (x-axis) in combination with CD19 (y-axis) are shown for patients UPN 13, 7, 12, and 1, respectively. For each case, expression of antigens at diagnosis (upper panels) and during follow-up (lower panels) are reported. Analysis of BM and PB samples are depicted in the left and the right panels, respectively. Red dots represent leukemic cells; black dots represent normal B cells. Modulation of antigens in follow-up samples can be observed for CD10, CD34, CD11a , but not for CD58. Dashed rectangles are drawn around the leukemic cells at diagnosis and then translated in the follow-up cytograms to facilitate the comparison of the red dots positions.

diagnosis and at day 8 (Figure 2 panel b). TdT was employed as MRD marker in only three patients. Nevertheless, we could observe a dramatic downmodulation of its expression in BM day 15 as compared to diagnosis; median MESF 18 729 vs 1451.

We then decided to investigate whether such a phenotypic modulation persists at later time points (day 33 and day 78) of remission induction therapy. Eight out of 30 patients were MRD positive at day 33; of these, six were suitable for quantitative analyses (ie they had more than 150 MRD + events) and only one of these was MRD positive also at day 78 (UPN18). As shown in Figure 3, CD10 (panel a) and CD34 (panel b) were further downmodulated until day 33 in 4/6 and 3/4 cases, respectively, while CD58 expression was decreased in 5/6 cases as compared to day 15 (panel c). CD19 and CD20 were further upmodulated, to variable extents, in 5/6 and 3/4 cases, respectively (data not shown). The only patient who showed a clear upmodulation of CD10 and CD34 at day 33 was the only one who remained MRD positive even at day 78 (UPN18). Of note, at this time point, patient UPN18 further increased CD10,

CD19, and CD58 expression (median MESF 642 vs 1278; 47 187 vs 62 054, and 12 307 vs 20 689, respectively), while CD34 remained virtually unchanged (117 700 vs 115 095).

In order to exclude that immunophenotypic modulation was not just a sign of cell death, we analyzed the scattergrams (FSC vs SSC) of the blasts undergoing immunophenotypic modulation, and we found that they were always located in the region where viable cells are normally found (data not shown). Furthermore, we measured the linear channel value of both FSC and SSC signals associated with blast cells at diagnosis and at day 15 ($n=30$), and we did not find any significant differences: the mean values of FSC channel were 357 (range 257.7–567) and 365.1 (range 264–481), respectively ($P=0.345$) and of SSC these were 86.2 (range 64.1–140) and 88.2 (range 56–167), respectively ($P=0.838$). Furthermore, we wanted to exclude that immunophenotypic modulation was related to technical artefacts. We performed parallel tests of (i) red cell lysis using both a commercial solution (BD) and NH$_4$Cl as well as (ii) of materials obtained by lysis and gradient-separation
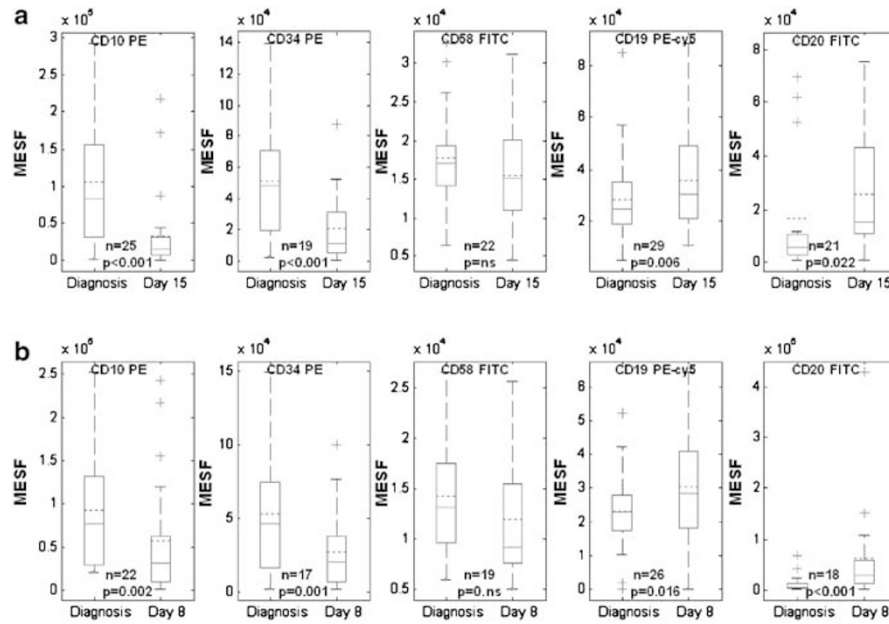
Figure 2 Quantitative expression of CD10, CD34, CD58, CD19, and CD20 antigens in leukemic cells at diagnosis and at early follow-up. Median values of MESF measurements in BM (panel a) and PB (panel b) are reported. Quantitative antigen expression was calculated at diagnosis and at follow-up. The box includes all the observations between the first and the third quartile; the bold line represents the median, whereas the dotted line is the mean. Whiskers extend from the edges of the box to 1.5 times the interquartile. Outlier values are indicated with ' + ' signs. The number of tested patients and the statistical significance are indicated.
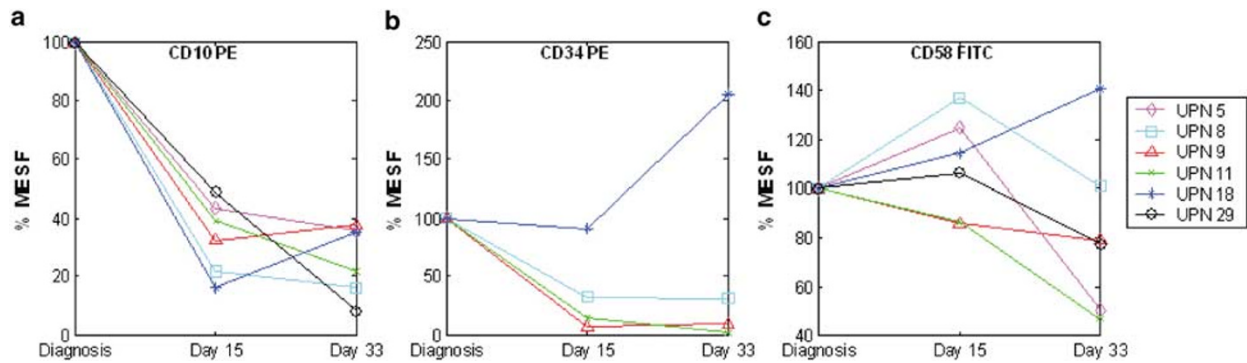
Figure 3 Relative variation of CD10, CD34, and CD58 expression as compared to diagnosis. The relative variations (%) of MESF value were calculated in the BM of day 15 and day 33, as compared to diagnosis, in six patients with MRD (more than 150 leukemic cells) at both these two time points. Relative expression of CD10 ($n = 6$), CD34 ($n = 4$), and CD58 ($n = 6$) are represented in panel a, b and c, respectively.

procedures. No differences in modulation of CD10, CD34, CD19, and CD58 were observed in samples of two patients (data not shown). Finally, to verify that this phenomenon was not related to a particular antibody clone nor fluorochrome, we simultaneously analyzed the expression of CD10 by using different reagent clones conjugated to PE and FITC, respectively. In paired BM samples from 30 patients, the median MESF of CD10 PE (clone SS2/36, Dako) at diagnosis was 82 720 (range 20 310–293 108) *vs* 15 725 at day 15 (range 696–217 284), $P < 0.001$; whereas CD10 FITC (clone ALB2, Coulter/Immunotech) was 25 414 at diagnosis (range 4958–205 168) *vs* 10 441 at day 15 (range 632–131 394), $P < 0.001$.

Antigen expression in normal cells

In order to investigate whether immunophenotypic modulation exclusively occurred in leukemic cells, we analyzed the quantitative expression of CD19, CD20, CD58 and CD3, CD4, CD8 in normal residual B and T cells from the same specimens. Normal B cells in diagnostic bone marrow samples were present in adequate number (at least 150 events) in 27 out of 30 analyzed patients: median number of events was 320.2 (range 40–1163), while the median % of normal B cells among total nucleated cells was 1.861 (range 0.130–1.88). Separation of such a normal B-cell clusters from the bulk of leukemic cells has been successfully performed in all cases considering their CD10 low/−, CD20 + +, CD34−, CD58 low, CD45RA + +, CD11a + + immunophenotype, compared to the CD10 +/ + +, CD20 low/−, CD34 +/ + +, CD58 + +, CD45RA low/ −, CD11a low/− leukemic immunophenotype (see Figure 1 for representative examples). As shown in Figure 4, the antigen expression patterns of normal mature B cells frequently appeared to undergo modulation. However, unlike in leukemic cells, both CD58 and CD20 underwent significant down-modulation in day 15 BM as compared to diagnosis ($P = 0.001$

and $P = 0.023$, respectively) and this result was confirmed when day 8 PB was compared to PB from diagnosis ($P = 0.0001$ and $P = 0.0153$, respectively). No significant modulation of CD19 was observed neither in BM ($P = 0.122$) nor in PB ($P = 0.0667$), while CD45RA and CD11a expression usually increased as in leukemic cells. Differences in expression of these latter two antigens were clearly obvious (data not shown), but statistical evaluation was not performed due to the low number of available samples.

Quantitative expression of antigens like CD3, CD4, and CD8 was measured in normal T cells collected from the PB of 23 patients at diagnosis and at day 8; As shown in Figure 4 panel c, we did not find any significant variations in the expression of these three antigens ($P = 0.234$; $P = 0.709$ and $P = 1.000$, respectively), indicating that T cells do not undergo significant immunophenotypic modulation.

Detection of MRD

We used three-colour antibody combinations to detect the residual leukemic cells in paired samples of PB day 8 and BM day 15, 33, and 78 from 30 B-cell precursor ALL patients, according to the leukemia-associated immunophenotypes identified at diagnosis. All of the analyzed PB day 8 and BM day 15 samples contained $\geqslant 1$ leukemic cells among 10^4 nucleated cells ($\geqslant 0.01\%$); the median proportion of leukemic cells in these cases ranged from 0.02 to 22.43% (median 0.43%) for PB samples and from 0.01 to 51% (median 0.56%) for BM samples. Eight of these 30 patients had more than 0.01% of leukemic cells also at day 33 (range from 0.01 to 2.32%, median 0.19%), and only one of them remained MRD positive at day 78 with 0.38% of leukemic cells among nucleated BM cells.

In spite of the immunophenotypic modulation, leukemic cells could unequivocally be identified as clusters of immature abnormal cells distinguishable from the very few residual mature normal B cells present in the same specimens, which invariably exhibited the mature $CD10^- CD20^{++}$ phenotype. Moreover, we built box graphs, corresponding to the area in which normal B cells are included, by overlapping multiple follow-up samples (PB day 8 and BM day 15) of both B-cell precursor ALL and T-ALL patients ($n = 3$), who were treated with the same protocol as the patients being studied. These templates of normal B-cell phenotypes were found to be identical in B-cell precursor and T-ALL patients, and no significant overlapping of residual leukemic cells occurred when plotting their localizations against them (Figure 5 left panels). Despite further antigen modulation occurred in CD10, CD34, and CD58 expression at day 33, MRD detection at this time point was also possible against the normal nonregenerating B-cell background, which was very similar to that observed at day 15 (data not shown). By contrast, when performing similar plotting experiments against phenotypic templates of normal B cells found in BM samples of healthy individuals ($n = 10$), leukemic cells with modulated phenotypes often overlapped the normal regions (Figure 5 right panels excluding the CD10/CD58 dot plot).

To further assess whether the immunophenotypic modulation could affect the flow cytometric detection of MRD, 82 out of 90 BM samples from the 30 patients under study were simultaneously analyzed by PCR amplification of antigen receptor genes (26 from day 15, 27 from day 33, and 29 from day 78). Qualitative comparison of MRD detection by the two techniques showed concordant results in 75/82 samples (91.4% overall: 42/82 contained MRD $< 0.01\%$ and 33/82 MRD $\geqslant 0.01\%$). In seven cases (8.5%), the two methods yielded discordant results: five samples had $\geqslant 0.01\%$ of leukemic cells by PCR, but $< 0.01\%$ by flow cytometry (3/5 at day 33 and 2/5 at day 78), whereas two samples had $\geqslant 0.01\%$ of leukemic cells by flow cytometry and negative PCR result (both at day 33). The median proportion of leukemic cells among nucleated cells in the 33 cases with concordant positive results was quite similar: 0.49% (range 0.01–51.1%) by flow cytometry and 0.90% (range 0.01–78%) by PCR technique.
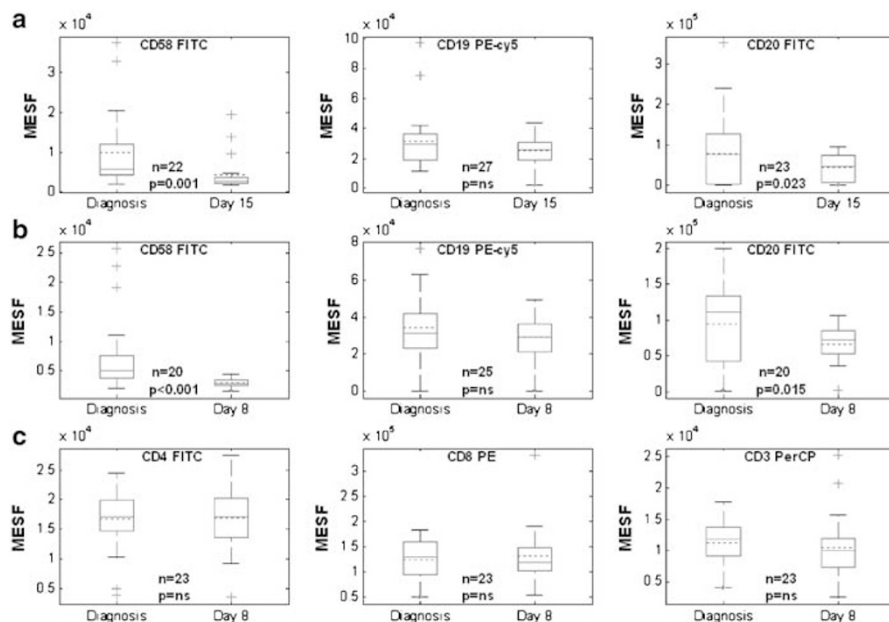

Figure 4 Quantitative antigen expression in normal B and T lymphocytes at diagnosis and during follow-up. Median values of MESF measurements in BM (panel a) and PB (panel b) are reported for CD58, CD19, and CD20, as expressed on normal B lymphocytes, at diagnosis and at follow-up. Expression of CD4, CD8, and CD3 on normal T cells are reported in panel c. Box and Whisker plots are as in Figure 2, and include the number of tested patients and the levels of statistical significance.

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Discussion

Within the context of a prospective flow cytometric MRD study in childhood ALL, we noticed the frequent occurrence of immunophenotypic changes in leukemic cells during early follow-up compared to diagnosis. In B-cell precursor ALL, almost all the highly relevant antigens like CD10, CD19, CD20, CD34, CD58, CD11a, and CD45RA were found to be involved. However, to the best of our knowledge, this phenomenon has not been described previously. We decided, therefore, to systematically analyze the phenomenon in B-cell precursor ALL, by quantitative three-colour flow cytometric measurements of antigen expression, which were converted to MESF values to allow for comparisons over time. Between samples from early follow-up (PB day 8; BM day 15) and the initial diagnostic sample, CD10 and CD34 were significantly downmodulated, and by contrast, CD19, CD20, CD45RA, and CD11a were upmodulated. Only CD58 expression was not significantly affected, confirming our recent observations.[27] Interestingly, this drug-induced modulation also affected normal B cells, but not the T cells from patients.

In order to assess whether this antigenic modulation was sustained in leukemic cells or only occurred during the steroid phase of treatment, we were able to investigate residual leukemic cells also in BM samples of day 33 (at steroid tapering) as well as of day 78 (after stopping of steroid treatment). Of note,
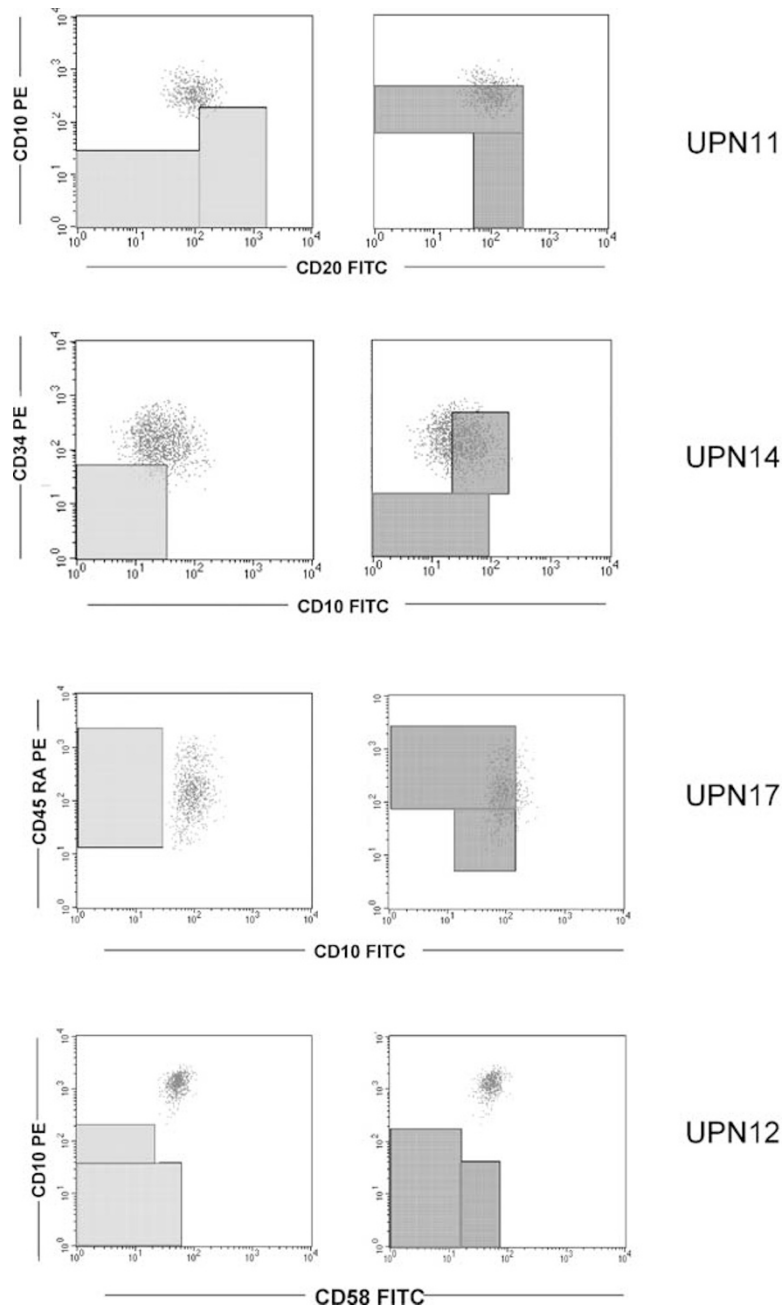
Figure 5 Flow cytometric analysis of minimal residual disease in B-cell precursor ALL. Dual-fluorescence dot plots showing modulated immunophenotypic patterns of residual leukemic cells in day 15 BM in four representative cases. Leukemic cells are partly overlapping regions of normal B cells, which are represented by geometric boxes. These phenotypic templates of normal B cells were obtained by analysis of BM from T-ALL patients in follow-up (left panels) and from healthy donors (right panels).

only few samples of our series were still MRD positive at these time points and thus available for the analyses (day 33, $n = 6$; day 78, $n = 1$). At day 33, CD10, CD34, and also CD58 were further downmodulated or remained at low levels similar to day 15 in 5/6 cases. Only one case showed significantly reincreasing expression of these antigens at day 33, and this (UPN18) was the only case who still exhibited MRD also at day 78 due to a poor treatment sensitivity. Notably, at day 78, these leukemic cells showed a further reincrease of CD10 and CD58 expression and stable CD34 expression.

Immunophenotypic changes, compared to diagnosis, have been reported in relapsed acute leukemias suggesting that MRD detection by immunological methods should be based on multiple marker combinations to minimize the possibility of false negative results (1,17–18). Such changes were usually realized late in the disease course of patients, and clonal selection or lineage switch (ie secondary leukemia) were mostly considered as causative. In the study presented herein, significant modulations of antigen expression occurred already within the first 2 weeks of therapy, and seem different from the former changes. Nevertheless, any change of leukemia-associated phenotype can affect the accuracy of the flow cytometric detection of MRD creating the possibility of an underestimation of MRD levels or of rendering falsely negative results. Combining multiple marker-combinations may also not solve the problem completely, since most antigens in our experience were modulated. Hence, we investigated whether immunophenotypic modulation interferes with the ability to accurately determine MRD. In our series, residual blasts, even bearing modulated immunophenotypes, were always located outside the dot-plot regions of residual normal B cells from the same follow-up sample. Notably, these residual normal B cells exhibited predominantly mature (CD20 + +) phenotypes. The accuracy of our normal templates (related to follow-up time points) was confirmed by coinvestigating residual normal B cells from patients with T-ALL, who were treated with the same therapy protocol. By contrast, BM samples collected from healthy donors or individuals without hematological malignant diseases, which are usually considered as useful background standards, showed significant overlap of modulated leukemic phenotypes with those of normal B cells. These latter BM samples contained also immature normal B precursor cells, which explains differences in phenotypic overlap. Hence, at least in early follow-up samples, phenotypic modulations do not seem to interfere with the ability to unambiguously distinguish leukemic from normal cells. This is highly relevant, since flow cytometric day 15 BM results can be used for low-risk definition in ALL patients treated according to BFM treatment protocols.[28] More generally, this is further corroborated by our finding of a strong concordance of MRD data obtained in parallel by flow cytometry and PCR technology in the same cohort of patients along all early time points of treatment. However, some differences have been observed, in particular the median levels of MRD detected by PCR resulted higher, whereas qualitative discordances corresponded mostly to PCR-positive/flow cytometry-negative samples collected at later time points in which hematogones may emerge. In particular, we failed to detect MRD in five BM samples (three at day 33 and two at day 78); whether these negative results by flow cytometry were due to the changes in antigen expression was not fully established: however, in 2/3 discordant day 33 samples (UPN 19 and UPN 28) the MRD level, as assessed by PCR, was at the lower limit of flow cytometry sensitivity (0.01%), in addition PCR positivity in these samples was not confirmed by a second molecular marker, thus suggesting a sensitivity problem rather than antigen

modulation. The third discordant day 33 sample (UPN 15) had MRD level of 0.06%; in this case, CD58 was not suitable for MRD detection and a strong modulation of CD10 and CD34, already present at day 15, could not be excluded as causative of the flow cytometric negativity. In the two discordant day 78 samples (UPN 5 and UPN 9), the level of PCR-based MRD ranged between 0.01 and 0.1%. As previously reported,[6] this time point corresponds to a significant repopulation of BM with normal immature B cells bearing close immunophenotypic resemblance to leukemic lymphoblasts. On the other hand, we have preliminary evidence that antigenic modulation occurs predominantly during the steroid phases of treatment, and that it may be quickly reversible thereafter (Dworzak MN, unpublished observations), suggesting that false negative results observed at day 78 could be mainly due to the background of hematogones regardless of the antigen modulation. Moreover, the incidence of MRD-positive patients at day 78 is low in our study (less than 10%, data not shown), and it seems that resistant diseases do not undergo significant modulation. For these reasons, further studies are necessary to fully address the question of how often the MRD detection would be impaired by changes in antigen expression against the B-cell background of regenerating BM.

In addition to antigen modulation as well as regenerating status of the BM, discordances with PCR technique may also be related to technical differences like: (i) usage of lysed whole blood samples for flow cytometry, and mononuclear cell preparations for PCR, (ii) debris exclusion by SYTO 16-stain with flow cytometry, while DNA sequences from shredded cells may have efficiently been amplified by PCR.

The possible mechanism underlying the immunophenotypic modulation was not directly investigated in this study. Nevertheless, we favor the hypothesis that it could include drug-induced regulation of the cellular machinery at different levels (antigen internalization, molecule transcription and translation, RNA and protein stability). In particular, we consider that the steroid hormones used during induction therapy, which are potent regulators of gene transcription, could be central to the phenomenon. In line with this, we observed modulation already on day 8 of treatment (ie after 1 week of prednisone therapy and one single intrathecal injection of methotrexate). Interestingly, downmodulation of CD10 was reported in the leukemic B-cell precursor cell line REH after treatment with various agents such as phorbol ester and protein kinase activators.[29,30] More recently, an in vitro prednisolone-induced downregulation of the number of CD10 molecules on ALL cells was also reported (Styczynski et al, Blood 2003; 102: 216b, abstract). Furthermore, antigen regulation is favored by our findings that normal mature B cells are also affected by modulation to some extent. Alternatively, it may be that the more mature leukemic cell subsets, which express low CD10 and CD34, and higher CD19, CD20, CD45RA, and CD11a, and which are poorly represented at diagnosis, could withstand the treatment and constitute the majority of residual disease during follow up, thus mimicking antigen modulation. Of note, the view of a selective depletion of more immature leukemic cell subpopulations has been recently considered by Stahnke et al; these authors demonstrated a higher sensitivity to drug-induced apoptosis in more immature CD34 + leukemic subsets as compared to CD34− subsets in both AML and ALL during remission induction chemotherapy.[31] Further studies including in vitro experiments are necessary to address all these questions. It may also be relevant to correlate the extent of modulation of certain markers with MRD regression in a large series of patients with treatment response, in order to establish whether antigenic modulation may reflect sensitivity to treatment.

In summary, our results indicate that specific modulation of immunophenotypes occurs in ALL during the earliest phases of treatment which are dominated by steroids, and that this phenomenon has to be taken into account by investigators in order to avoid pitfalls in MRD assessment.

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