Flow minimal residual disease monitoring of candidate leukemic stem cells defined by the immunophenotype, CD34^{+}CD38^{low}CD19^{+} in B-lineage childhoood acute lymphoblastic leukemia

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ABSTRACT

Flow cytometric minimal residual disease (MRD) monitoring could become more powerful if directed towards the disease-maintaining leukemic stem cell (LSC) compartment. Using a cohort of 48 children with B-lineage acute lymphoblastic leukemia (ALL), we sought the newly proposed candidate-LSC population, CD34^{+}CD38^{low}CD19^{+}, at presentation and in end of induction bone marrow samples. We identified the candidate LSC population in 60% of diagnostic samples and its presence correlated with expression of CD38, relative to that of normal B-cell progenitors. In addition, the candidate LSC was not detectable in all MRD positive samples. The absence of the population in 40% of diagnostic and 40% of MRD positive samples does not support the use of this phenotype as a generic biomarker to track LSCs and suggests that this phenotype may be an artifact of CD38 underexpression rather than a biologically distinct LSC population.
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Key words: childhood acute lymphoblastic leukemia, minimal residual disease, flow cytometry, leukemic stem cells.

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Introduction

In childhood acute lymphoblastic leukemia (ALL), the response to treatment, as measured by levels of minimal residual disease (MRD), has been shown to be an independent prognostic marker in several clinical trials with varying drug regimens. For example, in one of the first, large prospective studies, the 3-year cumulative incidence of relapse in children who had detectable MRD (>0.01%) at the end of remission induction was 32.5% compared to 7.5% for children who were MRD negative.[1] Thus, MRD assessment is currently being used to identify patients who may benefit from treatment intensification or treatment reduction. The two predominant methodologies for MRD monitoring in childhood ALL are molecular-based RQ-PCR analysis of rearranged immunoglobulin and/or T-cell receptor genes and flow cytometry of aberrant immunophenotypes. Both have predictive value.[2-5] In the latter technique, a leukemia-associated immunophenotype (LAIP) is identified which allows the discrimination of leukemic blasts from normal lymphocyte progenitors and relies on qualitative or quantitative differences in antigen expression between leukemic cells and their normal counterparts, such as aberrant expression of myeloid antigens or underexpression of CD38 or CD45.[6-8] Whether flow cytometric or PCR-based, both methods depend on the identification of *markers* characteristic of the predominant population(s) in the diagnostic sample which are then used to track leukemic cells throughout treatment.

A far more powerful, and perhaps more accurate approach to predict outcome, may be to quantify the leukemic stem cell (LSC) compartment since this is likely to be the source of relapse and may show different treatment response kinetics compared to the bulk blast population. In this context, an aberrant CD19^{+}CD34^{+} lymphoid cell that lacks CD38 expression has recently been proposed as a candidate LSC population in ALL.[9] Lentiviral transduction of normal cord blood cells with the ETV6-RUNX1 fusion gene (also known as TEL/AML) led to the formation of cells with this aberrant immunophenotype. Moreover, using samples of patients with an ETV6-RUNX1 fusion, CD34^{+}CD38^{Low}CD19^{+} blasts were able to re-initiate and sustain leukemic growth in immune-deficient NOD SCID mice.[10]

We therefore explored whether the candidate CD34^{+}CD38^{Low}CD19^{+} LSC phenotype would be a useful marker for flow cytometric MRD monitoring in a cohort of primary ALL samples.

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The Online version of this paper has a a Supplementary Appendix.

Design and Methods

Patient samples

Bone marrow aspirates were available from 48 children presenting with acute lymphoblastic leukemia at the Royal Victoria Infirmary, Newcastle upon Tyne, UK who were entered into the UKALL2003 clinical trial. Samples were taken from excess material leftover from diagnostic or staging bone marrow aspirates and ethical approval for the study was obtained (reference numbers 2002/111 and 07/H0906). Cytogenetic analysis was carried out on diagnostic bone marrow using standard procedures and FISH was performed for the presence of TEL/AML1, BCR-ABL fusions and MLL gene rearrangements. Patients were classified into 3 major cytogenetic subgroups: TEL/AML1 positive, High Hyperdiploidy, and Other. There was one patient with a BCR-ABL rearrangement. Four bone marrow aspirates, taken from children in continuous remission at the end of treatment (more than 2-3 years following diagnosis) served as *normal* comparison samples.

Flow cytometry

Flow cytometric analyses for the detection of MRD were performed as described previously.[11] Day 28 samples were considered positive if MRD was detectable at greater than or equal to 0.01%. In this study, we retrospectively interrogated flow cytometric data from diagnostic (50,000 events) and day 28 follow-up samples (500,000 events) for the $CD34^+CD38^{Low}CD19^+$ population. Samples were considered positive if at least 50 $CD34^+CD38^{Low}CD19^+$ events were visible. Diagnostic samples were also assessed for CD38 expression relative to normal B-cell progenitors, i.e. $CD10^+CD19^+CD34^+$ and $CD10^+CD19^+CD34^-$ using standard gates and classified as normal, overlapping or under-expressing.

Results and Discussion

The proposed candidate LSC population is found at varying frequencies across different cytogenetic subgroups

Given that a cell population, defined by $CD34^+CD38^{Low}CD19^+$ expression, has recently been reported to show cancer stem cell activity in TEL/AML1 ALL,[10] we sought this population in diagnostic samples from children with ALL (n=48) using multiparameter flow cytometry (Figure 1A). The cohort included the two major good risk cytogenetic groups, TEL/AML1 (n=10) and High Hyperdiploid (n=8), and also a heterogeneous group consisting of rare or no apparent cytogenetic abnormalities, which were classified as Other (n=30). The $CD34^+CD38^{Low}CD19^+$ population was detectable at 0.1% or higher in 60% of our patient cohort (n=29) with the level of the population varying from 0.2-80.3% (median, 5.6%), as a proportion of the total leukemic blasts. In the remaining 40% of patients (n=19), the $CD34^+CD38^{Low}CD19^+$ population was either absent or below the limit of detection. The incidence of the candidate LSC population varied between cytogenetic subgroups (Figure 1B). Within the TEL/AML1 group the $CD34^+CD38^{Low}$ $CD19^+$ population was present in all samples at levels ranging from 0.2-14.4% (median, 2.8%). For the High Hyperdiploid and Other cytogenetic subgroups, the population was evident in 63% (n=5; range 0.7-74.3; median, 2.3%) and 47% (n=14; range 0.2-80.3%; median, 10.2%), respectively. A table with all data is shown in *Online Supplementary Appendix Table S1*. There was no evidence of the $CD34^+CD38^{Low}CD19^+$ population in *normal* bone marrow samples (n=4).

CD38 expression on leukemic blasts is variable across cytogenetic subgroups and correlates with the presence of the candidate LSC population

Flow cytometric tracking of minimal residual disease relies on the identification of LAIPS at diagnosis that allow discrimination of leukemic blasts from normal progenitors and which can, therefore, be used to track disease throughout therapy. Underexpression of CD38 is a common feature of ALL blasts, thus diagnostic blasts were assessed for their CD38 expression relative to that of normal B-cell progenitors and were classified as Normal, Under or Overlap with mean MFIs of 132.67, 32.17 and 72.33, respectively (Figure 2A). CD38 expression varied

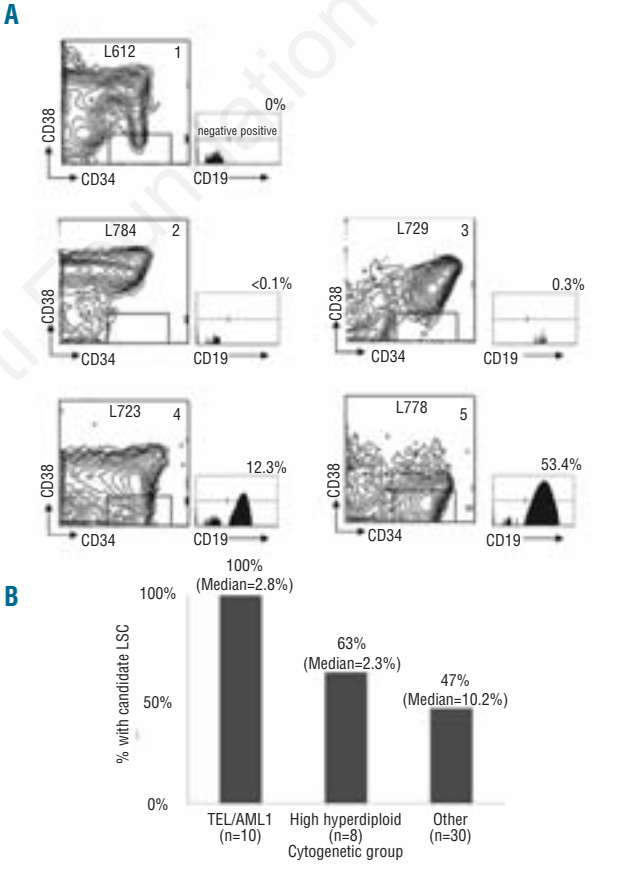

Figure 1. (A) Diagnostic ALL samples were investigated for the presence of the $CD34^+CD38^{Low}CD19^+$ population. Contour plots shown are already gated on a lymphoid light scatter profile and display CD34 and CD38 expression. The $CD34^+CD38^{Low}$ population has then been gated as shown to display CD19 expression in histogram form. Plot 1: normal bone marrow showing the normal pattern of maturation and the absence of the aberrant $CD34^+CD38^{Low}CD19^+$ population. Marker lines show the boundary of CD19 positive and negative events. The same gates were then used to assess diagnostic leukemic samples. Plot 2: ALL (CD38 normal expression) with no detectable $CD34^+CD38^{Low}CD19^+$ population (<0.1%). Plot 3: ALL (CD38 overlap expression) with low but detectable numbers of $CD34^+CD38^{Low}CD19^+$ cells (0.3%). Plots 4 and 5: ALL samples (CD38 underexpression) that clearly show the $CD34^+CD38^{Low}CD19^+$ population at higher levels (12.3% and 53.4%, respectively). (B) Bar chart showing the incidence of the candidate LSC across the 3 major cytogenetic subgroups.

across cytogenetic subgroups (Figure 2B). For TEL/AML1 cases, 90% showed underexpression (n=9) and 10% showed overlap of CD38 expression (n=1) and for the high hyperdiploid patients, 63% (n=5) were classified with CD38 underexpression and 37% (n=3) with overlap. For the Other cytogenetic subgroup, 37% (n=11) were classified as underexpressers, 10% (n=3) were overlap and 53% (n=16) had normal CD38 expression. Since most ALL blasts are both CD19 and CD34 positive, those which under-express CD38 are likely to house the $CD34^{+}CD38^{Low}CD19^{+}$candidate LSC population. Subsequent correlation of these two parameters revealed that almost all CD38 underexpressers (96%; 24 from 25) contained the $CD34^{+}CD38^{Low}CD19^{+}$ population, while patients classified as normal for CD38 expression were all negative (0%, 0 from 16) (Figure 2C). Of those with an overlapping CD38 immunophenotype, 71% (5 from 7) contained the $CD34^{+}CD38^{Low}CD19^{+}$ population. The only Philadelphia positive patient had high levels of the candidate LSC (72.3%) due to their blasts having a low CD38 and high CD34 expression; an immunophenotypic feature characteristic of BCR-ABL positivity in adult ALL.[12]

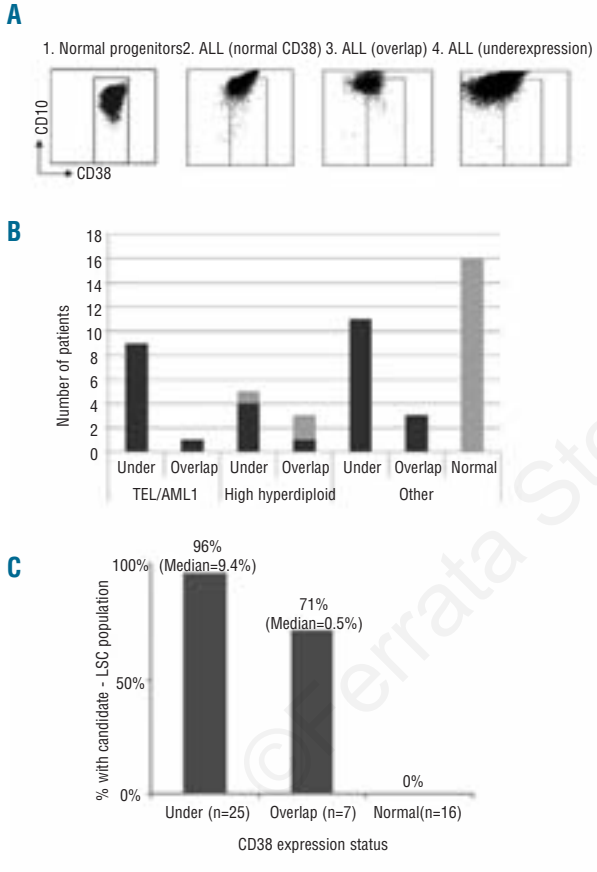

Figure 2. (A) **CD38 expression in ALL. Diagnostic samples were assessed for CD38 expression relative to that of normal B-cell progenitors using multiparameter flow cytometry. The dot plots show CD38 versus CD10 expression of events resulting from the sequential gating strategy; lymphoid scatter gate, then a $CD19^{+}$, low side scatter gate and finally a $CD19^{+}CD34^{+}$ gate. Plot 1 shows the CD38 expression levels of normal hematopoietic $CD19^{+}CD34^{+}$ B-cell progenitors outlined with a rectangle region which served as a reference template. Plot 2: a diagnostic ALL with CD38 expression similar to that of normal progenitors (Normal). Plot 3: an ALL in which a proportion of the population has underexpression. Plot 4: an ALL in which the bulk of the population shows CD38 underexpression (Under). (B) CD38 expression and cytogenetic subgroup. Also shown is the presence (dark shade) or absence (light shade) of the candidate LSC. (C) The incidence of the candidate-LSC population in relation to CD38 expression. The median percentage of the candidate-LSC within the blast population is also shown.**

The candidate LSCs are not found in all MRD positive samples but may persist and undergo enrichment

To prove useful as a marker of MRD, the $CD34^{+}CD38^{Low}CD19^{+}$ population would need to be traceable during therapy. Thus, we sought to determine if this candidate LSC population persisted through treatment by investigating samples taken 28 days after chemotherapy initiation which were positive by Flow MRD. Of our initial cohort, 90% (n=43) were assessed by Flow MRD at day 28 and 28% (12 of 43) were positive at a level of 0.01% or higher (range 0.01-25.4%). Of these 12 patients who were MRD positive, 92% (n=11) had initially contained the candidate LSC population at a detectable level at presentation (CD38 overlap, n=1 and CD38 underexpression, n=10). For 4 of 11 MRD positive patients (2 TEL/AML1, one High Hyperdiploid and one Other), there was no evidence of the candidate LSC, due to the MRD blasts being confined to the CD34 negative population, and thus there were no detectable $CD34^{+}CD38^{Low}CD19^{+}$ cells (a representative example is shown in Figure 3A). For the other 7 patients in which LSC population was detectable (2 High Hyperdiploid, 5 Other), we compared the proportion of the LSC within the blast population in both diagnostic and day 28 samples. For all 7, there was an increase in the proportion of the candidate LSC population during therapy with a mean of 38.5% at diagnosis compared to 86.3% at day 28 ($P<0.01$ by paired t-test). This was associated with a decrease in the CD38 mean fluorescent intensity from diagnostic compared to MRD blasts (21.03 compared to 5.76; $P<0.05$, paired t-test). Risk stratification and the induction regimen for these 7 patients are shown in *Online Supplementary Table S2*.

The finding that almost all MRD positive patients were positive for the presence of the candidate LSC at diagnosis and were CD38 under-expressers prompted us to compare the incidence of MRD positivity between cases with normal CD38 expression (of which there were no candidate LSC positive samples) and under/overlap expression (of which 90% were positive for the candidate LSC). From the former group, 0 samples were MRD positive, 7 samples were negative and 9 were indeterminate/not evaluable. In contrast, in the latter, 12 were positive, 16 were negative, and 4 were indeterminate/not evaluable. Thus, there was a correlation between CD38 expression and MRD status such that patients with leukemic blasts which under-express CD38 relative to normal B-cell progenitors were more likely to be MRD positive ($P=0.07$, Fisher's exact test).

CD38 underexpression is a common feature of ALL blasts and as such often makes up an important component of a patient LAIP, the aberrant leukemic phenotype that can be tracked throughout therapy and is easily distinguishable from normal bone marrow. In this study, we confirmed CD38 underexpression was common among our cohort, with 67% of patients showing a partial (overlap) or fully under-expressing immunophenotype. We found a strong correlation between the presence of the

candidate LSC population, $CD34^{+}CD38^{Low}CD19^{+}$ and underexpression of CD38. Thus, one has to question whether the existence of the candidate LSC population may be an artifact of CD38 underexpression rather than a biologically and functionally distinct LSC population, responsible for the hierarchical maintenance of the bulk blast population. However, without further analysis of the stem cell properties of this and other populations that constitute the leukemia, this cannot be determined. In 40% of the cohort, the candidate LSC population was either not present or was below the limit of detection (i.e.<0.1%), indicating that at least in about half of patients, this immunophenotype is not an appropriate biomarker of leukemic stemness.

Chemotherapy induced modulation of antigen expression, principally by glucocorticoids, is well recognized and the downregulation of CD34 and modulation of CD19 has been described in several studies[13,14] Thus, this phenotypic instability of 2 of 3 of the antigens necessary to track the candidate LSC population may hamper identification during treatment. This may be the explanation for the clear identification of an MRD population in some patients but with no evidence of the candidate LSC population, despite its presence at diagnosis. In MRD positive samples in which we identified $CD34^{+}CD38^{Low}CD19^{+}$ cells, we found that the proportion of these cells within the blast population was increased in follow-up samples from all patients, sometimes quite markedly. Whether this represents enrichment of a more drug resistant, candidate LSC, or may again be a reflection of drug induced antigen modulation, is unclear. Functional analyses of these cells may address this issue. There was a dramatic variation in

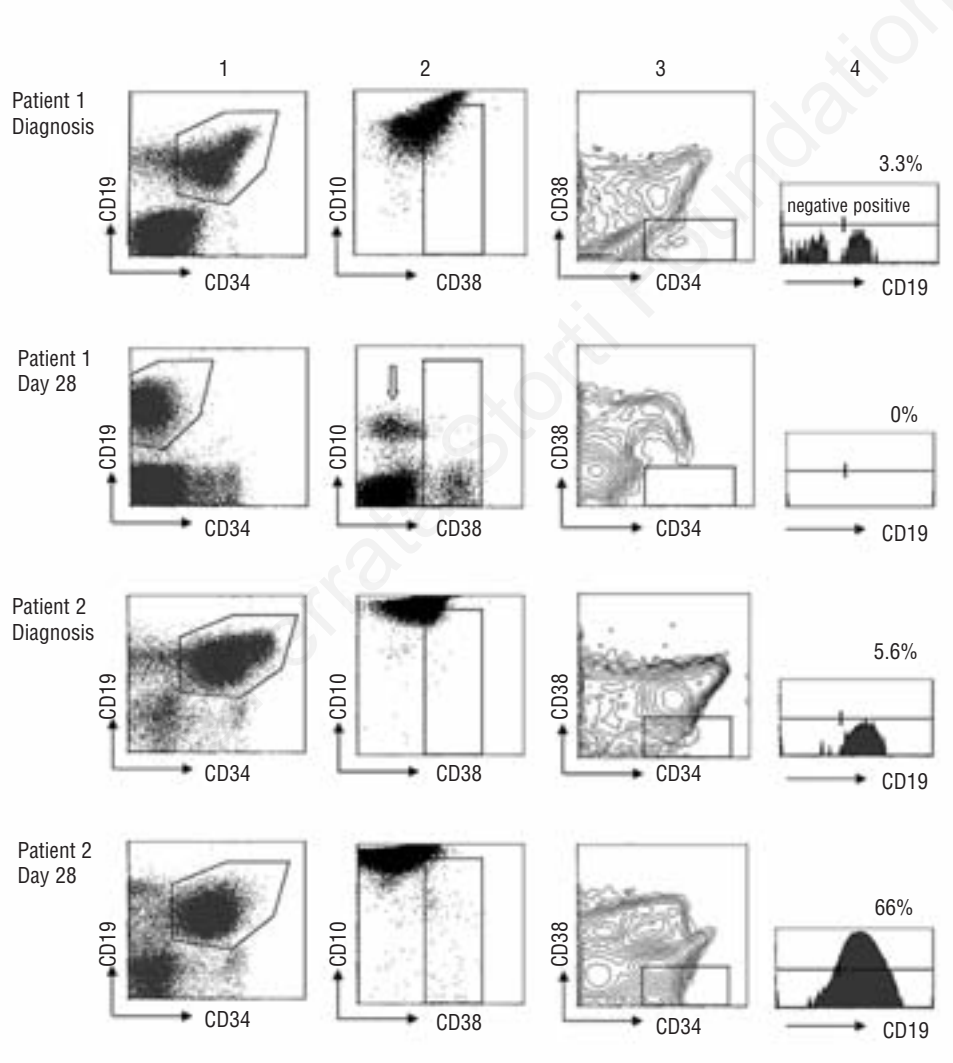

Figure 3 The $CD34^{+}CD38^{low}CD19^{+}$ population in diagnostic and MRD positive samples. (A) Row 1 plots show flow cytometric data of diagnostic samples. Plot 1: CD34/CD19 expression of diagnostic blasts already gated on a lymphoid scatter gate. Plot 2: CD38 versus CD10 expression of cells gated by the region shown in the CD34/CD19 plot with a rectangle showing the area in which normal progenitor cells are found. Plot 3: a contour plot of cells already gated on a lymphoid light scatter profile and CD34 and CD38 expression is shown. The $CD34^{+}CD38^{low}$ population is gated as shown to display CD19 expression in histogram form (Plot 4). Row 2 plots show data from the same patient analyzed at day 28 in which the MRD blasts (marked with arrow) are clearly identifiable but because they are CD34 negative, there is no evidence of the candidate LSC population. Similarly, Rows 3 and 4 show data from a second patient, gated in the same way which shows an increase in the candidate LSC population in the day 28 samples due to a decrease in CD38 expression in the MRD compared to the diagnostic blasts.

the size of the proposed LSC population from a rare population constituting <1% of the blasts through to samples where conversely much of the bulk blast population displayed the immunophenotype $CD34^{+}CD38^{Low}CD19^{+}$. In AML, the frequency of LSC at diagnosis, as defined by the immunophenotype $CD34^{+}CD38^{-}$, shows similar heterogeneity and the frequency correlates with MRD and poor survival.[15] Paradoxically, we found that all TEL/AML1 patients, a subgroup with a superior prognosis and low incidence of MRD positivity, housed the population.[16] Yet in the cohort overall, the LSC positive, CD38 underexpressing patients appeared to have a higher rate of MRD positivity. These observations require validation in a larger cohort but are supported by findings that high CD38 expression is associated with a favorable prognosis in both adult AML and ALL.[17]

In summary, given that the candidate LSC population is found in only 60% of patients at diagnosis and is not detectable in 40% of MRD positive samples, we conclude that the $CD34^{+}CD38^{Low}CD19^{+}$ phenotype is not appropriate as a generic marker for monitoring LSC-MRD. Currently, much debate surrounds LSCs in childhood ALL with evidence identifying candidate LSCs in both rare, immature populations as well as conversely, across several immunophenotypically distinct groups.[9,10,18-21] It is unlikely that we can clinically exploit current evidence to effectively re-direct flow cytometric MRD monitoring towards the LSC compartment until the immunophenotype of the cells which maintain the leukemia are more clearly defined.

Authorship and Disclosures

KW, JV and JI devised the study. KW, LM, MC, NB, SB, JJ and SL generated data. JI and JV co-ordinated the study and take primary responsibility for the paper. KW, JV and JI wrote the first draft of the paper and all other authors contributed to the final version.

The authors reported no potential conflicts of interest.

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