[1]Department of Hematology and Oncology, Skåne University Hospital, Lund, Sweden;
[2]Department of Laboratory Medicine, Lund University, Lund, Sweden;
[3]Department of Pediatrics, Institution for Clinical Sciences, Queen Silvia Children's Hospital, Gothenburg, Sweden;
[4]Department of Hematology, Sahlgrenska University Hospital Gothenburg, Gothenburg, Sweden;
[5]Department of Hematology, University Hospital Linköping, Linköping, Sweden;
[6]Hematology Center, Karolinska University Hospital, Stockholm, Sweden;
[7]Department of Hematology, Academic Hospital, Uppsala, Sweden;
[8]Department of Hematology, Sunderby Hospital, Luleå, Sweden;
[9]School of Medical Sciences, Örebro University Hospital, Örebro, Sweden and
[10]Department of Radiation Sciences, Umeå University, Umeå, Sweden
E-mail: gunnar.juliusson@med.lu.se

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Plastic CD34 and CD38 expression in adult B–cell precursor acute lymphoblastic leukemia explains ambiguity of leukemia-initiating stem cell populations

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is an aggressive hematologic malignancy of bone-marrow (BM)-derived lymphoid precursor cells at various stages of differentiation.[1] Although first-line therapy with chemotherapy and—in the case of BCR-ABL1 positive ALL—tyrosine kinase inhibitors is initially highly effective with remission rates of > 90%, the overall survival rate in adult patients is 40–50% across all risk groups.[1–3] Relapse originates from putative leukemia-initiating cells (LICs) that are intrinsically resistant to chemotherapeutic regimens, which may explain the poor long-term prognosis of patients with disease recurrence. Eradication of LICs thus is a principal aim of novel therapeutic approaches. A prerequisite for developing effective LIC-targeted treatments is the ability to identify and clinically monitor LICs in ALL, a goal that has to date been elusive. The existence, phenotype, biological properties and the hierarchical organization of LICs in BCP-ALL remain highly controversial.[4]

The prospective enrichment of LICs in ALL using the surface markers CD34 and CD38 (also in combination with other markers)—as well-established in acute myelogenous leukemia[5–8]—largely failed, resulting in highly variable results.[9–12] We investigated the reason for these ambiguous results by observing the expression of these markers even at single cell level in high temporal resolution.

We found that CD34 and CD38 are highly plastic on individual BCP-ALL cells and are up- or downregulated in one cell generation within hours, and may not be useful for prospective LIC isolation.

To investigate the plasticity of CD34 and CD38 surface marker expression in BCP-ALL, we utilized a unique ALL patient-derived long-term cell culture system (PDLTCs) established from patient PH.[13] The PDLTC-PH reflects the polyclonal propensity of the disease and remains genetically and functionally stable in culture for more than 6 months (Nijmeijer et al.[13] and data not shown). Furthermore, we achieved to generate isogenic clonal PDLTC-PH subcultures, which are invaluable to investigate relations between phenotypes and distinct function.

First, we confirmed the presence of cells with LIC activity in PDLTC-PH by injecting 5×10^6 unsorted bulk cells intravenously into sublethally-irradiated immune-compromised NSG mice, revealing human ALL cell engraftment and leukemia progression in the peripheral blood (PB) of the recipients via FACS as well as their survival (Figure 1a, Supplementary Methods). After 62 days, we found the first ALL cells in the PB, and the human ALL cell chimerism successively increased over time until the mice died of the disease after 119 days in average (± 4.6 days), with a high proportion of ALL cells in the BM (Figure 1a). After confirming the existence of LICs in PDLTC-PH, we checked for the cell surface expression of CD34 (APC clone 8G12, BD, Heidelberg, Germany) and CD38 (PE clone HB7 eBioscience, Frankfurt, Germany) in these PDLTCs via FACS (FACS Canto, BD). These are indicative surface markers to determine normal hematopoietic stem and progenitor populations, and LIC-enriched fractions in acute myelogenous
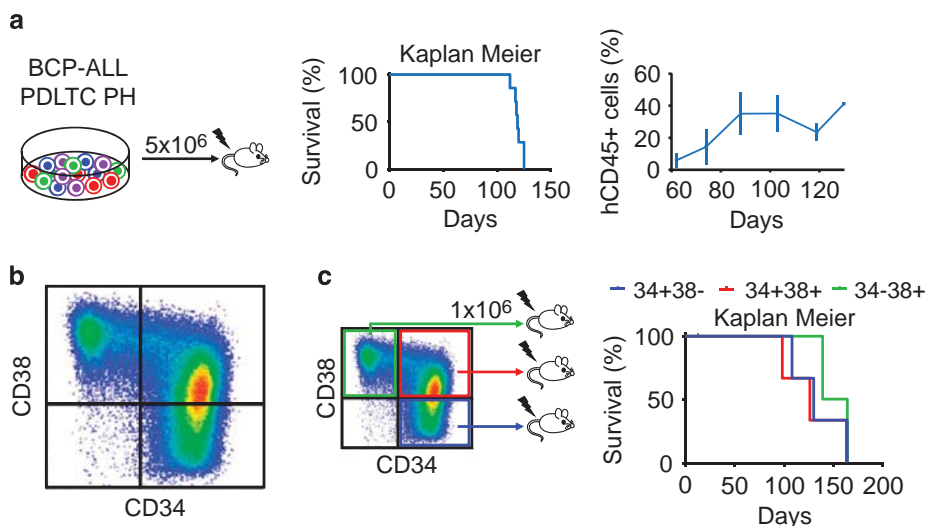
Figure 1. PDLTC-PH contains cells with leukemia-initiating potential *in vivo*. (**a**) Xenograft transplantation of PDLTC-PH cells. Survival and leukemic peripheral blood engraftment of NSG mice (*n* = 7) receiving bulk PDLTC-PH cells. (**b**) Surface CD34 and CD38 expression of PDLTC-PH cells via FACS. (**c**) Xenograft transplantation of prospectively FACS-enriched PDLTC-PH subpopulations. Survival of transplanted NSG mice (*n* = 3 mice per subpopulation). No significant difference calculated by the log-rank test. The experiment was performed in a randomized and blinded way. One mouse of the CD34 − CD38+ group died within the first week, caused by the transplantation procedure, and was excluded. The mean and s.d. are displayed.

leukemia.[5–8] We revealed a typical pattern of at least four subpopulations based on differential CD34 and CD38 expression (Figure 1b). Next, we prospectively isolated three subpopulations based on their differential CD34 and CD38 surface expression (CD34+CD38 − , CD34+CD38+, CD34 − CD38+) via FACS sorting (FACS Aria, BD) and transplanted 1×10^6 living cells into sublethally irradiated NSG recipients by tail vein injection (Figure 1c). As demonstrated before,[9–12] the markers CD34/CD38 did not help to enrich for LICs in PDLTC-PH. All three sub-populations showed very similar engraftments and leukemia progression *in vivo* (Figure 1c).

Despite the fact that PDLTC-PH consists of distinctive cell populations according to the well-described stem/progenitor markers CD34/CD38, all tested subpopulations have identical leukemogenic potential irrespective of their CD34/CD38 expression pattern. Next, we wanted to test whether these two surface markers are stably expressed over time. Therefore, we prospectively isolated four subpopulations based on their distinctive CD34/CD38 expression via FACS and cultured them for 3 months (Figure 2a). The FACS analysis after 30 days and 90 days of culture revealed a return of the FACS profile to the starting culture, all four subpopulations have re-emerged over time. Interestingly, only CD34+CD38 − cells remained more stable in their marker profile and a large proportion of cells maintained CD34+CD38 − over time (Figure 2a). This observation would argue against a more immature stage of these cells as anticipated by the HSPC-like CD34+/CD38 − expression pattern seen in normal hematopoiesis. To exclude the possibility that reversion of the marker profile was attributed to selection and outgrowth of contaminating cells resulting from cell sort impurities and to evaluate the dynamics of this process, we increased our temporal resolution and repeatedly checked the surface marker expression already a few hours after prospective cell isolation via FACS (Supplementary Figure S1). Strikingly, we noticed a successive increase in marker-reverted populations starting already 2 h after isolation (Supplementary Figure S1). To further confirm that the marker reversion truly originates from individual cells, we FACS-sorted hundreds of individual cells of all four CD34/CD38 quadrants and established single clonal-derived subcultures of PDLTC-PH (Figure 2b). The clonal outgrowth varied between 10.4% (CD34 − CD38 −) and

16.5% (CD34+CD38 −). Even from single cells, independent of their distinct CD34/CD38 phenotype at sorting, we determined that 100% of single cells gave rise to progeny representing at least three subpopulations, and 79% of them even gave rise to all 4 subpopulations after 48 days in culture (Figure 2b). This result clearly shows that the expression of CD34 and CD38 is not stable over time, and since all starting cells irrespective of their marker profile give rise to all four subpopulations, also speaks against a linear hierarchy of cells depicted by these markers.

In order to determine the plasticity of marker expression in individual cells over time, we continuously observed single PDLTC-PH cells by time-lapse epifluorescence microscopy-based cell tracking for several days (Figure 2c), as previously described.[14] This technology allows to measure surface marker up- or downregulation in living cells in real-time, using fluorescent antibodies against CD34 and CD38 at low concentrations in the culture medium,[14] without losing individual cell identity. Pre-stained cells for CD34 and CD38 with fluorescent antibodies were continuously observed until their first division *in vitro*. And indeed, we found changes in marker expression—both onset of markers in originally negative cells and downregulation of marker-positive cells at single cell resolution within one cell generation (Figure 2c; Supplementary Figure S2; Supplementary Movies S1 to S3). In particular, the majority of double negative prospectively isolated PDLTC-PH cells upregulated CD34 and/or CD38 successively within one cell generation, although with various kinetics and intensities (Figure 2c). This clearly indicates a highly fluctuating and transient expression of both markers, which does not require cell division.

Do these results indicate that there is no distinction of cells with different LIC properties within the PDLTC, and that consequently every cell has LIC activity? Since we failed to use CD34/CD38 enrichments for LICs, we changed the strategy and grew subclones derived from single FACS-sorted PDLTC-PH cells of all four FACS quadrants, as described earlier. The tail vein transplantation of 5×10^6 cells of individual subclones in sublethally irradiated NSG recipients clearly distinguished subclones, causing a fast progressing disease, whereas some subclones did not give rise to full blown leukemia at all within 155 days of observation (Figure 2d and Supplementary Figure S3). Importantly, all clonal cells homed and engrafted in the BM, even those that showed minimal BM outgrowth and no
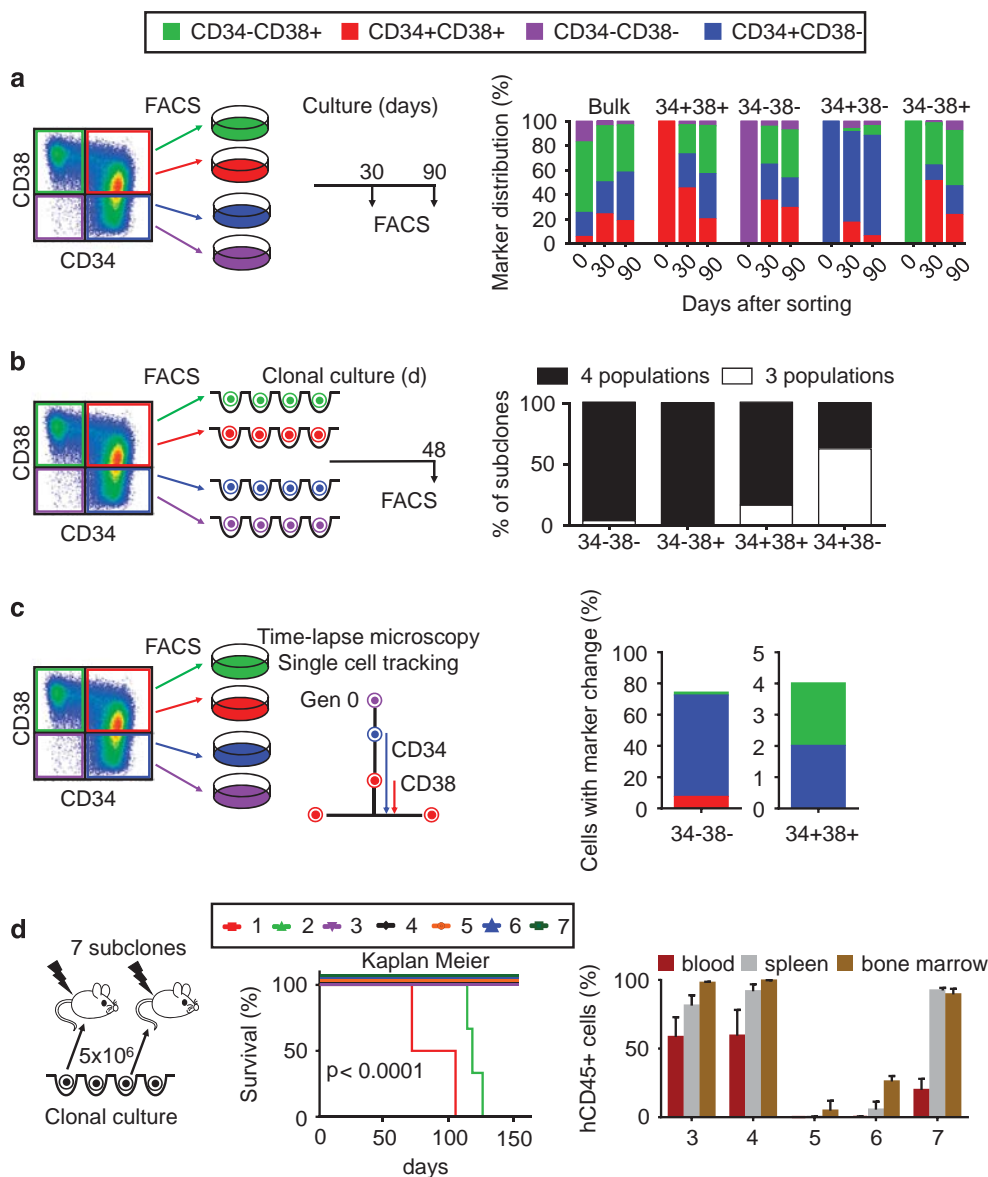
Figure 2. Plasticity in CD34 and CD38 expression counteracts a prospective enrichment of existing LIC clones. (**a**) Experimental scheme and FACS results of marker expression after prospective enrichment of subpopulations upon a 90-day-culture. (**b**) Experimental scheme and FACS results of marker expression of subclones after 48 days in culture. Hundred single-cell-derived subclones obtained via single cell FACS sort of cells from all four subpopulations were grown and the marker profile was analyzed via FACS. All clones consisted of at least three subpopulations of cells after 48 days in culture. (**c**) Experimental scheme and continuous microscopy-based tracking of marker expression at single cell level. Fifty single cells of all four subpopulations were observed until the mother cell divided. High proportion of marker reversion was found especially in CD34 − CD38 − cells. (**d**) Xenotransplantation of seven PDLTC-PH subclones. Survival of NSG mice receiving individual subclones ($n = 3$ mice per subclone). Only clone 1 and 2 resulted in early leukemic death of recipients, all other mice survived the observation period of 155 days. Significant difference calculated by the log-rank test. Leukemic engraftment in recipient mice that survived 155 days of observation. The mean and s.d. are displayed.

contribution to peripheral ALL cells (Figure 2d). These results again highlight the heterogeneity of individual ALL cells with variable ability to engraft and progress to leukemia in NSG mice and strongly propose the existence of LICs in BCP-ALL.

Is the, here described, plasticity of ALL cells restricted to the surface expression of some molecules, or do ALL cells change their molecular composition, functionality and even their identity over time? We need to find out whether this change is unidirectional and/or linear, following a differentiation path or whether some ALL cells may be able to dedifferentiate and switch between LICs and non-LICs. While in many tissues a hierarchical organization of cancer development is proposed, Ratajczak and coworkers reported similar

fluctuating expression of potential cancer stem cell surface markers CD24 and CD44 in an ovarian cancer cell line.[15] Our findings did not exclude the existence of a minor LIC population, which is uncovered due to the lack of respective markers. The clonal outgrowth and also the various leukemogenicity of established clonal subcultures highlight the fact that not every ALL cell has LIC potential. Only the use of CD34 and CD38 will not be sufficient to enrich for them.

The further analysis of this highly plastic ALL model, which revealed a non-hierarchical cellular development in ALL can be used to explore the properties of LICs in ALL, as the single clone system was capable to dissect between leukemogenic and non-leukemogenic single cells. These analyses are pivotal to find

proper markers for LIC identification in ALL, which is a prerequisite for the development of novel LIC-directed therapies. These novel strategies are urgently needed in order to improve long-term outcome and survival in ALL patients.

F Lang[1,7], B Wojcik[1,7], S Bothur[1,2,3], C Knecht[1], JHF Falkenburg[4], T Schroeder[5], H Serve[1,2,3], OG Ottmann[6] and MA Rieger[1,2,3]

[1]LOEWE Center for Cell and Gene Therapy and Department for Medicine, Hematology/Oncology, Goethe University Frankfurt, Frankfurt am Main, Germany;
[2]German Cancer Consortium (DKTK), Heidelberg, Germany;
[3]German Cancer Research Center (DKFZ), Heidelberg, Germany;
[4]Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands;
[5]Department of Biosystems Science and Engineering (D-BSSE), ETH Zurich, Basel, Switzerland and
[6]Division of Cancer and Genetics, School of Medicine, Cardiff University, Cardiff, UK
E-mail: m.rieger@em.uni-frankfurt.de
[7]These authors contributed equally to this work.

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Frequent evolution of copy number alterations in CLL following first-line treatment with FC(R) is enriched with TP53 alterations: results from the CLL8 trial

Malignant populations constitute a mixture of multiple genetically distinct clones, which can often be dramatically altered with therapy.[1] Clonal evolution was recently shown to be frequent in chronic lymphocytic leukemia (CLL) by sequential whole-exome sequencing of matched sequential samples taken at treatment initiation and first relapse following chemo(immuno)therapy from 59 individuals.[2] This finding raises important questions: Is evolution occurring as frequently during the preceding 'watch and wait' period, or is it primarily driven by therapeutic intervention? Are there clinical features associated with higher rates of evolution? Do the genetic variations that emerge over the course of evolution alter the clinical outcome?

To address these questions, we analyzed serial samples from 103 individuals treated within the CLL8 trial, in which patients uniformly received fludarabine and cyclophosphamide as first-line