Leukemia reconstitution *in vivo* is driven by cells in early cell cycle and low metabolic state

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ABSTRACT

In contrast to well-established hierarchical concepts of tumor stem cells, leukemia-initiating cells in B-cell precursor acute lymphoblastic leukemia have not yet been phenotypically identified. Different subpopulations, as defined by surface markers, have shown equal abilities to reconstitute leukemia upon transplantation into immunodeficient mice. Using a non-obese diabetes/severe combined immunodeficiency human acute lymphoblastic leukemia mouse model and cell cycle analysis annotating cells to distinct cycle phases, we functionally characterized leukemia-initiating cells and found that cells in all stages of the cell cycle are able to reconstitute leukemia *in vivo*, with early cycling cells ($G1b^{low}$ population) exhibiting the highest leukemia-initiating potential. Interestingly, cells of the G2/M compartment, i.e. dividing cells, were less effective in leukemia reconstitution. Moreover, $G1b^{low}$ cells were more resistant to spontaneous or drug-induced cell death *in vitro*, were enriched for stem cell signatures and were less metabolically active, as determined by lower levels of reactive oxygen species, compared to G2/M stage cells. Our data provide new information on the biological properties of leukemia-initiating cells in B-cell precursor acute lymphoblastic leukemia and underline the concept of a stochastic model of leukemogenesis.

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Introduction

According to a hierarchical stem cell model, continuous proliferation of progenitor and mature blood cells is sustained by hematopoietic stem cells (HSC) characterized by their ability to differentiate into all hematopoietic blood lineages while retaining self-renewal potential.[1] In leukemia, the presence of a leukemia-initiating cell (LIC) was proven by studies showing that only $CD34^+/CD38^-$ cells were able to transfer acute myeloid leukemia when transplanted into non-obese diabetes/severe combined immunodeficiency (NOD/SCID) mice, suggesting a hierarchical stem cell model in acute myeloid leukemia.[2,3] Recent work challenged this view and showed that $CD34^-$, $CD34^+/CD38^+$ and $CD123^+$ cells were also able to reconstitute acute myeloid leukemia.[4-8]

In B-cell precursor acute lymphoblastic leukemia (BCP-ALL), several studies have addressed the identification of LIC, demonstrating that more or less immature cells expressing different surface markers were all able to reconstitute leukemia in immunodeficient mice, supporting a stochastic stem cell model.[9-13] Thus, the capacity to propagate BCP-ALL clearly appeared not to be restricted to a phenotypically defined subpopulation, but may rather be defined at a functional level. Previously, we identified two distinct engraftment phenotypes, i.e. "Time To Leukemia short" (TTL^{short}) and "Time To Leukemia long" (TTL^{long}), reflecting early and late engraft-

ment of primary cells in a BCP-huALL model.[14] These two phenotypes were strongly associated with patients' outcome and the TTL^{short}/high-risk phenotype involved increased activation of the mammalian target or rapamycin (mTOR) pathway[14,15] and deficient apoptosis signaling.[16]

In this study, we identified annotations to distinct cell cycle compartments as a biological discriminator identifying a cellular subfraction with higher LIC activity capable of driving leukemia reconstitution in a NOD/SCID/huALL mouse model.

Methods

Twenty patient-derived xenograft samples established by transplantation of patients' ALL cells into NOD/SCID mice (NOD.CB17-*Prkdcscid/J*, Charles River) as previously described[14,16] were used in this study. The patients' samples were obtained with informed consent in accordance with the institution's (Ulm University) ethical review board; all animal experiments were approved by the appropriate authority (Regierungspräsidium Tübingen) and carried out following institutional and national guidelines on the care and use of laboratory animals. The characteristics of the patients and their malignancies are summarized in *Online Supplementary Table S1*.

For gene expression analysis, RNA was prepared from sorted cells and analyzed using Affymetrix Human Genome-U133 Plus 2.0 arrays. Gene-expression data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO series accession #GSE71836). Cell cycle staining was performed as previously described[17-19] labeling DNA/RNA with Hoechst (Molecular Probes, ThermoFisher Scientific) and pyronin Y (Polysciences, Hirschberg, Germany). Post-sorting analysis confirmed the positions of the sorted populations within the sorting gates. Viable cells (identified by trypan blue exclusion) were transplanted into NOD/SCID mice (10^5 viable cells/recipient); group sizes were chosen based on the availability of cells after sorting. Secondary recipients were transplanted with unsorted cells ($1x10^5$) isolated from mice with full-blown leukemia, which had been transplanted with G1b^{low} or G2/M sorted cells. For analysis of reactive oxygen species (ROS), cells were stained with CM-H2DCFDA (Invitrogen/ThermoFisher Scientific) and analyzed by flow cytometry. DNA was labeled with Hoechst. Post-sorting analysis confirmed high/low ROS levels. Viable cells (identified by trypan blue exclusion) were transplanted (10^5 viable cells/recipient); cells sorted based on FSC-A/SSC-A and FSC-A/FSC-H gates were used as control. Drug sensitivities (cell death upon prednisolone or cytarabine) were investigated in sorted cellular subfractions.

Statistical analyses were carried out using the Mann-Whitney test, the unpaired *t*-test with Welch correction (two-tailed), the one sample *t*-test or the log-rank test (two-tailed) as indicated. $P \leq 0.05$ was considered statistically significant.

Additional and detailed information on the methods used can be found in the *Online Supplementary Data*.

Results

Leukemia-initiating cell potential in B-cell precursor acute lymphoblastic leukemia is associated with cell cycle activity

We previously characterized the engraftment potential of primary BCP-ALL cells transplanted into NOD/SCID mice and found that a rapid engraftment and a short time to leukemia (TTL^{short}) are associated with poor patients' outcome.[14] The capacity of a cell to give rise to a phenotypically equal progeny *in vivo* is considered a stem cell's hallmark feature.[20] We evaluated LIC activities by transplanting decreasing numbers of four primograft samples (TTL^{short}, n=2; TTL^{long}, n=2) in limiting dilutions (10^5 to 10^1 cells) assessing leukemia engraftment 25 weeks after transplantation. Interestingly, higher LIC frequencies (ID04, LIC: 1/329, TTL: 9 weeks; ID05, LIC: 1/739, TTL: 10 weeks) were observed in TTL^{short}/poor prognosis

Table 1. High leukemia-initiating cell frequencies in TTL^{short}/poor prognosis acute lymphoblastic leukemia. Estimated leukemia-initiating cell (LIC) frequencies of 2 TTL^{short} and 2 TTL^{long} ALL samples. Limiting dilution analysis.

Sample	TTL	N. cells transplanted	Recipients transplanted	Recipients engrafted	LIC frequency
ID04	short	10^5	8	8	1/329
		10^4	8	8	
		10^3	8	7	
		10^2	8	4	
		10^1	8	0	
ID05	short	10^5	8	8	1/739
		10^4	8	8	
		10^3	8	6	
		10^2	8	1	
		10^1	8	0	
ID12	long	10^5	8	8	1/2159
		10^4	8	8	
		10^3	8	3	
		10^2	8	0	
		10^1	8	0	
ID11	long	10^5	8	6	1/74028
		10^4	8	1	
		10^3	8	0	
		10^2	8	0	
		10^1	8	0	

TTL: time to leukemia; N: number.

leukemias as compared to decreasing frequencies along with prolonged engraftment (ID012, LIC: 1/2159, TTL: 19 weeks ID11, LIC: 1/74028, TTL: 22 weeks) in TTL^{long} leukemias (Table 1). Accordingly, only TTL^{short} cells led to engraftment upon transplantation of 10^2 cells.

Next, we analyzed expression of the lineage and stem cell markers CD19, CD10, CD34 and CD38, previously described to be characteristic of cells with stem or initiating cell potential.[5,9-12] Altogether, 50 patients' ALL samples, which had been transplanted and characterized for their engraftment phenotype, were analyzed. No differences in marker expression were observed between the two phenotypes (Figure 1A); however, a trend of higher proportions of CD34$^+$ cells in TTL^{long}/good prognosis samples was seen, in line with earlier reports.[21,22] In order to look for stem cell features, which are different from expression of surface markers, we analyzed our previously obtained gene expression data[14] using gene set enrichment analysis. We identified 23 gene sets significantly enriched in the TTL^{short}/high risk profile (false discovery rate q-value ≤ 0.05), of which 17 were annotated to cell cycle functions, pointing to an association of cell cycle regulation with the TTL phenotype and, therefore, LIC activity in ALL (Figure 1B and *Online Supplementary Table S2*).

To further investigate these findings functionally, we analyzed the proportions of cells in active mitosis in all 20 samples (n=10 TTL^{short} and n=10 TTL^{long}), (*Online Supplementary Table S1*) by staining for phosphorylated histone H3 (Ser10). Significantly higher proportions of mitotic ALL cells were identified in TTL^{short} compared to TTL^{long} leukemias (Figure 2A), in line with our gene expression analysis results. Moreover, we investigated cellular proliferation of leukemia cells *in vivo* in one leukemia of each TTL phenotype. Dividing cells were marked with bromodeoxyuridine and huCD19/bromodeoxyuridine-positive cells were analyzed after labeling/pulse and during follow up/chase. At the end of the labeling (day 0), significantly higher percentages of huCD19/bromodeoxyuridine-positive cells were detected in spleen and bone marrow of TTL^{short} mice than in TTL^{long} mice (Figure 2B). Moreover, a clear reduction of bromodeoxyuridine positivity in human ALL cells was observed during chase in TTL^{short} in contrast to similar or slowly decreasing levels in TTL^{long} leukemias (Figure 2C). During the experiment, all animals showed similarly high leukemia loads (Figure 2D).

These findings indicate that the LIC frequency is related to a higher *in vivo* proliferation capacity. Moreover, despite variation in frequencies between different samples, we did not find that LIC in BCP-ALL are extremely rare, which further supports recent observations suggestive of a stochastic stem cell concept in ALL in which many cells possess leukemia-initiating potential.

Cells in early G1-S transition possess higher leukemia-initiating cell potential

Since we found that differences in LIC frequencies and cell cycle progression are associated with distinct engraftment capabilities, we hypothesized that leukemia cells in different cell cycle phases are characterized by a specific repopulating potential. We used a cell cycle "live" staining with simultaneous staining of DNA and RNA[17,19] distinguishing G0/G1, S and G2/M phases. In particular, cells in G0/G1 were further divided based on increasing RNA intensity, reflecting transition from G to S phases.[23,24] Early cell cycle annotated cells (G0/G1) were subdivided into G1a, G1b^{low} and G1b^{high} fractions according to progressively increasing RNA fluorescence. A fourth gate was placed on cells in G2/M (Figure 3A). Staining of G1a/G1b^{low}-sorted cells with the proliferation marker Ki-67 revealed that Ki-67-negative resting G0 cells are part of the G1a fraction in this analysis (*Online Supplementary Figure S1*).

All sorted subpopulations showed leukemia-initiating

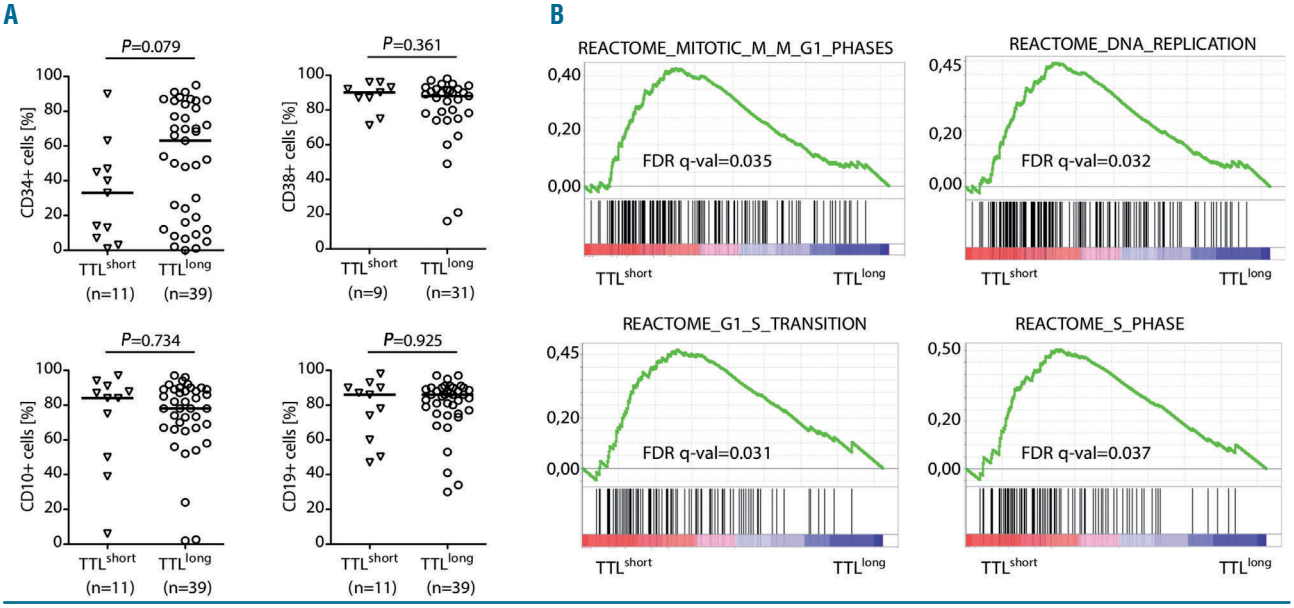

Figure 1. Leukemia engraftment is associated with cell cycle activity. (A) No difference in surface marker expression (CD34, CD10 and CD19 n=50; CD38 n=40) as measured by flow cytometry on primary patients' ALL cells with either TTL^{short} or TTL^{long} phenotype. Single and median values are indicated. Mann-Whitney test; *P*= statistical significance. (B) Representative gene set enrichment analysis plots of cell cycle annotated gene sets in the TTL^{short}/high LIC activity profile False Discovery Rate (FDR) (q-value ≤0.05).

activity upon transplantation into subsequent mice, irrespective of the originating cell cycle compartment and TTL phenotype (*Online Supplementary Table S3*), leading to full-blown leukemia of the initial common-ALL immunophenotype. Interestingly, of the four cell cycle fractions transplanted, G1b^{low} cells showed quickest engraftment and were associated with the shortest leukemia-free survival. This feature was observed in samples with both short and long TTL phenotypes, suggesting that the high leukemia repopulating and initiating capacity of G1b^{low} cells is a general feature of this early cell cycle leukemia cell subfraction. Importantly, despite these distinct repopulating activities of ALL cells from different cell cycle subgroups, the overall short or long leukemia engraftment of unfractionated leukemia cells was recapitulated in the sorted subfractions. Moreover, G2/M cells were always the last to engraft, leading to longer leukemia free survival of the recipient animals (Figure 3B). In addition, no differences in the expression of lineage/stem cell markers huCD19, huCD38, huCD10 or huCD34 were observed on ALL cells of either G1b^{low} or G2/M cell fractions (*Online Supplementary Figure S2*). Most interestingly, the distinct engraftment of G1b^{low} *versus* G2/M cells was also retained upon secondary transplantation of G1b^{low}- and G2/M-derived, unsorted bulk leukemia cells, indicating maintenance of LIC-capacities in the bulk of G1b^{low}- and G2/M-derived cells (Figure 3C and *Online Supplementary Table S3*).

G1b^{low} acute lymphoblastic leukemia cells are characterized by a stem cell signature

To further corroborate our observation of higher LIC activity in G1b^{low} leukemia cells as suggested by our functional *in vivo* data, we investigated transcriptional signatures of sorted G1b^{low} and G2/M leukemia cells (pairs of 4 samples; TTL^{short}= 1; TTL^{long}= 3). We identified 865 genes (1122 probe sets, false discovery rate q-value <0.05) as being differentially regulated between G1b^{low} and G2/M subpopulations irrespective of their TTL phenotype, with 330 up- and 535 down-regulated genes in G1b^{low}, which were mainly attributed to cell cycle regulation (Figure 4A and *Online Supplementary Tables S4 and S5*). Interestingly, gene set enrichment analysis identified a positive enrichment of 4 out of 16 gene sets previously associated with stem cell activity with the G1b^{low}/high LIC-enriched cells (Figure 4B and *Online Supplementary Table S6A*), whereas genes sets from short-term HSC or mature cells were found to be positively enriched in G2/M/low LIC[8,25-29] (Figure 4B and *Online Supplementary Table S6B*). Thus, these gene expression data further support the higher LIC activity of G1b^{low} cells observed upon transplantation.

Different cell death sensitivities of cell cycle annotated acute lymphoblastic leukemia cells

Cancer initiating or stem cells have been described to be characterized by an increased resistance to anti-tumor therapies. To investigate this issue, we studied the intrin-

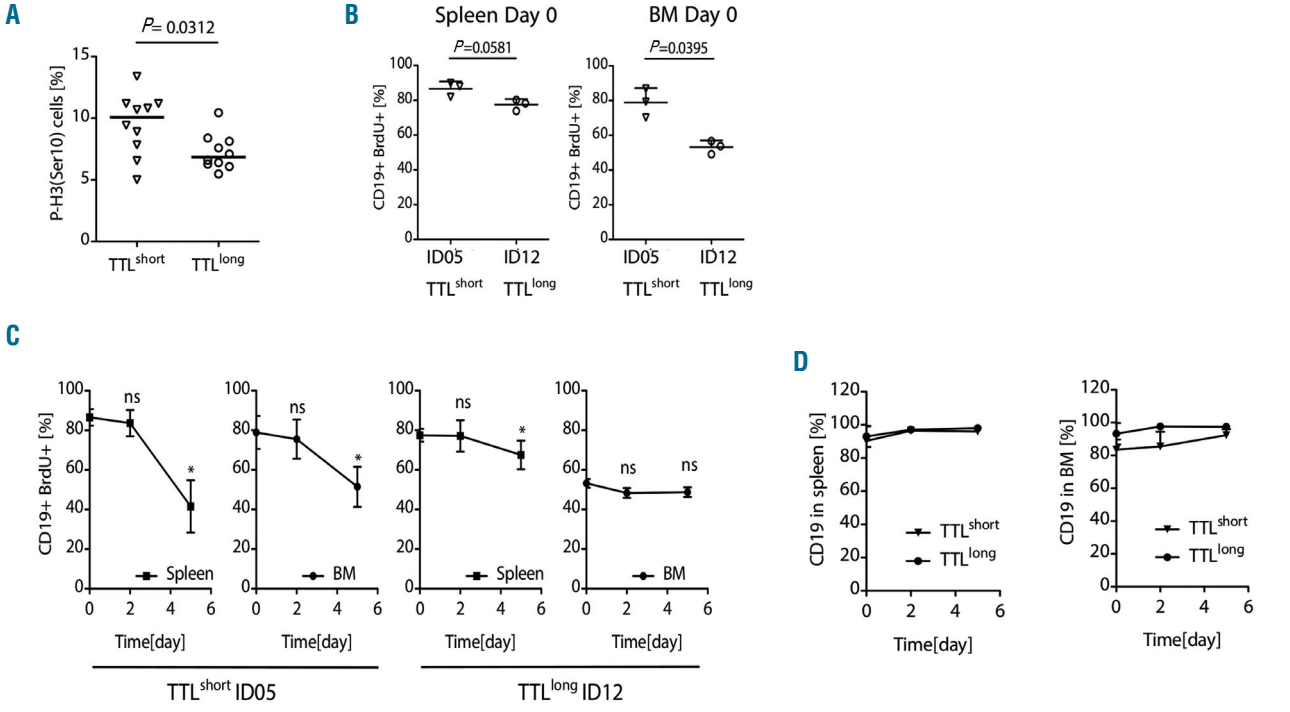

Figure 2. High leukemia-initiating cell activity is associated with increased cell cycle activity. (A) Higher phosphorylated histone 3 (P-H3; Ser10)-positive cells in active mitosis in TTL^{short} (n=10) as compared to TTL^{long} leukemia samples (n=10), Mann-Whitney U-test; the line represents the median; *P*=statistical significance. (B and C) Higher bromodeoxyuridine (BrdU) uptake (B) and increased decline (C) after *in vivo* labeling as detected by flow cytometry of ALL cells in TTL^{short}/high LIC frequency compared to TTL^{long}/low LIC frequency ALL bearing recipients (n=3/time point; biological replicates). Percentages of huCD19^{+}/BrdU^{+} cells in bone marrow (BM) and spleen of ALL bearing recipients (mean ±SD). Unpaired *t*-test with Welch correction (two-tailed); *P*= statistical significance; *≤0.05; n.s.: not significant. (D) Similar high leukemia load in recipients used for *in vivo* proliferation analysis; percentages of huCD19^{+} ALL cells in spleen and BM over time in recipients (n=3 per group; biological replicates) bearing a TTL^{short} or TTL^{long} leukemia (mean ± Standard Deviation).

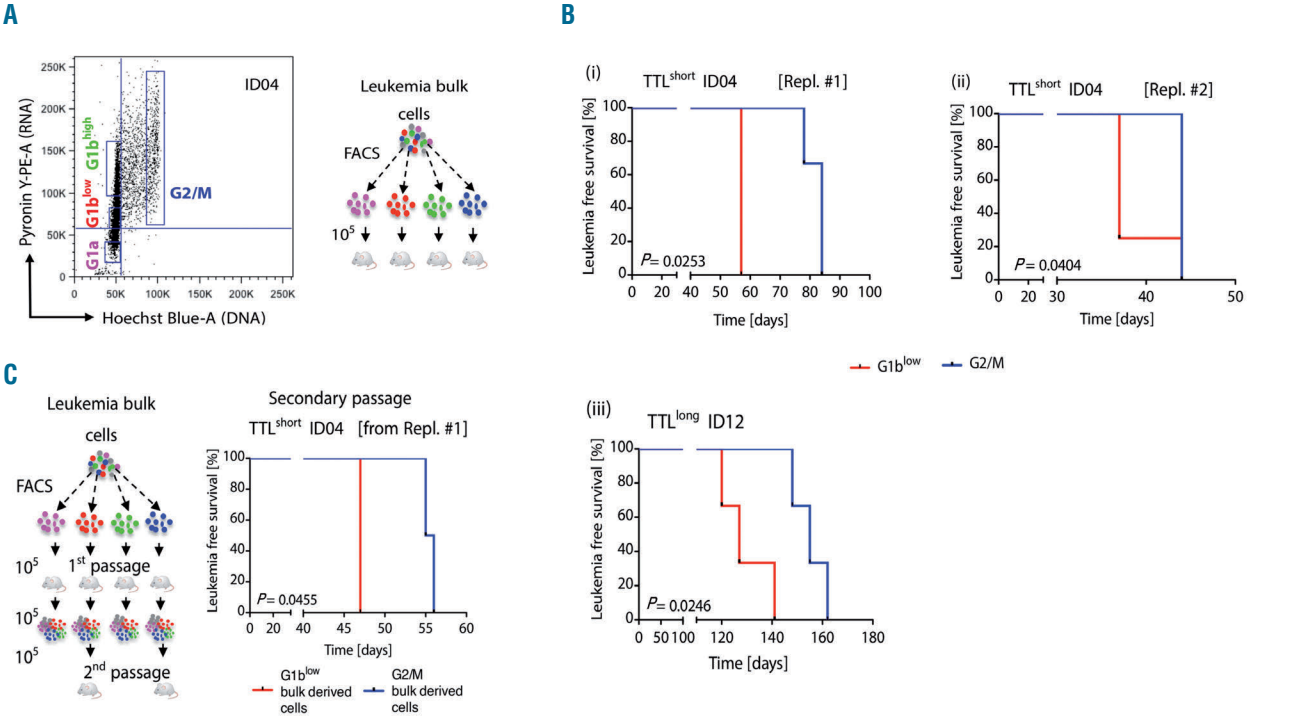

Figure 3. Higher leukemia-initiating cell potential of cells in early G1-S transition. (A) Cell cycle "live" staining: simultaneous DNA (Hoechst 33342) and RNA (pyronin Y) labeling of BCP-ALL cells and cell cycle annotated cellular subfractions G1a, G1b^{low}, G1b^{high} and G2/M analyzed by FACS. Sorted fractions were transplanted into NOD/SCID mice (10^5 cells/mouse) and leukemia engraftment was analyzed as weeks from transplantation until appearance of ≥1% huCD19$^+$ ALL cells in peripheral blood of the recipients. (B) Increased engraftment activity upon transplantation of G1b^{low} (red) compared to G2/M (blue) cells; (i, ii) short time to leukemia (TTL^{short}) ALL, two independent experiments, n=3 and n=4 mice/group; (iii) long time to leukemia (TTL^{long}) ALL, n=3 mice/group. (C) Maintained distinct engraftment in secondary recipients transplanted with unsorted bulk primograft ALL derived from primary recipients transplanted with G1b^{low} or G2/M sorted fractions, n=3 and n=2 mice/group, respectively. Log-rank test; *P*=statistical significance.

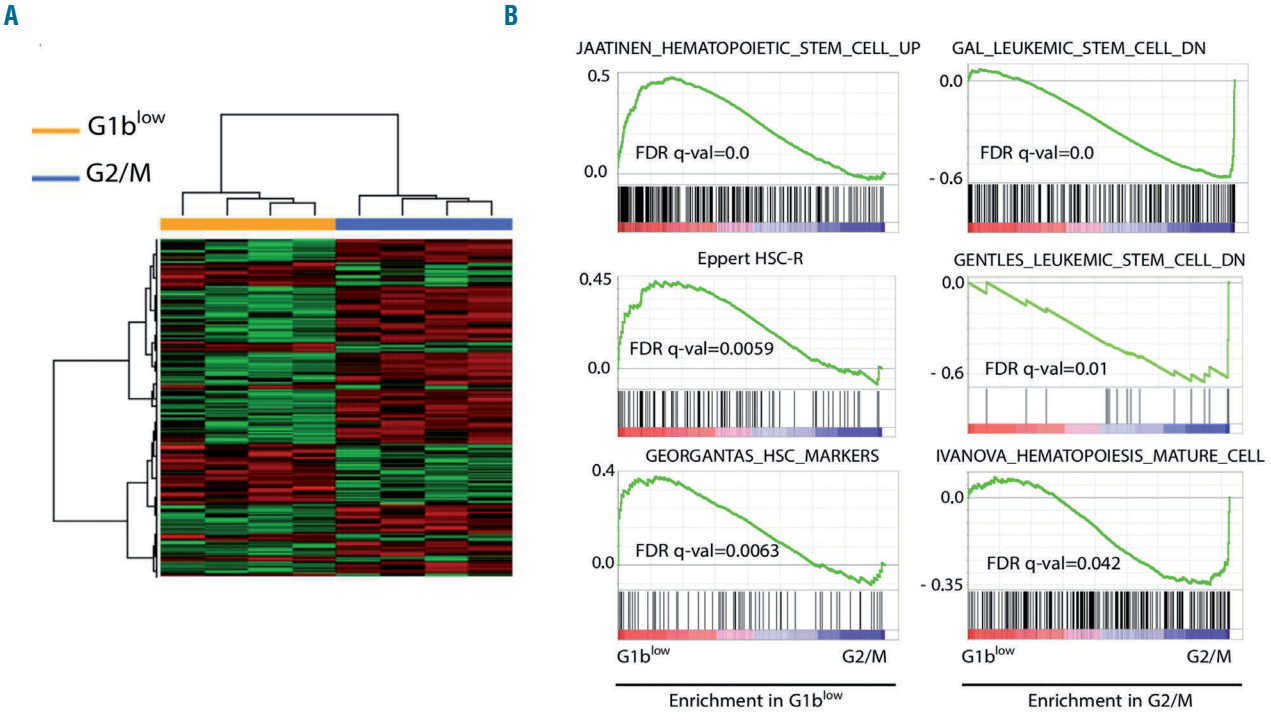

Figure 4. G1b^{low} acute lymphoblastic leukemia cells are characterized by a transcriptional stem cell program. (A) Unsupervised cluster analysis of 865 genes (1122 probe sets) differentially regulated (green: down-regulated; red: up-regulated) between G1b^{low} and G2/M sorted cellular subfractions False discovery rate (FDR) q-val<0.05 (B) Positive (left) enrichment of gene sets attributed to stemness or negative enrichment of sets annotated to mature or short-term stem cells (right) with the G1b^{low}-profile (gene set enrichment analysis, NOM *P*-value ≤0.05 and FDR q-value ≤0.05).

sic propensity of primograft ALL cells of G1b^{low} and G2/M subfractions to undergo cell death, both upon *ex vivo* culture and in response to prednisone and cytarabine, two drugs used in protocols to treat pediatric ALL patients. Interestingly, all 4 primograft samples analyzed showed lower rates of spontaneous and drug-induced cell death in G1b^{low} as compared to G2/M cells (Figure 5A and B).

Leukemia-initiating activity in acute lymphoblastic leukemia is characterized by distinct cellular oxidative states

Recently, low levels of ROS were described in cellular subfractions associated with stem cell properties.[30-32] We, therefore, investigated ROS activity in xenograft samples of TTL^{short} or TTL^{long} phenotypes. Lower ROS activities were observed in rapidly engrafting, TTL^{short}/poor prognosis leukemia cells (Figure 6A) and in G1b^{low}-sorted cells compared to G2/M-sorted cells (Figure 6B).

We observed that cells with low ROS activity (ROS^{low}) were almost exclusively allocated to G0/G1 cell cycle phases, whereas cells with high levels of ROS activity (ROS^{high}) included those in later S and G2/M phases (Figure 7A and B), suggesting that the ALL cell's oxidative state is indicative of its leukemia-initiating activity. To further address this hypothesis on a functional level, we investigated 3 primograft ALL samples and sorted cellular subfractions according to high or low ROS levels (upper or lower 15% fluorescence intensity, ROS^{high} and ROS^{low} cells, respectively). Upon transplantation into recipient animals, both sorted subfractions led to leukemia engraftment. However, in all three leukemias, ROS^{low} cells displayed a higher repopulating activity and were associated with significantly shorter leukemia-free survival in contrast to prolonged engraftment and survival in mice transplanted with ALL cells with a high oxidative state (ROS^{high}) (Figure 7C). In HSC, a ROS-MAP kinase axis has been implicated in negative regulation of the life span of the cells.[30,33] A high LIC potential of the ROS^{low} subtype was also found in T-ALL and functionally linked to expression of PKC-θ as a consequence of deregulated NOTCH signaling.[34] However, the analysis of MAP kinase p38α/β-expression did not reveal significant differences between the engraftment phenotypes (*Online Supplementary Figure S3A* and *B*) and, in contrast to the T-ALL data reported, we did not detect PKC-θ expression in our BCP-ALL cells *(Online Supplementary Figure S3C)*.

Taken together, we observed that LIC are not rare in BCP-ALL, pointing to a stochastic stem cell concept in this type of leukemia. We identified distinct LIC activities in cell cycle annotated cellular subfractions, with early cycling (G1b^{low}) cells possessing the highest LIC potential independently of the overall engraftment phenotype and high/low relapse risk. Moreover, early cycling (G1b^{low}) cells with high LIC potential are characterized by a transcriptional stem cell profile, cell death resistance, and a low oxidative state, which in turn results in higher LIC

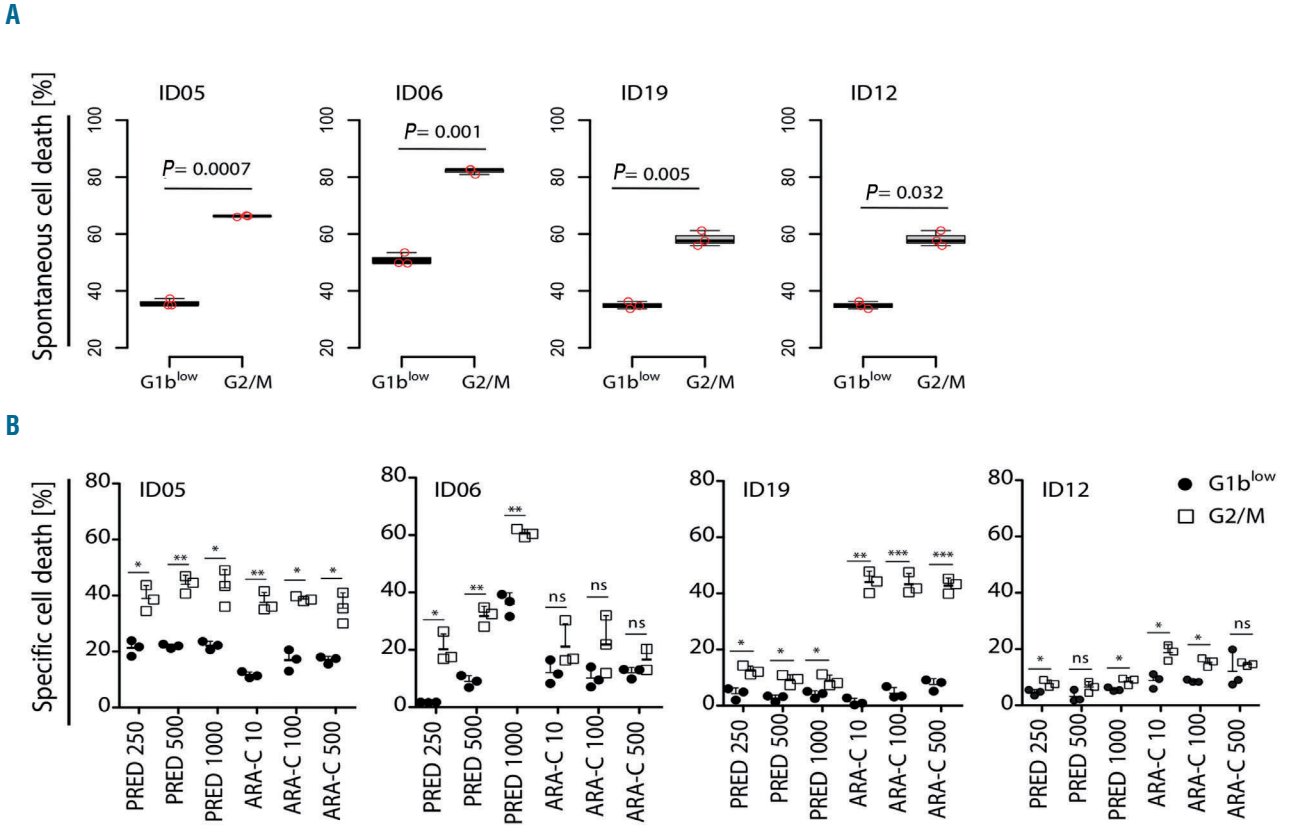

Figure 5. G1b^{low} acute lymphoblastic leukemia (ALL) cells are characterized by cell death insensitivity. (A and B) Low sensitivity for G1b^{low} sorted ALL cells for spontaneous (A) and drug induced (B) cell death measured by flow cytometry. Cell death upon culture or exposure (24 h) to prednisolone (PRED, 250, 500 or 1000 μg/mL) or arabinosylcytosine (ARA-C, 10, 100, 500 μg/mL) in cell cycle annotated, sorted subfractions, (mean ± Standard Deviation). Data points represent technical replicates within 4 individual leukemia samples, unpaired *t*-test with Welch correction; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; n.s.: not significant.

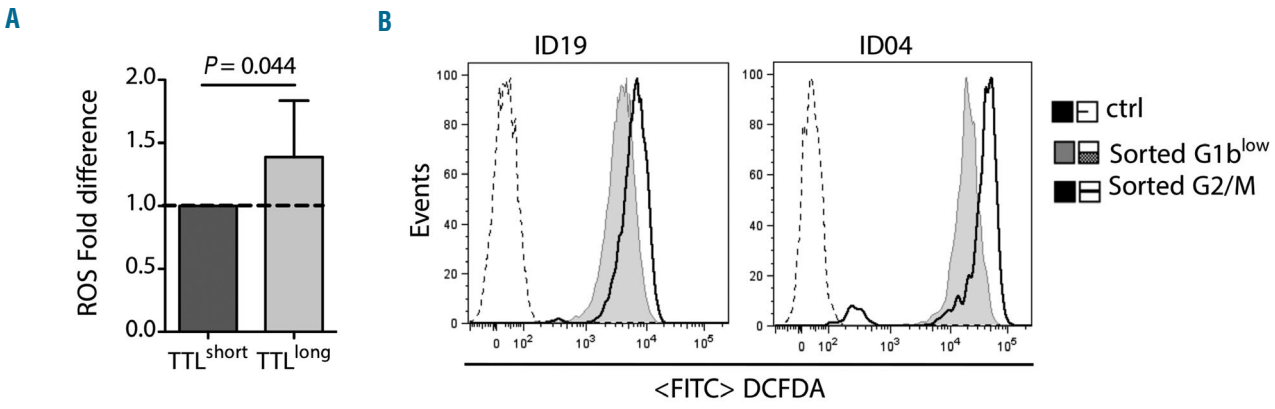

Figure 6. Reactive oxygen species (ROS) activity is associated with different cell cycle phases and cycling potential. (A) Low ROS activity in TTL^{short}/high proliferating (n=8) compared to TTL^{long}/slow proliferating (n=8) acute lymphoblastic leukemia samples: fold-change difference, (mean ± Standard Deviation) measured by flow cytometry. (B) Low ROS activity in sorted G1b^{low} annotated primograft leukemia cells compared to G2/M cells measured by flow cytometry. TTL^{short}: Time To Leukemia short; TTL^{long}: Time To Leukemia long; ctrl: control.

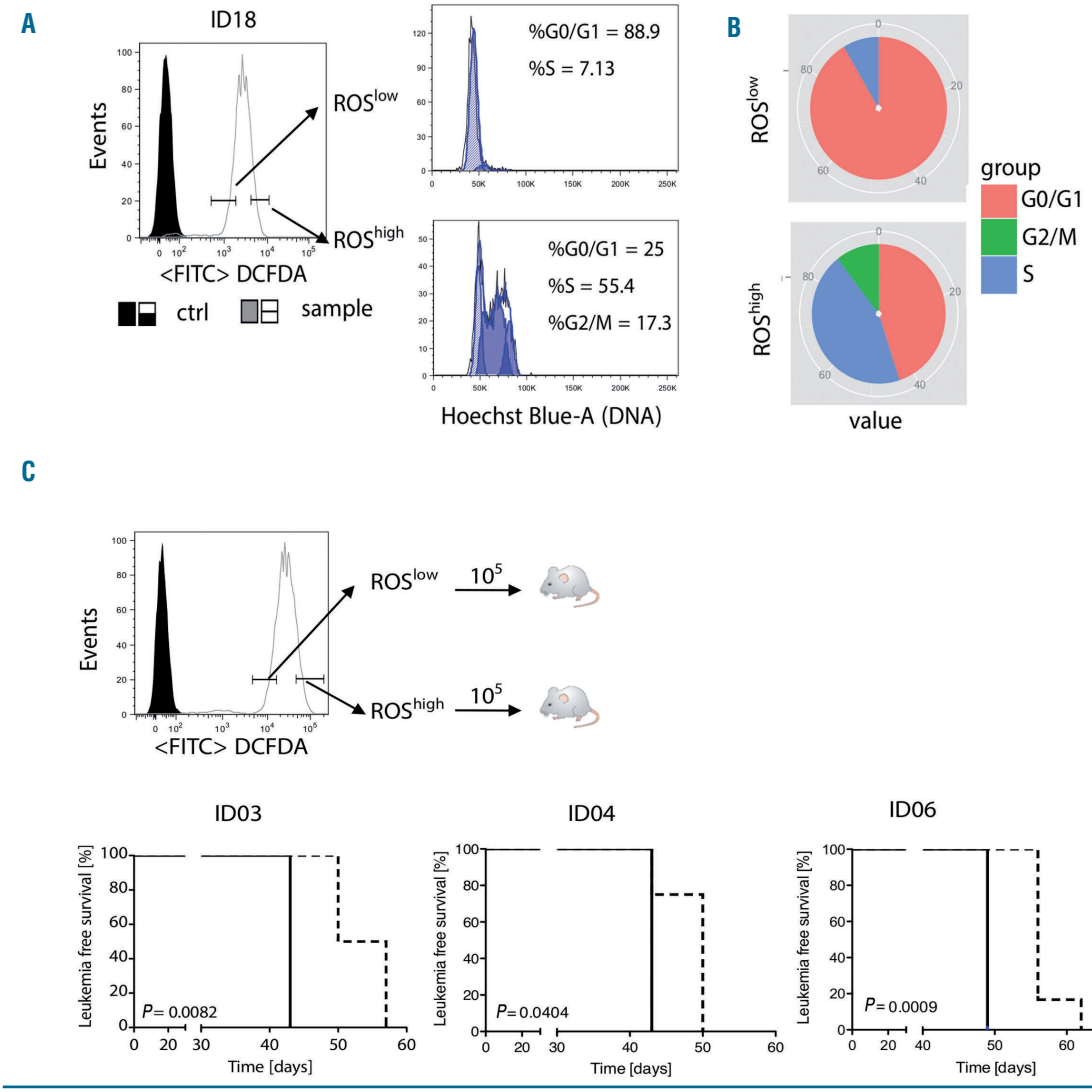

Figure 7. High leukemia initiating-cell activity in acute lymphoblastic leukemia (ALL) is associated with low reactive oxygen species (ROS) activity. (A) ALL cells with low ROS levels (ROS^{low}) are predominantly in early G0/G1 cell cycle phases, whereas cells with high ROS (ROS^{high}) include later S-G2/M phases. Simultaneous analysis of ROS activity and cell cycle distribution; cell cycle analysis in gated subpopulations of low or high (lower or upper 15%) ROS levels; one representative example of six analyses is shown. (B) Cell cycle distribution according to flow cytometry analysis in ROS^{low} and ROS^{high} subpopulations of 6 primograft ALL samples. (C) Increased engraftment activity of ROS^{low} ALL cells. Sorted ROS^{high} or ROS^{low} subfractions were transplanted (10^5 cells/mouse) and leukemia engraftment was analyzed as weeks from transplantation until appearance of ≥1% huCD19$^+$ ALL cells in peripheral blood of the recipients. N=4 (ID03, ID04), n=6 (ID06) mice/group, log-rank test; *P*= statistical significance.

activity in functional repopulation experiments, opening up ROS modulation as a perspective for treatment.

Discussion

In this study, we used engraftment phenotypes identified in our NOD/SCID/huALL model to investigate characteristic features of LIC in BCP-ALL. First, we did not obtain any evidence of preferential accumulation of LIC in subpopulations defined by surface markers. Since high-risk leukemia was characterized by higher LIC frequencies and higher cell cycle progression *in vivo*, we analyzed leukemia-initiating capacities of cells in different cell cycle compartments. Using a vital DNA/RNA staining method, we found that: (i) all cells possess LIC potential *in vivo* independently of the cell cycle phase of origin (i.e. G1a, G1b^{low}, G1b^{high} and G2/M); (ii) early G1b cells entering into S phase (G1b^{low}) lead to the most rapid engraftment, suggesting that G1b^{low} cells are the first engrafting leukemia-initiating cells; (iii) G2/M cells possess significantly lower engraftment potential; (iv) these features were maintained independently of the leukemia cell engraftment phenotypes; and (v) G1b^{low} LICs exhibit low metabolic activity and ROS potential, probably associated with increased cell death resistance. These findings have implications for the characterization of the putative leukemic stem cell in ALL, the heterogeneity of leukemic clones and sensitivity and/or resistance of functionally defined subpopulations for treatment approaches using targeted or conventional therapy.

Two models have been proposed for cancer stem cells or LIC. According to the hierarchical concept, a few immature cells harboring stem cell properties are considered to be able to generate their progeny and give rise to leukemia. While a number of data have supported this hierarchical model in acute myeloid leukemia,[2,3] for ALL and particularly for BCP-ALL a more stochastic model, in which literally every cell, including more mature and differentiated phenotypes, is considered to be able to initiate leukemia, appears to be valid. This implies that the establishment of the leukemia phenotype in patients and upon transplantation in mice may be an intrinsic capacity of individual cells independently of surface phenotype and maturation stage.[9-13] This concept corresponds to the findings of leukemia initiation by low cell numbers and by all subfractions seen in our huALL mouse model. Another issue along this line is whether or not LIC are derived from quiescent, cycling or dividing subpopulations. The data from our *in vivo* labeling experiments indicate that increased proliferation is associated with rapid engraftment and rapid development of full-blown leukemia resulting in the TTL^{short} phenotype defining a poor prognosis subgroup. Thus, one may have expected that actually dividing cells (G2/M) harbor the highest leukemogenic potential upon transplantation. However, the resting/early recruitment compartment (G0, G1) contained similar reconstitution potential and the early recruitment phenotype (G1b^{low}) exhibited the highest leukemogenic potential in the transplantation experiments.

The potential of both G0/G1 and G2/M cells to reconstitute the leukemia phenotype *in vivo* is a new finding compared to previous data on human and murine HSC. Indeed, human and murine HSC have been reported to be heterogeneous with respect to the cell cycle: while only cells in G0/G1 sufficiently reconstituted hematopoiesis in sublethally irradiated mice,[18,35-38] cells in S-G2/M did not repopulate the bone marrow at all or had only a minimal engraftment potential.[35,39]

The higher *in vivo* leukemogenic activity of G1b^{low} cells suggests that these cells are likely the first out of the leukemic bulk to engraft in recipients. The higher "stem cellness" of this subpopulation is also supported by gene signatures previously assigned to stem cell activity. In contrast, G2/M cells with lower LIC potential were negatively associated with stem cell-like profiles or were enriched for genes characteristic of short-term HSC or mature cells.[8,25-27,29] Importantly, the G1b^{low} and G2/M features are maintained irrespective of the engraftment phenotype suggesting that these characteristics are conserved features of BCP-ALL LIC. However, the engraftment potential of both subpopulations, irrespective of stem cell signatures, emphasizes the stochastic nature of LIC in ALL, in line with data recently reported on similar engraftment activities of slowly or rapidly dividing BCP-ALL cells.[40]

LIC frequencies calculated in our NOD/SCID/huALL model showed variations associated with the patients' outcome and engraftment phenotype, suggesting that speed of ALL repopulation is a measure of LIC activity, as suggested before.[41] Thus, we analyzed repopulation times to evaluate LIC activities of sorted cells. In both TTL^{short}/poor prognosis ALL samples, engraftment was observed upon transplantation of down to 100 cells, in line with reported high LIC frequencies in studies including poor outcome BCP-ALL, even in the more immunodeficient NSG mouse strain.[9,40,42] However, higher minimum cell numbers of up to 10^3 cells were required to initiate leukemia in both TTL^{long} samples, of which one also showed hyperdiploidy, similar to numbers observed in studies including favorable prognosis ALL.[10-13,43,44] Accordingly, TTL^{long} phenotypes were always observed in ALL with the favorable prognostic features hyperdiploidy or *ETV6/RUNX1* rearrangements,[14] suggesting lower LIC frequencies in good outcome BCP-ALL.

In TTL^{short} leukemia, gene expression, more cells in active mitosis and the *in vivo* bromodeoxyuridine labeling data indicate a higher proliferation rate, including activated mTOR signaling.[15] Along this line, effects of the mTOR pathway on cell cycle progression and particularly regulation of the G1 phase have already been described.[45,46] Accordingly, in addition to a distinctive transcriptional program,[14] different basal mTOR activation[15] and deficient apoptosis signaling[16] were found in the TTL^{short} *versus* TTL^{long} phenotypes.

The higher LIC capacity of G1b^{low} appears to result from a favorable functional status. G1b^{low} cells were less prone to undergo spontaneous and drug-induced cell death *ex vivo* whereas G2/M slowly engrafting cells showed a greater predisposition to both intrinsic and induced cell death. Increased intrinsic cell death resistance may be a consequence of a block in cell death pathways or alteration of the metabolic state. Recent work showed that different levels of ROS and a lower mitochondrial mass distinguished cells with higher LIC activity.[31,32] We found that G1b^{low} cells were characterized by lower ROS activity compared to G2/M cells. Moreover, when analyzing ROS levels in cell cycle compartments, we observed that ROS^{low} cells were almost exclusively found in G0/G1, while ROS^{high} cells were progressing not only through the G0/G1 but also the S and G2/M phases of the cell cycle.

Interestingly, ROS^{low} cells possessed an engraftment advantage with the transplanted mice having a shorter leukemia-free survival. Therefore, as already reported for HSC,[30,33] our data suggest that increased ROS levels are associated with reduced repopulating stem cell activity in BCP-ALL. Along this line, it is interesting to note that higher endogenous ROS levels were detected in TTL^{long}/good prognosis samples. While an increased ROS level in HSC was directly linked to MAP kinase activation, we could not detect differential expression of p38α/β in the TTL^{long}/TTL^{short} subgroups characterized by different engraftment properties and ROS levels. In T-ALL, a different pathway for modulating the ROS status involving PKC-θ was described. However, we could not detect PKC expression at all in our leukemia cells. Thus, the molecular mechanism and/or association of specific pathways with different ROS states is unclear, but may be linked to different activation of survival pathways, as indicated by the gene profile.

In addition to cell intrinsic mechanisms, neighboring cells in the bone marrow environment interact with residing hematopoietic or leukemia cells and contribute to regulation of cellular programs including proliferation and cell cycle,[47] and homing and interaction of leukemia cells with the environment are modulated by the expression of adhesion molecules.[48] Topographically, in ALL a rare subfraction of cells was described to preferentially reside close to the endosteum,[40] the assumed site of the hematopoietic niche in the bone marrow.[49] Similar to our findings, these cells were characterized by low proliferation and insensitivity to chemotherapy, but did not show higher LIC activity compared to the corresponding non-dormant bulk leukemia cells.[40] It appears very likely, that the ability of a cell to initiate leukemia is determined by different factors including niche interactions contributing to the phenotype of early cycling cells with low metabolic activity identified in our study. Interestingly, recently presented data showed that HSC are able to transfer mitochondria into adjacent stromal cells, thereby lowering their intracellular ROS levels.[50] High mTOR expression,[15] lower cellular propensity to undergo apoptosis,[16] and the higher cycling capacity are cell intrinsic characteristics leading to the high proliferative potential of leukemia cells in poor prognosis patients, independently of the capacity of all leukemia cells to engraft in mice as described in this work, and the even higher LIC potential distinguishing G1b^{low} leukemia cells.

Our work adds new information in the search for LIC in BCP-ALL, at least in experimental models of human BCP-ALL. The ability to initiate leukemia seems not to be associated with particular subtypes of the heterogeneous leukemia subfractions and also appears to be independent of the cell cycle compartment from which the leukemogenic cells are transplanted. This argues strongly against the concept that leukemogenesis is restricted to quiescent clones and absent or reduced in cycling cells or *vice versa*. Thus, this finding has consequences for therapeutic strategies, which aim to target exclusively quiescent or dividing cells. Interestingly however, cells in quiescence or the early phase of the cell cycle appear to be more resistant to cell death induction than cells in other compartments of the cell cycle. The high LIC potential and low metabolic activity associated with cell death resistance may also indicate that strategies to activate these cells to exit quiescence or the early phase compartment may decrease intrinsic cell death resistance with resulting consequences for treatment sensitivity.

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