HHS Public Access

Author manuscript

Exp Hematol. Author manuscript; available in PMC 2025 May 19.

Published in final edited form as:

Exp Hematol. 2022 November ; 115: 1–13. doi:10.1016/j.exphem.2022.09.001.

Ex Vivo Expansion of Phenotypic and Transcriptomic Chronic Myeloid Leukemia Stem Cells

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Abstract

Despite decades of research, standard therapies remain ineffective for most leukemias, pushing toward an essential unmet need for targeted drug screens. Moreover, preclinical drug testing is an important consideration for success of clinical trials without affecting non-transformed stem cells. Using the transgenic chronic myeloid leukemia (CML) mouse model, we determine that leukemic stem cells (LSCs) are transcriptionally heterogenous with a preexistent drug-insensitive signature. To test targeting of potentially important pathways, we establish ex vivo expanded LSCs that have long-term engraftment and give rise to multilineage hematopoiesis. Expanded LSCs share transcriptomic signatures with primary LSCs including enrichment in Wnt, JAK-STAT, MAPK, mTOR and transforming growth factor β signaling pathways. Drug testing on expanded LSCs show that transforming growth factor β and Wnt inhibitors had significant effects on the viability of LSCs, but not leukemia-exposed healthy HSCs. This platform allows testing of multiple drugs at the same time to identify vulnerabilities of LSCs.

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm, a clonal malignancy, identified by a translocation of chromosomes 9 and 22 forming a constitutively active tyrosine kinase fusion protein, BCR-AB [1,2]. The leukemia stem cells (LSCs) in CML share phenotypic hematopoietic stem cell properties [3-5]. CML is treated primarily with tyrosine kinase inhibitors (TKIs) that eliminate the bulk of the disease; however, these fail

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SBP designed, planned and performed experiments, analyzed data, and wrote the article. VK, AMF and VRM helped perform experiments. MAB helped with data analysis. RSW performed experiments, supervised the study, and assisted with data interpretation and writing of the article.

Conflict of interest disclosure

The authors have declared that no conflict of interest exists.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.09.001.

to eradicate LSCs, leading to disease relapse or progression to a more aggressive blast phase leukemia [6-9]. Therefore, it is essential to identify mechanisms of LSC survival to target LSCs without affecting healthy hematopoiesis.

One of the reasons for the TKI insensitivity of LSCs is inherent heterogeneity and clonal selection [10-13]. CML is a model disorder in which to study these heterogeneous LSCs, and the mouse model resembles many of the clinical manifestations seen with the human disease [5,14,15]. Importantly, LSCs within this preclinical model are a well-defined population that can be identified using cell surface markers, making it possible to determine the best therapeutic regimen targeting LSCs that can be translatable to human patients [5,8]. Additionally, it is known that TKIs eliminate the bulk of the disease, but in most cases, not the quiescent LSCs because of the activation of alternative signaling pathways [8,16,17]. Thus, even though a molecular response to treatment is seen in most patients, less than half can discontinue treatment [18-20]. These well-established phenotypes, hierarchies, and observations make CML an ideal disorder to study and identify better methodologies to target LSCs.

Many pathways have been identified to target and eliminate CML LSCs, including transforming growth factor β (TGF), tumor necrosis factor α (TNFα), and JAK–STAT [8,15,17,21-25]. These signaling pathways are enriched in human CML $CD34^+$ stem and progenitor cells (HSPCs) obtained from patients responding poorly to TKI [12]. However, these studies were conducted using HSPCs because of the limiting numbers of phenotypic LSCs, making it challenging to characterize and identify LSC-specific targets. Surface markers specific for CML LSCs, including CD33, interleukin-1 receptor accessory protein (IL1-RAP), CD93, CD25, and CD26, have been discovered to circumvent this [11,26-31]. However, not all LSCs express these markers owing to their heterogeneity [32]. Thus, the challenge of obtaining adequate numbers of LSCs for functional characterization and subsequent identification of novel targets and response to treatment persists.

Ex vivo expansion of CML LSCs is beneficial for identifying and testing drug targets that eliminate LSCs without affecting non-transformed stem cells [33,34]. Although different ex vivo stem cell culture techniques have been established, they promote differentiation, require technical expertise, and are labor intensive [35-38]. Moreover, these culture methods have been developed to expand stem cells for bone marrow transplants as a therapeutic option [39-41]. We have adapted a recently established hematopoietic stem cell (HSC) ex vivo expansion assay for CML LSCs that share the same phenotype as HSCs [40]. These cultures significantly expand phenotypic LSCs that share transcriptional similarities with freshly isolated CML LSCs. We found that the ex vivo expansion of LSCs from the CML transgenic mouse serves as a promising preclinical tool with which to carry out drug screens. These cultures consider cellular heterogeneity and identify targets specifically for eliminating phenotypic LSCs, bringing the field a step closer to achieving complete remission.

METHODS

Mouse models

All mice are housed in the University of Alabama at Birmingham (UAB)'s animal facility and experiments were performed under Institutional Animal Care and Use Committee-approved protocol. Double transgenic BCR–ABL × SCL–tTA mice were used as a CML model at ~60% myeloid cells in peripheral blood, tested by flow cytometry as well as HemaVet [8,14,15]. B6-GFP mice were used for competition assays. Males and females were equally distributed throughout the study [42].

Bone marrow transplant

To test the engraftment potential of expanded stem cells, 8-week-old CD45.1 mice were sublethally irradiated (450 rad), and retroorbitally transplanted with one well of expanded CD45.2 HSCs or CML LSCs along with 200,00 CD45.1 whole bone marrow competitor cells, each. Peripheral blood was obtained from mice every 4 weeks for up to 6 months to test chimerism as previously described. At the end of 6 months, spleen and bone marrow were isolated to test for chimerism, stem and progenitor cells, and myeloid and lymphoid lineages. For single-cell RNA sequencing, 8-week-old CD45.1 mice were sublethally irradiated (450 rad), and retroorbitally transplanted with 5×10^6 bone marrow cells from CD45.2 CML or control mice. Imatinib treatment was started ~4 weeks posttransplant as previously described [8,15].

FACS staining and single-cell sorting

For fluorescence-activated cell sorting (FACS) profiles of expanded stem cells, whole bone marrow, spleen, and peripheral blood, a single-cell suspension was stained with a cocktail of monoclonal antibodies conjugated to fluorophores: Lineage markers included CD3 (17A2, phycoerythrin [PE]–Cy5), CD19 (6D5, PE–Cy5), B220 (RA36B2), CD11b (ICRF44, PE–Cy5), Gr-1 (RB6-8C5, PE–Cy5), and Ter-119 (TER-119, PE-Cy5), F4-80 (BM8, allophycocyanin [APC]–Cy7), Ly6G (1A8, fluorescein isothiocyanate [FITC]), Ly6C (HK1.4, PE–Cy7), CD115 (AFS98, PE). Stem and progenitor markers included cKit (2B8, APC–Cy7), Sca1 (D7, APC), CD135 (A2F10, PE), CD48 (HM48-1, Pacific Blue), CD150 (SLAM, PE–Cy7), ESAM (1G8, PE), endothelial protein C receptor (EPCR) (1560, APC), FcγRII/III (93, PE), and CD41 (HIP8, FITC), CD45.2 (104), and CD45.1 (A20) for chimerism. 4,6-Diamidino-2-phenylindole (DAPI) was used as a viability marker. Apoptosis was assessed with Annexin V (ThermoFisher Scientific, Waltham, MA) and DAPI as a counter stain using standard protocol. BD Fortessa with Diva Software was used to acquire data, and FlowJo 10.7.2 (Tree Star) was used for analysis. For cell sorting, the whole bone marrow was depleted for lineage marker-positive cells using biotin and streptavidin beads on the autoMACS sorter. The lineage-negative fraction was then stained; stem cells were sorted using a FACSAria II (BD Biosciences, San Diego, CA).

Cell cycle and proliferation assay

Fourteen-day expanded HSCs and CML LSCs were pulsed with 10 μmol/L EdU for 6 hours. The cells were then harvested, fixed, stained, and analyzed according to the manufacturer's protocol for the Click-iT EdU flow cytometric assay kit (Invitrogen, Waltham, MA).

Ex vivo expansion

We adapted the previously described polyvinyl alcohol (PVA)-based ex vivo HSC culture to expand LSCs [40,43]. Fifty LSCs or HSCs (Lineage-cKit$^+$Sca1$^+$Flt3$^-$CD48$^-$CD150$^+$) were directly sorted per well of a fibronectin-coated 9 6-well plate. The medium was supplemented with 10 ng/mL stem cell factor (SCF), 100 ng/mL thrombopoietin (TPO), 0.1% PVA, 1 × HEPES, 1 × insulin–transferrin–selenium–ethanolamine (ITS-X) and 1 × L-glutamine. The first medium change was carried out 4 days after sorting, with subsequent medium changes carried out every 2 days. For competition assays, 50 HSCs from B6-UBC-GFP and 50 LSCs from the CML mouse model were sorted into each well, and the culture was maintained as described above.

Bulk RNA sequencing

Twenty thousand expanded HSCs and CML LSCs in triplicates were lysed using Trizol. RNA was isolated using the RNeasy Plus mini kit (Qiagen, Hilden, Dusseldorf, Germany). Libraries were prepared with NexteraXT library construction (Illumina, San Diego, CA) according to the manufacturer's protocol. The quality and size of indexed libraries were determined using BioAnalyzer (Agilent, La Jolla, CA) and then sequenced. Analysis was performed using Partek Flow software. Briefly, double-ended sequencing reads were aligned to the mouse (mm10) using Spliced Transcripts Alignment to a Reference (STAR 2.7.3a). Aligned reads were then quantified to the transcriptome (Ensembl Transcripts release 101) and normalized based on counts per million. Identified differentially expressed genes were used for hierarchal clustering and pathway analysis. Pathway analysis was carried out using Enrichr [44].

Single-cell RNA sequencing

Small conditional (sc) RNA sequencing experiments were conducted using inDrop, a 3′ biased droplet-based microfluidic platform on stem and progenitor cells (LSK, Lineage-Sca1$^+$cKit$^+$) from the CML chimeric mouse model [45]. In brief, we encapsulated 3,000–9,000 cells per sample. The protocol for library prep was followed as described before [46]. The quality and size distribution were determined on the BioAnalyzer (Agilent High Sensitivity DNA kit).

Analysis of scRNA sequencing

Bowtie Version 1.1.1 was used with parameter *-e* 80, and reads were aligned to Ensembl release 85 *Mus musculus* GRCm38 reference. Each library was filtered to include only abundant barcodes (>500 total counts). Next, we excluded putatively stressed or dying cells based on mitochondrial genes, and normalization of data was carried out [46]. We also used Scrublet to identify clusters of cell doublets that coexpressed marker genes of distinct cell types [47]. Sample independent graph-based clustering to remove cells expressing

transcripts for committed cells [48] followed by differentially expressed gene identification and pathway analysis was done using Partek Flow software (Partek Inc., Chesterfield, MO).

Statistical analysis

Analyses were performed depending on the spread of the variable and reported as SEMs. Outliers were removed using the interquartile range with data points 1.5 below Q1 or 1.5 above Q3 eliminated from the data set. A Shapiro–Wilk test was used to determine normal versus abnormal distributions, and all continuous variables were tested for mean differences. Depending on the spread of variable, both nonparametric (Mann–Whitney U test, analysis of variance (ANOVA) Kruskal–Wallis test, Wilcoxon test) and parametric (Student's *t* test and ANOVA) tests were used. For ANOVA, Tukey's or Sidak's posttest was used to compare groups (Version 7.0, GraphPad Prism, San Diego, CA).

Data sharing statement

These data are deposited in the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus and accessible through GEO Series Accession Nos. GSE189368 and GSE189369.

RESULTS

Heterogenous clusters of CML stem and progenitor cells activate distinct pathways

Chronic myeloid leukemia is a clonal disorder with favorable treatment outcomes; however, patients often relapse and fail therapy. Various targets have been identified and advanced for clinical trials with limited success. It was essential to consider LSC heterogeneity to identify unique pathways to target LSCs without affecting the healthy HSCs [10-12,49]. Thus, we performed scRNA sequencing on freshly isolated HSPCs obtained from transplanted CML mice (Figure 1A). The control cells (WT) were recipient HSPCs ($CD45.1^{+}$) present in the same inflammatory environment as the donor CML HSPCs, in other words, leukemia exposed. Using this approach, we were able to identify unique signatures masked by inflammation and mimic human patient samples (Figure 1A) [15]. Differential gene expression in CML HSPCs compared with bystander WT HSPCs (Supplementary Figure E1A,) revealed that the top 25 differentially expressed genes in CML HSPCs are mostly AP1 transcription factors; one of their regulators includes STAT3, which plays a vital role in CML drug persistence [8]. Of these, Fos and Dusp1 are essential for TKI insensitivity in CML [50]. Pathway analysis of these differentially expressed genes revealed activation of MAPK and IL-17 signaling along with enrichment of the citric acid cycle (Supplementary Figure E1B). These are consistent with the previous publications that reported that BCR–ABL activates the MAPK pathway and that CML stem and progenitor cells rely on oxidative phosphorylation (OxPhos) over glycolysis [8,51-53].

To identify different subsets of stem and progenitor populations, namely, HSCs and multipotent progenitors 2–4 (MPPs 2–4), we used published transcriptomic data sets for HSCs, MPP2, MPP3, and MPP4 from healthy mice to demarcate these populations and applied it to our non-transformed and CML HSPCs (Supplementary Figure E1C-F, Supplementary Table E1) [54]. We observed that the HSC transcriptional signature is

overrepresented (39%) in CML HSPCs compared with WT, even though there are fewer in phenotypic LSCs (Figure E1C, D; Supplementary Table E1) [8]. Consequently, *K*-means clustering was applied to CML HSPCs, and four unique clusters were identified. The unique CML HSPC signature was differentially expressed compared with leukemia-exposed non-transformed HSPCs comprised of clusters 3 and 4 (Figure 1B; Supplementary Figure E1A; Supplementary Table E1). We found that MPP2 and MPP4 were overrepresented in cluster (C) 1, whereas MPP3 was found primarily in C2. Conversely, C3 comprises 70% transcriptional HSCs while cluster 4 (C4) contained only (100%) HSCs, where C4 represents 4% of all cells and C3 represents 42.7% (Figure 1B; Supplementary Figure E1E, F). This indicates that targeting clusters C3 and C4 would be a good therapeutic option as these are HSC-like clusters.

To determine whether the HSC-like clusters (C3 and C4) share a transcriptional signature with the CML LSCs, we applied bulk RNA sequencing data obtained from CML LSCs (LSK CD4 $8^{-}CD150^{+}$) [8]. We observed that our recently published CML LSC signatures, correlated with both C3 and C4, suggesting the heterogeneity of LSCs distributed over two clusters (Figure 1C; Supplementary Table E1) [8]. To determine differential pathways activated in these clusters, we performed nonparametric ANOVA differential gene expression analysis followed by KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis. We observed enrichment in the IL-17 signaling pathway, specifically in C3 driven by mitogen-activated protein kinase (MAPK), while hypoxia-inducible factor 1 (HIF-1) signaling was enriched in both C3 and C4 (Figure 1D). Furthermore, we observed significant enrichment of the Wnt signaling pathway and oxidative phosphorylation in C4 (the cluster that is mostly LSCs), an important source of energy for CML HSPCs. These pathway analyses point toward activation of different pathways in heterogenous LSC clones, one of the probable reasons for the failure of prior attempts targeting a single signaling pathway. Overall, these data suggest heterogeneity of LS Cs, with each cluster having a distinct gene expression profile and pathway activation compared with bystander healthy cells that can be exploited to eliminate CML LSCs.

Imatinib treatment transcriptionally rewires a preexistent TKI-persistent signature

The first line of therapy for CML patients is TKIs (imatinib); however, the LSCs have been reported to persist following this treatment [7,8,55,56]. To identify signatures unique to these persistent LSCs, we performed scRNA sequencing on CML HSPCs obtained from CML mice treated with TKIs (Figure 2A). On exposure to TKI for 4 weeks, we found that transcriptional patterns of the transformed stem and progenitor cells were altered, consisting of several genes involved in myeloid differentiation, cellular structure, and cell–cell interaction (Supplementary Figure E2A. Compared with CML HSPCs, the top genes in the CML-TKI cluster were S100A8–S100A9. By applying published transcriptomic data sets for HSCs, MPP2, MPP3, and MPP4 from healthy mice, as in Supplementary Figure E1C, we observed that the HSC transcriptional signature is underrepresented (1%) in CML-TKI HSPCs compared with WT and CML (Supplementary Figure E2B,C; Supplementary Table E1) [8]. On the contrary, CML-TKI HSPCs are overrepresented by MPP3 transcriptional signature (37%) compared with WT and CML (Supplementary Figure E2B,C; Supplementary Table E1). *k*-Means clustering of CML-TKI HSPCs identified two

clusters, suggesting less heterogeneity of CML HSPCs post-imatinib treatment (Figure 2B). Cluster 1 (T1) was transcriptionally composed (46%) of steady-state MPP3 signature while cluster 2 (T2) comprised a 39% MPP2 and ~5% HSC signature (Supplementary Figure E2D; Supplementary Table E1). Applying the CML-TKI LSC bulk RNA sequencing gene signature to the CML–TKI HSPCs revealed a strong correlation with T1 (mean = 1.05); meanwhile, the primary and expanded CML LSC signature was found enriched in only a few cells of T2 (mean = 0.46) (Figure 2C, Supplementary Figure E2, Supplementary Table E1) [8]. This suggests that the LSCs, on exposure to imatinib, transcriptionally rewire [8]. Pathway analysis on the differentially upregulated genes in TKI-resistant clusters revealed that cluster T1 was enriched in metabolic pathways such as carbon metabolism, NF-κB signaling, and HIF-1 signaling pathways (Supplementary Figure E2F). This corroborates with $CD34^+$ stem and progenitor cells obtained from TKI-treated CML patients [57-60]. Moreover, cluster T1 was also enriched in genes regulating drug metabolism, which implies reduced TKI treatment efficacy in these cells and a potential mechanism of TKI persistence in CML LSCs (Supplementary Figure E2F). These pathways provide a window of finding targets without affecting the healthy cells and suggest transcriptional reprogramming of CML LSCs on exposure to TKI [8].

To determine if a transcriptional drug-persistent clone of LSCs preexists or a clone of cells reprograms on drug exposure [61], we applied the CML–TKI LSC signature to the CML HSPCs and found that it was associated with C2 and C4 (Figures 1B and 2D; Supplementary Table E1) [8]. The LSC signature also marked C4, indicating that this HSC-like population is shared in both CML and CML–TKI. This also suggests that the TKI-persistent signature potentially preexists in the LSCs before drug treatment. Interestingly, we observed that the JAK–STAT, HIF-1, Wnt, and mTOR signaling pathways are shared among the CML-TKI LSCs as well as cluster C4 from the LSCs (Figure 2E). The JAK–STAT signaling pathway in this instance is attributed to the upregulation of AP1 transcription factors. Targeting STAT3, one of the regulators of AP1 transcription factors and the Wnt signaling pathway, has been reported to induce apoptosis of the TKI-persistent CML stem and progenitor cells [8,24,62-65]. Moreover, we also observed upregulation of cell adhesion molecules known to support and protect the CML cells from TKI treatment (Figure 2E) [65-67]. This indicates that the preexistent TKI-persistent signature seen in the LSCs becomes the predominant clone on exposure to TKI. These pathways can hence be targeted in patients who have been treated with imatinib, potentially leading to treatment-free remission. However, preclinical drug testing on LSCs specifically has been technically challenging, which is essential to identify specific targets with limited effect on healthy cells.

Ex vivo expansion of leukemic stem cells

To study LSCs with the goal of pre-clinical drug testing, we worked on expanding LSCs ex vivo since in CML, LSCs comprise ~0.01% of the whole bone marrow [5,8]. The rarity of these cells makes it difficult to obtain adequate numbers of LSCs for characterization using assays that require more than 100,000 cells and identify targetable vulnerabilities that do not affect the non-transformed hematopoietic stem cells. We adapted the PVA-based ex vivo HSC culture to expand phenotypically well-characterized CML LSCs (Figure 3A) [5,8,14,15,40]. Flow cytometry analysis revealed exponential growth of both healthy HSCs

and LSCs. After day 12, HSC growth began to plateau while LSC growth declined (Figure 3B; Supplementary E3A,B. Based on these analyses, we decided to use days 12–14 of phenotypic LSC expansion as the time point for further experiments. Moreover, we found that CD48 does not delineate progenitors from HSCs in vivo; thus, we used CD150 to define phenotypic stem cells (Figure 3C). Flow cytometry of the expanded LSCs on day 12 revealed that 22.39 ± 3.09%) of the well was Lineage^{-}Sca1^{+}cKit^{+} (LSK) leukemic cells, and 71.29 ± 6.4% of these were CD150^{+} LSCs (Figure 3C; Supplementary Figure E3C). Meanwhile, in freshly isolated CML whole bone marrow, 14.82 ± 0.62% of viable cells are LSK cells, of which 2.81 ± 0.35% are LSCs (CD150^{+}CD48^{-}) (Supplementary Figure E3D). Importantly, we observed a 460 ± 125-fold expansion of LSCs by day 12 (Figure 3D). Expansion of LSCs was significantly less than that of HSCs; however, the proliferation rates of HSCs and LSCs were similar (Figure 3E). Meanwhile, apoptosis revealed more deaths of LSCs than HSCs (Supplementary Figure E3E,), which could account for the reduced output. To further characterize the expanded LSCs, we ran flow cytometry for human and murine markers known to be expressed in CML [10,28,29]. ESAM^{+}, EPCR^{+}, and CD34 describe more primitive murine stem cells, and we see conservation of this phenotype in our cultures of both HSCs and LSCs (Figure 3F) [30,68]. Although not significantly different from the HSCs, the expanded LSCs express CD41, CD90, FcgRII/III, TNFR II, and PDL1, the former four receptors previously identified in CML, while the latter is an immune checkpoint (Figure 3F; Supplementary Figure E3F) [10-12,64,69-71]. On the other hand, we did not see marked expression of CD25, CD26, and CD105 receptors and other immune checkpoint markers such as Lag3, CTLA4, and Tim3 (Figure 3F; Supplementary Figure E3F) [28,29,67,69]. Apart from surface marker expression, we also observed transcript expression of genes important for stemness such as Ctnna1, Fzd3, Gata1, Ikfz1, Itgam, Myc, and Mycn was maintained in the expanded LSCs, similar to HSCs (Supplementary Figure E3G) [48,54,72]. In summary, we found that phenotypic LSCs can be expanded ex vivo.

Ex vivo leukemic stem cells maintain engraftment and multilineage potential

Leukemic stem cells are defined by their potential to self-renew and engraft as well as being drivers of leukemia. Thus, to test the function of expanded LSCs, we transplanted one well of 14-day expanded LSCs per sublethally irradiated recipient mouse (Figure 4A). Of the mice transplanted with expanded HSCs and LSCs, we observed 100% of mice engrafted with HSCs, while 77.8% of mice transplanted with LSCs exhibited long-term engraftment, as measured by CD45.2 expression in peripheral blood (PB) (Figure 4B; Supplementary Figure E4A [73,74]. At week 12, HSCs exhibited 21.13 ± 7.47% chimerism while LSCs exhibited 2.80 ± 1.76% chimerism in PB (Figure 4B; Supplementary Figure E4A). Mature cell screening of the PB revealed trilineage potential, with HSCs giving rise to 7.26 ± 2.18% myeloid cells (defined by Gr1^{+}CD11b^{+}) and LSCs giving rise to 8.96 ± 3.77% (Figure 4C). Although we did not see a significant difference in the myeloid cell lineage between the HSCs and LSCs, we observed a significant reduction in PB CD3^{+} T cells and B220^{+} B cells from the transplanted CML mice, as is observed in primary CML mice (Figure 4C) [5,15]. Additionally, we saw 0.41 ± 0.31% engraftment of LSCs in the BM and 2.27 ± 1.84% in the spleen as compared with 3.96 ± 1.66% of HSCs in the marrow and 10.54 ± 4.51% in the spleen (Figure 4D). Although we did not see significant myeloid skewing in the bone marrow or spleens of the mice transplanted with LSCs, we

did observe reduced lineage-negative cells or HSPCs in the bone marrow as previously described (Supplementary Figure E4B,C) [5,8]. However, five of the seven mice engrafted with LSCs survived 6 months post-transplant; splenomegaly was observed in the two mice that had died (Figure 4E). Heterogeneity of CML LSCs has been previously reported, where even though LSCs engraft, not all mice develop leukemia after primary and secondary transplants [10]. Consistently, we found that expanded LSCs retained part of their functional potential even after ex vivo expansion.

Expanded LSCs are transcriptionally similar to freshly sorted LSCs and so could be used for drug screens

Ex vivo culture forces cells to proliferate and induce cultural artifacts, which could pose potential problems for drug screens. Thus, to validate that expanded LSCs are comparable to freshly isolated LSCs, we sought to determine if these populations share transcriptional similarity using bulk RNA sequencing. Differential gene expression comparing expanded healthy HSCs and expanded LSCs revealed that genes upregulated in LSCs were significantly enriched in TNFα, TGFβ, and STAT5 signaling pathways as well as inflammatory response (Figure 5A). These pathways have been proven important for CML, where STAT5 is activated by BCR–ABL tyrosine kinase and required for initiation and maintenance of CML [25,75]. TNFα and TGFβ signaling pathways are signatures of CML stem and progenitor cells correlated with poor response to TKI treatment [12,50]. To identify transcriptional similarities and differences between expanded and freshly sorted LSCs, we compared independent differential gene lists of both LSCs using Venn analysis (Supplementary Figure E5A and Table E2,). One of the pathways differentially activated in the expanded LSCs, but not in the freshly isolated LSCs, was KRAS signaling, a known mediator of cell division while the latter had distinct activation of Notch signaling, a yet understudied pathway in CML (Supplementary Figure E5A and Table E2) [76], whereas both LSCs were enriched for reactive oxygen species (ROS) pathways. CML HSPCs are known to accumulate reactive oxygen species (ROS), leading to oxidative stress and subsequently BCR–ABL mutation-mediated drug resistance [77]. Furthermore, we found that TNFα, TGFβ, and STAT5 signaling pathways were also enriched among the 460 genes commonly expressed between freshly isolated LSCs and expanded LSCs when each was previously compared with their respective healthy controls (Figure 5B; Supplementary Figure E5A and Table E2). This suggested that the expanded LSCs are transcriptionally comparable to freshly isolated LSCs, proving that this expansion assay can be used to characterize LSCs. Moreover, the single-cell profiles and pathways are shared with the ex vivo expansion of LSCs and hence is a promising tool to validate drug targets (Supplementary Figure E5B,C and Table E1).

To test drugs with the aim of eliminating LSCs without affecting non-transformed leukemia-exposed HSCs, we established a competition assay where we sorted 50 HSCs and 50 LSCs per well of a 96-well plate (Supplementary Figure E5D). Fourteen days after expansion, we noticed that, as expected, the HSCs outcompeted CML LSCs, with ~40% of the well comprising LSCs (Supplementary Figure E5D). We exposed our competition-based expanded stem cells with imatinib treatment for 4 days to determine if our ex vivo model is reproducible in patients and primary CML murine models. We confirmed

that LSCs persisted through imatinib treatment, unless treated with a nonphysiologically high dose of 5 mmol/L (Figure 5C; Supplementary Figure E5E) as we and others had previously reported using primary CML mice as well as patient samples [8,55,56]. To validate the targets identified by our bulk and scRNA sequencing, we tested drugs for the pathways identified for LSCs. After competition-based 14-day expansion culture, we subsequently treated the cells with JAK1/2 inhibitors (AZD1480 and ruxolitinib), mTOR inhibitor (rapamycin), TGFβ inhibitor (SB 431542), MAPK inhibitor (U0126), and Wnt signaling pathway inhibitor (iCRT14) (Figure 5D, Supplementary Figure E5F) [51,78-81]. We observed that among all these treatments, only iCRT14, SB 431542, AZD1480, and ruxolitinib were significantly able to reduce the count of LSK CD150$^+$ CML cells with no difference observed in relative frequency (Figure 5D; Supplementary Figure E5F). However, AZD1480 and ruxolitinib also affected the nonmutant LSK CD150$^+$ cells, suggesting that targeting the JAK signaling pathway could potentially lead to toxic effects in patients (Figure 5D; Supplementary Figure E5F). On the other hand, inhibiting the Wnt signaling pathway, previously found to eliminate CML LSCs, would be a good target for clinical trials [63-65,82]. We thus found that ex vivo expanded LSCs can serve as a preclinical tool for testing drugs and dosages and validating drug targets.

DISCUSSION

Leukemic stem cells (LSCs) are functionally similar to HSCs, defined by their ability to initiate and propagate all lineages while self-renewing [83]. CML has a well-defined LSC population, phenotypically defined as HSCs. Even though progress has been made in CML treatment, LSCs can evade drug treatment because of their heterogeneous properties. scRNA sequencing of CML stem and progenitor cells revealed heterogeneity of LSCs with enrichment of metabolic and signaling pathways in one cluster and cytokine–chemokines in the other cluster. scRNA sequencing on imatinib-treated CML HSPCs revealed that a persistent signature preexists and undergoes transcriptional rewiring, forming a new clone after imatinib treatment. Wnt, Jak–STAT, MAPK, mTOR, and TGFβ signaling pathways are commonly upregulated in LSCs and imatinib-persistent LSCs. CML LSCs form a minor part (<0.01%) of the whole bone marrow [3-5,84]; therefore, in this study, we adapted a protocol for long-term ex vivo expansion of phenotypical and functional LSCs [40]. We found that these cultures result in an ~450- to 1000-fold expansion of LSCs and HSCs, which maintain engraftment and multilineage potential, a measure of stem cell function. Moreover, expanded LSCs share most transcriptional signatures with primary LSCs. Drug testing using our ex vivo expansion culture revealed that inhibition of Wnt and TGFβ signaling pathways is a promising target. These pathways have been previously identified in CML, thus validating our ex vivo expansion cultures. Using the primary CML transgenic mouse model as an in vivo setting and the ex vivo expansion of LSCs provides assays requiring more cells that have previously not been feasible to carry out with LSCs, such as large drug screens.

Ex vivo cultures, such as colony assays, and liquid cultures with cytokine cocktails have been used for years as functional progenitor readouts and drug testing [74]. The most widely used systems are 2-D suspension cultures and cocultures, 3-D organoids, and, more recently, organ-on-a-chip [33-38]. Although the latter two culture methods mimic a complex

in vivo system, they are technically challenging and labor intensive. On the other hand, suspension cultures are cost-effective; however, they sustain LSCs for a short time before they start differentiating, limiting the maintenance of a stemlike property. Additionally, all these cultures have been optimized for stem and progenitor cells, limiting our understanding of LSCs. Ex vivo expansion cultures are cost-effective for expanding LSCs for downstream assays. Importantly, we found that expanded LSCs retain engraftment potential and maintain hematopoiesis. This assay can also be used for clonal studies, initiating the culture with a single cell, thus also considering the heterogeneity of LSCs. This would be especially useful for human patient samples in which surface markers are still being studied to differentiate the mutant LSCs from the nonmutant HSCs [40].

Heterogeneity of LSCs is an aspect now appreciated to be responsible for transcriptional rewiring, drug persistence, and even relapse [12]. CML LSCs with higher thrombopoietin receptor expression and CD93 and CD26 expression are insensitive to TKI treatment [10,31]. On the other hand, low expression of CD7 and increased expression of elastase and proteinase 3 have been considered markers of improved patient survival [85]. Moreover, single-cell RNA sequencing analysis of human CML $CD34^+$ cells obtained at diagnosis or after TKI treatment reveals a persistent population that is quiescent and enriched in TGFβ and TNFα inflammatory signaling pathways and JAK–STAT signaling pathways [12]. However, stem cell phenotype in humans is still under study, making it difficult to extrapolate the findings to LSCs. Hence, using our transgenic CML mouse model and scRNA sequencing, we consistently found that a small subset of CML LSCs have a preexistent TKI-persistent signature. Subsequently, this preexistent clone is selected with TKI treatment and is transcriptionally rewired, leading to disease relapse and even progression to blast. This can be corroborated by our recent work reporting transcriptional and metabolic reprogramming via STAT3 signaling [8]. Additionally, we found that the transcriptional profiles of expanded CML LSCs are similar to those of the freshly sorted CML LSCs compared with their respective non-transformed controls. Drug screens inhibiting pathways commonly upregulated in the CML and persistent CML LSCs revealed that although these pathways are potential targets [52,58,59,62,78,82], not all can be carried forward for clinical trials because of the effect on nonmutant HSCs found in the same inflamed environment as the LSCs. In this study, we thus found that ex vivo expansion is a preclinical tool for identifying and validating drug targets before moving to more complex, time-intensive, and expensive modeling.

These expansion cultures can be used for high-throughput proteomic screens such as Western blot and immunoprecipitation assays using LSCs as transcripts do not always correlate to the presence of functional protein. Moreover, we can now use LSCs to expand scalable cells for functional genomic studies, such as knockouts and knockins, and metabolic assays, such as seahorse and metabolite flux tracing, that require many more cells. Additionally, these expansion cultures can also be optimized for human LSCs to validate results obtained from murine LSCs. Ex vivo expansion culture can hence be used to answer questions that have been impractical to conduct with rare LSCs that share phenotypic characteristics with HSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Samuel L. Wolock and Dr. Virginia Camacho for their help with data collection and feedback, respectively. This project was supported by National Institutes of Health (NIH) Grant 1PO1HL131477; startup funds from the Division of Hematology/Oncology at the University of Alabama at Birmingham (UAB); American Cancer Society–Institutional Research Grant Junior Faculty Development Grant (2019); the American Society of Hematology Bridge Grant (2018); and Leukemia Research Funding (2019). Flow cytometry was supported by the UAB Center for AIDS Research (CFAR), an NIH-funded program (P30 AI027767-31).

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HIGHLIGHTS

- A means to expand leukemic stem cells from murine CML where they phenocopy their primary HSCs is described.
- Both transcriptional and functional similarities to primary LSCs exist that lead to identification of targetable pathways, replicating other's findings and suggesting additional pathways for potential therapeutic intervention.
- This approach provides a unique means to obtain significant numbers of CML LSCs and could assist in drug screens and mapping of targetable pathways in the future.

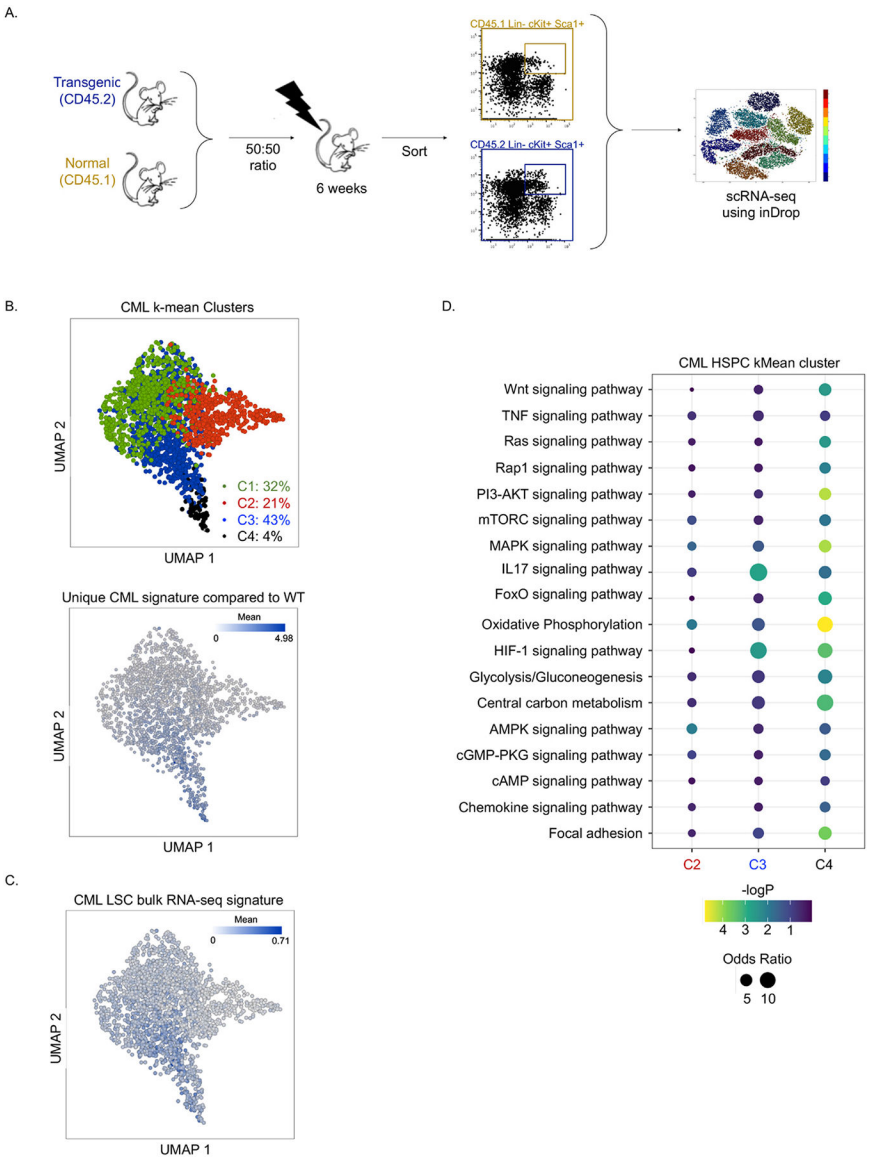

Figure 1.

Heterogenous chronic myeloid leukemia (CML) hematopoietic stem and progenitor cells (HSPCs) upregulate distinct pathways compared with leukemia-exposed HSPCs. **(A)** Experimental design for small conditional (sc)RNA sequencing on $Lin^{-}cKit^{+}Sca1^{+}$ (LSK) stem and progenitor cells (HSPCs) obtained from transplanted CML mice. **(B)** *k*-Means clustering of CML HSPCs (top) and uniform manifold approximation and projection (UMAP) depicting expression of differentially upregulated CML transcriptional signatures compared with leukemia-exposed HSPCs (bottom). See Supplementary Table E1. **(C)** UMAP depicting expression of differentially upregulated bulk RNA sequencing transcriptional signature of CML leukemic stem cells (LSCs) applied to CML HSPC scRNA sequencing k-means clusters. **(D)** KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis for genes uniquely expressed in clusters C2, C3, and C4 obtained by nonparametric analysis of variance analysis. See Supplementary Figure E1.

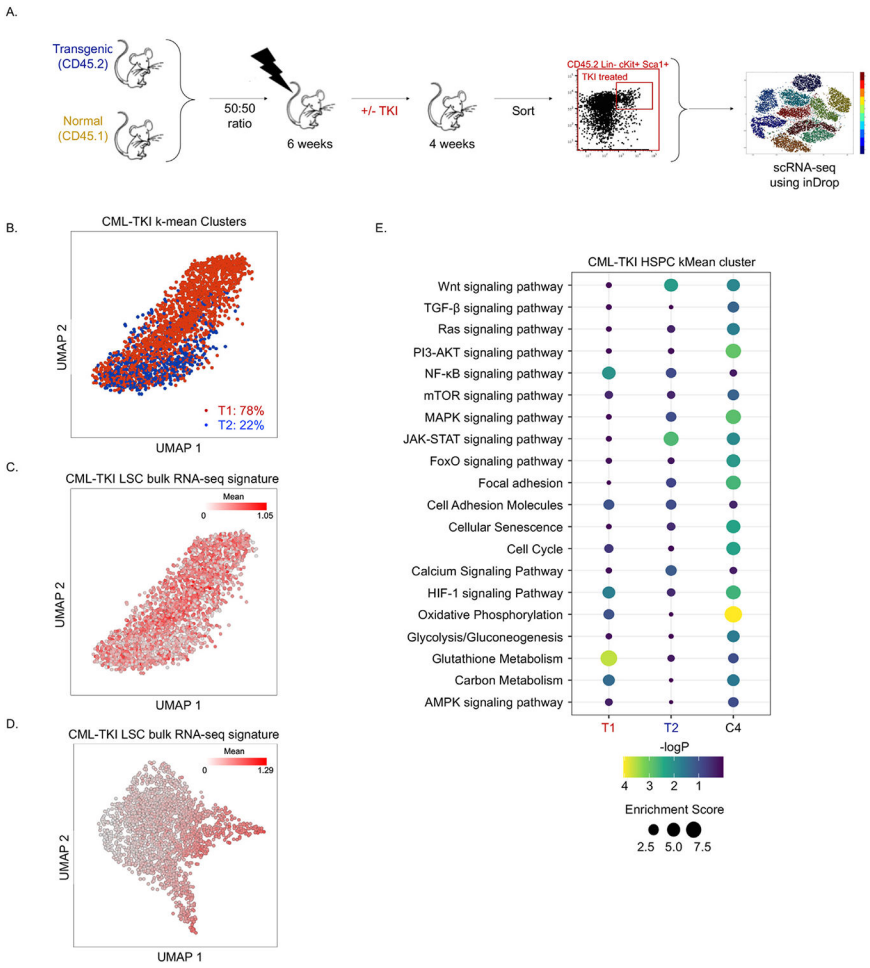

Figure 2.

A tyrosine kinase inhibitor (TKI)-persistent transcriptional leukemic stem cell (LSC) signature preexists in chronic myeloid leukemia (CML) hematopoietic stem and progenitor cells (HSPCs). **(A)** Experimental design for scRNA sequencing on HSPCs obtained from transplanted CML mice treated with 200 mg/kg imatinib for 4 weeks. **(B)** k-Means clustering of CML-TKI HSPCs. **(C)** Uniform manifold approximation and projection (UMAP) depicting expression of differentially upregulated bulk RNA sequencing transcriptional signature of CML–TKI LSCs applied to CML-TKI HSPC small conditional (sc) RNA sequencing *k*-means clusters. See Supplementary Figure E1. **(D)** UMAP depicting expression of differentially upregulated bulk RNA sequencing transcriptional signature of CML–TKI LSCs applied to CML HSPC scRNA sequencing *k*-means clusters. See also Supplementary Table E1. **(E)** KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis for genes uniquely expressed in CML–TKI clusters T1 and T2 and CML cluster C4. See Supplementary Figure E2.

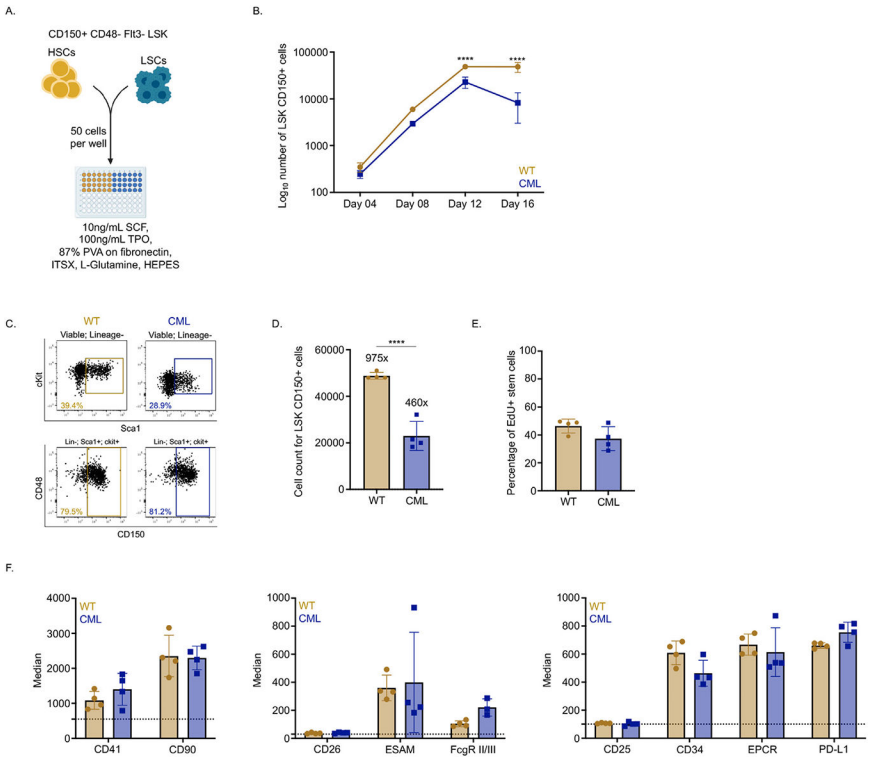

Figure 3 .

Leukemic stem cells (LSCs) maintain stem cell phenotype on ex vivo expansion. **(A)** Experimental layout for ex vivo expansion of normal hematopoietic stem cells (HSCs) and chronic myeloid leukemia (CML) LSCs. **(B)** Growth curve of HSCs and LSCs over 2 weeks. Cultures were initiated with 50 cells, and $Lin^{-}cKit^{+}Sca1^{+}$ (LSK) $CD150^{+}$ cells were counted using flow cytometry-based counting beads every 4 days. **(C)** Representative flow cytometry dotplot of LSK and LSK $CD150^{+}$ cells from ex vivo expanded HSC and LSC cultures. The values represent percentages of parent. **(D)** Total number of $CD150^{+}$ LSK cells obtained after 12 days of ex vivo expansion of 50 HSCs and LSCs. **(E)** Percentage EdU incorporation over 4 hours in LSK $CD150^{+}$ cells after 12 days of expansion of 50 HSCs and LSCs. **(F)** Median expression of stem cell surface markers on ex vivo expanded HSCs and LSCs after day 12. The data are means ± SEM representative of three independent experiments. An unpaired Student t test or two-way analysis of variance was used to determine statistical significance with Tukey's multiple comparison. p Values < 0.05 were considered to indicate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns = not significant. See also Supplementary Figure E3.

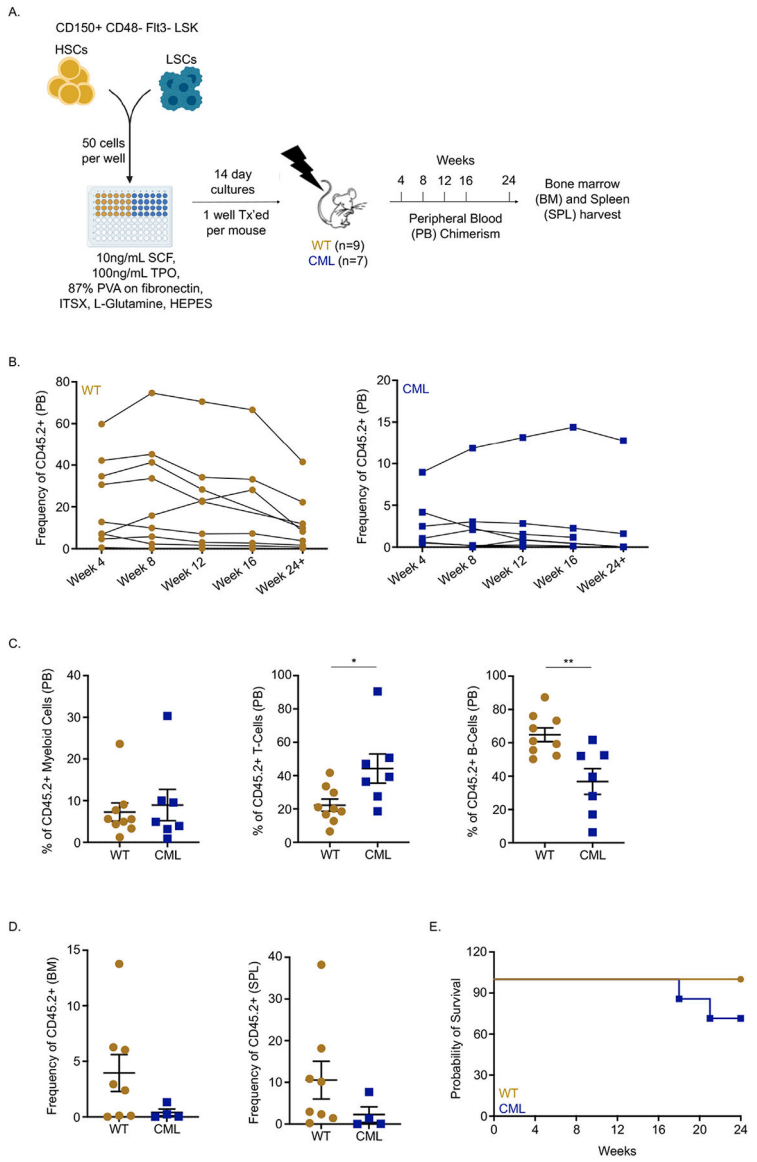

Figure 4.

Ex vivo expanded leukemic stem cells (LSCs) engraft and reconstitute hematopoiesis. **(A)** Schematic of the transplant experiment carried out after 14 days of expansion of HSCs and LSCs ($n = 9$ for the WT and $n = 7$ for the CML transplants). **(B)** Line graph tracking frequency of $CD45.2^+$ cells in the peripheral blood obtained every 4 weeks posttransplant. **(C)** Frequency of myeloid, T, and B cells in the $CD45.2^+$ fraction of peripheral blood obtained 12 weeks posttransplant. **(D)** Frequency of $CD45.2^+$ cells in the bone marrow and spleen obtained 24 weeks post-transplant. **(E)** Survival probability of mice tracked for 6 months transplanted with ex vivo expanded HSCs and LSCs. The data are means ± SEM representative of two independent experiments. A Student *t* test or two-way analysis of variance was used to determine statistical significance with Tukey's multiple comparison. *p* values < 0.05 were considered to indicate statistical significance. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. ns = not significant. See Supplementary Figure E4.

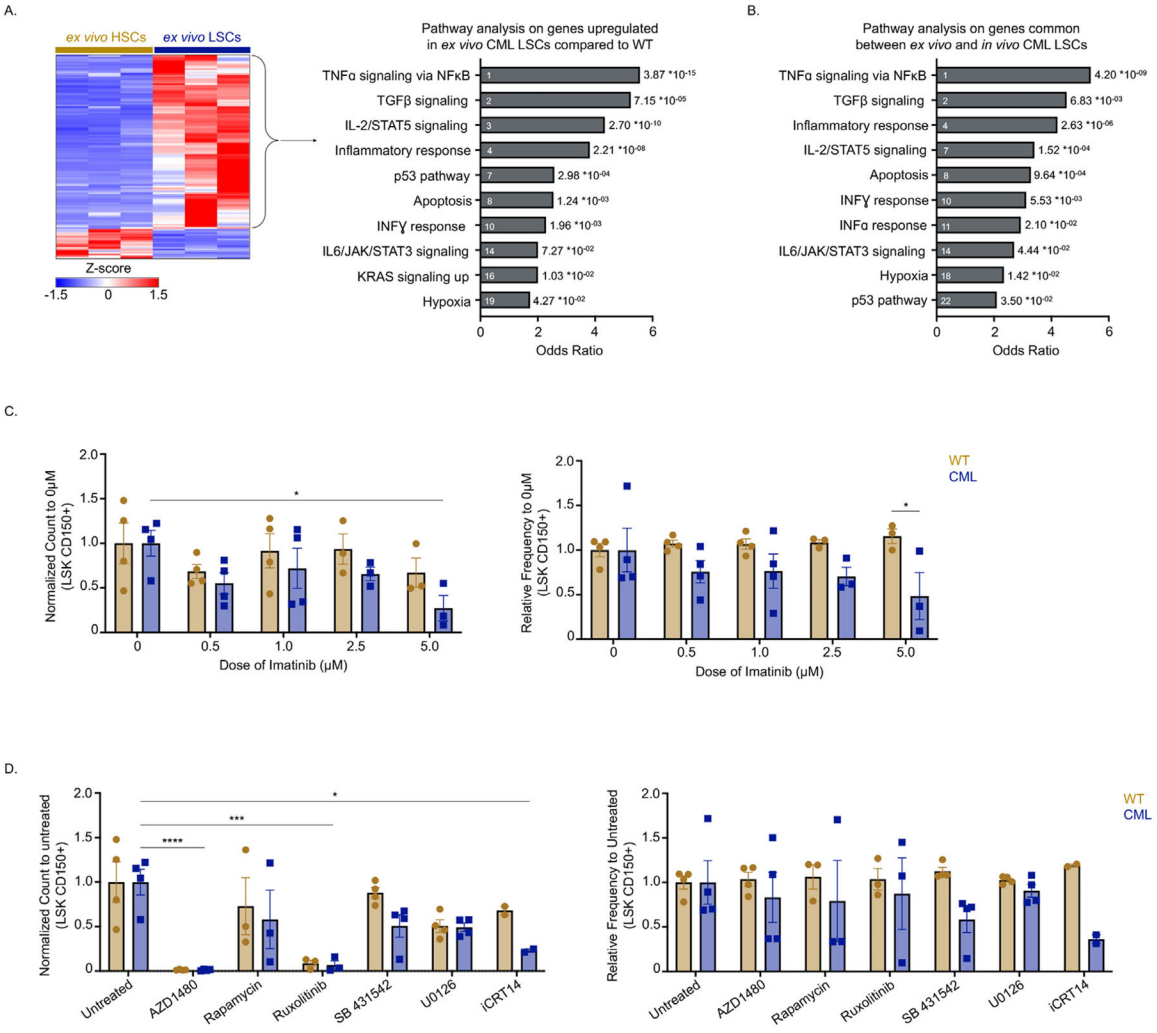

Figure 5.

Drug screens on expanded leukemic stem cells (LSCs), which are transcriptionally similar to freshly isolated LSCs. **(A)** Hallmark pathway analysis of differentially upregulated bulk RNA sequencing transcriptional signature of ex vivo expanded chronic myeloid leukemia (CML) $Lin^{-}cKit^{+}Sca1^{+}$ (LSK) $CD150^{+}$ cells compared with wild type (WT). See Supplementary Table E1. **(B)** Hallmark pathway analysis of genes common to differentially upregulated bulk RNA sequencing transcriptional signature of ex vivo expanded CML LSK $CD150^{+}$ cells and CML LSCs. See Supplementary Table E2. **(C)** Normalized count (left) and relative frequency (right) of LSK $CD150^{+}$ cells obtained by flow cytometry after 4-day treatment of 14-day expanded hematopoietic stem cells (HSCs) and LSCs with mentioned doses of imatinib. **(D)** Normalized count (left) and relative frequency (right) of LSK $CD150^{+}$ cells obtained by flow cytometry after 4-day treatment of 14-day-expanded HSCs and LSCs with 2 μmol/L AZD148, 2 μmol/L ruxolitinib, 20 nmol/L rapamycin, 2 μmol/L SB 431542, 5 μmol/L U0126, and 20 μmol/L iCRT14. The data are means ± SEM and are representative of three independent experiments **(C,D)**. An unpaired Student *t* test or two-way analysis of variance was used to determine statistical significance with Tukey's multiple comparison. *p*

Values < 0.05 were considered to indicate statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns = not significant. See Supplementary Figure E5.