**Plasticity of B-acute lymphoblastic leukemia stem cells**

Vivian Morris1+, Dahai Wang2+,William Marion1, Travis Hughes3-5, Patricia Sousa1, Taku Harada6, Shannan Ho Sui7, Sergey Naumenko7, Jeremie Kalfon4, Prerana Sensharma1,2, Renan Vinicius da Silva8, Yana Pikman5,6, Marian Harris5,9, Maxim Pimkin4,5,6, Stuart H. Orkin5,6,10, Alex K. Shalek3,4, Trista E. North1,5, George Q. Daley1,5, Edroaldo Lummertz da Rocha11†, R. Grant Rowe1,2,5,6†\*

1Stem Cell Program, Boston Children’s Hospital, Boston, MA 02115 USA

2Stem Cell Transplantation Program, Department of Hematology, Boston Children’s Hospital, Boston, MA 02115 USA

3Institute for Medical Engineering and Science, Department of Chemistry, and Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; Ragon Institute for MGH, MIT, and Harvard, Cambridge, MA 02139

4Broad Institute of MIT and Harvard, Cambridge, MA 02142

5Harvard Medical School, Boston MA 02115 USA

6Cancer and Blood Disorders Center, Dana-Farber Cancer Institute and Boston Children’s Hospital, Boston, MA 02115 USA

7Harvard Chan Bioinformatics Core, Harvard T.H. Chan School of Public Health, Boston, MA 02115

8Graduate Program of Pharmacology, Center for Biological Sciences, Federal University of Santa Catarina, Florianópolis, SC, 88040-900, Brazil

9Department of Pathology, Boston Children’s Hospital, Boston, MA 02115 USA

10Howard Hughes Medical Institute, Boston MA 02115 USA

11Department of Microbiology, Immunology and Parasitology, Federal University of Santa Catarina, Florianópolis, SC, 88040-900, Brazil

+†Equal contribution

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Correspondence

R. Grant Rowe MD PhD

Karp Family Research Building 7

1 Blackfan Circle

Boston MA 02115

[Grant\_rowe@dfci.harvard.edu](mailto:Grant_rowe@dfci.harvard.edu)

Phone: 617-919-6288

**SUMMARY**

High-risk forms of B-acute lymphoblastic leukemia remain a therapeutic challenge. Leukemia stem cells (LSCs) self-renew to drive leukemic growth and spark relapse and therefore have been the subject of intensive investigation. Here, we aimed to better understand LSCs in high-risk B-ALL by using single-cell transcriptomics and quantitative xenotransplantation. Although we identify multipotent self-renewing LSCs in human *MLL*-rearranged B-ALL, we observe that LSCs emerge from more differentiated blast populations to replenish the entire cellular diversity. We find that activation of MYC drives this dedifferentiation process, and, that relative to most blasts which depend on oxidative phosphorylation, LSCs adopt a hypoxic, glycolytic state that is a potential therapeutic vulnerability. These findings highlight the plasticity of high-riskB-ALL LSCs, explain the aggressive nature of this form of leukemia and provide a rationale for targeting metabolism.

**INTRODUCTION**

B-acute lymphoblastic leukemia (B-ALL) is heterogeneous, with certain genetic subtypes bearing poor prognosis despite the great progress made over the preceding decades in deriving otherwise effective chemotherapy regimens. B-ALL with rearrangement of the *MLL* locus (*MLL*-r) constitutes about 80% of B-ALL of infancy with incidence diminishing in childhood but later increasing in adulthood (Boissel and Baruchel, 2018). *MLL*-r B-ALL is a very unfavorable form of B-ALL, often presenting with high leukemic burden, corticosteroid resistance, and central nervous system infiltration contributing to poor long-term outcomes (Pieters et al., 2019; Pieters et al., 2007). This aggressive clinical behavior is associated with distinctive underlying biology including coexpression of myeloid markers and a capacity to undergo a B-lymphoid-to-myeloid lineage switch (Orbach et al., 2013; Pieters et al., 2007; Rossi et al., 2012). Based on these clinical observations, we hypothesized that the aggressive nature of human *MLL*-r B-ALL is underpinned by a high content of LSCs with undifferentiated hematopoietic stem cell (HSC)/multipotent progenitor (MPP)-like multipotency programs.

LSCs possess the capability to fully reconstitute fulminant leukemia at relapse, or, experimentally, upon xenotransplantation. This definition was initially formed based on the foundational observation that only a specific minority of human acute myeloid leukemia (AML) cells could sustainably engraft immunodeficient mice, establishing engraftment in xenotransplantation as the gold standard to detect LSCs (Lapidot et al., 1994). Through the lens of this model, LSCs have been defined in many forms of leukemia (Aoki et al., 2015; Bonnet and Dick, 1997; Cobaleda et al., 2000; Cox et al., 2004; Kong et al., 2008; Somervaille and Cleary, 2006). Although LSCs have been previously accepted to be primitive and rare, recent advances suggest that such paradigms of LSC biology might be subject to revision. The use of improved immunodeficient mouse strains with heightened sensitivity for human stem cell engraftment has revealed that LSCs often show heterogeneity in both their oncogenetics and differentiation state (Ho et al., 2016; McKenzie et al., 2019; Sarry et al., 2011). These findings could explain the limited clinical success of interventions targeting LSCs as has been suggested in solid tumors where stem cells often show phenotypic plasticity (Gupta et al., 2019; Pollyea and Jordan, 2017). Although such findings have led to reassessment of classical models of leukemic ontogeny, it remains generally accepted that leukemia cells are functionally heterogeneous with variable xenotransplantation capacities. Since the frequency of engraftable cells within individual leukemias – a readout of LSC content - is reproducibly of prognostic importance, better understanding of mechanisms of LSC heterogeneity has the potential to lead to the development of new therapies (Monaco et al., 2004; Paczulla et al., 2017).

Here, we used single cell RNA sequencing (scRNA-seq) combined with xenotransplantation assays to define the cellular diversity of *MLL-r* B-ALL. We report that the LSC state in B-ALL is plastic. Although LSCs are enriched in the most phenotypically primitive fraction of *MLL*-r B-ALL, LSCs can emerge from more differentiated, relatively LSC-depleted blast populations to reconstitute the full leukemic cellular diversity. We find that MYC signaling drives upward reconstitution of leukemic diversity from the most phenotypically differentiated LSCs. *MLL*-r B-ALL cells are heterogeneous with respect to their metabolic states, with LSCs maintaining a glycolytic phenotype compared to more differentiated blasts, and that targeting cellular metabolic pathways can impact differentiation state and LSC function. Together, our findings define new mechanisms of LSC heterogeneity and plasticity and advance a new candidate approach to therapy for high-risk forms of ALL.

**RESULTS**

**Xenotransplantation model of *MLL*-r B-ALL with B-lymphoid/myeloid multipotency**

The ability to switch lineage from the B-lymphoid to myeloid lineage at relapse suggests that *MLL*-r B-ALL LSCs possess lympho-myeloid multipotency characteristic of primitive HSC/multipotent progenitor (MPP) identity (Pieters et al., 2007; Rossi et al., 2012). We therefore aimed to elicit both B-lymphoid and myeloid differentiation at the single-cell level. To this end, we employed early passage patient derived xenografts and primary patient cells (Table S1)(Townsend et al., 2016). We defined the mutational complement of these specimens (Table S1)(Kluk et al., 2016). Upon xenotransplantation, human B-ALL cells infiltrate the liver, spleen, lymph nodes, and central nervous system of unconditioned NOD.Cg-*PrkdcscidIl2rgtm1Wjl* (NSG) recipient mice, recapitulating the clinical behavior of *MLL*-r B-ALL in humans (Fig. S1A). *MLL*-r B-ALL cells nearly uniformly express the B-cell marker CD19 with rare cells expressing the myeloid marker CD33 while maintaining the morphology of immature lymphoblasts (Fig. S1B). Following culture on pro-myeloid MS5 stromal cells, cells differentiated toward the myeloid lineage with most cells expressing surface CD33 and showing primitive myeloid morphology (Fig. S1C)(Doulatov et al., 2010; Issaad et al., 1993). We confirmed that single cells possess multipotency in this system (Fig. S1D-E). These results indicate B-lymphoid/myeloid multipotency consistent with HSC/MPP-like programs in *MLL*-r B-ALL.

**Single cell RNA sequencing identifies candidate LSCs in *MLL*-r B-ALL**

To gain further insight into LSCs, we performed single-cell RNA sequencing (scRNA-seq) on two human *MLL*-r B-ALL specimens (Gierahn et al., 2017; Klein et al., 2015). We obtained 5,153 viable, human CD45+ single cells from leukemia 1 (*MLL-AF4*, primary patient peripheral blood blasts, sorted based on CD45-low phenotype and forward/side scatter profile, inDrop platform) and 6,230 cells from leukemia 2 (*MLL-ENL*, early passage xenograft, SeqWell platform), for analysis after performing quality controls (see Methods). In leukemia 1, unsupervised graph-based clustering identified 14 transcriptional groups with unique signatures (compared with 9 clusters in leukemia 2), which were visualized using t-stochastic neighbor embedding (t-SNE; Fig. 1A, Fig. S2A, D). We used the SingleCellNet algorithm to compare each subpopulation to normal hematopoietic cell benchmarks and found that most cells classified strongly as pro-B cells, indicating that the observed transcriptional heterogeneity is independent of the broader leukemic differentiation state (Fig. S2B, G)(Tan and Cahan, 2019; van Galen et al., 2019). We next used the StemID algorithm to assign a multipotency label to each cell (Grun et al., 2016). In this leukemia, we found that clusters 1 and 9 were most highly enriched in putative multipotent cells, with such cells found only rarely in other clusters (Fig. 1B). Consistent with this prediction, we also found that a validated primitive HSC transcriptional signature was enriched in clusters containing multipotent cells (Fig. 1C)(Ivanova et al., 2002). We observed similar results in leukemia 2 (Fig. S2E-G).

To define the differentiation state of candidate LSC-enriched clusters, we analyzed the expression of cell surface markers in our datasets. We found that the primitive hematopoietic cell marker CD34 as well as the cancer stem cell marker CD44 were expressed in the multipotency-enriched cluster 1 (Fig. 1D)(Al-Hajj et al., 2003). These cells generally did not express the lineage-restricted progenitor marker CD38, the committed B-cell markers CD86, CD79A, and CD79B, or the myeloid markers CD33 and CD58 (Fig. 1D). This suggested a relatively dedifferentiated state in cluster 1 (Doulatov et al., 2012). Given this finding and considering the multipotency programs of *MLL*-r B-ALL, we analyzed expression of CD34 and CD38 as well as the additional human HSPC markers CD90 and CD45RA by flow cytometry. We found that *MLL*-r B-ALLs contained three distinguishable populations that we designated based on their surface immunophenotype: rare, undifferentiated CD34+ CD38- CD90- CD45RA+ cells (34+38-), more abundant CD34+ CD38+ CD90- CD45RA+ cells (34+38+), and the remaining CD34- blasts, all of which could be readily sorted (34-; Fig. 1E-F, Fig. S2C,H).

**Functional cellular heterogeneity in *MLL*-r B-ALL**

Since LSCs are typically quiescent relative to other leukemic cells, we first analyzed the cell cycle status of each population (Guan et al., 2003; Saito et al., 2010; Terpstra et al., 1996). We found that the primitive 34+38- fraction contained the highest proportion of cells in G0 phase (Fig. 2A-B and Fig. S3A-B). Next, we used MS5 assays to quantify leukemic progenitors, finding that more actively cycling 34+38- cells possessed the highest frequency of clonogenic cells (Fig. 2C, Fig. S3C). We used limiting dilution xenotransplantation to quantify LSCs (Hu and Smyth, 2009). Using the onset of terminal leukemia as our endpoint, as we initially hypothesized, we observed that unfractionated human *MLL*-r B-ALL possessed a remarkably high frequency of LSCs (1/426 cells (1/1417 – 1/128 95% confidence interval) compared to acute myeloid leukemia (AML; 347-fold more frequent than the most efficiently engrafting AML reported previously in NSG mice (Sarry et al., 2011)). We found that the 34+38- population caused leukemia with the shortest latency and contained the highest frequency of LSCs in limiting dilution analysis, with human bone marrow chimerism correlating with disease status at the endpoint (Fig. 2D-F, Fig. S3D-H**,** Fig. S4A-B; Table S2). We corroborated this finding using primary cells sorted from patient peripheral blood (Fig. S4C-D). In line with this finding, using RNA sequencing (RNA-seq) of FACS-purified populations, we found that the 34+38- fraction bore the strongest primitive HSC signature (Fig. S4E-F).

**Plasticity of *MLL*-r B-ALL LSCs**

Although the primitive 34+38- fraction contained abundant LSCs, we observed that terminal leukemia developed in recipients of the more differentiated 34+38+ and 34- fractions, albeit at lower efficiency (Fig. 2E-F, Fig. S3D-F). When we compared the cellular content of leukemia derived from 34+38-, 34+38+, and 34- cells, we found that the full diversity emerged from each source at terminal disease (Fig. 3A). Moreover, by secondary xenotransplantation, we found that leukemia derived from each source contained serially transplantable, self-renewing LSCs (Fig. 3B). To directly observe this plasticity during leukemic reconstitution in vivo, we transplanted purified 34- cells into NSG mice and isolated bone marrow at defined time points prior to the expected onset of terminal leukemia, finding that 34- cells gradually replenished the CD34+ compartment (Fig. 3C).

Next, we cultured sorted populations of 34+38-, 34+38+, and 34- cells on MS5 stroma and monitored population flux. We observed that the CD34+ content of 34+38+ and 34+38- cultures decreased such that the proportion of CD34+ cells was similar in cultures from each of the three input populations by the end of the 15-day culture period (Fig. S5). To exclude an artifactual effect of the patient-derived xenograft model, we used cells isolated directly from patient peripheral blood corresponding to the xenografts used in this study and observed similar LSC plasticity in vivo and in culture down to the single-cell level, where we also observed latent myeloid potential in single-cell clones from all phenotypic populations (Fig. S6).

To better track surface marker profile and primitive stem cell signatures, we used single cell cellular indexing of transcriptomes by sequencing (CITE-seq). With this approach, we found that cells with primitive HSPC signatures were present throughout the cellular ontogeny, suggesting that even cells with the most differentiated phenotypes can bear latent LSC signatures (Fig. 3D). Together, these data show that engraftable LSCs are plastic, consistent with bidirectional hierarchical organization of LSC ontogeny.

**Activation of MYC during leukemic reconstitution from CD34- LSCs**

To investigate mechanisms of LSC plasticity, we performed RNA-seq on the most differentiated 34- cells – which are relatively LSC-deplete - either transplanted alone and actively dedifferentiating or growing in vivo in the presence of 34+38+ and 34+38- cells (Fig. 4A). Despite their identical immunophenotype, regenerating 34- cells (34-r) and steady-state 34- cells bore divergent transcriptional profiles (Fig. 4B). We found that 34-r cells activated signatures of oxidative phosphorylation and MYC target gene expression relative to 34- cells (Fig. 4C). MYC signatures are enriched in multipotent scRNA-seq clusters, suggesting that 34-r cells are activating programs existing in LSCs (Fig. 4D). By performing chromatin immunoprecipitation with sequencing (ChIP-seq) in *MLL*-rearranged B-ALL cell lines MV4;11 and RS4;11, we found that transcripts of genes bound by MYC were enriched in 34-r cells (Fig. 4E).

To determine MYC’s role in LSC plasticity, we treated cells with IBET-151, an inhibitor of MYC activity known to impair leukemic growth and monitored population flux in vitro and in vivo (Dawson et al., 2011). As expected, we found that IBET-151 diminished MYC levels (Fig. 4F). Treatment of 34- cells either undergoing dedifferentiation in culture or in vivo with IBET-151 blocked the acquisition of the CD34+ state, indicative of a role for MYC in mediating LSC plasticity (Fig. 4G-J). We next generated a doxycycline-inducible, GFP-tagged vector for ectopic MYC expression (Fig. 4K). Following transduction and sorting of GFP+ cells, culture in the presence of doxycycline for 14 days preserved CD34+ content (Fig. 4L). These results implicate MYC in LSC plasticity in *MLL*-r B-ALL.

**Inhibition of oxidative metabolism promotes leukemic dedifferentiation and recruitment of LSCs**

LSCs have been reported to rely on mitochondrial oxidative phosphorylation (Jones et al., 2018; Lagadinou et al., 2013). Using the Mitotracker dye to quantify mitochondrial activity in *MLL*-r B-ALL cells, we confirmed that 34-r cells contained a higher level of mitochondrial activity compared to 34- cells with a trend toward higher mitochondrial numbers (Fig. 5A-B, Fig. S7A-B). We also found that MYC modulated mitochondrial metabolism in *MLL*-r B-ALL cells (Fig. S7C-I). Therefore, to determine if targeting oxidative metabolism affected emergence of *MLL*-r B-ALL LSCs, we used tigecycline, an inhibitor of mitochondrial translation, which has been advanced as a candidate therapeutic in AML and chronic myeloid leukemia (CML) targeting LSCs (Kuntz et al., 2017; Skrtic et al., 2011). We found that tigecycline diminished viability of *MLL*-r B-ALL cells in culture (Fig. 5C-D). We next treated 34- cells actively dedifferentiating in vivo with a course of tigecycline, finding that this intervention decreased disease burden (Fig. 5E-H). However, we were surprised to observe that, at completion of the treatment course, tigecycline promoted dedifferentiation with emergence of CD34+ cells (Fig. 5I-J). By following xenotransplanted mice for survival, we found that despite the early effect of tigecycline on chimerism, there was no survival benefit (Fig. 5K). Secondary transplantation of vehicle- or tigecycline-treated leukemia revealed that tigecycline treatment promoted LSC emergence from 34- cells in vivo (Fig. 5L).

***MLL*-r B-ALL LSCs maintain a glycolytic state**

Since blockade of mitochondrial translation facilitates leukemic dedifferentiation and LSC emergence, we next sought to understand the energy state of 34+38- LSC-enriched cells. Using GSEA, we found that 34+38- cells bore glycolytic signatures relative to other populations (Fig. 6A). 34+38- cells showed lower mitochondrial activity compared to 34- cells (Fig. 6B). Relative to other populations, 34+38- cells tended to express higher level of core glycolysis transcripts (Fig. 6C). 34+38- cells were also enriched for signatures of hypoxia, and hypoxia preserved of 34+38- cells in culture (Fig. 6D-E). Consistent with these findings, in vivo, 34+38- bore lower levels of reactive oxygen species compared to other fractions (Fig. 6F-G). Using ChIP-seq, we found that MYC-bound peaks were enriched in gene ontology terms related to both oxidative phosphorylation and the hypoxic response and that MYC directly bound key glycolysis-associated loci (Fig. 6H-I)(Fang et al., 2019). Inhibiting MYC with IBET-151 impaired glycolytic activity in MV4;11 cells (Fig. 6J-K). Together, these data indicate that the LSC-enriched 34+38- fraction maintains a hypoxic, glycolytic state, and that MYC maintains glycolytic activity.

**Inhibiting glycolysis to target *MLL*-r B-ALL LSCs**

Inhibition of glycolysis has been an approach to cancer therapy as a means of targeting the Warburg effect, and so we next examined glycolysis as a target in *MLL*-r B-ALL. 3-bromopyruvate (3-BP) is a direct inhibitor of glycolysis (Chapiro et al., 2014). 3-BP decreases viability of *MLL*-r B-ALL PDX cells, and both MV4;11 and RS4;11 show sensitivity to 3-BP in culture (Fig. 7A-B). Dichloroacetate (DCA) is an inhibitor of pyruvate dehydrogenase kinase that serves to shunt pyruvate into the TCA cycle (Bonnet et al., 2007). As expected, DCA increased oxidative metabolism in MV4;11 cells (Fig. 7C). Treatment of MV4;11 with either DCA or 3-BP effectively diminished leukemic progenitor cells in colony formation assays (Fig. 7D). In 34+38- *MLL*-r B-ALL cells, DCA increased mitochondrial membrane potential and active mitochondria, consistent with loss of a quiescent stem cell state (Fig. 7E)(Nakamura-Ishizu et al., 2020). DCA decreased the viability of *MLL*-r B-ALL cells selectively under hypoxic conditions where cells would be more reliant on glycolysis and also promoted differentiation to the CD34- state (Fig. 7F-G). Treatment of mice xenografted with 34+38- *MLL*-r B-ALL cells with DCA resulted in a significant survival benefit (Fig. 7H). We also found that treatment of *MLL*-r B-ALL cells with 3-BP ex vivo could diminish LSCs (Fig. 7I). Since LSCs also showed signatures of hypoxia, we treated xenografts with the HIF1α inhibitor echinomycin (Wang et al., 2011). We found that two weeks of treatment diminished overall disease burden and CD34+ content in vivo (Fig. 7J-L). Together, these findings indicate the possibility of targeting glycolysis as a means of depleting LSCs in *MLL*-r B-ALL.

**DISCUSSION**

In this study, we aimed to understand LSCs in high-riskB-ALL, and, using LSC-rich *MLL*-r B-ALL as a model, begin to gain further insight into essential properties of LSCs more broadly. Although the heterogeneous cells of *MLL*-r B-ALL follow a hierarchical organization in that LSCs are enriched in the most undifferentiated fraction, engraftable, self-renewing cells emerge from all levels of this hierarchy. We find that LSCs in *MLL*-r B-ALL bear hallmarks of cancer stem cells - some of which were until recently thought to be specific to solid tumors - including phenotypic and metabolic plasticity (Gupta et al., 2019; Jones et al., 2018; Lee et al., 2017). Our findings uncover new aspects of the biology underlying the aggressive clinical behavior and unfavorable outcomes of this high-risk form of B-ALL, and, more broadly, provide mechanistic insight into LSC plasticity.

Since the initial supposition of LSCs, models of leukemic ontogeny have been built upon paradigms of normal hematopoiesis (Bonnet and Dick, 1997; Kreso and Dick, 2014; Pollyea and Jordan, 2017). Based on seminal studies performed in AML, the ‘classic’ conclusions that LSCs are both uniform and rare akin to normal HSCs have gradually been revised (Pollyea and Jordan, 2017). At first glance, our data would appear to be consistent with this ‘classic’ model in that we found distinct populations stratified from least to most differentiated based on transcriptional profiles, immunophenotypes and engraftment capacities. In B-ALL, though LSCs have been reported in multiple phenotypic populations, their relative frequency in each of these apparent differentiation states has not previously been quantified (Aoki et al., 2015; Bardini et al., 2015; Kong et al., 2008; Lapidot et al., 1994; le Viseur et al., 2008). Use of more immunodeficient mouse models with heightened sensitivity for human engraftment has suggested plasticity in AML LSCs similar to our findings, although the molecular mechanisms conferring this plasticity were unclear (Ng et al., 2016; Sarry et al., 2011). The consensus is emerging that across many forms of leukemia, leukemic ontogeny departs from the strict stratification and unidirectional differentiation of healthy hematopoiesis. Despite LSC paradigms undergoing constant revision, the notion that engraftable LSC content predicts leukemia outcome highlights the importance of LSCs in leukemic pathobiology and provides an ongoing impetus for investigation of the determinants of their state (Monaco et al., 2004; Paczulla et al., 2017).

Our data reinforce the notion that LSC identity can be fluid, as has been described in stem cells of solid tumors and sporadically in LSCs (Fumagalli et al., 2020; Gupta et al., 2019). Use of NSG mice led to the first evidence that AML LSCs within CD34- fractions could reconstitute the full cellular diversity of the original AML specimen, although the mechanism of this cell state interconversion was unknown (Sarry et al., 2011). A recent study reported that acute promyelocytic leukemia cells undergoing differentiation driven by retinoic acid could re-acquire the LSC state following drug withdrawal (McKenzie et al., 2019). In B-ALL, we detected LSC emergence from all immunophenotypic populations - even the most differentiated CD34- blasts – with the capability of reconstituting the full cellular diversity of the original leukemia. Moreover, using CITE-seq, we found that cells bearing stem cell transcriptional signatures existed in all phenotypic states, demonstrative of discordance of surface marker profiles and stem cell signatures.

Our efforts to uncover mechanisms of LSC plasticity led us to MYC signaling and implicated metabolic rewiring in dedifferentiation of 34- cells. Oncogenic MYC activity is a downstream effector of transforming MLL translocations and mitochondrial turnover is important for LSC homeostasis (Dawson et al., 2011; Pei et al., 2018). MYC can drive both mitochondrial oxidative metabolism and glycolysis in cancer (Fang et al., 2019; Lee et al., 2017). We impaired MYC activity through inhibition of BET bromodomain proteins, finding that this intervention perturbed cellular metabolism and blocked the conversion of CD34- to CD34+ cells (Bardini et al., 2018; Dawson et al., 2011; Delmore et al., 2011). Although we found that direct inhibition of mitochondrial translation transiently impeded growth of cells in vivo, this intervention promoted the emergence of dedifferentiated CD34+ cells containing transplantable LSCs, suggesting that inhibition of oxidative phosphorylation facilitates dedifferentiation to the quiescent, glycolytic LSC state. Our data indicate that the LSCs are quiescent and glycolytic with low levels of oxidative phosphorylation and reactive oxygen species compared to other populations. In AML, LSCs also contain relatively low levels of reactive oxygen species; however, in contrast to our findings, AML LSCs seem to have low glycolytic capacity and are susceptible to inhibition of oxidative phosphorylation (Jones et al., 2018; Lagadinou et al., 2013). The metabolic state of *MLL*-r B-ALL LSCs seems to be co-opted from normal long-term HSCs and could explain the concomitant retention of multipotency and self-renewal programs (Nakamura-Ishizu et al., 2020; Qiu et al., 2021; Spencer et al., 2014; Takubo et al., 2010). MYC seems to fulfill context-specific roles in B-ALL cells both as an activator of oxidative phosphorylation to promote growth of differentiated blasts as well as to maintain glycolysis to implement and support the LSC state.

Our conclusion that the LSC state is plastic can be connected to the clinical behavior of this form of high-risk B-ALL and may have therapeutic implications. We find latent myeloid transdifferentiation potential within single-cell-derived clones from each stratum of the leukemic hierarchy, consistent with a multipotent cell state (Rossi et al., 2012). The coexisting metabolic, lineage and LSC plasticity in *MLL*-rB-ALL provides two distinct mechanisms of chemotherapy evasion. We conclude that the high functional LSC content and plasticity in both lineage and the LSC state likely contribute to the poor outcomes of *MLL*-r B-ALL (Pieters et al., 2019). Our approach to understanding the mechanisms of LSC plasticity uncovered glycolysis as a potential vulnerability in B-ALL. Improved understanding of the molecular basis of LSCs in other forms of leukemia relative to normal hematopoiesis could lead to new candidate therapies.

Overall, we show that LSCs in high-risk B-ALL 1) maintain an HSC/MPP-like undifferentiated state and retain multipotency programs; 2) are frequent compared to reported LSC frequencies in AML (Sarry et al., 2011); 3) are relatively quiescent compared to more differentiated populations; and 4) are glycolytic; a state that can be implemented by inhibition of oxidative phosphorylation to drive LSC emergence from more differentiated blasts, contrasting with dependency on oxidative phosphorylation in myeloid leukemias (Kuntz et al., 2017; Lagadinou et al., 2013; Skrtic et al., 2011). Our findings in *MLL*-r B-ALL reinforce the notion that LSCs are plastic and adaptable, providing possible explanations as to why therapies targeting LSCs have yet to prove widespread efficacy despite being the object of intense investigation for over two decades (Pollyea and Jordan, 2017; Saygin et al., 2019). Although aspects of LSCs in *MLL*-r B-ALL might prove to be disease-specific, placement of our results within the broader context of the prevailing knowledge of LSCs illustrates the ongoing revision of classical LSC paradigms. Further incremental innovation in immunocompromised mouse models, genomics and single-cell-level readouts could continue to improve understanding of LSC pathobiology.

**STAR METHODS**

**Mice and xenotransplantation**

Unconditioned NSG mice (Jackson Laboratory stock 005557) were transplanted with the indicated cell sources at the indicated doses by tail vein injection. Mice were followed to the onset of terminal leukemia as assessed by the onset of morbidity, judged by investigators blinded to the experimental conditions.

For tigecycline treatment, mice were treated for two days with 25 mg/kg tigecycline (LKT labs) dissolved in 10% DMSO/90% (5% Kleptose HPB in PBS). The dose was then escalated to 50 mg/kg for three days and then 100 mg/kg for 7 days. After 48 hours, mice were euthanized for endpoint analysis. For IBET-151 treatment, mice were treated with IBET-151 (R and D Systems) at a dose of 30 mg/kg in 10% DMSO/90% (5% Kleptose HPB in PBS) for 5 days per week for two weeks followed by euthanization for analysis. For in vivo dicholoroacetate treatment, mice were engrafted with the indicated leukemias and dichloroacetate added to the drinking water to target a dose of 100 mg/kg/day(Bonnet et al., 2007).

**Cell culture**

Patient derived leukemia cells were cultured on MS5 stromal cells in the presence of 50 ng/ml recombinant human stem cell factor (SCF), 50 ng/ml recombinant human thrombopoietin (TPO), 10 ng/ml recombinant human FLT3 ligand (FLT3L), and 10 ng/ml recombinant human interleukin-7 (IL-7, all from R and D Systems)(Issaad et al., 1993). Leukemia 1 was most amenable to in vitro culture and so was used for most culture-based experiments. For in vitro LDA experiments, 5,000 MS5 cells were plated in wells of gelatin-coated Nunc 96 well plates (Fisher Scientific) with cytokines 48 hours prior to FACS-based sorting of leukemia cells directly into the wells.

Human MV4;11 and RS4;11 cells were cultured in RPMI with 10% fetal calf serum supplemented with penicillin and streptomycin.

**Flow cytometry and cell sorting**

Data were acquired on either BD LSR Fortessa or LSR II Instruments (BS Biosciences). Cells were sorted on a BD FACS Aria (BD Biosciences) with a 100 μm nozzle. Mitotracker Green was purchased from Thermo.

**Morphology**

For morphologic analysis, leukemia cells were spun onto slides and stained with May-Grunwald and Giemsa stains (Sigma) sequentially. Transmission electron microscopy was performed at the electron microscopy core at Harvard Medical School.

**Recombinant DNA**

The human *MYC* cDNA was purchased from Addgene (pDONR223\_MYC\_WT, a gift from Jesse Boehm and Matthew Meyerson and David Root (plasmid # 82927; <http://n2t.net/addgene:82927> ; RRID: Addgene\_82927). The *MYC* cDNA was cloned into the pCW57.1 vector (gift from David Root (Addgene plasmid # 41393; <http://n2t.net/addgene:41393> ; RRID:Addgene\_41393) using LR clonase (Thermo). The purified plasmid was used to generate lentivirus in HEK-293T cells, which was used to transduce MV4;11 cells. A stable transduced polyclonal line was selected with 0.5 μg/ml puromycin (Thermo). The *MYC* cDNA was also cloned into pINDUCER21 (ORF-EG) which was a gift from Stephen Elledge and Thomas Westbrook (Addgene plasmid # 46948 ; <http://n2t.net/addgene:46948> ; RRID: Addgene\_46948). Gene expression was confirmed by Western blotting following doxycycline exposure.

**Seahorse Assay**

The Seahorse assays were performed using the Agilent Seahorse XF Cell Mito Stress Test kit using injections of 1 μ*M* oligomycin, 1 μ*M* carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) and 0.5 μ*M* each of antimycin A and rotenone at the intervals indicated. The instrument is located at the Seahorse Core at Brigham and Women’s Hospital.

**Single cell RNA sequencing**

For scRNA-Seq using the Seq-Well platform, 20,000 cells were applied to Seq-Well devices pre-loaded with mRNA capture beads as previously described (Gierahn et al., 2017). Following hybridization and reverse transcription, random second-strand synthesis was performed to generate double stranded cDNA. PCR was performed using the following primer sequence 5’ – AAGCAGTGGTATCAACGCAGAGT – 3’. Sequencing libraries were generated using the Illumina Nextera XT protocol using custom N700 sequencing indices. Libraries were sequenced using Next-Seq 75 cycle high output sequencing kits with 20 base read 1 sequence and 50 base read 2 sequence.

For inDrops-seq the cells were encapsulated in 2-3 nl droplets using a microfluidic device and the libraries were made following a previously described protocol (Klein et al., 2015; Zilionis et al., 2017), with the following modifications in the primer sequences. RT primers on hydrogel beads-

5’CGATTGATCAACGTAATACGACTCACTATAGGGTGTCGGGTGCAG[bc1,8nt]GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[bc2,8nt]NNNNNNTTTTTTTTTTTTTTTTTTTV- 3’

R1-N6 primer sequence (step 151 in the library prep protocol in (Zilionis et al., 2017))- 5’TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNN-3’

PCR primer sequences (steps 157 and 160 in the library prep protocol in(Zilionis et al., 2017))-

5’-AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC-3’, where XXXXXX is an index sequence for multiplexing libraries.

5’- CAAGCAGAAGACGGCATACGAGATGGGTGTCGGGTGCAG-3’

With these modifications in the primer sequences, custom sequencing primers are no longer required. Single-cell RNA-Seq was library preparation was performed by the Single Cell Core at Harvard Medical School, Boston, MA.

For CITE-seq, cells were pre-stained with barcoded antibodies against CD38 and CD34 (Biolegend) and inDrop libraries were prepared as above.

**Bulk RNA sequencing library prep**

Cells were sorted by FACS and lysed in Trizol reagent (Thermo). RNA was isolated using RNAeasy columns (Qiagen) and low input libraries prepared in collaboration with the Molecular Biology Core at the Dana-Farber Cancer Institute.

**Data analysis**

*InDrop Single-cell RNA-sequencing*

Raw sequencing reads were processed using the inDrop pipeline (https://github.com/indrops/indrops) using default parameters (Klein et al., 2015) The GRCh38 reference genoma was used for alignment of sequencing reads. We used scImpute to account for dropout rates in single-cell RNA-seq data and obtain an imputed count matrix that was used for all downstream analysis described(Li and Li, 2018). We used scImpute with the parameter ‘Kcluster = 10’. To analyze imputed single-cell inDrop data we performed quality control, dimensionality reduction, clustering and differential expression analysis using CellRouter (Lummertz da Rocha et al., 2018). For this leukemia, we applied the following quality control metrics: all genes that were not detected in at least 20 cells were excluded. All cells with less than 200 genes detected were also excluded. As expression of ribosomal or mitochondrial genes is indicative of technical variation in single-cell RNA-seq data we also removed cells where the proportion of the transcript counts derived from mitochondrial genes was greater than 10% (Ilicic et al., 2016). After such quality control of the imputed count matrix, we retained 5,153 cells with a median of 15,214 genes detected per cell.

The data was then scaled and used for dimensionality reduction. We performed a principal component (PC) analysis using all genes (34,747 genes) and selected the top 20 PCs using the elbow method. These PCs were used for graph-based clustering to identify clusters of transcriptionally similar cells in our dataset. We also used the top 20 PCs to perform spectral t-stochastic neighbor embedding (t-SNE) analysis and visualize the underlying cluster structure in a space of reduced dimensionality.

*SeqWell single-cell RNA-sequencing*

Read alignment was performed as described (Macosko et al., 2015). Briefly, for each NextSeq sequencing run, raw sequencing data was converted to FASTQ files using bcl2fastq2 that were demultiplexed by Nextera N700 indices corresponding to individual samples. Reads were first aligned to HgRC19, and individual reads were tagged according to the 12-bp barcode sequence and the 8-bp UMI contained in read 1 of each fragment. Following alignment, reads were binned and collapsed onto 12-bp cell barcodes that corresponded to individual beads using Drop-seq tools (http://mccarrolllab.com/dropseq). Barcodes were collapsed with a single-base error tolerance (Hamming distance = 1), with additional provisions for single insertions or deletions. An identical collapsing scheme (Hamming distance = 1) was then applied to UMIs to obtain quantitative counts of individual mRNA molecules. We also used scImpute to impute the raw counts matrix obtained, with the parameter of ‘Kcluster = 6’. For this leukemia, we removed all genes expressed in less than 10 cells and also removed all cells expressing less than 500 genes. Cells with transcript counts derived from mitochondrial genes larger than 10% were also removed. After QC, we retained 6,320 cells with a median of 2,588.5 genes detected per cell.

*Normalization*

Both inDrop and Seq-Well data were analyzed with CellRouter. In CellRouter, transcript counts are normalized using a global scaling normalization method that normalizes expression measurements for each cell by the total expression, multiplied by a scale factor of 10,000, and log-transformed the result.

*StemID analysis*

First, with the raw counts data obtained from the inDrop sample, we performed an initial quality control removing cells not expressing at least 200 genes or genes not expressed in at least 20 cells. This filtered, not normalized counts matrix, was used as input for scImpute with “Kcluster=10”. After data imputation, we used the imputed count matrix for StemID analysis. Briefly, we removed cell cycle genes and performed the StemID analysis setting the following parameters: mintotal=0.01, minexpr=0, minnumber=0, maxexpr=Inf, downsample=TRUE, dsn=1 in the “filterdata” function, outminc=5,outlg=2,probthr=1e-3,thr=2\*\*-(1:40),outdistquant=.95 in the “findoutliers” function.

*SingleCellNet analysis*

We downloaded scRNA-seq data from the GEO accession number GSE116256 (van Galen et al., 2019). This study performed a random sampling of hematopoietic cells in the normal and leukemic bone marrow (BM) ecosystem. We reanalyzed five healthy BM samples published with this study and used cell types identified by the authors to train machine learning models of cell type identity of BM cells using SingleCellNet. After training, we classified each single cell in our leukemia samples as belonging to any of the classes in our training dataset.

*Signature Scores*

We downloaded gene lists from <https://www.gsea-msigdb.org/gsea/msigdb>. Specifically, we downloaded the following gene sets: HALLMARK\_MYC\_TARGETS\_V1.txt, HALLMARK\_OXIDATIVE\_PHOSPHORYLATION.txt and IVANOVA\_HEMATOPOIESIS\_STEM\_CELL.txt. Then, we used CellRouter to calculate signature scores for each cell and plotted the distribution of these scores.

*CITE-seq*

We converted binary files from the Illumina run to fastq files corresponding to 4 reads of indrops3 library with bcl2fastq/2.18.0.12 without read trimming. [https://support.illumina.com/sequencing/sequencing\_software/bcl2fastq-conversion-software.html](https://urldefense.proofpoint.com/v2/url?u=https-3A__support.illumina.com_sequencing_sequencing-5Fsoftware_bcl2fastq-2Dconversion-2Dsoftware.html&d=DwMF-g&c=WO-RGvefibhHBZq3fL85hQ&r=fSG3DCavH_rchaYXU5zYQYij-zUNg1EFo8eMW7lXhEE&m=-Jd5UAA5tw4qeT-SNQY_fdzILlw70JNCds0c-GMptvY&s=mxUYcYzFEQtCZqphIqhu4qBWEIgDCudxUcKTMiaw6q4&e=). We created a single fastq file containing cell and sample barcode information in the header of the read with umis fastqtransform <https://github.com/vals/umis>. We then wrote a custom python script to count antibody barcodes and assign the counts to a proper cell/sample pair. The input parameters of the script were antibody barcodes (tags), a fastq file from step2, and a list of sample barcodes. We ran the script using the wrapper [https://github.com/hbc/rowe2020\_indrop\_citeseq\_hbc03948/blob/master/04.cite\_seq\_count.sh](https://urldefense.proofpoint.com/v2/url?u=https-3A__github.com_hbc_rowe2020-5Findrop-5Fciteseq-5Fhbc03948_blob_master_04.cite-5Fseq-5Fcount.sh&d=DwMF-g&c=WO-RGvefibhHBZq3fL85hQ&r=fSG3DCavH_rchaYXU5zYQYij-zUNg1EFo8eMW7lXhEE&m=-Jd5UAA5tw4qeT-SNQY_fdzILlw70JNCds0c-GMptvY&s=ckScSKhSia_4ZfenAu4flftf-UDnNKXzIELI7FIPMYU&e=).

*Bulk RNA-sequencing*

Fastq files containing single-end RNASeq reads were aligned with Tophat 2.0.12 against the UCSC hg28 reference genome using Bowtie 2.2.4 with default settings (Kim et al., 2013; Langmead and Salzberg, 2012). Gene level counts were obtained using the subRead featureCounts program (v1.5.1) using the parameter “--primary” and gene models from the UCSC hg28 Illumina iGenomes annotation package (Liao et al., 2014). Read counts were normalized using size factors as available by the DESeq2 package (Love et al., 2014).

**ChIP-seq**

ChIP-seq was performed as previously described and in accordance with the Encode guidelines (Chapuy et al., 2013; Landt et al., 2012). The following antibodies were used for ChIP: MYC (CellSignaling 13987T. ChIPseq libraries were prepared using Swift S2 Acel reagents (Swift 21096) on a Beckman Coulter Biomek i7 liquid handling platform from approximately 1 ng of DNA according to manufacturer’s protocol and using 14 cycles of PCR amplification. Sequencing libraries were quantified by Qubit fluorometer and Agilent TapeStation 2200. Library pooling and indexing was evaluated by shallow sequencing on Illumina MiSeq. Subsequently, libraries were sequenced on NovaSeq targeting 40 million 100 bp read pairs by the Molecular Biology Core facilities at the Dana-Farber Cancer Institute. Sequencing reads were processed using the AQUAS pipeline (<https://github.com/kundajelab/chipseq_pipeline>) with minor modifications and according to the ENCODE3 guidelines. Reads were aligned to the hg38 genome build using BWA-ALN and peaks were called using MACS2.

*Data availability*

The RNA sequencing data acquired in this paper were uploaded to Gene Expression Omnibus under accession number GSE147862.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, R.G.R and E.L.d.R.; Methodology, R.G.R and E.L.d.R.; Software, E.L.d.R.; Formal analysis, J.K., S.H.S., S.N., R.G.R and E.L.d.R.; Investigation, V.M., D.W., E.L.d.R., R.V.d,S, W.M., T.H., P.S., P.S., and R.G.R.; Resources, Y.P., M.H., and A.K.S.; Data curation, T.H., R.G.R, M.P., T.H., and E.L.d.R.; Writing – original draft, R.G.R. and E.L.d.R.; Writing – revising and editing, A.K.S., T.E.N., and R.G.R.; Supervision, T.E.N., S.H.O., G.Q.D., and R.G.R.; Project administration, T.E.N., G.Q.D., and R.G.R.; Funding acquisition, R.G.R.

**COMPETING INTERESTS**

The authors declare no competing interests.

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**FIGURE LEGENDS**

**Fig. 1. Single cell heterogeneity of *MLL*-r B-ALL.** (**A**) Human CD45+ *MLL*-r B-ALL cells were isolated from patient peripheral blood and analyzed by scRNAseq (results shown are for leukemia-1*/MLL-AF4*)*.* t-distributed stochastic neighbor embedding (t-SNE) was used to visualize 14 distinct populations. (**B**) The StemID algorithm was used to annotate each single cell with a multipotency score, with results overlaid on the t-SNE plot and proportion of predicted multipotent cells in each cluster shown (Grun et al., 2016). (**C**) t-SNE plot showing enrichment of a validated HSC signature. (**D**) The expression of the indicated cell surface markers in each population is shown. (**E**) Representative flow cytometry distributions of cells based on the indicated markers. (**F**) The indicated populations were isolated from leukemic marrow (leukemia 1/*MLL-AF4*) by FACS and morphology examined following May-Grunwald-Giemsa staining (scale = 10 μm).

**Fig. 2**. Functional hierarchy in *MLL*-r B-ALL. (**A-B**) The indicated populations were isolated by FACS and cell cycle state analyzed by flow cytometry following staining for Ki67 and DNA content with DAPI. Cell cycle distribution of each population was quantified (results aggregated over two independent experiments, n = 5 xenografted mice tested; \* p < 0.05 compared to L-MLPs by unpaired student’s t-test). (**C**) The indicated populations were sorted onto MS5 stromal layers at doses of 1, 2, or 4 cells per well. After 4 weeks, outgrowths at each dose were tabulated, and progenitor cell content quantified by limiting dilution analysis. Estimated progenitor cell frequency is presented. Results are aggregated over two independent experiments (for 34+38- versus 34+38+ Χ2 = 64.3, p = 1 x 10-15; for 34+38- versus 34- Χ2 = 14.9, p = 0.0001; for 34+38+ versus 34- Χ2 = 18.1, p = 0.00002). (**D**) 400 cells of the indicated fraction were xenotransplanted into unconditioned NSG recipients, and the incidence of terminal leukemia monitored over time (results aggregated over two independent transplantation experiments; p = 0.008 for 34+38- versus 34+38+, 0.005 for 34+38- versus 34-, and 0.9 for 34+38+ versus 34- comparisons by log rank test). (**E**) NSG mice were transplanted with the indicated doses of cells from each population, and the incidence of terminal leukemia quantified at each dose aggregated over two experiments. (**F**) LSC content of each population was quantified by in vivo limiting dilution analysis. Results are plotted over 400 and 40 cell doses and aggregated over two independent xenotransplantation experiments (for 34+38- versus 34+38+ Χ2 = 10.7, p = 0.001; for 34+38- versus 34- Χ2 = 10.2, p = 0.001; for 34+38+ versus 34- Χ2 = 0.005, p = 0.9).

**Fig. 3**. **Plasticity in the *MLL*-r B-ALL LSCs.** (**A**) Representative flow cytometry profiles of terminal leukemias derived from the indicated transplanted cell populations from leukemia 1 and leukemia 2, gated on viable and human CD45+ cells within the mouse bone marrow. Proportions of each cell type in leukemias derived from the indicated transplanted cell populations are presented (p = NS comparing each outcome population between leukemia sources except in leukemia 1 where p < 0.05 comparing the 34+38+ and 34- fractions of leukemias derived from 34+38+ to both 34+38- and 34- derived leukemia). (**B**) Terminal leukemias derived from the indicated cell populations of leukemia 1/*MLL-AF4* in primary transplantation were transplanted into secondary recipients at the indicated cell doses, and the onset of terminal leukemia was monitored in the secondary recipients. (**C**) 34- cells were purified from leukemia 1 by FACS and transplanted into recipient mice. Reconstitution of CD34+ populations was monitored by flow cytometry (gated on viable human CD45+ cells) and quantified over time. (**D**) CITE-seq was performed on leukemia 1. Cells were scored for enrichment of HSPC signatures (95th percentile cutoff) and plotted versus CD34 and CD38 expression determined from barcoded antibodies (Ivanova et al., 2002; Jaatinen et al., 2006).

**Fig. 4**. **MYC regulates *MLL*-r B-ALL LSC plasticity.** (**A**) Schematic showing the input populations sorted for RNA-seq at steady state (left) and from regenerating 34- cells (right) isolated from the bone marrow of NSG mice. (**B**) RNA-seq was performed on FACS-sorted 34- cells growing in an active leukemia and mice transplanted with sorted 34- cells actively repopulating CD34+ populations (34-r) at 5 weeks post transplantation, and differential gene expression analyzed. Representative heatmap of differentially expressed gene is shown across three replicates. (**C**) GSEA was used to identify differentially enriched signatures in 34-r versus 34- cells. (**D**) The indicated expression signatures were analyzed in the context of the corresponding scRNA-seq data. (**E**) MYC ChIP-seq was performed in MV4;11 and RS4;11 cells and peaks associated with promoters and transcriptional start sites identified. GSEA was performed using preranked lists comparing transcript expression of 34-r versus 34- cells. (**F**) MV4;11 cells were treated for 48 hours with 50 n*M* IBET-151 at which time MYC protein was measured by Western blot. (**G-H**) FACS-sorted CD34- cells were cultured on MS5 stroma for 14 days with DMSO vehicle or 50 n*M* IBET-151, at which time the CD34+ content of the cultures was quantified (n = 5 independent samples). (**I-J**) CD34-r cells were engrafted into NSG mice for three weeks, at which time a two-week treatment course with IBET-151, after which the human CD45+ content of the bone marrow was analyzed by flow cytometry with the indicated markers (n = 5 mice per group). (**K**) K562 cells were transduced with a doxycycline inducible MYC vector and treated with or without doxycycline for 72 hours, at which time MYC levels were measured by Western blotting. (**L**) *MLL*-r B-ALL cells were transduced or not transduced with the doxycycline-inducible MYC vector, sorted for GFP+ cells, and cultured on MS5 stroma with cytokines with or without doxycycline for 14 days, at which time CD34+ content of the cultures was measured by flow cytometry. In all experiments results are presented as mean ± SEM and results compared by student’s t-test, with p-value shown.

**Fig. 5. Targeting oxidative phosphorylation drives LSC emergence in *MLL*-r B-ALL.** (**A-B**) Active mitochondria were quantified in the 34- fraction of bulk leukemia or 34-r cells using Mitotracker green, with representative flow cytometry results presented compared to background of unstained cells (n = 8 34- and 6 34-r biologic replicates over two independent experiments). (**C**) 34- human *MLL*-r B-ALL cells were cultured in the presence of stroma and cytokines with tigecycline (Tig, 2.5 μ*M*) or DMSO vehicle for 14 days, at which time percentage viable human CD45+ cells was measured by flow cytometry n = 4 biologic replicates over two experiments). (**D**) Cell lines were exposed to the indicated concentrations of tigecycline for 48 hours, at which time viable cells were quantified using Cell Titer Glo (n = 3 replicates over two experiments). (**E-F**) NSG mice were engrafted with cells for three weeks, at which time treatment with tigecycline commenced and continued for two weeks, when the mice were euthanized and mouse/human chimerism within the bone marrow analyzed (n = 8 subjects in vehicle and tigecycline groups, results compared by paired student’s t-test). (**G-H**) Spleens were isolated and weighed at the endpoint from (**E**). (**I-J**) CD34+ content of the xenografts isolated in (**E**) was analyzed by flow cytometry (n = 8 subjects in vehicle and tigecycline groups). (**K**) Xenotransplanted NSG mice were treated with the same treatment course of tigecycline as in (**E**) but followed to the onset of terminal leukemia (n = 7 subjects in vehicle and tigecycline groups, compared with log-rank test). (**L**) NSG mice were secondarily transplanted with primary grafts from (**E**) and survival monitored and compared by log-rank test (n = 5 subjects in each group). In all experiments, unless otherwise stated, results are presented as mean ± SEM and results compared by student’s t-test, with p-value shown.

**Fig. 6**. ***MLL*-r B-ALL LSCs maintain a glycolytic state.** (**A**) GSEA was used to query enrichment of a glycolysis gene expression signature in the indicated populations. (**B**) Relative Mitotracker signal was quantified in the indicated populations from two independent leukemia sources (n = 6 samples for leukemia 1 and 5 samples for leukemia 2 compiled over three independent experiments). (**C**) Heatmap showing relative normalized counts for the indicated genes in the indicated populations. (**D**). GSEA was used to query enrichment of a hypoxia gene expression signature in the indicated populations. (**E**) Human *MLL*-r B-ALL cells were cultured for 10 days under either 21% oxygen or 5% oxygen, and the composition of the cultures was measured by flow cytometry (result combined across three independent experiments from two independent donors). (**F-G**) CellRox was used to stain for reactive oxygen species in *MLL*-r B-ALL cells and relative signal quantified (n = 8 specimens combined from two independent donors). (**H**) Visualization of MYC ChIP-seq peaks associated with the indicated glycolytic genes in MV4;11 and RS4;11, with control input for each shown. (**I**) Gene ontology analysis was used to query for terms enriched in MYC ChIP-seq peaks in RS4;11 cells. (**J-K**) MV4;11 cells were culture in the presence or absence of 50 n*M* IBET-151 for 72 hours, at which the extracellular acidification rate (ECAR) was measured using the Seahorse assay. In all experiments results are presented as mean ± SEM and results compared by student’s t-test, with p-value shown.

**Fig. 7. Targeting glycolysis and hypoxic signaling in LSCs.** (**A**) 34+38- human *MLL*-r B-ALL cells were cultured for 8 days in the presence or absence of the indicated concentrations of 3-BP and viable human CD45+ in the stromal coculture assays were quantified by flow cytometry (n = 6 leukemia specimens aggregated over two independent experiments). (**B**) MV4;11 or RS4;11 cells were cultured in the presence of the indicated concentrations of 3-BP for 72 hours at which time relative viable cells were quantified using Cell Titer Glo. (**C**) MV4;11 cells were cultured in the presence or absence of dichloroacetate (DCA; 2 m*M*) for 72 hours at which time the oxygen consumption rate (OCR) was measured using the Seahorse assay. (**D**) 1,000 MV4;11 cells were cultured in the presence or absence of the indicated compounds for 96 hours at which time cells were placed in methylcellulose colony formation assays. After 8 days, colonies were enumerated (n = 6 independent experiments). (**E**) 34+38- human *MLL*-r B-ALL cells were sorted onto stromal cells and treated with or without DCA (0.25 m*M*) for 5 days at which time Mitotracker or tetramethylrhodamine methyl ester (TMRM) were used to quantify active mitochondria or ΔΨmt, respectively, by flow cytometry (n = 5 specimens over two independent experiments). (**F-G**) Human *MLL*-r B-ALL cells were cultured on MS5 stroma in the presence or absence of 2 m*M* DCA under normoxic or hypoxic conditions for 10 days, at which time viable human cells and CD34- content were quantified by flow cytometry (n = 12 specimens derived from two leukemic donors collected over two independent experiments). (**H**) NSG mice were xenotransplanted with 34+38- *MLL*-r B-ALL cells. Two weeks following transplantation, treatment with DCA commenced and survival was monitored (n = 7 mice in each group; results compared by log-rank test). (**I**) *MLL*-r B-ALL cells were treated in culture with or without 3-BP (10 μ*M*) and then transplanted into NSG mice, with survival monitored (n = 5 mice in each group; results compared by log-rank test). (**J-L**) NSG mice were transplanted with *MLL*-r B-ALL cells (*MLL-ENL*). Three weeks following engraftment, mice were treated with echinomycin for two weeks at which time human CD45 and CD34 chimerism were analyzed. In all experiments, unless otherwise stated, results are presented as mean ± SEM and results compared by student’s t-test, with p-value shown.