B-lineage acute lymphoblastic leukemia causes cell-autonomous defects in long-term hematopoietic stem cell function

Many of the clinical manifestations of leukemia, including infections, anemia and hemorrhage, reflect a progressive disruption of normal blood cell development. While chronic lymphoid leukemia impairs the function of hematopoietic stem cells (HSC),[1] acute myeloid leukemia[2] and T-lineage acute lymphoblastic leukemia (T-ALL)[3] primarily target lineage-restricted progenitor compartments. Data obtained from a xenograft model of B-lineage acute lymphoblastic leukemia (B-ALL) suggested that leukemia expansion results in a reduced expression of the chemokine (C-X-C motif) ligand 12 (CXCL12), causing defective HSC homing and reduction of $CD34^+$ cell populations in the bone marrow (BM).[4] However, single-cell RNA sequencing from a mouse B-ALL model indicated a substantial presence of cells with the RNA expression pattern of HSC in the BM even at advanced stages of leukemia.[5] Thus, the impact of B-ALL on HSC function and residency in the BM remains unclear.

We have developed a murine B-ALL model based on transplantation of primary tumor cells from mice carrying combined heterozygote mutations in the *Pax5* and *Ebf1* genes.[6] *Pax5* and *Ebf1* encode two transcription factors frequently targeted in human B-ALL and a significant proportion of the trans-heterozygote mice develop monoclonal or oligoclonal B-ALL before 40 weeks of age.[6] To determine the impact of B-ALL progression on hematopoiesis, we transplanted 150,000 primary mouse tumor cells from the lymph node of a leukemic *Cd45.2*$^{+/+}$*Ebf1*$^{+/-}$*Pax5*$^{+/-}$ mouse to wildtype CD45.1 mice by tail vein injection (Figure 1A). The transplants were performed without pre-conditioning allowing us to determine the impact of B-ALL on steady-state hematopoiesis (Figure 1B). Disease was manifested by palpable accessory axillary or subiliac lymph nodes 3-4 weeks after transplantation. At this time the tumor burden in the BM of the transplanted mice was over 90% (Figure 1C) and the absolute numbers of CD45.1 cells in the BM reduced (*Online Supplementary Figure S1A*). To determine the composition of the remaining BM population, the lineage negative (Lin^-)$CD45.1^+KIT^+$ host populations were divided into lineage-restricted $Lin^-KIT^+SCA1^-$, (LK) progenitors and $Lin^-KIT^+SCA1^+$ (LSK) multipotent progenitors including HSC (Figure 1D). Sub-fractionation of the LK compartment[7] (Figure 1D) revealed 10- to 100-fold reductions in both relative and absolute numbers of myeloid lineage-restricted progenitors in tumor transplanted mice as compared to the numbers in sham transplanted control mice (Figure 1E). This included the pre-granulocyte/monocytes, granulocyte/monocyte progenitors, pre-megakaryocyte/erythrocytes, megakaryocyte progenitors, pre-colony-forming unit erythrocytes and colony-forming unit erythrocytes (Figure 1E). The multipotent LSK population was fractionated into $CD150^-$ short-term and $CD150^+$ long-term HSC[8] (Figure 1D, E). The frequency as well as absolute numbers of LSK were reduced in leukemic mice (Figure 1E). However, even though the absolute numbers of $CD150^+$ HSC were reduced 1.6-fold, the relative frequency of the most primitive progenitors was increased. Transplantation of two other independently generated pro-B-ALL tumors generated similar results as we detected reduced frequency of lineage restricted progenitors but no reduced frequency of $CD150^+$LSK in mice transplanted with tumor (*Online Supplementary Figure S1B-D*).

To further analyze the cellular composition of the leukemic BM we performed single-cell RNA-sequencing analysis of $Lin^-KIT^+CD45.1^+$ cells in control and leukemic mice (GSE207819). The data were processed using Seurat whereby the control and leukemic samples were integrated using Harmony prior to clustering. The cells were assigned an identity using a previously generated data set from hematopoietic progenitors[9] (*Online Supplementary Figure S2A, B*) employing SingleR. This revealed an 18-fold enrichment of HSC and a 5-fold reduction in megakaryocyte/erythroid progenitors in the BM from tumor-exposed mice as compared to the numbers in control mice (*Online Supplementary Figure S2C*). Even though the single-cell RNA-sequencing analysis did not quite recapitulate the dramatic loss of linage-restricted progenitors detected by fluorescence activated cell sorting analysis, the data support the idea that the HSC compartment is relatively well conserved while the frequencies of lineage-restricted progenitors are reduced in B-ALL.

To determine the status of the progenitor compartments in B-ALL patients, we analyzed flow cytometry data from the BM of 19 patients diagnosed with B-ALL (median age, 6.5; range, 0-59 years).[10] Cells were gated on high expression of CD34, a marker for early progenitor cells,[11] and lack of CD19, a defining B-ALL marker (Figure 2A). The $CD34^+$ population was gated negative for CD20 and CD66 (*Online Supplementary Figure S3*) and was highly heterogeneous for expression of CD38 (Figure 2A). This allowed us to investigate leukemia-associated changes in the ratios between $CD38^{high}$ cells, reported to be largely lineage-re-

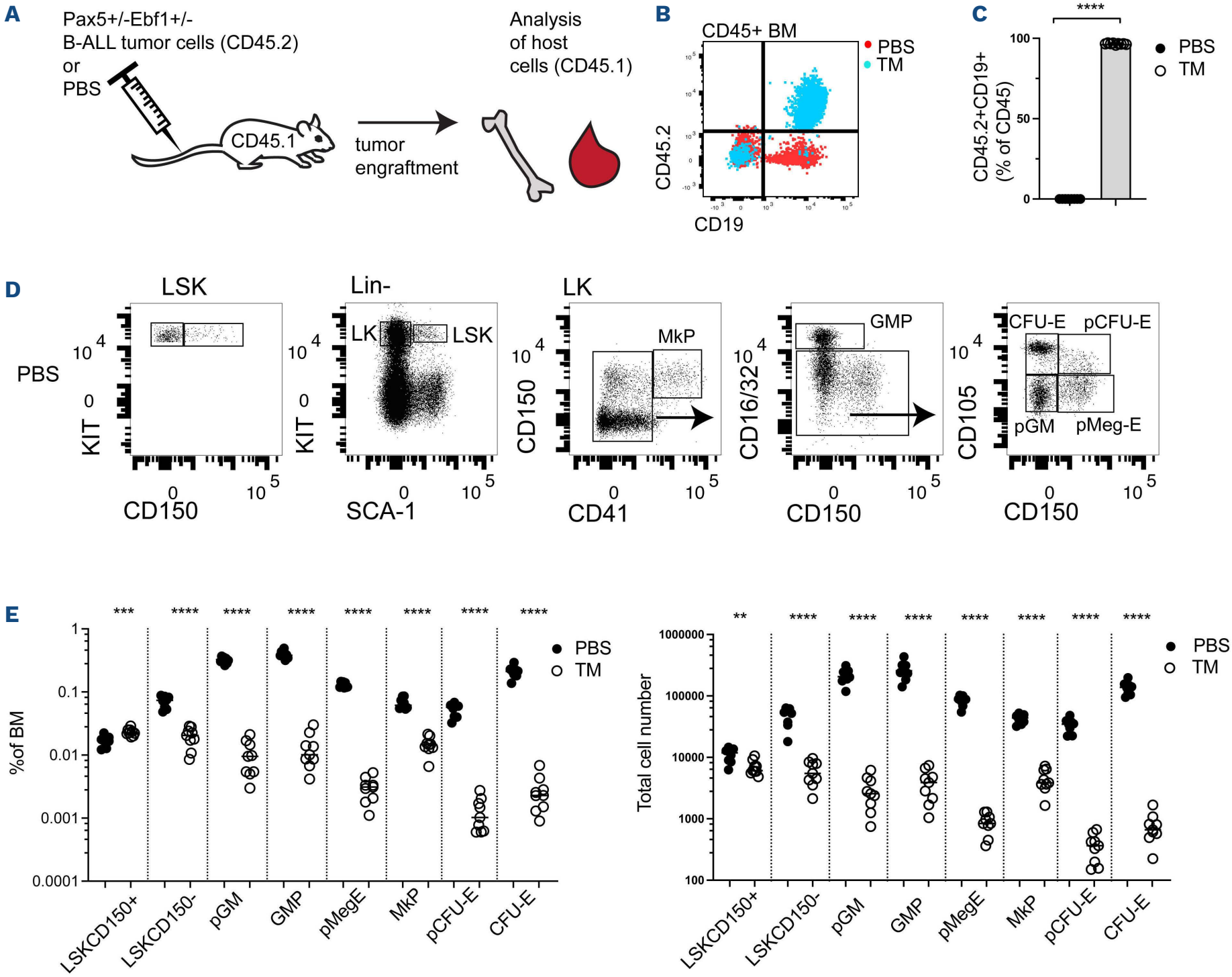


Figure 1. Leukemia affects the formation of lineage-restricted progenitor cells rather than the phenotypic stem cell compartment. (A) Schematic model of the leukemic cell transplants in which either phosphate-buffered saline (PBS) or 150,000 CD45.2$^+$ leukemic lymph node cells were transplanted into non-irradiated CD45.1 recipients to determine the impact of leukemia on recipient hematopoietic progenitors. (B) Fluorescence activated cell sorting (FACS) overlay plot of the distribution of cells with expression of CD19 and CD45.2 in CD45.1 mice injected with tumor (TM, blue) or PBS (red). (C) Percentage tumor engraftment (CD45.2$^+$CD19$^+$) in the bone marrow of either PBS (PBS group) or leukemic lymph node (tumor, TM group)-injected CD45.1 mice 22 days after transplantation. ****P<0.0001 (Student t test), from a total of eight PBS and nine tumor (TM) samples per condition obtained from two experiments. (D) FACS profiles of whole bone marrow displaying CD45.1$^+$ lineage-negative (CD11b/Mac1$^-$Gr1$^-$TER119$^-$CD3$^-$CD11c$^-$NK1.1$^-$CD19$^-$)SCA1$^+$KIT$^+$ (LSK) cells stained with antibodies against CD16/32, CD105, CD150 and CD41 to identify progenitor populations. Propidium iodide was used as a marker of viability and gates for each indicated cell population were set according to fluorescence minus one (FMO) controls. (E) Percent of bone marrow progenitors and numbers of progenitors in the bone marrow of PBS- or TM-injected non-irradiated CD45.1 recipients. **P<0.01, ***P<0.001, ****P<0.0001 (Student t test), from a total of eight PBS and nine tumor samples per condition from two experiments. B-ALL: B-lineage acute lymphoblastic leukemia; BM: bone marrow; MkP: megakaryocyte progenitors; GMP: granulocyte/monocyte progenitors; CFU-E: colony-forming unit erythrocytes; pCFU-E: pre-colony-forming unit erythrocytes; pGM: pre-granulocyte/monocytes; pMeg-E: pre-megakaryocyte/erythrocytes.

stricted progenitors, and CD38$^{low/-}$ progenitors, enriched for multipotent HSC.[11] While the relative frequency of CD34$^+$ cells was reduced in the patients' BM as compared to control samples (Figure 2B), the frequency of CD34$^+$CD38$^{low/-}$ cells was not significantly different. Re-analysis of single-cell RNA-sequencing data from leukemia patients[12] using SingleR as above provided additional support for the fact that the frequency of HSC was not reduced in leukemia patients (Figure 2C). Analyzing BM samples from B-ALL patients at diagnosis and at days 15, 29 and 78-79 after initiation of treatment, according to the NOPHO2008 protocol,[13] revealed a rapid reduction in the frequency of CD19$^+$

cells (Figure 2D), and increased abundance of CD34⁺CD38⁺ lineage-restricted progenitors (Figure 2E). The frequency of CD34⁺CD38⁻/low cells did not change to a significant degree throughout the treatment (Figure 2F). These data reveal that the earliest progenitor compartments are well preserved even in advanced stages of disease and the rapid recovery after initiation of treatment suggests that they retain a substantial functional capacity for blood cell production.

To determine the functional capacity of leukemia-exposed HSC, we sorted CD150⁺LSK from leukemic and control CD45.1 mice and transplanted 250 cells into lethally irradi-

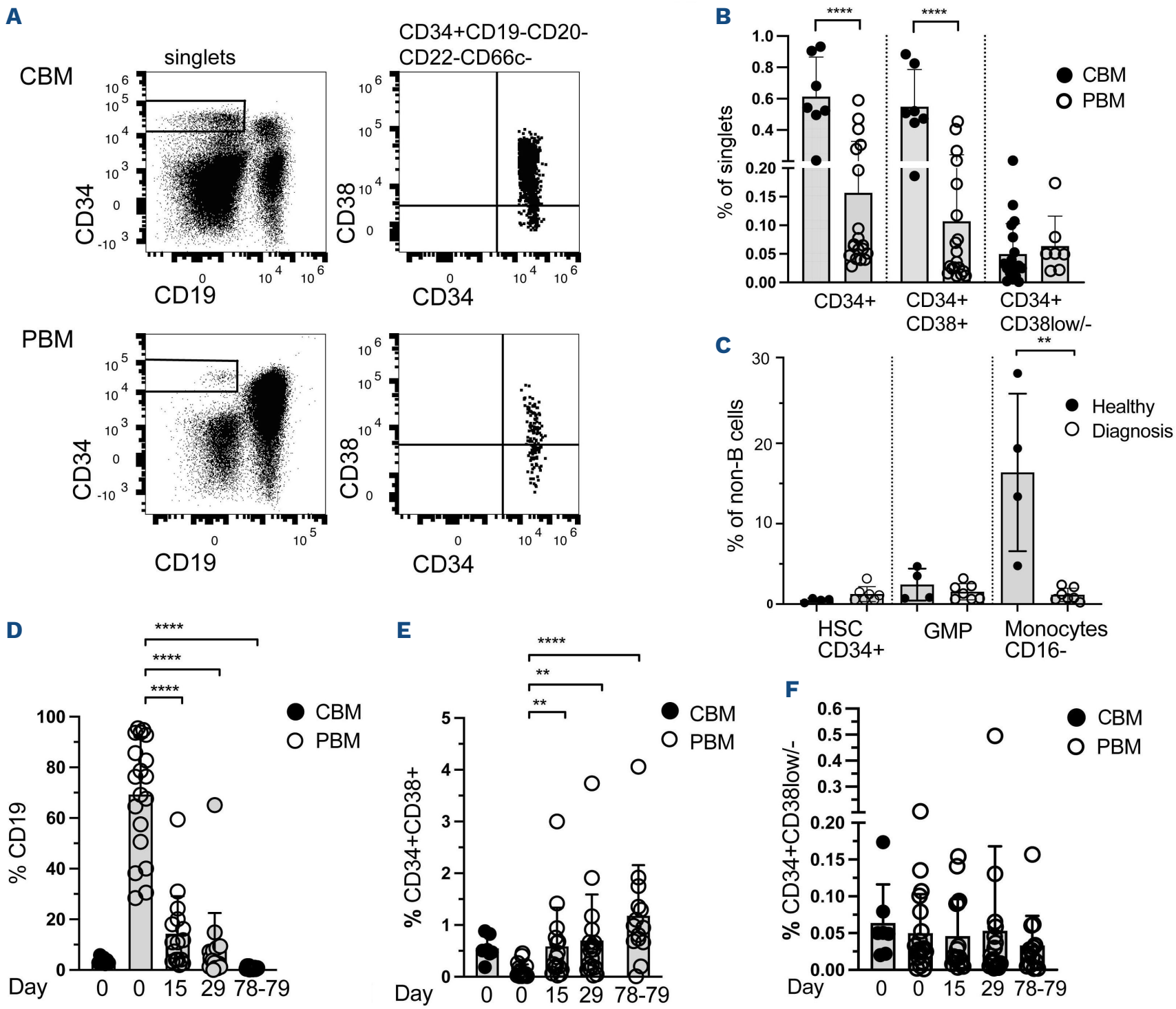
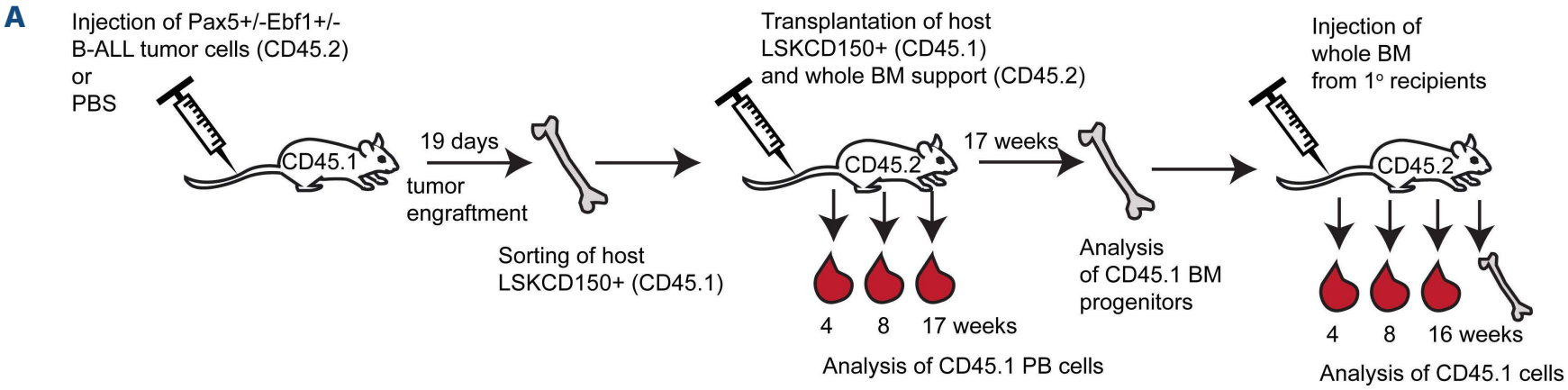
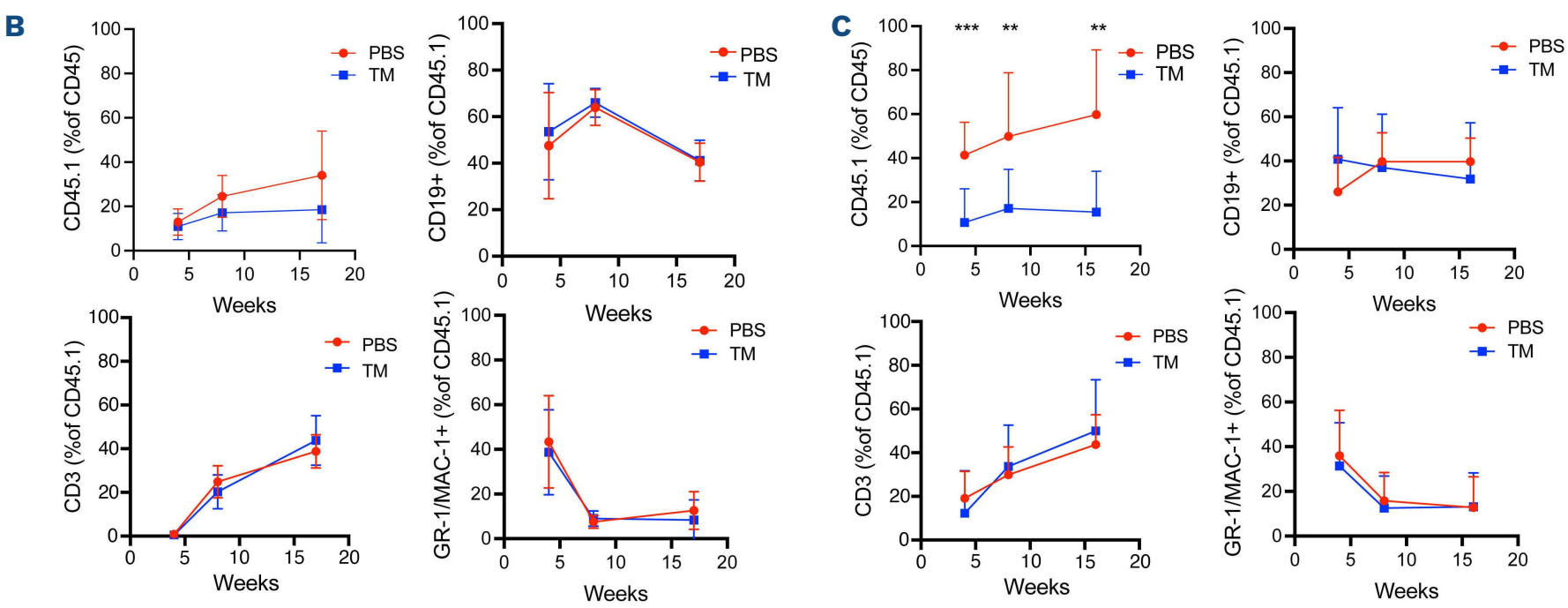


Figure 2. Leukemia patients retain CD34⁺CD38⁻ early progenitors while the frequency of CD34⁺CD38⁺ lineage-restricted progenitors is reduced. (A) Representative flow cytometry profiles. (B) Frequencies of bone marrow (BM) progenitors of total cells in diagnostics BM samples (PBM) from patients with B-lineage acute lymphoblastic leukemia (B-ALL) or control BM (CBM). ****P<0.0001 (Student t test), from a total of seven controls and 19 PBM. (C) Diagrams displaying a re-analysis of the human B-ALL dataset, GSE130116. Scanpy v1.8.2 was used to merge mtx files from GSE130116 into a single anndata object. The object was read into R using zellkonverter v1.4.0 readH5AD function. Scuttle version 1.4.0 was used to normalize the data, and SingleR version 1.8.1 was used to predict cell types using the HumanPrimaryCellAtlasData as the reference. The percentages of BM progenitor populations were calculated after cells assigned to B-cell classes were removed. The proportions of assigned hematopoietic stem cells (HSC), granulocyte/monocyte progenitors (GMP) and CD16⁻ monocytes collected from a single-cell RNA dataset of B-ALL patients and controls are shown as fractions of non-B lineage BM cells. **P<0.01 (Student t test), from a total of four healthy controls and seven diagnostic B-ALL samples (GSE130116). (D-F) Frequencies of CD19⁺ B cells (D), CD34⁺CD38⁺ BM multipotent progenitors (E) and CD34⁺CD38⁻/low HSC and early progenitors (F) in B-ALL diagnostic BM samples (PBM) or control BM (CBM) at diagnosis (day 0) and throughout the course of treatment. **P<0.01, ****P<0.0001 (Student t test), from a total of seven CBM and 19 patients at day 0, 16 patients at day 15, 18 patients at day 29 and 14 patients at day 78-79. All patients with a verified B-ALL diagnosis and treatment response were included in the analysis.

ated (9 Gy) CD45.2 mice together with 200,000 CD45.2 BM support cells (Figure 3A). Analysis of peripheral blood 4 and 8 weeks after transplantation identified a comparable fraction of CD45.1$^+$ cells generated from both control and leukemia-exposed HSC (Figure 3B). We were unable to detect any significant lineage bias as both groups of mice presented comparable ratios of CD19$^+$, CD3$^+$ and GR1/MAC1$^+$ cells (Figure 3B). Seventeen weeks after transplantation,





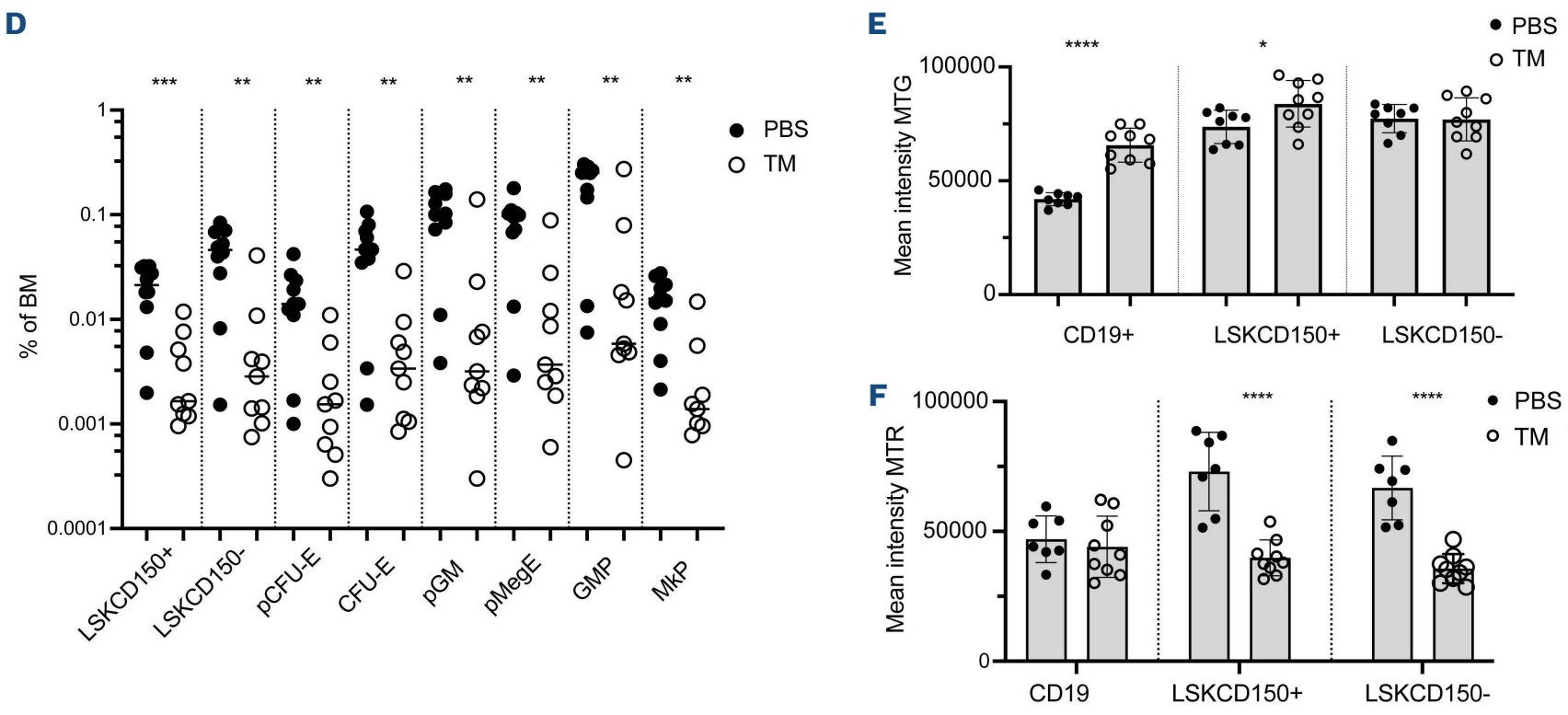


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Figure 3. Hematopoietic stem cells exposed to leukemia display a cell-autonomous defect in long-term reconstitution activity. (A) A schematic representation of the serial transplantation experiment. CD45.1 mice were injected with phosphate-buffered saline (PBS) or CD45.2$^+$ leukemic lymph node (tumor, TM) cells followed by leukemia development over 19 days. At day 19, bone marrow (BM) was harvested, LSKCD150$^+$ cells were sorted and 250 cells were transplanted into lethally irradiated (9 Gy) CD45.2$^+$ mice together with 200,000 CD45.2$^+$ whole BM support cells. Peripheral blood (PB) and BM were analyzed by fluorescence activated cell sorting (FACS) at the depicted time-points. At 17 weeks 10% of the extracted BM was serially transplanted into lethally irradiated CD45.2 mice followed by analysis of PB and BM as outlined. (B) Graphs display the contribution of transplanted LSKCD150$^+$ cells to total CD45.1 and the contribution of B cells (CD19), T cells (CD3), and myeloid cells (CD19$^-$CD3$^-$GR1/MAC1$^+$) within the CD45.1$^+$ compartment in PB at 4, 8 and 17 weeks after transplantation. Data were collected from a total of ten PBS-injected and ten TM-injected mice per condition from two experiments. (C) Graphs display total CD45.1, and the contribution of B cells (CD19), T cells (CD3), and myeloid cells (CD19$^-$CD3$^-$GR1/MAC1$^+$) within CD45.1$^+$ compartment in PB at 4, 8 and 16 weeks after serial transplantation of BM from LSKCD150$^+$ transplanted mice. Data were collected from a total of ten PBS-injected and ten TM-injected mice per condition from two independent experiments. (D) A graph displaying FACS data of the BM engraftment of CD45.1$^+$ hematopoietic stem cells (HSC) and myelo-erythroid progenitors, as identified in Figure 1) at 16 weeks after serial transplantation of BM from LSKCD150$^+$ transplanted mice as the percentage of total BM. $^{**}P<0.01$ (Student *t* test), from a total of ten PBS-injected and nine tumor (TM)-injected mice per condition from two experiments. (E) FACS data displaying the mean florescent intensity of Mitotracker Green (MTG) in HSC populations (primary tumor transplanted cells as in Figure 1) of control (PBS) and leukemia (TM)-transplanted mice. Samples were stained with MTG (30 nM) and 50 nM verapamil for 30 min at 37°C before analysis. (F) Mitotracker CMXRos (MTR) staining of HSC from tumor-transplanted or control mice was performed with 25 nM MTR and incubation with verapamil as above. $^{*}P<0.05$, $^{****}P<0.0001$ (Student *t* test), from a total of seven or eight PBS-injected mice and nine tumor TM-injected mice from two experiments. B-ALL: B-lineage acute lymphoblastic leukemia; pCFU-E: pre-colony-forming unit erythrocytes; CFU-E: colony-forming unit erythrocytes; pGM: pre-granulocyte/monocytes; pMeg-E: pre-megakaryocyte/erythrocytes; GMP: granulocyte/monocyte progenitors; MkP: megakaryocyte progenitors.

we noted a tendency towards a reduced level of reconstitution in mice transplanted with B-ALL-exposed HSC (Figure 3B). To determine whether the phenotypic stem cells retained long-term reconstitution potential, we performed serial transplantation of total BM from the mice reconstituted with control or leukemia-exposed HSC into lethally irradiated CD45.2 mice and followed the generation of CD45.1$^+$ cells (Figure 3C). Even though the lineage distribution was comparable between the two groups of serially transplanted mice, the reconstitution level was significantly reduced, already 4 weeks after transplantation, in mice that obtained BM containing tumor-exposed hematopoietic progenitors (Figure 3C). This effect became even more prominent at 16 weeks after the secondary transplantation and upon analysis of CD45.1$^+$ progenitors in the BM we detected a relative reduction in frequency of all progenitor populations, including the CD150$^+$LSK compartment, in mice transplanted with BM containing leukemia-exposed HSC (Figure 3D). Hence, even though exposure to leukemia does not have a major impact on the presence of HSC in the BM, the remaining stem cells display a defective ability for long-term reconstitution.

To identify B-ALL-induced changes in gene expression patterns, we compared RNA levels in cells defined as part of a given population in the SingleR analysis (*Online Supplementary Figure S2*) in mice injected with phosphate-buffered saline or leukemia (Figure 1A). These changes were mined for significantly enriched gene ontology terms using ToppCluster (*https://toppcluster.cchmc.org/*) and displayed in a heatmap. A major part of the gene ontology terms were related to mitochondrial function and electron transport (*Online Supplementary Figure S2D, E*). To determine the total mitochondria content in progenitor cells we stained HSC from control and leukemic mice (Figure 1A) with Mitotracker green. While CD150$^+$LSK contained a slightly higher mitochondria mass in leukemic as compared to control mice, no such difference was found in the CD150$^-$LSK population (Figure 3E). To compare mitochondrial membrane potential in control and leukemia exposed cells, we stained the LSK cells with Mitrotracker CMXRos (MTR). The retention of MTR was reduced in both the CD150$^+$ and CD150$^-$ cells from the leukemic BM as compared to control cells (Figure 3F) indicating that the B-ALL-exposed cells harbor a deficiency in mitochondria function.

The impact of leukemia on normal blood cell development is reported to involve displacement as well as changes in the microenvironment causing disruptions in stem and progenitor cell function.[1-4] Our data show that even though the phenotypic HSC are well preserved in the BM in B-ALL, the cells display a defect limiting their ability for long-term reconstitution. As we were unable to detect pathological expansion of CD45.2$^+$CD19$^+$ cells or symptoms of leukemia in the mice transplanted with stem cells, we believe that the functional defect is HSC autonomous and not a consequence of contaminating leukemia cells. Furthermore, in contrast to our observation in leukemic mice (Figure 1E), the stem cell-transplanted animals show a clear reduction in the frequency of HSC (Figure 3D). The functional impairment was associated with defective mitochondria function in a manner resembling that observed in aged HSC[14] likely arising from cellular stress in the leukemic BM. Even if such a defect would not have an impact on the initial recovery of blood cell production after treatment of a leukemia patient, it could potentially contribute to late effects such as clonal hematopoiesis linked to primary hematologic malignancies.[15] We believe that our findings open an interesting path for further studies of the underlying causes of secondary cancers and long-term medical conditions in leukemia patients.

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Disclosures

No conflicts of interest to disclose.

Contributions

CJ, JÅ, AP, JTG, RS, JU, and MS designed, conducted and analyzed the experiments. SS, SL, and CJ performed bio-informatics analysis of data. All authors contributed to writing the manuscript.

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Data-sharing statement

The RNA-sequencing data generated are deposited in GEO (GSE207819). Excel files with FACS data from mouse experiments will be shared upon request. Detailed materials and methods will be provided upon request.

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