Kinetics of normal hematopoietic stem and progenitor cells in a Notch1-induced leukemia model

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Running Title: stem cells in leukemic environment

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ABSTRACT

The predominant outgrowth of malignant cells over their normal counterparts in a given tissue is a shared feature for all types of cancer. However, the impact of a cancer environment on normal tissue stem and progenitor cells has not been thoroughly investigated. We began to address this important issue by studying the kinetics and functions of hematopoietic stem and progenitor cells in mice with Notch1-induced leukemia. While hematopoiesis was progressively suppressed during leukemia development, the leukemic environment imposed distinct effects on hematopoietic stem and progenitor cells, thereby resulting in different outcomes. The normal hematopoietic stem cells in leukemic mice were kept in a more quiescent state but remained highly functional upon transplantation to non-leukemic recipients. In contrast, the normal hematopoietic progenitor cells in leukemic mice demonstrated accelerated proliferation and exhaustion. Subsequent analyses on multiple cell cycle parameters and known regulators (such as p21, p27 and p18) further support the above paradigm. Therefore, our current study provides definitive evidence and plausible underlying mechanisms for hematopoietic disruption but reversible inhibition of normal hematopoietic stem cells in a leukemic environment. It may also have important implications for cancer prevention and treatment in general.
INTRODUCTION

The predominant outgrowth of malignant cells over their normal counterparts in a given tissue is a shared feature for all types of cancer. While many intrinsic and extrinsic factors have been implicated in cancer development, the impact of a cancer environment on normal tissue stem and progenitor cells has been poorly understood. Like other cancers, leukemia is caused by both intrinsic factors, such as the aberrant expression of oncogenes or tumor suppressors, and extrinsic factors, such as immune dysfunction, neo-vasculature and other tumor-promoting microenvironmental cues in the hematopoietic system. Leukemia stem cells (LSCs) are thought to play a key role in the initiation, and possibly the maintenance, of leukemia. During leukemogenesis, LSCs outcompete their normal counterparts, namely hematopoietic stem cells (HSCs), and become dominant by acquiring an increased capacity for self-renewal coupled with decreased levels of cell death or a disrupted differentiation program. Thus, the clinical manifestations of leukemia are largely determined by the competition between LSCs and normal HSCs.

HSCs give rise to all types of mature blood and immune cells, and these mature cells provide environmental factors that can also influence leukemogenesis. Deregulated hematopoiesis can lead to significantly decreased numbers of blood cells and to anemia, infection and hemorrhage, all of which are directly responsible for the poor life quality and increased mortality of cancer patients. Moreover, because autologous HSC transplantation (auto-HSCT) has been used with chemotherapy and radiotherapy to treat hematological malignancies and other solid tumors, the
quality of normal HSCs from a cancer patient may serve as a critical parameter for the ultimate success of auto-HSCT for the patient. Therefore, the impact of a leukemic environment on normal HSCs and hematopoietic progenitor cells (HPCs) during leukemogenesis is an issue of high significance. Studies along this line may have implications for other cancers as well.

In this study, we have examined the kinetics of normal HSCs and HPCs in the Notch1-induced murine T cell leukemia model. Our results demonstrate that hematopoietic cells in the leukemic environment are progressively decreased as measured by both phenotype and function. However, the effects of the leukemic environment on HSC and HPC are distinct in that the repopulation potential of HSCs from the leukemic environment is preserved whereas mature blood cells cannot be produced due to the exhaustion of HPCs. Therefore, these data yield new insights into the pathogenesis of Notch1-involved T cell leukemia and may guide better clinical management for this type of malignancy.

MATERIALS AND METHODS

Mice

Wild type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and B6.SJL-PtprcaPepcb/BoyJ mice (B6.SJL) were purchased either from Taconic (Hudson, NY) or the Jackson Laboratory. CD45.1+/CD45.2+ mice were the F1 generation of C57BL/6J and B6.SJL-PtprcaPepcb/BoyJ mice. All mice were maintained in the certified animal facility of the Hillman Cancer Center, University of Pittsburgh. The procedures involved in the animal work
were approved by the Institutional Animal Care and Use Committee at all participating institutions in this study.

**Transduction of murine primary bone marrow cells**

The retrovirus vector containing the cDNA-encoding intracellular domain of Notch1 (ICN1) was kindly provided by Dr. David Scadden (Harvard University, Boston, MA). The plasmid (MSCV-ICN1-IRES-GFP) was co-transfected into package cell line 293T (bestowed by Dr. Wan Yong, University of Pittsburgh, Pittsburgh, PA) with pCMV-VSV-G and pKAT, using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Supernatant was harvested 48h and 72h after transfection.

Lineage negative (Lin⁻) cells from the bone marrow of female C57BL/6J or B6.SJL mice were enriched with Biotin-conjugated lineage antibodies [CD3(RM3415-3), CD4(RM2515-3), CD8(RM2215-3), CD11b(Mac-1, RM2815-3), Gr-1(Ly-6G, RM3015-3), CD45R(B220, RM2615-3), Ter-119(MTER15-3), (CALTAG Laboratories, Burlingame, CA)], and selected with Microbeads-conjugated streptavidin (Miltenyi Biotec, Auburn, CA) using manufacture’s protocol. Then the Lin⁻ cells were re-enriched for expression of Sca-1 using the EasySep™ murine Sca-1 positive selection kit (Miltenyi Biotec, Auburn, CA). Transduction of lineage negative Sca-1 positive (Lin⁻Sca-1⁺) cells with ICN1 plasmid were performed as previously described with minor modifications. The transduction efficiency was measured by flow cytometry.
Transplantation of the transduced cells and analysis of leukemia-bearing mice

Bone marrow nucleated cells (BMNCs; $1 \times 10^7$/host) from B6.SJL mice at the age of 6-8 weeks were transplanted into lethally-irradiated (10Gy) female C57BL/6J recipients (6-8 weeks old) with or without $1 \times 10^6$ ICN1 plasmid-transduced Lin$^{-}$Sca-1$^+$ cells from C57BL/6J mice as indicated in Fig.1A. Due to the \textit{in vitro} transduction and culture procedures, the majority of the cells transplanted into the recipients (detailed below) should be the progenies of Lin$^{-}$Sca-1$^+$ cells, not the Lin$^{-}$Sca-1$^+$ cells themselves. The recipients were followed up physically on a daily basis. Peripheral blood (PB) sampling from the lateral tail vein was collected starting at 1 week post-transplantation for white blood cell (WBC) count, and the percentage of CD45.2$^+$GFP$^+$ cells was analyzed by flow cytometry using PE-Cy5.5-CD45.1 and PE-CD45.2 antibodies (eBioscience, San Jose, CA).

Flow cytometric analysis and cell sorting

Mouse bone marrow cells were obtained by flushing ilias, femurs, tibias and humeri as described. The immunophenotypes for murine long-term repopulating HSCs (CD34$^-$Lin$^-$c-Kit$^-$Sca-1$^+$, CD34$^-$LKS), short-term repopulating HSCs (CD34$^+$Lin$^-$c-Kit$^+$Sca-1$^+$, CD34$^+$LKS) and Lin$^-$c-Kit$^-$Sca-1$^-$ (LKS$^-$) \cite{7,8} were used to quantify these different cell types within the normal cell populations that were separated with different congenic markers. All the antibodies were purchased from BD Biosciences unless otherwise noted. For detection of HSC/HPC, we used streptavidin conjugated with PE-TxRed (to stain for biotinylated CD34), PE-Cy7 conjugated with a mixture of lineage antibodies [anti-CD3, CD4, CD8, B220, Gr-1,
Mac-1, Ter-119 (eBioscience, San Diego, CA)], PE-conjugated Sca-1, APC-conjugated c-Kit, PE-Cy5.5-conjugated CD45.1, and FITC-conjugated CD45.2 (eBioscience). The method used to quantitatively measure the frequency of primitive cells was as previously described. For cell cycle analysis, cells were stained with Hoechst-33342 (Molecular Probes, Eugene, OR) and Pyronin-Y (Sigma Aldrich, St. Louis, MO), or APC-BrdU Flow kit with HSC/HPC cell surface markers. Analyses were performed on an LSR (BD).

For HSC/HPC isolation, the cells were then enriched for c-Kit expression by immunoselection with CD117-conjugated micromagnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Then the enriched cells were stained with PE-Cy7 conjugated with a mixture of lineage antibodies, PE-Sca-1, APC-c-Kit. CD45.1+LKS cells were directly sorted into different tubes and lysed for gene expression analysis. For normal hematopoietic cell sorting, CD45.1+GFP− cells were sorted with BD FACSAria+ sorter (BD). During the sorting procedure, 4,6-diamidino-2-phenylindole (DAPI) was used to exclude the dead cells. For normal hematopoietic cell (CD45.1+) sorting, the whole bone marrow cells from mice in leukemia-bearing or control groups were each pooled together, respectively. To discriminate transplanted normal hematopoietic cells (CD45.1+GFP+) from endogenous (CD45.2+GFP+) and leukemia cells (CD45.2+GFP+), the cells were stained with PE-Cy5.5-conjugated CD45.1 and PE-conjugated CD45.2 antibodies. To exclude the possibility of leukemia cell contamination in CD45.1+GFP− cell sorting, double sorting was used in the sorting strategy. The purity of CD45.1+GFP− cells was more than 99.99% (Fig. 3A).
**In vitro clonal assay**

CD45.1^GFP^- cells from the leukemia and control groups were sorted at 2 weeks after transplantation for *in vitro* clonal assay and *in vivo* cBMT, respectively. For the CFC assay, the cells were placed in methylcellulose medium M3234 (StemCell Technologies, Vancouver, BC, Canada) supplemented with a mixture of recombinant cytokines including 50 ng/mL murine stem cell factor, 20 ng/mL murine interleukin-3, 20 ng/mL murine interleukin-6 (Peprotech, Rocky Hill, NJ) and 3 U/mL human erythropoietin (Amgen, Thousand Oaks, CA). Cells were plated in 24-well plates with a 0.5mL volume at a density of 2x10^4 cells/mL with 4 replicated wells. At day 10, the CFC colonies were counted under an inverted microscope and recorded as colony number in a specific lineage. For 5-fluorouracil (5-FU) exposure assay *in vivo* 72 hours after transplant, 150 mg/kg 5-FU was given (intravenous) at 12 hours before BM was harvested. CD45.1^GFP^- cells were sorted for CFC assay as described above.

The frequency of CAFC at day 35 was determined using limiting dilution assay as described. Briefly, sorted CD45.1^GFP^- cells were plated on an irradiated (15Gy) primary murine stroma in 96-well plates containing 100μL M5300 (StemCell Technologies, Vancouver, BC, Canada) supplemented with 10^-6 M hydrocortisone (Sigma, St Louis, MO) at 6 different concentrations with 20 replicates per concentration. The cells were fed with half-medium change weekly. The cobblestones were counted at day 35, and recorded as negative or positive for each well. Based on the Poisson distribution of the negative wells, the frequency of long-term initiating cells was
calculated with L-Calc software (StemCell Technologies, Vancouver, BC, Canada).

**Apoptosis assay**

Two weeks after transplantation, the whole bone marrow cells were isolated from each group and stained for PE-Cy7 conjugated with a mixture of lineage antibodies and PE-Annexin-V with congeneric marker. The proportion of apoptosis cells was determined using flow cytometry.

**Competitive and serial bone marrow transplant**

The competitive bone marrow transplantation (cBMT) procedure was detailed in our previous publication.\(^{11}\) Briefly, normal hematopoietic cells (CD45.1^+GFP^-) were sorted from the BM of leukemia or control mice 2 weeks after transplantation. 2x10^6 sorted CD45.1^+GFP^- cells (test cells) along with an equal number of CD45.1^+/CD45.2^+ BMNCs (competitive cells) were co-transplanted into lethally-irradiated (10Gy) female C57BL/6J recipients (n=5/group, 6-8 weeks old). After transplantation, blood was collected monthly. The endpoint was 6 months. The relative contribution of test cells (CD45.1^+) and competitive cells (CD45.1^+/CD45.2^-) in reconstituted recipients was measured by flow cytometry using PE-conjugated anti-mouse CD45.1 and FITC-conjugated CD45.2 antibodies (eBioscience, San Jose, CA).

**BrdU detection and cell cycle analysis**

For *in vivo* analysis, the mice were given a single pulse administration of 5-bromo-2-deoxyuridine (BrdU) (BD Biosciences) at 72h after transplantation; an intraperitoneal injection of 100µg/g of BrdU was given at 2h before harvesting the BM cells. BrdU staining was
quantitated using flow cytometry by combining surface staining to define BM subset with intracellular staining using the BrdU-APC staining kit following the manufacturer’s instructions (BD Biosciences). For in vitro cell cycle analysis, the whole bone marrow cells from the leukemic or control group were isolated at 14 days after transplantation. Then the cells were permeabilized and stained with 1.67μmol/L Hoechst-33342 followed by 1μg/ml Pyronin-Y. The proportion of CD45.1+LKS cells in G0 phase was determined by flow cytometry with quantitation of DNA and RNA.

**Cell proliferation tracing in vivo**

Whole bone marrow cells from B6.SJL mice were labeled with 1μm of CFSE (Molecular Probes) as described.12 1x10^8 CFSE labeled CD45.1+cells were injected into lethally-irradiated C57BL/6J recipients with or without 5x10^6 developed ICN1 over-expressing leukemic cells (CD45.2+). Seventy-two hours after transplantation, the recipients were sacrificed and the bone marrow cells were harvested and stained with PE-Cy5.5-conjugated CD45.1, PE-conjugated Sca-1, APC-conjugated c-Kit, and a mixture of PE-Cy7-conjugated lineage markers. Dead cells were identified with DAPI. The BD LSRII was used for data acquisition, and ModFit LT software (version 3.0, Verity Software House) was used for cell proliferation analysis.

**Real-time RT PCR**

3000 CD45.1+LKS cells were sorted directly into the lysis buffer (Strategene, La Jolla, CA). Total RNA was extracted with the RNA nanoprep kit according to the manufacturer’s instructions.
(Strategene, La Jolla, CA). Reverse transcription was achieved by using oligo-dT
(12-18) and M-MLV reverse transcriptase (Ambion, Austin, TX). Real-time PCR reaction was done with
SYBR green Master Mix (Finnzymes, Finland), 0.3µM of specific forward and reverse primers
and normalized cDNA. The parameters for the thermal cycling of PCR were as follows: 15s at
95°C and 60s at 60°C, 45 cycles. All the primer sequences are listed in supplemental Table 2.

RESULTS

Notch1 overexpression induces T-ALL with 100% penetrance.

We chose the Notch1-induced T acute lymphoblastic leukemia (T-ALL) model because the
Notch1 signaling is thought to be a conserved pathway for self-renewal in HSCs and LSCs, and
the retrovirus-mediated overexpression of the intracellular domain of Notch1 (ICN1) was shown
to be potent in T-cell leukemogenesis. We established the mouse T-ALL model in which
lethally-irradiated recipients (C57BL/6J) were transplanted with 1×10^6 cells that were transduced
with the MSCV-ICN1-IRES-GFP vector (starting with Lin−Sca-1− cells) from C57BL/6J and 1×10^7
bone marrow nucleated cells (BMNCs) from B6.SJL mice (Fig.1A). Those recipients
accumulated a double positive population (CD4+CD8+) of immature T cells in the peripheral
blood (PB) and bone marrow (BM) from the Notch1-transduced cells as early as 7 days after
transplantation (Supplemental Fig.1). Two to 4 weeks after transplantation, white blood cells
(WBCs) in PB and BM cellularity dominated by GFP+ cells were increased in a temporal fashion
(Fig. 1B-D). Gross examination of tissues from the sick mice revealed splenomegaly,
hepatomegaly, and lymphadenopathy, due to extensive organ infiltration by CD4+CD8+
lymphoblasts. This robust leukemia model, in which 100% of the mice that received Notch1 over-expressing cells developed T-ALL within 6 weeks (Fig. 1E), permitted us to study the kinetics of co-transplanted normal hematopoietic cells (CD45.1) in a leukemic host from the onset of the disease.

**Hematopoietic suppression at the stem cell and progenitor cell levels in leukemic mice.**

To document the disrupted growth of normal hematopoietic cells during leukemia development, we monitored the kinetics of co-transplanted normal hematopoietic cells that expressed CD45.1 (Fig. 1A). Using the Notch1-induced leukemia model, we examined the percentage of CD45.1+ cells in both PB and BM one week after transplantation. The results showed that in contrast to the rapid proliferation of the GFP+ leukemic cells, the percentage of CD45.1+ cells declined dramatically (Fig. 2A-B). The average percentage of CD45.1+ cells in PB was 43.67% ± 4.14% at 2 weeks post transplantation, and 10.83% ± 5.67% at 4 weeks post transplantation. As expected, the level of CD45.1+ cells in the PB of the control group was stable at around 90%. A similar level was observed in BM.

To investigate whether the under-representation of normal hematopoietic CD45.1+ cells was due to the direct negative impact of the leukemic environment on HSCs and HPCs, we quantified the frequencies of hematopoietic primitive cells at different time points after transplantation (Fig. 2C-F). At one week after transplantation, the frequency of the HSC-enriched Lin-c-Kit+Sca-1+ (LKS) cell population in the BM of leukemic hosts was similar to that of the control, whereas the
frequency of the HPC-enriched Lin− population was statistically higher than that in the control group. After the second week, the frequencies of both the LKS and Lin− cell populations became lower in the leukemia group than that in the control group (Fig. 2C-D), while LKS cells in both groups continued to proliferate. At the fourth week, CD34−LKS (Long-term repopulating HSC, LT-HSC), CD34+LKS (short-term repopulating HSC, ST-HSC) and LKS− (differentiating HPC) were significantly lower in the leukemic BM than in the control group as measured by either the frequency or the absolute yield of each population (Fig. 2E-F). The input (CD45.1+CD34−LKS) and output (CD45.1+LKS) cells harvested 2 weeks after transplantation were measured. The yield of LKS cells per initial CD34−LKS in the leukemic host was 5 times lower than that in the non-leukemic but irradiated recipients (Supplemental Table 1). An independent assessment for HSC quantitation with the SLAM markers also confirmed the significant inhibition of HSC growth in the leukemic host (Supplemental Fig. 2). More specifically, to exclude the possibility that the decreased frequency of the primitive cells in the leukemic marrow could be solely attributed to the absence of competition, we transplanted 1x10^7 BMNCs from B6.SJL (CD45.1+) along with a 100-time higher dose of BMNCs from C57BL/6J (1x10^8, CD45.2+) than that of the leukemic cells into the lethally-irradiated control recipients (C57BL/6J). The frequency of HSCs (defined as CD150+CD48−LKS) in the leukemic group was still statistically lower than that in the control group (Supplemental Fig. 2).

We also performed in vitro clonal functional assays. CD45.1+ cells from leukemic and control groups 4 weeks after transplantation were isolated for the assessment of colony-forming cells
(CFCs) and cobble-stone area forming cells (CAFCs) (Fig. 3A). Notably, the frequency of CFCs was dramatically lower in the leukemic group (Fig. 3B). Moreover, both groups showed detectable CAFC activity in the long-term culture system, but the CAFC frequency at day 35 during the culture from leukemia-bearing mice was approximately 4 times lower than that of the cells from the control mice ($P<0.05$) (Fig. 3C). Together with the phenotypic analysis (Fig. 2 C-D), these data provide direct evidence for the progressive suppression of hematopoietic cell growth during leukemia development, although the kinetics of HSC and HPC seemed to differ at the early stage.

**Functional preservation of HSCs in leukemic marrow.**

HSC is best defined by its ability to reconstitute the hematopoiesis of a lethally-irradiated recipient.\(^\text{16}\) Given the significant suppression of normal HSCs and HPCs during leukemia development as assessed by phenotypic analysis and *in vitro* culture, we next tested whether the repopulating ability of normal hematopoietic cells from a leukemic environment was decreased in a new non-leukemic host. We co-transplanted equal numbers of CD45.1\(^+$\) cells isolated from either a leukemic or a control group (2 weeks after transplantation) along with competitor cells (BMNCs freshly isolated from mice with a CD45.1\(^+$\)/CD45.2\(^+$\) phenotype) into lethally-irradiated C57BL/6J recipients. Because the repopulation unit (RU) in an unmanipulated C57BL/6J mouse has been well documented,\(^\text{16}\) a fixed number of competitor cells ($2\times10^6$) can serve as a standard measure against which the input cells from both groups can be compared. PB was collected 1-6 months after competitive bone marrow transplantation (cBMT), and the relative
contribution to hematopoiesis of CD45.1+ cells isolated from both the leukemic and control groups was quantified using flow cytometry based on distinct congenic surface markers (CD45.1+ vs. CD45.2+). Unexpectedly, CD45.1+ cells isolated from the leukemic hosts engrafted better than the cells isolated from the non-leukemic control recipients. The level of CD45.1+ cells gradually increased after transplantation and stabilized at 3 months. Six months after cBMT, the level of CD45.1+ hematopoietic cells from the leukemic environment was more than 3 times higher than that from the control group (38.45% vs 11.40%) (Fig. 4A). This indicates no apparent defect in the hematopoietic potential of the residual normal HSCs isolated from the leukemic environment. A multilineage analysis of PB from cBMT recipients was performed to determine whether the HSCs in the leukemic environment maintained their ability to give rise to both myeloid and lymphoid lineages. The myeloid cells (granulocytes and monocytes) were immunophenotypically defined by Mac-1+, the T cells by CD3+, and the B cells by B220+ (Fig. 4B). At the endpoint of 6 months after cBMT, the average level of CD45.1+ cells isolated from leukemic BM was about 10-fold higher than that of cells from the irradiated but non-leukemic environment (44.87% vs. 4.68%) (Fig. 4C-D). Consistent with this finding, the number of HSCs/HPCs from the leukemic environment was significantly higher than that from the control environment in the new recipients (Fig. 4E).

**Increased proliferation of normal hematopoietic progenitors upon exposure to leukemic marrow.**

The marked decrease of normal hematopoietic cells in leukemic marrow may be due to
alterations in cell proliferation or apoptosis. We examined the proliferation of the affected HPC populations with multiple assays. We first measured cell divisions of an immunophenotypically-defined HPC population in lethally-irradiated recipients either in the presence or absence of leukemic cells. We used the dye 5-(and 6-)carboxy-fluorescein diacetate succinimidyl ester (CFSE) to label the normal BMNCs from B6.SJL animals before transplantation,\textsuperscript{17} and surface markers of hematopoietic primitive cells were used to co-stain BMNCs that were harvested 72 hours after transplantation. Due to the undetectable level of c-Kit expression (Shen H. et al., manuscript in preparation) on cells shortly after transplantation,\textsuperscript{18} we used the Sca-1 marker to define a relatively primitive state within the Lin\textsuperscript{−} population and found that 72 hours after transplantation, the CD45.1\textsuperscript{+}Lin\textsuperscript{−}Sca-1\textsuperscript{+} cells co-transplanted with the leukemic cells underwent more cell divisions than the control cells (Fig. 5A). This suggests that HPCs in the leukemic environment have a more active cycling status.

To determine the actual cycling status of the hematopoietic primitive cells under leukemic stress, mice in the leukemia and control groups were then pulsed with 5-bromo-2-deoxyuridine (BrdU) 72 hours after transplantation of the leukemic cells. CD45.1\textsuperscript{+}Lin\textsuperscript{−}Sca-1\textsuperscript{+} cells were analyzed for BrdU incorporation 2 hours after injection. As shown in Fig. 5B, BrdU\textsuperscript{+}Lin\textsuperscript{−}Sca-1\textsuperscript{+} cells were significantly more abundant in the leukemic group than in the control group. Furthermore, we utilized the anti-metabolic reagent 5-fluorouracil (5-FU) to functionally measure the cells in S-phase. Seventy-two hours after transplantation, CD45.1\textsuperscript{+}GFP\textsuperscript{−} cells were isolated for CFC assay. While the CFC frequency increased significantly in leukemic marrow, there was a more
dramatic reduction of the CFC yield after 5-FU treatment (Fig. 5C), thus confirming the result of BrdU incorporation (Fig. 5B). These cell cycle measurements, together with the CFC yields, are also consistent with the finding that CD45.1+Lin- cells were more abundant in leukemic mice at the early stage (Fig. 2C).

To exclude the contribution of apoptosis, we then stained for the apoptotic marker, Annexin V, in the cell population after transplantation and found no statistical difference in the apoptotic fraction between the hematopoietic cells from the leukemic environment and the cells from the control environment (Supplemental Fig. 3). This suggests that apoptosis may not be the main reason for the decrease of hematopoietic cells in the leukemic environment.

**Increased quiescence of normal HSCs in leukemic hosts**

It is known that relative quiescence is associated with the quality of stem cells in adults. To understand why HSCs from leukemic BM displayed higher levels of reconstitution than those isolated from the control mice when transplanted into non-leukemic new recipients, the quiescent fraction of HSCs in cell cycle was examined using the RNA dye pyronin Y (PY), which has been used as a measure for stem cell quiescence (G0 phase in cell cycle). While there was no significant difference in the cycling fraction (S + G2/M) as assessed by the DNA dye Propidium Iodide (PI) (Fig. 6A), CD45.1+LKS cells from leukemia-bearing hosts demonstrated a larger fraction in G0 phase (PYlow in the continuum) as opposed to that in G1 phase (Fig. 6B), thereby suggesting that the leukemic environment is able to prevent quiescent HSCs from moving into
Potential molecular players underlying the increased quiescence of the HSC pool were explored by examining several known cell cycle inhibitors including p18\textsuperscript{INK4C}, p21\textsuperscript{Cip/Waf1} and p27\textsuperscript{Kip1} (p18, p21 and p27 hereafter) in tissue stem and progenitor cells.\textsuperscript{10,12,21,22} As demonstrated in our previous studies,\textsuperscript{11,21} p21 maintains HSC quiescence in at least some mouse strains, whereas p27 primarily controls the proliferation of HPC. However, p18 and p21 play opposite roles in regulating HSC self-renewal. CD45.1+LKS cells were harvested 2 weeks after initiation of leukemogenesis (Fig. 1A) for the analysis with real-time RT PCR. There was a decrease in p18 expression in the cells from leukemic hosts compared to the control cells. However, p21 expression was significantly increased in the LKS cells isolated from a leukemic environment. Consistent with this, its upstream regulator, Gfi-1,\textsuperscript{23} was more dramatically increased. In contrast, there was no statistical difference in p27 expression between the cells from leukemic and control marrow (Fig. 6C). Therefore, the altered expression of these cell cycle regulators may contribute to an underlying molecular basis for the more quiescent state and preserved self-renewal potential of HSCs in leukemic marrow. This mechanism could prevent normal HSCs from overly reacting to the leukemic environment and better maintain the potentiality of HSCs (Supplemental Fig. 4).

DISCUSSION

In summary, normal HSCs and HPCs are progressively suppressed during leukemogenesis.
While HPCs rapidly overreact to leukemic bone marrow with a more actively cycling status, it causes the exhaustion of HPC pools. In contrast, the function of HSC is better preserved at least in part due to increased quiescence in cell cycle. Therefore, HSCs and HPCs have distinct responses to a leukemic environment. Our findings may not only be able to model Notch1-induced leukemia, which is associated with more than 50% of T-ALL in children, they may also have implications to studying other types of leukemia or cancer via a unique approach.

Our study demonstrates that the reduction in the number of blood cells in a leukemic environment is largely due to the impairment of HSC/HPC repopulation. Evolving leukemic cells cause disruption to the BM structure, which may interrupt the physiological interaction between HSCs and their niches. As a consequence, the responsiveness of normal HSCs and HPCs to cytokines may be altered. It has been reported that leukemic cell growth disrupts normal HPC niches in BM and creates an abnormal response to microenvironments that sequester transplanted human HPCs in a xeno-graft model. Based on our current study, this response could be heightened during the early stages of leukemogenesis (Fig. 2C, Fig.5) and decreased at a later stage (Fig. 2C-F). Importantly, our study documents for the first time that normal HSCs can be reversibly suppressed by a leukemic environment. The compartment of normal HSCs in a leukemia host can be kept in a more quiescent state with preserved self-renewal potential, while normal HPCs may be exhausted after undergoing an accelerated proliferation. Therefore, when more quiescent HSCs from a leukemic environment are seeded into a non-leukemic or perhaps minimally leukemia-loaded hematopoietic microenvironment,
they re-enter the cell cycle and the self-renewal potential of HSCs can be fully revealed (Supplemental Fig. 4). This model underscores the importance of host environment or condition in dictating a specific functional state of tissue stem cells and more specifically, it is also consistent with the rapid recovery of normal hematopoiesis in leukemia patients after an effective chemotherapy.

It should be noted that in our current study, young mice were used and the period of latency of leukemia was short. Thus, we cannot generalize our conclusions to leukemia development in elderly mice. Our previous work, in which HSCs and T leukemic cells were transplanted for multiple rounds over a 2 to 3 year period, demonstrated that normal HSCs in leukemic mice were no longer functional at later stages as assessed by both in vitro and in vivo assays. In our present study, normal HSCs recovered from the leukemic environment were still highly functional after transplantation into new non-leukemic hosts. The difference in performance between the HSCs in these 2 studies is likely due to the different proliferation history of the stem cells. HSCs that remain in a leukemic environment may experience less proliferation than control cells, which may contribute to their ability to better repopulate after the second transplantation. In addition, in our current study, we used lethally-irradiated recipients, which may impact the engrafted HSCs via the bystander effect of irradiation (Shen H. et al., Manuscript under submission). But this potential confounding factor should be minimized due to our proper control, in which normal hematopoietic cells alone were transplanted into irradiated recipients (Fig. 1A).
While hematopoietic suppression is a general feature of leukemogenesis, the mechanisms that lead to the suppression and especially to the better performance of donor HSCs in secondary recipients may be context-dependent. A previous study by others showed that the size of the normal HSC compartment was unaffected in the B-ALL induced by ETV6-RUNX1 or p190 BCR-ABL, whereas it was significantly reduced in the B-ALL induced by p210 BCR-ABL. Therefore, the actual effects of a cancer environment on normal tissue stem cells are likely determined by multiple factors, including the type and stage of leukemia, the patient age, and the specific therapeutic regimen. Future studies on the underlying molecular pathways, especially in the context of a specific cancer environment, are needed. Such studies may eventually offer complementary approaches for the prevention and treatment of cancer.
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**Authorship**

Contribution: X.H. performed most experiments, analyzed the data and wrote the paper; H.S. performed the flow cytometry work and analyzed the data; C.T. participated in leukemia modeling and data analyses; H.Y. performed the transplant experiment; G.Z. contributed to leukemia model and data analyses; R.XF. helped perform the transplant experiment; Z.J. performed some of the flow cytometry work; J.X. contributed to retroviral transduction and plasmid preparation; J.W. analyzed the data and co-supervised X.H.’s research work; T.C. came out with the original idea, designed the experiments, analyzed the data, wrote the paper and oversaw the overall research project.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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REFERENCES


9. Yuan Y, Yu H, Boyer MJ, et al. Hematopoietic stem cells are not the direct target of


17. Oostendorp RA, Audet J, Eaves CJ. High-resolution tracking of cell division suggests similar


FIGURE LEGENDS

Figure 1. Experimental design and establishment of a murine leukemia model by Notch1 overexpression. Lin^Sca-1^ cells (CD45.2^) were isolated from BM of C57BL/6J mice. 1×10^7 BMNCs (CD45.1^) from B6.SJL mice were transplanted into lethally-irradiated recipients (10 Gy, C57BL/6J) either with (leukemia group) or without (the control group) 1×10^6 cells that were transduced with the MSCV-ICN1-IRES-GFP vector. The transplanted normal hematopoietic cells (CD45.1^GFP^) from the recipients were analyzed and sorted by flow cytometry at different time points (A). Resultant data is shown in all the figures except Figure 5. An additional control with a higher dose of normal congenic BMNCs was included in some experiments (Supplemental Fig. 2). A T-ALL feature of the affected mice is shown by an immature T cell phenotype (Supplemental Fig. 1). The hosts receiving Notch1-transduced cells displayed leukemia symptoms, including increases of total leukemic cells (CD45.2^GFP^) in PB (B), WBCs counts in PB (C) and BM cellularity (D). Values are mean ± standard deviation (s.d.). *P<0.05 (t-test). The 100% penetrance of the leukemic development is shown in the survival curve (E). P<0.0001 (n=15/each, Kaplan-Meier analysis).

Figure 2. Growth kinetics of normal hematopoietic cells in leukemia-bearing mice. The percentages of normal hematopoietic cells (CD45.1^) in the leukemia group were monitored in both PB (A) and BM (B) 2 and 4 weeks after transplantation. In addition, the frequencies of GFP^CD45.2^CD45.1^Lin^ and GFP^CD45.2^CD45.1^LKS cells in BM were measured 1, 2 and 3 weeks after transplantation (C-D). P<0.05 (n=5/each, t-test). Frequencies and absolute
numbers of different hematopoietic cell populations within GFP^CD45.2^CD45.1^ BMNCs 4 weeks after transplantation are shown in the graphs (E-F). "P<0.05; "**P<0.01 (t-test). The BM cellularity of tibia, femur, iliac and humerus was calculated as 40% of the total BM cellularity of a young adult C57BL/6J mouse (based on our own data). The data shown here represent one of four independent experiments (n=6-7/group). Each value is mean ± s.d.

Figure 3. In vitro clonal growth of normal hematopoietic cells from a leukemic environment. Two weeks after transplantation, the mice were euthanized and BM was harvested. The normal hematopoietic cells (CD45.1^GFP^-) were doubly sorted with near 100% purity for in vitro clonal assays (A). For the CFC assay to measure committed HPCs (B), the sorted CD45.1^GFP^- cells were cultured in the defined methylcellulose medium supplemented with a cytokine cocktail. Mix, GM, G, M and E represent CFC-mix (more than two lineages), CFC-granulocyte/monocyte, CFC-granulocyte, CFC-monocyte and BFU-erythrocyte, respectively. Each value is the mean ± s.d. "P<0.05; "**P<0.01 (t-test). In addition, the cobblestone area-forming cell (CAFC) assay with limiting dilution at day 35 during the long-term culture was used to measure the more primitive hematopoietic cells and the result is shown as a representative data set from 3 experiments with similar results (C).

Figure 4. Long-term reconstitution of normal hematopoietic cells from leukemic marrow in new non-leukemic hosts. The hematopoietic regeneration of the normal hematopoietic cells from leukemic or control mice were examined in secondary non-leukemic recipients using
the competitive bone marrow transplantation (cBMT) assay, in which equal numbers of test (CD45.1⁺GFP⁻) and competitor cells (CD45.1⁺/2⁺) were co-transplanted into lethally-irradiated congenic recipients (CD45.2⁺). The overall reconstitution levels of normal HSCs from the primary recipients were monitored within 6 months after transplantation (A). Multi-lineage differentiation of the engrafted cells was analyzed 6 months after transplantation (B). GM, T and B indicate lineages for myeloid (Mac-1⁺), T (CD3⁺) and B (B220⁺) cells, respectively. Six months after cBMT, the overall representation of CD45.1⁺GFP⁻ cells, multilineage analysis, and different hematopoietic cell subsets in BM were also quantified (C-E). *, P<0.05; **, P<0.01 (n=5-7/each group, t-test). Data shown is from 1 of 3 experiments with similar results.

Figure 5. Proliferative response of normal HPCs to leukemic cells in vivo. (A). CFSE assay. 1×10⁸ BMNCs (C57BL/6J, CD45.2⁺) labeled with CFSE were co-injected with or without 5×10⁶ Notch1-induced leukemia cells (CD45.2⁺GFP⁺) into lethally-irradiated recipient mice (SJL.B6, CD45.1⁺). BMNCs were harvested 72 hours after the transplantation to assess the number of cell divisions. BMNCs were stained with lineage markers and Sca-1, and CFSE-labeled cells were analyzed in the gate for CD45.1⁺Lin⁻Sca-1⁺. A representative figure of the flow cytometric analysis is shown. Blue peaks on the right indicate undivided cells (parent cells) and each peak towards the left side represents one cell division or generation. The figure shown is from 1 of 4 experiments with similar results. The proliferation index¹² in the CD45.1⁺Lin⁻Sca-1⁺ population is shown in the graph (*P<0.05). (B). BrdU assay. BrdU was injected via IP 72h after transplantation. BM cells were analyzed 2 hours later and the
proportion of BrdU^+CD45.1^+Lin^−Sca-1^− cells were analyzed with flow cytometry. A representative plot is shown, and the figure shown is from 1 of 6 experiments with similar results. Each value is mean ± sd, ** P<0.01 (n=3 mice /group, t-test). (C). 5-FU assay. 150mg/g 5-FU was injected 12h before BM was harvested and then CD45.1^+GFP^− cells were isolated for the CFC assay 72h after transplantation. The total CFC colonies were counted at day 14 with microscopy. The data shown is from 1 of 2 independent experiments (n=3-5 wells), **, P<0.01.

Figure 6. Mitotic quiescence of the primitive hematopoietic cells in the leukemic hosts and expression of several cell cycle regulators. As illustrated in Fig. 1A, 1×10^7 BMNCs from B6.SJL mice were transplanted with or without 1×10^6 Notch1-induced leukemia cells (CD45.2^+GFP^+) into lethally-irradiated C57BL/6J recipients. At the 2-week time point, LKS cells from CD45.1^+BMNCS were sorted for staining with Propidium Iodide (PI) to assess the general cell cycle profile (G0/G1 vs. S/G2+M) or staining with Pyronin-Y (PY) in conjunction with Hoechst-33342 (HO) to specifically determine the portion of cells in G0 vs. G1 with flow cytometry. An aliquot of the cells was also used to examine the expression of several cell cycle regulators with real time RT PCR. (A). PI staining. A representative figure is shown from 1 of 2 experiments with similar results. (B). PY staining. Cells residing in G0 appear at the bottom of the G0/G1 peak as shown in the representative plot. Each value is mean ± s.d., **P= 0.017 (t-test). (C). Expression of cell cycle regulators. The CD45.1^+LKS cells were sorted directly into lysis buffer for real-time RT-PCR analysis. Data shown are the ratios between leukemic and control groups from 1 of 2 independent experiments with similar results. Each value is
mean ± s.d., *, *P<0.05; ** *P<0.01 (t-test).
**Fig. 1**

**B**

CD45.2\(^+\)GFP\(^+\) in PB (\%)

Weeks after transplantation

**C**

WBC (×10^6/ml)

Weeks after transplantation

**D**

BM cellularity (×10^6/ml)

BM: Control
Leukemia

Weeks after transplantation

**E**

Survival (%)

Days after transplantation
Fig. 2

E

F

Primitive cell frequency ($\times 10^3$)

Primitive cells per harvest ($\times 10^2$)

- Control
- Leukemia

CD34-LKS
CD34+LKS
LKS-

CD34-LKS
CD34+LKS
LKS-

5.1 X
7.6 X
2.5 X

113 X
159 X
65 X

* P < 0.05
** P < 0.01
**Fig. 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>95% CI</th>
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<tbody>
<tr>
<td>Control</td>
<td>1/258,127</td>
<td>1/(177,727~578,138)</td>
</tr>
<tr>
<td>Leukemia</td>
<td>1/1,014,936</td>
<td>1/(411,082~3,295,657)</td>
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</table>
Fig. 4

A

CD45.1+ cell engraftment (%) in PB

Month(s) after transplantation

Control
Leukemia

B

Engraftment (%) in CD45.1+ cells

Mac-1
B220
CD3

*P<0.05, **P<0.01
Fig. 4

C

D

CD45.1+ engraftment in BM (%)

44.87**

Leukemia

Control

Engraftment in CD45.1(%)

Mac-1

B220

CD3

Control

Leukemia
Fig. 4

E

Primitive cells in BM (×10^2)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leukemia</th>
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<tbody>
<tr>
<td>Lin^-</td>
<td></td>
<td>X 8 **</td>
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<tr>
<td>Lin^-Sca-1^+</td>
<td></td>
<td>X 6.6 *</td>
</tr>
<tr>
<td>LKS</td>
<td></td>
<td>X 19.6 **</td>
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</table>

* p < 0.05
** p < 0.01
Fig. 5

B

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20% 33%

Control Leukemia

BrdU 7-AAD

% BrdU+ cells

Control Leukemia

**

0 5 10 15 20 25 30 35 40

Control Leukemia

**
Fig. 5

C

CFCs/10^4 CD45.1+ BMNCs

5-FU

-  +

Control

Leukemia

**↓ 45%

**↓ 55%
Fig. 6

Graph showing relative expression levels of p18, p21, p27, and Gfi-1.
Kinetics of normal hematopoietic stem and progenitor cells in a Notch1-induced leukemia model

Xiaoxia Hu, Hongmei Shen, Chen Tian, Hui Yu, Guoguang Zheng, Richard XuFeng, Zhenyu Ju, Jing Xu, Jianmin Wang and Tao Cheng

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