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Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML

Yoriko Saito¹, Naoyuki Uchida², Satoshi Tanaka³, Nahoko Suzuki¹, Mariko Tomizawa-Murasawa¹, Akiko Sone¹, Yuho Najima¹, Shinsuke Takagi^{1,2}, Yuki Aoki¹, Atsushi Wake², Shuichi Taniguchi², Leonard D. Shultz⁴, and Fumihiko Ishikawa¹

¹Research Unit for Human Disease Models, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan

²Department of Hematology, Toranomon Hospital, Tokyo, Japan

³Nippon Becton Dickinson Company, Tokyo, Japan

⁴The Jackson Laboratory, Bar Harbor, Maine, USA

The concept of cancer stem cells has been proposed in various malignancies including acute myeloid leukemia (AML)^{1–3}. Human AML leukemia stem cells (LSCs) resemble normal hematopoietic stem cells (HSCs) in their ability to engraft, produce progeny long-term and self-renew in vivo⁴. We previously reported that CD34+CD38– human primary AML LSCs are relatively cell cycle quiescent and CD34+CD38– LSCs residing within the bone marrow (BM) endosteal region are chemotherapy-resistant, suggesting that cell cycle quiescence underlies chemotherapy resistance of human AML LSCs, leading to AML relapse⁵. Here we demonstrate that the BM endosteal region is enriched for quiescent human AML LSCs and in vivo cytokine treatment induces their entry into cell cycle. Using an in vivo treatment strategy in NOD/SCID/IL2r^γnull human AML xenotransplantation model, significantly enhanced elimination of human primary AML LSCs was achieved through in vivo cell cycle modification, suggesting functional modification of LSCs as an effective therapeutic strategy.

We generated human AML-engrafted NOD/SCID/IL2r^γnull recipients by transplantation of purified hCD34+CD38– LSCs from seven AML patients. The mean PB and BM hCD45+ cell engraftment level of recipients were 77.6 ± 5.9% and 95.0 ± 1.8% (mean ± SEM, n=48), respectively, indicating that the recipient BM was nearly completely replaced by human AML cells. Flow cytometry plots of BM from representative recipients engrafted with each case of AML are shown in Fig. 1a (Cases 3 and 5) and Supplementary Fig. 1a (Cases 1, 2, 4, 6 and 7). All hCD45+ cells in the recipient PB and BM were also hCD33+, indicating that all CD45+ human hematopoietic cells in these recipients are human AML cells (Supplementary Fig. 1b). The injection of purified hCD34+CD38– cells resulted in repopulation of recipient BM with hCD34+hCD38–, hCD34+CD38+ and hCD34– cells (Fig. 1a, Supplementary Fig. 1c). Successful secondary engraftment (Supplementary Fig. 1d) demonstrated self-renewal capacity of hCD34+hCD38– cells, thereby satisfying the criteria for malignant stem cells (long-term engraftment capacity, capacity to develop leukemia in

Correspondence should be addressed to F.I. (f_ishika@rcai.riken.jp).

Note: Supplementary information is available on the Nature Biotechnology website.

AUTHOR CONTRIBUTIONS

Y.S., L.D.S. and F.I. designed the study, analyzed the data and wrote the manuscript. N.U., A.W. and S. Taniguchi provided clinical samples, information and discussion. Y.S., S. Tanaka, M.T., N.S., A.S. and F.I. performed the experiments. S. Takagi and Y.A. analyzed the data. Y.N. analyzed the data and wrote the manuscript.

vivo, the generation of non-stem AML cells and self-renewal capacity). While there was case-dependent variability, the majority of recipient BM LSCs was in G0 and G0/G1 phases of cell cycle, significantly higher frequencies compared with hCD34+CD38+ cells (Supplementary Table 1).

Next we examined the relationship between LSC cell cycle status and cytotoxic effect of a chemotherapeutic agent cytarabine (AraC), a key chemotherapeutic agent used both in remission induction and post-remission therapy for AML patients. Mean pre-treatment PB engraftment level as measured by %hCD45+ cells were similar between AraC-treated and control AML-engrafted recipients (74.9 $\pm$ 4.2%, and 67.6 $\pm$ 7.1%, respectively, n=15 for each group, p=0.4041 by two-tailed t-test). Representative cell cycle analyses following BrdU incorporation are shown in Supplementary Fig. 1e. When primary AML-engrafted NOD/SCID/IL2r γ ^{null} recipients were given AraC, BM CD34+CD38- AML cells in S-phase of cell cycle were preferentially eliminated (%S = 0.1 $\pm$ 0.1 in AraC treated group and %S = 10.6 $\pm$ 0.9 in control group, respectively, n=15 for each group, p < 0.0001 by two-tailed t-test). This was accompanied by the enrichment of non-cycling LSCs in G0/G1 phases of cell cycle (%G0/G1 = 91.7 $\pm$ 2.3 in AraC treated group and %G0/G1 = 80.9 $\pm$ 1.5 in controls, n=15 for each group, p = 0.0011 by two-tailed t-test). These findings demonstrate that within hCD34+CD38- LSC population, cell cycle quiescence is associated with AraC-resistance. Therefore, we hypothesized that entry of quiescent LSCs into cell cycle would increase their susceptibility to chemotherapeutic agents. To test this hypothesis, we examined whether granulocyte colony-stimulating factor (G-CSF) induces human LSCs to enter cell cycle in vivo. While induction of human and mouse normal HSC cell cycle entry by G-CSF has been described, the effect of G-CSF on LSCs has not been defined^{6,7}. Hoechst/PyroninY (Fig. 1a,b) and BrdU incorporation (Supplementary Fig. 1e) assays were used to document the induction of cell cycle in LSCs. In all seven primary AML cases, G-CSF treated AML-engrafted recipient BM LSCs showed significant reduction in the fraction of cells in G0 phase with concomitant increase of LSCs in S and G2/M phases (Fig. 1c).

We previously demonstrated that chemotherapy-resistant CD34+CD38- LSCs are enriched within the BM endosteal region, while CD38+ AML cells resided mainly in the central region of the BM⁵. Therefore, we performed Ki67 labeling in primary human AML-engrafted recipient bone sections to directly examine the cell cycle status of LSCs within the BM endosteal region, adjacent to the bone endosteum lined with osteopontin-positive osteoblasts (Supplementary Fig. 2). Ki67 is a nuclear protein expressed in proliferating cells during late G1, S, G2 and M phases but is negative in quiescent cells in G0 phase of cell cycle. In a representative AML-engrafted recipient (with a post-sacrifice BM human AML chimerism of 98.5%) human leukemic cells in the central zone of the BM were strongly Ki67 positive indicating that they are highly proliferative, while the majority of AML cells abutting the endosteum were in G0 phase as evidenced by the negative Ki67 labeling (Fig. 2a,b). AML cells in the perivascular region, one of the physiological niches for normal murine HSCs^{8,9}, were actively cycling as demonstrated by positive Ki67 staining (Fig. 2d,e). Z-stack images showing vertical slices of the bone section further confirmed the relative paucity of Ki67-expressing cells in the region adjacent to the endosteum (Fig. 2c,f). This endosteal enrichment of G0 AML cells combined with our previous observation that chemotherapy-resistant LSCs preferentially localize within the BM endosteal region suggest that AML relapse may be attributable to cell cycle quiescence within the BM niche.

We then examined whether in vivo cytokine treatment induces quiescent LSCs within the endosteal niche to enter cell cycle. Fig. 2g-i and Supplementary Fig. 3a-c show bone sections from a representative human AML-engrafted recipient treated with G-CSF (with post-sacrifice BM human AML engraftment of 97.8%). Following five-day in vivo administration of G-CSF, human CD45+ AML cells in the endosteal region became Ki67-

positive, indicating their cell cycle entry (Fig. 2g–i). The central zone of the BM from the same recipient also demonstrates numerous Ki67-positive CD45+ human AML cells (Supplementary Fig. 3a–c). Three-dimensional reconstruction of the bone section clearly demonstrated cell cycle entry by human CD45+ AML cells within the endosteal region following cytokine treatment (Fig. 2j,k, Supplementary movies 1 and 2). Great majorities of BM cells in AML-engrafted recipients examined were hCD45+CD33+ AML cells (93.2 $\pm$ 1.3% for G-CSF-treated group, n=37 and 96.2 $\pm$ 0.6% for control group, n=45). Co-labeling for human CD34 and Ki67 demonstrated that human CD34+ AML cells in the endosteal region enter cell cycle in response to in vivo cytokine treatment (Supplementary Fig. 3d,e,g,h). The results of BrdU staining were consistent with Ki67 labeling, showing cell cycle induction of human AML cells within the endosteal region in cytokine-treated recipients (Supplementary Fig. 3f,i). Quantification of human CD45+Ki67+ cells within the endosteal region demonstrated statistically significant increase in cycling LSCs following in vivo cytokine treatment (Supplementary Table 2). Taken together, these findings indicate that in vivo administration of G-CSF induces quiescent LSCs residing within the endosteal niche to enter cell cycle.

Next, to demonstrate that entry into cell cycle increases susceptibility of human LSCs to chemotherapy in vivo, we developed an in vivo treatment model using AML-engrafted recipients. After chemotherapy (AraC) alone or chemotherapy following cell cycle induction by G-CSF, the recipient BM was evaluated through: 1) Flow cytometric assessment of LSC apoptosis as evidenced by active caspase 3 expression, 2) histological examination of BM in situ to directly examine LSC apoptosis within the BM niche by TUNEL staining and 3) functional determination of the frequency and AML-initiating capacity of remaining LSCs via limiting dilution serial transplantation. Mean pre-treatment human AML engraftment as measured by PB %hCD45+ was similar between chemotherapy alone and chemotherapy with cell cycle induction groups (31.6 $\pm$ 4.1% with a range of 10.6–67.5% and 28.5 $\pm$ 4.3% with a range of 9.9–78.4%, respectively, p=0.6031 by two-tailed t-test).

The degree of apoptosis in LSCs was quantified by flow cytometric measurement of active caspase 3 expression within BM CD34+CD38– cells (Fig. 3a, Supplementary Fig. 4). Chemotherapy with cell cycle induction significantly reduced the frequency of viable non-apoptotic hCD34+CD38– LSCs in recipients engrafted with AML originating from each of the seven primary AML cases examined. While this effect is variable among the seven AML cases reflecting biological heterogeneity, it is statistically significant in each case.

In situ examination of the BM demonstrated that in recipients treated with chemotherapy alone, TUNEL-negative viable AML cells remained at the endosteum (femoral section from a representative recipient shown in Fig. 3b, upper panels). In contrast, chemotherapy with cell cycle induction resulted in increased apoptosis in the endosteal region (as well as the central region), as evidenced by decreased cellularity by HE staining and positive TUNEL staining of the residual cells (femoral section from a representative recipient shown in Fig. 3b, lower panels). In BM of AML-engrafted recipients post-treatment, great majorities of BM cells were hCD45+CD33+ AML cells (95.9 $\pm$ 0.8% for chemotherapy alone group, n=32 and 91.4 $\pm$ 1.3% for chemotherapy with cell cycle induction group, n=29). Therefore, the majority of the cells present in the histological sections shown are human AML cells, not residual mouse hematopoietic cells. Taken together, these findings indicate that chemotherapy-induced LSC apoptosis within the endosteal niche is enhanced by cell cycle induction of LSCs in vivo.

We found significant reductions in both proportion and number of viable hCD34+ cells in the BM of recipients following chemotherapy with cell cycle induction with the absolute number of BM hCD34+ cells decreasing by 1.5- to 7-fold compared with those receiving

chemotherapy alone (Supplementary Table 3). While the reduction in hCD34+ cells indirectly demonstrates the reduction of LSC frequency and number, in vivo evaluation of AML-initiation is required to definitively prove the reduction in LSC frequency and function. Therefore, we performed limiting dilution serial transplantation of purified viable BM hCD34+ cells at doses of 2×10^2 , 2×10^3 , 2×10^4 and 2×10^5 cells per recipient from AML-engrafted recipients treated with either chemotherapy alone or chemotherapy with cell cycle induction. CD34+ cells, not CD34+CD38- cells, were used as the graft for serial transplantation given the possibility that CD38 expression level may alter with exposure to G-CSF. PB engraftment time course and overall engraftment efficiency of serial transplant recipients at each human CD34+ LSC graft dose are shown in Fig. 3c and Supplementary Table 4. At the graft dose of 200 cells, none of the 18 recipients of LSCs from cytokine combined with AraC-treated AML-engrafted mice developed AML at 24 weeks post-transplantation. In contrast, 13/18 (72.2%) recipients of 200 LSCs from AraC only-treated AML-engrafted mice developed AML. At the dose of 2×10^4 and 2×10^5 , all recipients of LSCs from AraC-only treated AML-engrafted recipients developed AML by week 24 (n=38 and n=14, respectively) while 15/31 and 10/15 recipients of LSCs from cytokine+AraC-treated AML-engrafted mice developed AML. Although the engraftment kinetics varied among LSC samples, at each dose level, the frequency of recipient mice not developing AML was significantly higher in cytokine-combined chemotherapy group compared with chemotherapy alone (Fig. 3c and Supplementary Table 4). The frequency of LSCs was estimated by Poisson statistics using the method of maximum likelihood, a standard methodology used in the estimation of HSC frequencies in limiting dilution transplantation assay^{10,11}. Estimated LSC frequency within BM hCD34+ cells was significantly lower in the recipients following chemotherapy with cell cycle induction (Supplementary Table 5, overall frequencies 1 in 55,076 vs. 1 in 560, $p=0.0001$ by two-tailed t-test). Furthermore, at 24 weeks post-transplantation, 7.2% (9 out of 125) of recipients of BM hCD34+ cells from AML-engrafted recipients treated with chemotherapy alone survived while 74.3% (81 out of 109) of the recipients of BM hCD34+ cells from AML-engrafted recipients exposed to cytokine prior to chemotherapy survived (Fig. 3d). Taken together, these findings indicate that in vivo cell cycle entry potentiates the elimination of chemotherapy-resistant human LSCs.

Despite increasing understanding of the pathogenesis and biology of AML, patient outcomes remain poor, with a median survival of approximately one year with standard treatment due to high rates of disease relapse¹². Recent studies have demonstrated that LSCs play a central role in AML pathogenesis, suggesting that failure to eradicate LSCs is an important factor in patient outcomes¹³. Therefore, we aimed to develop a therapeutic strategy against LSCs through in vivo functional modification of primary human AML LSCs.

Using the newborn NOD/SCID/IL2 γ ^{null} xenotransplantation model, we demonstrated in situ that primary AML LSCs are cell cycle quiescent within the endosteal niche. These quiescent LSCs within the BM endosteal region survive in vivo chemotherapy despite efficient elimination of cycling AML cells, consistent with the gap between successful remission induction and overall survival in AML. Furthermore, cell cycle quiescence of human AML LSCs can be modified by in vivo G-CSF administration, leading to significantly enhanced chemotherapy sensitivity of human AML LSCs in vivo. These findings provide proof-of-concept that cell cycle inducers such as G-CSF can directly modify the functional behavior of LSCs in vivo, increasing their responsiveness to chemotherapy (Fig. 3e,f).

The roles of G-CSF as an inducer of myeloid maturation, differentiation and proliferation have been successfully exploited to accelerate granulocyte recovery in the setting of myelosuppressive chemotherapy, and its function as mobilizer of HSCs has proven effective

for PBHSC collection¹⁴. G-CSF was also found to metabolically activate AML and CML cells enhancing the effects of chemotherapy in vitro, leading to clinical studies examining G-CSF during induction chemotherapy for AML^{15,16}. In a large randomized controlled study of 640 newly diagnosed AML patients, Lowenberg and colleagues reported statistically significant improvements in the rate of relapse and disease-free survival at four years in all patients¹⁷. Subsequently, five other randomized controlled studies examining the addition of G-CSF during induction chemotherapy reported equivocal results^{18–22}. The effects of cytokine-combined chemotherapy on LSCs were unevaluated in these studies.

It is difficult to interpret the results of these studies as a composite since the treatment regimens and patient populations were highly diverse with two studies consisting of relapsed/refractory patients and three studies consisting of the elderly. LSC-targeted therapy is unlikely to affect the rates of remission induction significantly, since LSCs are not expected to constitute a substantial portion of the total body AML burden at the time of induction. Therefore, LSC-targeted therapy likely has the greatest positive impact in the setting of post-remission therapy, whether as a part of consolidation therapy or as pre-transplant conditioning. This is consistent with uncontrolled single center experiences reporting improved patient outcomes associated with pre-transplant conditioning regimen containing G-CSF^{23,24}.

We observed considerable variability in the response of LSCs to cell cycle induction and the frequency of residual functional LSCs following chemotherapy with cell cycle induction, reflecting biological heterogeneity of AML. Further studies are required to determine if LSC response to cell cycle modifying agents correlates with patient characteristics such as age, cytogenetics, presence of specific mutations and response to prior therapies. In addition, the LSC responsiveness to cell cycle induction itself as a prognostic indicator must be investigated.

Agents that modify cell cycle or disrupt stem cell-niche interactions are also expected to affect normal HSCs. To begin to address this issue, we examined in vivo survival of normal HSCs following chemotherapy alone compared with cytokine-combined chemotherapy in NOD/SCID/IL2 γ ^{null} recipients reconstituted with normal human hematopoiesis³⁴. We found that the rate of apoptosis in hCD45+CD34+CD38– human HSCs was not significantly increased following cytokine-combined chemotherapy compared with chemotherapy alone (Supplementary Fig. 5). These findings are consistent with clinical observations that hematologic recovery was not adversely affected in patients receiving cytokine-combined chemotherapy^{17–22}. More extensive evaluation of the effects of the proposed therapy including long-term repopulation and self-renewal capacity of human HSCs is required. To optimize the effect of cytokine-combined anti-LSC therapy, normal HSC rescue may be required to assure patient safety.

In a model of murine leukemia, restriction of cell cycle entry appear to contribute to LSC maintenance, further supporting the essential role of cell cycle regulation in LSC function²⁵. Therefore, agents that promote LSC cell cycle entry and/or disrupt the interactions between LSCs and the BM niche, including modifiers of CXCL12/CXCR4, VLA-4/fibronectin and Gro β /CXCR2 axes, may effectively alter LSC chemotherapy responsiveness^{26–28}. Recently, Andreeff and colleagues demonstrated that a CXCR4 antagonist mobilizes murine leukemic cell lines and human AML cells²⁹. Similarly, DiPersio and colleagues showed mobilization and chemosensitization of murine leukemia with a CXCR4 antagonist³⁰. In addition, recent studies have elegantly demonstrated that interferon- α induces the proliferation of murine normal HSCs and that the suppression of interferon- α signaling protects murine normal HSCs from exhaustion through the maintenance of cell cycle quiescence^{31,32}. These findings has led to the hypothesis that the mechanism of interferon- α action against CML

may be related to its effects on cell cycle status of CML stem cells³³. Whether these agents are effective in altering cell cycle status and chemotherapy responsiveness of LSCs must be investigated in various hematologic malignancies.

Here we demonstrated that functional modification of human primary AML LSCs facilitates their effective elimination in vivo. Recent studies have also demonstrated that cell surface markers expressed on LSCs may be effective targets for anti-LSC therapy^{35,36}. Multifaceted investigational approach integrating functional modification of LSCs with discovery of LSC-specific target molecules will be required for the development of effective LSC-targeted therapies for AML.

METHODS

Patient samples

All experiments were performed with authorization from the Institutional Review Board for Human Research at RIKEN RCAI. Following written informed consent, AML cells were collected from patients with French-American-British (FAB) classification system subtype M1 (Case 4), M2 (Cases 3, 6, 7), M4 (Cases 1, 2) and MDS/AML (Case 5). BM mononuclear cells (BMMNCs) were isolated using density-gradient centrifugation.

Mice

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tmlWjl}/Sz (NOD/SCID/IL2 γ ^{null}) mice were developed at The Jackson Laboratory (Bar Harbor, ME) by backcrossing a complete null mutation at the *Il2rg* locus onto the NOD.Cg-*Prkdc*^{scid} (NOD/SCID) strain. Mice were bred and maintained under defined flora with irradiated food and acidified water at the animal facility of RIKEN and at The Jackson Laboratory according to guidelines established by the Institutional Animal Committees at each respective institution.

Xenogeneic transplantation

Newborn (within two days of birth) NOD/SCID/IL2 γ ^{null} recipients received 150cGy total body irradiation using a ¹³⁷Cs-source irradiator, followed by intravenous injection of AML cells within two hours. For primary transplantation (to generate human AML-engrafted mice to be used for subsequent experiments), 10³ – 5×10⁴ sorted 7AAD⁻lineage (hCD3/hCD4/hCD8)⁻hCD34⁺hCD38⁻ AML patient BM cells per recipient were injected, as described previously⁵. For secondary transplantation (to determine the frequency of AML-initiating cells remaining after AraC alone or AraC following G-CSF), 2×10², 2×10³, 2×10⁴ or 2×10⁵ sorted 7AAD⁻hCD45⁺hCD34⁺ BM cells from treated AML-engrafted recipients were transplanted into newborn NOD/SCID/IL2 γ ^{null} recipients following the same irradiation and injection procedure as described above. For fluorescence-activated cell sorting (FACS), AML patient BMMNCs were labeled with fluorochrome-conjugated mouse anti-hCD3, anti-hCD4, anti-hCD8, anti-hCD34 and anti-hCD38 monoclonal antibodies (BD Biosciences, San Jose, CA) and recipient BMMNCs were labeled with mouse anti-hCD45, anti-hCD34 and anti-hCD38 monoclonal antibodies (BD Biosciences) followed by cell sorting using FACSaria (BD Biosciences). Doublets were excluded by analysis of FSC/SSC-height and FSC/SSC-width. The purity of hCD34⁺hCD38⁻ and hCD34⁺ cells was higher than 98% after sorting.

AraC and G-CSF treatments

For experiments comparing no treatment, G-CSF alone, AraC alone and AraC following G-CSF, primary human AML-engrafted recipients at 12–24 weeks post-transplantation were used. For each experiment comparing treatment groups, recipient pairs were chosen from the

same litter, transplanted with the same primary AML sample at the same dose on the same day, to control for variability among litters and variability due to differences in engraftment levels. Recombinant human G-CSF (PeproTech, Rocky Hill, NJ) was given as follows: 300µg/kg subcutaneously qd × 5 days. AraC (Biogenesis, Poole, UK) was given as follows: 1g/kg intraperitoneally qd × 2 days. AraC following G-CSF treatment was given as follows: G-CSF 300µg/kg subcutaneously qd × 5 days with concurrent administration of AraC 1g/kg intraperitoneally qd × 2 days on treatment days 4 and 5. The recipients were sacrificed 16 hours after the final injection. For recipients undergoing cell cycle analysis by BrdU incorporation, 1.5mg BrdU (BD Biosciences) per recipient was injected intraperitoneally immediately after the final injection of G-CSF and/or AraC.

Flow cytometry

Recipients were bled retro-orbitally every 3 weeks starting week 6 post-transplantation for the evaluation of human AML engraftment. For the analysis of AraC alone and AraC following G-CSF treated recipients, BM was harvested by flushing two tibiae and one femur and BM cell count was performed manually and by automated blood cell analyzer (Celltac α, Nihon Kohden, Japan), to estimate the absolute number of BM nucleated cells from each recipient. The absolute number of human CD34⁺ cells per mouse (from two tibiae and one femur) was determined by multiplying %BM 7AAD-hCD45⁺hCD34⁺ cells with total BM nucleated cell count thus obtained. For quantification of cells in G0/G1, S and G2/M phases of cell cycle, *in vivo* BrdU incorporation was measured using the BrdU flow kit (BD Pharmingen, San Diego, CA). For quantification of cells in G0 phase of cell cycle, cells were labeled with Hoechst 33342 and Pyronin Y followed by surface staining using standard procedures. Intracellular staining for active caspase-3 using rabbit anti-active caspase-3 monoclonal antibody (BD Pharmingen) was performed for the quantification of apoptotic cells. For surface labeling, mouse anti-human CD45, CD34 and CD38 monoclonal antibodies were used (BD Biosciences). The analyses were performed using FACS Aria and FACSCanto II (BD Biosciences).

Histological analysis

Paraformaldehyde-fixed, decalcified, paraffin-embedded sections were prepared from femurs of primary human AML-engrafted recipients. Primary antibodies used for labeling were mouse anti-human CD45 monoclonal (Immunotech, France), anti-human CD34 monoclonal (Beckman Coulter, Fullerton, CA), rabbit anti-human Ki67 polyclonal (Spring Bioscience, Fremont, CA), mouse anti-BrdU monoclonal (DAKO, Denmark) and rabbit anti-mouse osteopontin polyclonal (IBL, Japan) antibodies. Hematoxylin-eosin (HE) staining was performed using standard methodology. TUNEL staining was performed according to standard procedures using ApopTag peroxidase *in situ* apoptosis detection kit (Intergene, Purchase, NY). Light microscopy was performed using Zeiss Axiovert 200 (Carl Zeiss Microimaging GmbH, Germany). Laser-scanning confocal imaging was obtained using Zeiss LSM 710 (Carl Zeiss). Three-dimensional reconstructions of bone sections were performed by analyzing serial sectional confocal images using the IMARIS software (BITPLANE, Switzerland).

Statistical analysis

Otherwise noted, numerical data are presented as mean ± SEM and differences are examined by two-tailed t-test (GraphPad Prism, GraphPad, San Diego, CA). The difference in survival was analyzed by the log-rank (Mantel-Cox) test (GraphPad Prism, GraphPad, San Diego, CA). LSC frequency was estimated through Poisson statistics using the method of maximum likelihood and analyzed using two-tailed t-test (StemSoft Software, Vancouver, Canada).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Quiescent LSCs enter cell cycle following in vivo cytokine treatment

(a,b) Representative contour plots of hCD34+CD38⁻ LSCs in BM of recipients engrafted with LSCs isolated from Case 3 and 5. In **a** and **b**, histograms demonstrate nearly complete replacement of the recipient BM with human AML cells (left panels). The phenotypic isolation of hCD34+CD38⁻ LSCs is shown in the middle panels. (a) At baseline, the majority of BM LSCs are quiescent G0 cells, as demonstrated by Hoechst 33342-low PyroninY-negative phenotype. (b) After in vivo G-CSF treatment, LSCs enter cell cycle as indicated by increase in PyroninY-positive cells (indicating RNA synthesis), Hoechst 33342-high cells (indicating DNA synthesis), resulting in decreased frequency of cells in G0 phase. (c) Recipient BM LSCs in G0 phase decreased with G-CSF treatment (open circles) compared with control recipients (filled circles). Horizontal bars indicate means + SEM. Recipients engrafted with LSCs from AML Case 1: Control n=7, G-CSF n=6, p=0.0010; Case 2: Control n=9, G-CSF n=6, p<0.0001; Case 3: Control n=10, G-CSF n=5, p<0.0001; Case 4: Control n=6, G-CSF n=4, p=0.0016; Case 5: Control n=5, G-CSF n=6, p=0.0043; Case 6: Control n=5, G-CSF n=5, p=0.0050; Case 7: Control n=3, G-CSF n=5, p=0.0004, each by two-tailed t-test.

Figure 2. Quiescent human AML cells within the BM endosteal region enter cell cycle following *in vivo* cytokine treatment

Immunofluorescence labeling for human CD45 and Ki67 (**a–c**) in the peripheral and (**d–f**) the central zones of BM from a human primary AML recipient with BM human AML chimerism of 98.5%. The peripheral zone contains both the endosteal and perivascular regions and the central zone contains the perivascular region, framed by white rectangles on the images. (**a–c**) CD45+ human AML cells in the endosteal region are largely Ki67 negative (cell cycle quiescent), while in the perivascular region, CD45+ human AML cells are found Ki67 positive (cycling). Serial vertical sectional images through the bone section confirm that the endosteal region is nearly devoid of Ki67-expressing nuclei. (**d–f**) In

contrast, the central zone of the BM, including perivascular region, is enriched for Ki67 positive cycling human CD45+ AML cells, again confirmed by serial vertical sectional images. **(g–i)** Immunofluorescence labeling for human CD45 and Ki67 in the peripheral zone of BM from a recipient with BM human AML chimerism of 97.8%. The peripheral zone contains both the endosteal and perivascular regions, framed by white rectangles on the images. CD45+ human AML cells in the endosteal region enter cell cycle following in vivo cytokine treatment, as demonstrated by their Ki67 expression. Serial vertical sectional images through the bone section confirm that nuclei of human CD45+ AML cells adjacent to the endosteum express Ki67. **(j,k)** Three-dimensional reconstruction of recipient bone sections was performed using serial sectional images. **(j)** At steady state, the AML cells adjacent to the BM endosteum are cell cycle quiescent (Ki67 negative). **(k)** Following in vivo cytokine treatment, AML cells within the BM endosteal region became Ki67 positive, indicating that they had entered cell cycle. CD45 (red), Ki67 (green), DAPI (blue) and merged images are shown. **(a,d,g)** 20× magnification, scale bars, 50µm. **(d,e,h)** 40× magnification, scale bars, 20µm.

Figure 3. In vivo cell cycle entry potentiates chemotherapy-induced apoptosis of LSCs, reduces LSC frequency and leads to superior survival of secondary recipients

(a) Reduced LSC survival as measured by % active caspase-3 negative cells in BM hCD34+CD38- LSCs from recipients following chemotherapy with cell cycle induction (open circles) compared with chemotherapy alone (filled circles). Horizontal bars indicate means + SEM. Recipients engrafted with AML from Case 1: n=3 each, p=0.0127; Case 2: n=4 each, p=0.0006; Case 3: Chemotherapy alone n=7, chemotherapy with cell cycle induction n=4, p=0.0001; Case 4: n=4 each, p<0.0001; Case 5: n=4 each, p=0.0005; Case 6: n=5 each, p<0.0001; Case 7: Chemotherapy alone n=5, chemotherapy with cell cycle induction n=5, p=0.0003, each by two-tailed t-test. (b) HE and TUNEL staining of bone

sections from recipients following chemotherapy alone reveal apoptosis in the central region of the BM while cells abutting the endosteum remain viable(*). In contrast, BM of recipient following chemotherapy with cell cycle induction shows apoptosis in the endosteal region (+) in addition to the central zone. Scale bars, 10 μ m. (c) PB hCD45+ AML cell engraftment time course of serial transplant recipients at graft doses of 2 $\times$ 10², 2 $\times$ 10³, 2 $\times$ 10⁴ and 2 $\times$ 10⁵ hCD34+ AML cells, from 6 to 24 weeks post-transplantation. The findings are summarized in Supplementary Table 4. Blue and red symbols and lines respectively indicate recipients of BM hCD34+ cells from AraC alone- and AraC post-cytokine-treated AML-engrafted mice. Case 1: Open circles; Case 2: Open squares; Case 3: Open upward triangle; Case 4: open downward triangle; Case 5: Open diamonds; Case 6: Closed circles; Case 7: Closed squares. (d) From AML-engrafted mice receiving either chemotherapy alone or chemotherapy with cell cycle induction, BM hCD34+ cells were harvested and re-transplanted at doses of 2 $\times$ 10², 2 $\times$ 10³, 2 $\times$ 10⁴ and 2 $\times$ 10⁵ into new recipients that were followed for 24 weeks. Recipients of BM hCD34+ cells from AML-engrafted mice treated with chemotherapy with cell cycle induction (red) demonstrated higher overall survival compared with recipients of chemotherapy alone-treated mouse BM hCD34+ cells (blue) as estimated by the Kaplan-Meier method ($p < 0.0001$ for comparison of recipients within a given graft dose and for all recipients combined). (e,f) Central role of cell cycle-quiescent human primary AML LSCs in AML relapse and induction of cell cycle entry as a therapeutic strategy targeting LSCs. (e) In the past, disease relapse was attributed to the inability to completely eliminate AML cells by chemotherapy. (f) The current model maintains that chemotherapy-resistant LSCs that survive and repopulate the BM prompt AML relapse. One mechanism through which LSCs successfully evade chemotherapy is their cell cycle quiescence within the BM endosteal niche. Induction of cell cycle entry sensitizes these LSCs to chemotherapy, leading to reduced potential for relapse.