Acute myeloid leukemia does not deplete normal hematopoietic stem cells but induces cytopenias by impeding their differentiation

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Acute myeloid leukemia (AML) induces bone marrow (BM) failure in patients, predisposing them to life-threatening infections and bleeding. The mechanism by which AML mediates this complication is unknown but one widely accepted explanation is that AML depletes the BM of hematopoietic stem cells (HSCs) through displacement. We sought to investigate how AML affects hematopoiesis by quantifying residual normal hematopoietic subpopulations in the BM of immunodeficient mice transplanted with human AML cells with a range of genetic lesions. The numbers of normal mouse HSCs were preserved whereas normal progenitors and other downstream hematopoietic cells were reduced following transplantation of primary AMLs, findings consistent with a differentiation block at the HSC–progenitor transition, rather than displacement. Once removed from the leukemic environment, residual normal hematopoietic cells differentiated normally and outcompeted steady-state hematopoietic cells, indicating that this effect is reversible. We confirmed the clinical significance of this by ex vivo analysis of normal hematopoietic subpopulations from BM of 16 patients with AML. This analysis demonstrated that the numbers of normal CD34⁺CD38[−] stem-progenitor cells were similar in the BM of AML patients and controls, whereas normal CD34⁺CD38⁺ progenitors were reduced. Residual normal CD34⁺ cells from patients with AML were enriched in long-term culture, initiating cells and repopulating cells compared with controls. In conclusion the data do not support the idea that BM failure in AML is due to HSC depletion. Rather, AML inhibits production of downstream hematopoietic cells by impeding differentiation at the HSC–progenitor transition.

xenotransplant | anemia | thrombocytopenia | neutropenia

Hematopoiesis is tightly regulated under normal circumstances to ensure adequate production of mature blood cells. At steady state hematopoietic stem cells (HSCs) are relatively quiescent and the majority of proliferation occurs downstream of HSCs. Hematopoietic stresses such as bleeding or bone marrow (BM) damage from chemotherapy induce HSCs to enter the cell cycle to replenish mature blood cells (1–3).

BM failure (reduced production of neutrophils, red cells, and platelets) is almost universal at diagnosis of acute myeloid leukemia (AML) and contributes significantly to morbidity and mortality by inducing severe infections and bleeding. These complications often compromise the delivery of intensive chemotherapy and lead to a high frequency of induction death (4).

A common assumption is that marrow failure occurs due to displacement of normal hematopoietic cells from the marrow by AML cells, resulting in depletion of normal hematopoietic cells. However, AML has a more profound impact on BM function than many other types of hematologic malignancy (e.g., chronic

lymphocytic leukemia, follicular lymphoma) even where there is a similar degree of diffuse infiltration of BM by leukemia/lymphoma cells.

To clarify how AML suppresses normal hematopoiesis, we investigated the impact of AML on residual normal hematopoietic subpopulations, using primary patient samples and a xenograft model of AML.

Results

Engraftment of Human AML Does Not Reduce Numbers of Murine HSCs in a Xenograft Model of AML. Immunodeficient mice transplanted with human AML develop marrow failure as evidenced by peripheral cytopenias (5, 6) and pallor of the BM in association with reduced BM erythrocyte numbers (Fig. S1 A and B). We used nonobese diabetic/severe combined immunodeficiency/interleukin-2 receptor γ -chain null (NSG) mice to investigate the effect of human AML on normal murine hematopoietic populations.

Ten human AML samples with a range of genetic abnormalities (Table S1) were transplanted into 111 unirradiated NSG mice to determine the impact of AML on mouse CD45⁺ cells, hematopoietic progenitors (CD45⁺Lineage[−]c-kit⁺), and HSCs (CD45⁺ Lineage[−]c-kit⁺CD150⁺CD48[−]) (7) (Fig. 1 A and B). A median of 3 AML-transplanted mice and 3 control mice were killed at different time points posttransplantation to allow changes in mouse hematopoietic populations to be studied as AML proliferated.

The percentage of AML in the BM increased with time, although the growth rates of AML varied from sample to sample. The growth rate of AML was not related to the cytogenetic risk group (Fig. S1C). We observed changes in mouse HSCs and progenitors in three discrete phases, which we designated early, mid-, and late phases. In the early phase there was no significant difference in mouse progenitor ($P = 0.4$) or HSC ($P = 0.4$) numbers in mice transplanted with AML compared with controls. In the midphase HSC numbers were preserved whereas

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Fig. 1. Effect of AML on mouse hematopoietic populations in a xenograft model. (A and B) The gating strategy for identifying mouse hematopoietic progenitors and HSCs in BM is shown for a control mouse (A) and a mouse transplanted with AML (B). (C) The early (blue bars), mid- (pink bars), and late (gray bars) phases are seen following transplant of AML sample 1 (Left) whereas only early and midphases are seen following transplant of sample 3 (Right). (D) Summary of data from all experiments showing numbers of mouse CD45⁺ cells, progenitors, and HSCs in mice with AML as a percentage of values in controls in the three phases. The gray line represents the controls. (E) The mean AML percentage is shown for each of the time points and its relation to the phase. (F) The cellularity of BM in the ilium is not reduced in midphase following transplant of AML samples (three examples, Right) compared with control (Left) (20× objective magnification). *The HSCs and progenitors are expressed as a percentage of total CD45⁺ cells (AML plus mouse). [†] $P < 0.05$ and [‡] $P < 0.05$, comparing mean percentages in mice with AML to control values at each time-point prenormalization, using a paired *T* test.

mouse progenitors were significantly reduced ($P < 0.0001$). In the late phase both mouse progenitors ($P = 0.008$) and HSCs ($P = 0.009$) were significantly reduced. Two representative examples are shown in Fig. 1C and the full data are presented in Table S2. We have expressed the data for all 10 AML samples as a percentage of the values in control mice (to normalize the data) and present the collated data in Fig. 1D. Reductions in mouse CD45⁺ cells paralleled changes in mouse progenitors (Fig. 1D).

We observed that the early phase was present where AML infiltration was less than 15% of BM CD45⁺ cells ($n = 19/111$ mice transplanted with AML) (Fig. 1D and E). The midphase was seen in the majority of mice ($n = 69/111$ mice) across a wide range of AML infiltration (22–84%) (Fig. 1D and E) and occurred following transplant of all 10 AML samples, indicating it is unrelated to the cytogenetic subtype of the AML (Table S1). The late phase was seen only at very high levels of AML infiltration in 5 of the 10 AML samples ($n = 23/111$ mice) (Fig. 1C–E and Table S2). Late phase may have occurred in all experiments if the experiments were continued for longer as there was movement of HSC numbers in a downward direction at the last time point in some experiments in which late phase was not reached (Fig. S1D). Paraplegia due to AML infiltrating the spinal cord developed in midphase in some experiments, preventing longer follow-up.

Mouse HSCs (and progenitors) were expressed as a percentage of total BM CD45⁺ cells (human CD45⁺ AML cells plus mouse CD45⁺ cells) as the best available estimate of total HSC

numbers (Fig. 1A and B). Total HSC numbers are a product of the percentage of HSCs and the overall cellularity of the BM. We examined BM sections from mice in midphase to exclude the possibility that overall BM cellularity was reduced in mice transplanted with AML. As seen in the majority of human cases of AML, cellularity was at least as high (if not indeed increased) in mice with AML in midphase compared with controls (Fig. 1F). We conclude that the observed maintenance of HSC percentage represents a true preservation of absolute HSC numbers. Notably, we detected an actual increase in HSC numbers in midphase despite a reduction in progenitors in some experiments (Fig. 1D and Table S2). Therefore, BM failure in the mouse model in midphase is not due to depletion of HSCs.

To control for the effect of transplantation of cells we injected mice with AML cells from three AML samples that do not proliferate well in NSG mice (grafting 15% or less of BM cells). At 14 wk there was no difference in HSC ($P = 0.2$) or progenitor numbers ($P = 0.7$, Fig. S2). These data in combination with those from the early phase indicate that transplantation of cells per se does not induce changes in HSC or progenitor numbers.

In midphase there were significantly fewer progenitors per HSC in mice transplanted with AML (54 ± 10 progenitors per HSC) compared with controls (199 ± 23 progenitors per HSC) ($P = 0.0002$). This is consistent with the hypothesis that AML induces BM failure by impeding differentiation at the HSC–progenitor transition, leading to a failure of progenitor production. By contrast, there were significantly more mouse CD45⁺ cells per progenitor in mice transplanted with AML (43 ± 6 mouse

Fig. 3. Assessment of normal residual hematopoietic populations in humans with AML. (A) The gating strategy displayed was used to define normal HSPCs and progenitors in the BM of a control (Upper) and a patient with subtype A phenotype AML (Lower). (B) Numbers of phenotypically defined HSPCs are preserved ($P = 0.6$) in the BM of patients with subtype A AML ($n = 16$) whereas progenitors are reduced compared with controls ($n = 42$) ($P < 0.0001$). (C) A significantly greater proportion of BM CD34⁺CD38⁻ cells from patients with subtype A AML ($n = 7$) were CD49f⁺CD45RA⁻Rhodamine123low compared with controls ($n = 7$) ($P = 0.003$). (D) The percentage of HSPCs in cell cycle was lower in subtype A AML ($n = 7$) BM than controls ($n = 9$) ($P = 0.002$). (E) Similar numbers of colonies were observed after culture of CD34⁺ cells in methylcellulose from three subtype A AML BMs and three controls. On replating, increased numbers of colonies were seen from CD34⁺ cells derived from AML marrow. (F) More LTC-ICs were present in the CD34⁺ cells from two subtype A AML BMs than in controls. Error bars indicate 95% confidence intervals. (G) More LTC-ICs were present in the CD34⁺CD38⁻ cells from three subtype A AML BMs than in controls. Error bars indicate 95% confidence intervals. (H) More human CD45⁺ cells were detected in mice transplanted with CD34⁺ from one subtype A AML sample ($n = 3$) than in controls ($n = 4$) whereas similar numbers were seen following transplant of another sample ($n = 4$ for each arm). (I) More CD45⁺ cells were seen in the BM of secondary recipients ($n = 3$ per arm)

AML is due to increased apoptosis in the downstream hematopoietic progenitors ($P = 0.5$, Fig. S6B).

To look for evidence that the reduced numbers of progenitors are due to reduced production by HSPCs we determined the cell cycle profile of phenotypically defined normal HSPCs in the BM of patients with subtype A phenotype AML, using Ki67 staining. A lower percentage of HSPCs from AML BM were in cell cycle compared with controls ($P = 0.002$), indicating that HSPCs are more quiescent in AML (Fig. 3D). The results support the conclusion that HSPCs are failing to produce sufficient progenitors in the context of AML.

Normal CD34⁺ Hematopoietic Cells from Patients with AML Are Enriched in Long-Term Culture, Initiating Cells and Repopulating Cells.

To provide independent quantification of residual normal primitive hematopoietic cells, we tested CD34⁺ cells from the BM of subtype A phenotype AMLs in colony-forming unit, long-term culture (LTC), and repopulating assays. We confirmed the normal nature of the progeny of these CD34⁺ cells through genotyping consistent with earlier publications (Table 1) (8, 9). Similar numbers of normal myelo-erythroid colonies were produced by sorted CD34⁺ cells in the initial plating from BM from AML or controls ($P > 0.1$ for all, Fig. 3E and Table 1). On replating, significantly more normal colonies were seen from normal CD34⁺ cells from AML marrow ($P < 0.03$ for all, Fig. 3E), suggesting that they are enriched in primitive hematopoietic cells. The results are comparable to those from analogous experiments from midphase in the xenograft model (Fig. 2C). Similar results were also seen following coculture of AML and normal hematopoietic cells in vitro (Fig. S6C and D). Consistent with these data, we detected more LTC initiating cells (LTC-ICs) per CD34⁺ cell ($P < 0.0001$) and per CD34⁺CD38⁻ cell ($P < 0.002$) from subtype A AML than from control BM (Fig. 3F and G and Table 1), indicating that the normal residual hematopoietic stem-progenitor compartment is skewed toward the most primitive hematopoietic cells in patients with AML.

To test the repopulating potential of residual normal hematopoietic cells in AML, CD34⁺ cells from BM from two subtype A AML samples were transplanted into NSG mice. There were more human CD45⁺ cells in the mice transplanted with CD34⁺ cells from one AML sample than from its control ($P < 0.001$) and although there was no significant difference between the second AML sample and its control ($P = 0.3$) (Fig. 3H), more human CD45⁺ cells were detected in secondary recipients from both subtype A AML samples ($P < 0.02$ for both) (Fig. 3I). These grafts from the AML samples lacked the leukemia-specific genetic lesions (Table 1). These experiments indicate that the normal CD34⁺ compartment in subtype A AML is enriched in long-term repopulating cells compared with control BM.

The primary and secondary grafts from AML BM constituted a predominant B lymphoid population (B lymphocytes were $88\% \pm 5\%$ and $91\% \pm 2\%$ of human cells from control and AML, respectively, $P = 0.5$) and a smaller population of CD33⁺ cells (Fig. 3J), similar to that seen after transplant of normal BM, providing evidence of normal differentiation of CD34⁺ cells once removed from the leukemic marrow.

Normal CD34⁺ Cells Are Not Displaced from the Paratrabeular Region by AML.

The above experiments demonstrated that hematopoietic stem-progenitor cells are not reduced in number at

transplanted with 4 million human CD45⁺ cells derived from subtype A AML than in controls in both experiments. (J) The plots show human grafts with predominant B lineage populations and smaller myeloid populations in secondary recipients injected with human CD45⁺ cells derived from CD34⁺ BM cells from a subtype A AML sample (Upper) and from control CD34⁺ BM cells (Lower). * $P < 0.05$.

Table 1. Leukemia-specific genetic lesions are not present in CD34⁺ cells or their progeny from subtype A AML samples

Figure in which results are displayed and experiment	Genetic lesion in primary AML sample	Fraction	Postsort frequency of leukemic mutation	Post-CFC/LTC/xenograft frequency of leukemic mutation	Method
Fig. 3 E, i	None identified	CD34 ⁺	NA	NA	NA
Fig. 3 E, ii	<i>NPM</i> mutant	CD34 ⁺	ND	<0.1%	<i>NPM</i> PCR
Fig. 3 E, iii	<i>NPM</i> mutant	CD34 ⁺	0%	0%	<i>NPM</i> PCR
Fig. 3 F, i	<i>NPM</i> mutant	CD34 ⁺	ND	0%	<i>NPM</i> PCR
Fig. 3 F, ii	<i>NPM</i> mutant	CD34 ⁺	ND	0%	<i>NPM</i> PCR
Fig. 3 G, i	t(11;17)	CD34 ⁺ CD38 ⁻	0/100 cells	0/100 cells	<i>MLL</i> FISH
Fig. 3 G, ii	<i>NPM</i> mutant	CD34 ⁺ CD38 ⁻	0%	0%	<i>NPM</i> PCR
Fig. 3 G, iii	<i>NPM</i> mutant	CD34 ⁺ CD38 ⁻	<0.1%	<0.1%	<i>NPM</i> PCR
Fig. 3 H, i	t(11;17)	CD34 ⁺	ND	0/100 cells	<i>MLL</i> FISH
Fig. 3 H, ii	<i>NPM</i> mutant	CD34 ⁺	ND	0%	<i>NPM</i> PCR

CFC, colony forming cells; FISH, fluorescence in situ hybridization; LTC, Long-term culture; MLL, mixed-lineage leukemia; NA, not applicable; ND, not determined; NPM, nucleophosmin.

diagnosis of AML. However, AML might dislocate them from their usual position within the BM, impairing their function. Human stem-progenitor cells are found at increased numbers near the trabecular surface of the bone in BM (12). We therefore determined whether subtype A AML displaces normal stem-progenitor cells from their usual paratrabecular position, using immunostaining of BM biopsies from patients with AML or controls. The distribution pattern of dual-positive (CD45⁺CD34⁺) normal stem-progenitor cells was not different in subtype A AML compared with controls ($P > 0.1$, Fig. 4), suggesting that there is no gross displacement.

Together the immunophenotyping and functional studies on patient BM support the conclusion that the numbers of HSPCs are preserved in the BM at diagnosis (and progenitors are depleted) and that these cells differentiate effectively once removed from their leukemic environment. The results closely parallel the data from the midphase of the xenograft model. The cumulative data lead us to propose a model in which BM failure occurs due to a failure of production of progenitors by HSCs through a differentiation block at the HSC–progenitor transition.

Discussion

BM failure is a prominent clinical feature of AML but little is known about how AML affects the residual normal hematopoietic subpopulations. One potential explanation is that BM failure is due to HSC depletion caused by AML displacing normal hematopoietic cells from the BM. In our xenograft experiments mouse HSC numbers were not reduced whereas hematopoietic progenitors and other downstream hematopoietic cells were significantly depressed, indicating that BM failure in this model is not simply due to HSC depletion. We were careful to include functional assays, including the gold standard long-term repopulating assay, to produce robust data on HSC quantification. These experiments demonstrate that BM failure is not simply due to HSC depletion.

A potential problem with the xenograft model is that the interaction of human AML with mouse hematopoietic cells in an immunodeficient setting might not reflect the interaction of AML cells with human hematopoietic cells in patients. However, our studies that quantified normal HSCs and progenitors in bone marrow from patients at diagnosis of AML suggest that this model recapitulates what occurs in humans. Future planned experiments include investigation in syngeneic mouse models.

We studied AML samples with a range of cytogenetic abnormalities (including those from good, intermediate, and poor risk categories) and phenotypes (French, American, and British classification types, CD34 positive and CD34 negative) and all had a similar effect on residual hematopoiesis at a given level of AML infiltration (Fig. 1E). Thus, there appears to be a

common mechanism unrelated to the genetic lesions that drive the disease.

The mechanism of BM failure involves a block in differentiation at the HSC–progenitor transition as the number of progenitors per HSC was significantly reduced in humans and in the

Fig. 4. Normal CD34⁺ cells are not displaced from the paratrabecular region by AML. (A) Normal stem-progenitor cells, expressing dual CD45 and CD34, were identified in BM biopsy sections from patients with subtype A AML ($n = 4$) and controls ($n = 8$). CD45⁺ and CD34⁺ cells were visualized using Fluorescein and AlexaFluor 546, respectively. An example from a patient with AML is shown. Two CD45⁺CD34⁺ cells are indicated by white arrowheads. (B) The distance of normal stem-progenitor cells (CD45⁺CD34⁺) from the nearest trabecular bone was measured. Six stem-progenitor cells are seen with distance from the trabecular bone indicated in microns. (C) There was no significant difference in the distribution of stem-progenitor cells between AML and controls. Error bars indicate SD.

xenograft model. There was no evidence for a block in differentiation downstream of progenitors and therefore the mechanism appears to be specific for differentiation at the HSC–progenitor transition.

Hematopoietic stresses normally induce HSCs to enter the cell cycle to replenish mature blood cells (1–3). Therefore, the hematopoietic stress of BM failure induced by AML might be expected to induce HSC cycling to maintain blood cell numbers. The opposite (i.e., reduced HSC cycling) occurred in the xenograft model and in patient BM. This observation supports the notion that AML is impairing the function of HSCs rather than just depleting HSC numbers. HSCs might show reduced cycling if they are displaced from their niche but we could find no evidence of this, using our methodology.

The reduced cycling of HSCs may seem at odds with data from some xenograft experiments in which HSCs were actually increased in midphase (Fig. 1D and Table S2). However, this might reflect a relative increase in the number of daughter cells retaining an HSC phenotype after HSC division as a consequence of the differentiation block at the HSC–progenitor transition. The relatively small reduction in HSC cycling implies that BM failure is principally due to failure of differentiation with a lesser contribution related to reduced HSC cycling.

Our data are consistent with clinical observations. Recovery of normal hematopoiesis is usually rapid after successful blast clearance by induction chemotherapy (~20 d) (13). Time to regeneration is similar to that seen after conventional allogeneic BM transplantation (14), consistent with the notion that HSCs are preserved. If HSCs were severely depleted at diagnosis, one might expect greater delays in regeneration of hematopoiesis. Occasionally patients do, however, experience prolonged aplasia despite leukemia cell clearance by chemotherapy. These patients may have HSC depletion as observed in late phase in the xenograft experiments and may reflect delayed presentation to medical services.

In some patients with AML HSCs exist, termed preleukemic HSCs, that are functionally normal but contain some but not all of the mutations seen in the AML clone (15). Although we could not detect key driver mutations in the CD34⁺ cells from subtype A AML, we cannot exclude the possibility that some of these cells contain other mutations and might be preleukemic HSCs. Preleukemic HSCs may have a growth advantage over unmutated HSCs and this might have explained the superior functional ability of CD34⁺ cells from patients with subtype A AML (Fig. 3). Against this, similar data were seen in the xenograft model presented here and colony formation was similar from

preleukemic HSCs and control HSCs in the work from Stanford University (15).

Laboratory studies from the 1970s suggested that AML inhibited normal hematopoiesis (16, 17) but the tools to identify hematopoietic subpopulations had not yet been developed. Our work has elucidated the defects in the normal hematopoietic populations induced by AML but further work is needed to clarify the mechanism by which HSC differentiation is blocked. If the differentiation block can be reversed, the residual HSCs may be used to ameliorate BM failure. This may be of benefit for older patients who tolerate intensive chemotherapy poorly as well as for younger patients with refractory disease.

In conclusion we demonstrate that BM failure in AML is not due to depletion of HSC numbers but rather that HSCs from leukemic BM fail to produce sufficient progenitors as a result of a differentiation block at the HSC–progenitor transition. These findings may be exploited to provide treatments for one of the key complications of AML.

Materials and Methods

Quantification of Hematopoietic Cells in Patient BM. A standard operating procedure was used to ensure that the BM samples were obtained from the first pull of the aspirate and in a small volume (less than 0.5 mL) to reduce hemodilution for quantification of normal progenitors and HSPCs. Stem-progenitor cells were quantified in fresh BM, using surface marker staining combined with CountBright Absolute Counting Beads (Invitrogen), according to manufacturer's instructions.

Quantification of Mouse Repopulating Cells. B2M mice were transplanted with AML cells. After 2 mo mice were killed and the residual mouse CD45⁺ cells were purified and transplanted into irradiated (375 cGy) NSG mice. Different doses of CD45⁺ cells were transplanted to allow calculation of repopulating cell frequency by limiting dilution analysis (LDA) (Fig. S4). Mice were bled at 4, 8, 16, and 24 wk and the number of mice with a B2M graft was determined. Antibody against mouse histocompatibility complex (MHC) class I antigens was used to distinguish donor B2M cells (lacking MHC class I) from recipient NSG cells (expressing MHC class I) in the blood.

Statistical Analysis. Data are presented as means $\pm$ SE of mean (SEM) unless otherwise stated. Error bars indicate SEM unless otherwise stated.

Further details of materials and methods are given in *SI Materials and Methods*.

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