Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34− fraction

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**MYELOID NEOPLASIA**

Leukemia-initiating cells (LICs) in acute myeloid leukemia (AML) are believed to be restricted to the CD34+ fraction. However, one of the most frequently mutated genes in AML is nucleophosmin (NPM), and this is associated with low CD34 expression. We, therefore, investigated whether NPM-mutated AMLs have LICs restricted to the CD34+ fraction. We transplanted sorted fractions of primary NPM-mutated AML into immunodeficient mice to establish which fractions initiate leukemia. Approximately one-half of cases had LICs exclusively within the CD34− fraction, whereas the CD34+ fraction contained normal multilineage hematopoietic repopulating cells. Most of the remaining cases had LICs in both CD34+ and CD34− fractions. When samples were sorted based on CD34 and CD38 expression, multiple fractions initiated leukemia in primary and secondary recipients. The data indicate that the phenotype of LICs is more heterogeneous than previously realized and can vary even within a single sample. This feature of LICs may make them particularly difficult to eradicate using therapies targeted against surface antigens. (Blood. 2010;115:1976-1984)

**Introduction**

Normal hematopoiesis is organized as a hierarchy with hematopoietic stem cells (HSCs) at the apex and differentiated blood cells at the base. Acute myeloid leukemia (AML) is thought to be organized in a similar way, with leukemia-initiating cells (LICs) at the top of the hierarchy giving rise to variably differentiated blasts.1 LICs are thought to be critical to the growth of AML; hence, elimination of these is important to obtain a cure. To study and target LICs, one must know their phenotype. Two publications suggest that they all reside within the CD34+ fraction.4,7,13 Although many AML samples are termed “CD34+,”4,7,13 many samples that are classified as “CD34−”20% of blast cells expressing CD34. Thus, many samples that are defined as CD34+ may have a small population of CD34− cells, and this may contain the LICs as previously reported. However, we have observed normal human hematopoietic cells in the bone marrow of mice transplanted with the CD34+CD38− fraction of one AML with mutated NPM, suggesting that this fraction may contain normal hematopoietic progenitors in some AML specimens.3 This led us to question whether LICs express CD34 in all NPM-mutated AML specimens.

To investigate this, we tested CD34+ and CD34− cells from NPM-mutated AMLs using genomic and functional assays. CD34+ and CD34− cells were transplanted into immunodeficient mice to identify which fraction contained LICs. We used the most immunodecient mice available and used measures to abrogate the effect of antibody-mediated clearance.7 The CD34− cells alone initiated leukemia from approximately half of samples (that we termed subtype A), whereas the CD34+ cells gave rise to normal multilineage hematopoiesis. Most other samples had LICs within both CD34+ and CD34− fractions, and these were capable of transmitting leukemia to secondary recipients. The data indicate that one cannot blindly rely on the CD34+CD38− phenotype to identify LICs from all AMLs. We also show that the phenotype of LICs can change and this provides a probable explanation for the presence of LICs in multiple fractions from some AMLs.

**Methods**

**Primary cells**

Cord blood and AML cells were obtained after informed consent at St Bartholomew’s and the Royal London Hospitals. The protocol was approved by the appropriate institutional research ethics committees. Primary cells were cultured as described.14 The online version of this article contains a data supplement.

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CD34 sorting cells pretransplantation CD34 was performed on a BD Aria after staining the cells as described above. Gates were set up to exclude nonviable cells and debris. Cell sorting was performed in triplicate. Standard curves for NPM and albumin were established by amplifying a serial dilution of NPM mutants from 50 000 to 5 cells per reaction. A standard curve was created with each run (supplemental Figure 1; available on the Blood website; see the Supplemental Materials link at the top of the page of the online article). The assay was able to detect 5 cells reliably. The percentage of mutated NPM was determined by dividing the value for NPM mutation by the albumin value. Percentages greater than 100% were treated as 100%.

**Immunomagnetic depletion and enrichment of CD34+ cells**

Easysep Human CD34 Selection Cocktail and Easysep magnet (StemCell Technologies) were used according to the manufacturer’s instructions to enrich CD34+ cells from AML samples. The procedure resulted in an increase in percentage of CD34+ cells by more than 30-fold. Unbound CD34 depleted cells were obtained from the residual supernatant after CD34 enrichment. This depleted 88% plus or minus 8% of the CD34+ cells in the R1 gate (see Figure 2Ai) from subtype A samples and 97% of the fractions were plated in triplicate in 1 mL of MethoCult GF (StemCell Technologies) were used according to the manufacturer’s instructions to increase in percentage of CD34+ cells by more than 30-fold. Unbound CD34 depleted cells were obtained from the residual supernatant after CD34 enrichment. This depleted 88% plus or minus 8% of the CD34+ cells in the R1 gate (see Figure 2Ai) from subtype A samples and 97% of the colonies were scored using an inverted microscope. Cells were harvested and washed twice with PBS 2% FCS before analysis by quantitative polymerase chain reaction (PCR).

**Colony-forming assays**

Two to 500 x 10^3 cells from the CD34-enriched and CD34-depleted fractions were plated in triplicate in 1 mL of MethoCult GF (StemCell Technologies) in 35-mm tissue culture dishes. On day 14 of culture, the numbers of colonies were scored using an inverted microscope. Cells were harvested and washed twice with PBS 2% FCS before analysis by quantitative polymerase chain reaction (PCR).

**Table 1. Characteristics of AML patient samples**

<table>
<thead>
<tr>
<th>AML sample</th>
<th>FAB</th>
<th>NPM mutation</th>
<th>FLT3</th>
<th>Karyotype</th>
<th>Total CD34+ %</th>
<th>Subtype</th>
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</table>

The subtype refers to the classification based on the pattern of expression of CD34 and CD38. All samples except samples 25 and 26 have wild-type Wilms tumor 1 exons 7 and 9 (sample 18 was not tested).

FAB indicates French-American-British classification; NPM, nucleophosmin; FLT3, FMS-like tyrosine kinase; WT, wild type; ITD, internal tandem duplication; T, thymine; C, cytosine; G, guanine; and A, adenine.

approved by the East London and City Research Ethics Committee. The samples were collected at untreated presentation (n = 26) or relapse (n = 1). The median white blood count was 103 (range, 12-313) x 10^9 cells per liter. Details of the patient samples are listed (Table 1). Peripheral blood was used as a source of AML cells.

**Mutation analysis**

NPM3, FMS-like tyrosine kinase 3 (FLT3), and Wilms tumor 1 mutation sequencing was performed using previously published methods.

**Immunophenotyping and cell sorting**

All antibodies were obtained from BD Biosciences. AML cells were stained with phycoerythrin (PE)–conjugated anti-CD34, fluorescein isothiocyanate (FITC)–conjugated anti-CD38, and allophycocyanin (APC)–conjugated anti-CD3 antibody before resuspension in a 4,6-diamidino-2-phenylindole (DAPI) containing solution of 2% fetal calf serum (FCS) with phosphate buffered solution (PBS). Analysis was performed on a BD LSR2 or a BD Aria. Gates were set up to exclude nonviable cells and debris. Cell sorting was performed on a BD Aria after staining the cells as described above. For sorting cells pretransplantation CD3+ cells were excluded (none of the samples expressed CD3 on the blasts). Purity checks were performed to ensure sort quality. The purity of the CD34+CD38+ fraction and the CD34+CD38– fraction was 97.1% plus or minus 1.9% and 97.2% plus or minus 3.7%, respectively. The purity of the CD34+CD38+ fraction and the CD34+CD38– fractions were 92.8% plus or minus 6.2% and 91.4% plus or minus 1.9%, respectively.

**Real-time quantitative polymerase chain reaction assay for NPM exon 12**

DNA was extracted from 560 to 50 000 sorted cells using a QIAamp DNA mini kit (Qiagen) according to the manufacturer’s instructions with minor modifications as reported previously. To improve DNA yield, sorted cells were centrifuged at 3800g. Real time quantitative analysis of NPM exon 12 mutations was done using previously published methods. The total amount of DNA present was determined by quantitation of NPM gene copies in 50 000 to 5 cells per reaction. A standard curve was created with each run (supplemental Figure 1; available on the Blood website; see the Supplemental Materials link at the top of the page of the online article). The assay was able to detect 5 cells reliably. The percentage of mutated NPM was determined by dividing the value for NPM mutation by the albumin value. Percentages greater than 100% were treated as 100%.

**Colony-forming assays**

Two to 500 x 10^3 cells from the CD34-enriched and CD34-depleted fractions were plated in triplicate in 1 mL of MethoCult GF (StemCell Technologies) in 35-mm tissue culture dishes. On day 14 of culture, the numbers of colonies were scored using an inverted microscope. Cells were harvested and washed twice with PBS 2% FCS before analysis by quantitative polymerase chain reaction (PCR).
Mice
Nonobese diabetic/severe combined immunodeficiency disease/β2-microglobulin–null (NOD/SCID/β2m−/−) and NOD/SCID/interleukin-2 receptor γ chain–null (NOD/SCID/IL2rγ−/−) mice were a kind gift of Dr Leonard Shultz (The Jackson Laboratory) and were used as detailed previously.19,20 All animal experiments were performed in accordance to Home Office and CRUK guidelines. To abrogate antibody-mediated clearance of cells, all mice received a total of 1 mg/kg of human immunoglobulin (IVIG; Bio Products Laboratory) as described before.1 Mice received a sublethal dose of radiation (330–375 cGy) from a 137cesium source 24 hours before transplantation. Direct intra–bone marrow injection (as previously described)21 was the preferred route of administration unless more than 10⁶ cells were administered, for which the intravenous route was preferred.

Assessment of engraftment
Engraftment was assessed by immunophenotyping as described before.19 Briefly, normal multilineage engraftment was defined by the presence of separate CD45⁺CD33⁺ and CD45⁺CD19⁺ populations with the appropriate scatter characteristics. AML engraftment was defined by the presence of a single CD45⁺CD33⁺ population greater than 0.1% of live cells. The phenotype of engrafted cells was determined by staining bone marrow with Peridinin-chlorophyll protein (PerCP)–conjugated anti-CD45, PE-Cyanin 7 (PE-Cy7)–conjugated anti-CD14, APC-conjugated anti-CD15, and PE-conjugated anti-CD36 antibodies. In addition, the percentage of NPM-mutated cells in the graft was determined by quantitative genomic PCR. This was performed on sorted or unsorted cells from the bone marrow (there was no statistically significant difference between the approaches). We performed the assay on bone marrow cells (n = 15) from nontransplanted mice to determine nonspecific amplification and defined negativity based on this.

AML samples were screened for ability to generate a graft in immunodeficient mice. Only those samples capable of generating a leukemic graft were used in experiments to determine the phenotype of LICs: 21 of 25 NPM mutant AMLs were capable of engrafting immunodeficient mice.

Serial transplantation
Bone marrow cells from mice transplanted with fractions of AML were stained with APC-conjugated anti–mouse CD45 antibody and PE-conjugated anti–human CD33 antibody before resuspension in a DAPI-containing solution of 2% FCS with PBS. Human CD33⁺ cells were sorted on a BD Bioscience FACS Aria before transplantation into irradiated mice. In some experiments, particularly where engraftment of leukemia was high, bone marrow cells were transplanted without cell sorting.

Statistics
Results are expressed as mean with standard deviation unless stated. The Student t test was used to assess the significance of any differences. The chi-square test was used to assess the significance of differences between FLT3 mutation frequency in different subgroups. Extreme Limiting Dilution Analysis software (available from the Bioinformatics section of the Walter and Eliza Hall Institute of Medical Research, http://bioinf.wehi.edu.au/software/elda/index.html) was used to estimate the frequency of LICs from limiting dilution assays and differences between fractions.22

Results
Classification of AML with NPM mutation according to phenotype
Immunophenotyping was performed on 27 AML samples, all of which had mutated NPM (Table 1). The median CD34⁺ expression was 0.66% (range, 0.05%–65.3%), and this was significantly lower (P < .001) than that seen in 30 AMLs with wild-type NPM (median CD34⁺ expression was 24.5%; range, 0.07%–90%; data not shown). The NPM-mutated leukemias were classified into 3 distinct subtypes based on their CD34 and CD38 expression. Subtype A samples (13 of 27 AMLs) were predominantly CD34⁻, with a total CD34⁺ less than 0.5% (Figure 1A). Subtype B and C samples have a total CD34⁺ greater than 0.5%. The subtype B samples (3 of 27 samples) have a small CD34⁺ fraction (less than 0.1%) and distinct from the CD34⁺/CD38⁺ population (Figure 1B). Although the subtype C samples (11 of 27 samples) may have a small CD34⁺/CD38⁺ population, it is never separated clearly from the other fractions (Figure 1C). The subtype C samples had a higher incidence of FLT3 ITD than subtype A samples (80% vs 31%, respectively; P = .02).

The majority of cells within the CD34⁺/CD38⁻ fraction of subtypes A and B do not contain mutated NPM
Earlier studies suggested LICs are enriched within the CD34⁺/CD38⁻ fraction of AML. To investigate whether CD34⁺/CD38⁻ cells from NPM-mutated AML are leukemic or normal, we determined the percentage of mutated NPM in DNA from sorted cells from 12 AML samples (supplemental Figure 1). Four cell fractions were tested (CD34⁺/CD38⁻, CD34⁺/CD38⁺, CD34⁺/CD38⁻, and CD34⁻). The CD3⁺ cells and CD34⁺/CD38⁻ cells (this latter population comprises the majority of cells from most of the AML samples) served as negative and positive controls, respectively. We used the region R1 in Figure 2Ai to sort CD34⁺/CD38⁻ and CD34⁺/CD38⁻ fractions from 8 subtype A samples. We also tested the distinct CD34⁺/CD38⁻ population of cells (region R2 in Figure 2Aii) found in 5 of 13 subtype A samples. The data summarized in Table 2 suggest that the majority of cells in the defined CD34⁺/CD38⁻ and CD34⁺/CD38⁻ fractions of pattern A

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Figure 1. Classification of NPM mutant AML samples. The expression of CD34 and CD38 on 8 AML samples is shown. (A) Subtype A samples have CD34 expression less than 0.5% of cells. The phenotypes of 3 subtype A samples are shown (left, sample 1; middle, sample 3; and right, sample 4). (B) Subtype B samples have CD34 expression greater than 0.5%, but the CD34⁺/CD38⁻ population is small (<0.1%) and distinct from the CD34⁺/CD38⁺ and CD34⁻ populations. The phenotypes of samples 14 (left) and 15 (right) are shown. (C) Subtype C samples are more heterogeneous than the other subtypes. The CD34⁺ fraction is greater than 0.5%, but there is no distinct and small CD34⁺/CD38⁻ population. The phenotypes of samples 17 (left), 19 (middle), and 21 (right) are displayed.
samples contain wild-type *NPM*. There was no significant difference between the percentage of mutated DNA in the CD3+ cells and the CD34+CD38− and CD34+CD38+ fractions from subtype A samples. Given that the T lymphocytes are thought to be genetically normal in *NPM*-mutated AML, we interpret the finding of small amounts of mutated DNA in these 3 populations as being indicative of low-level contamination of each population by leukemic cells during sorting. 100% (sample 8) and 76% (sample 9) of cells in the CD34dim population contained mutant *NPM*. All fractions except the CD3+ from one subtype C sample contained mostly mutated *NPM*.

### The CD34+ fraction from subtype A samples contain all the erythroid colony-forming cells

To provide additional evidence that many CD34+ cells are normal in *NPM*-mutated AML, we determined whether CD34+ cells produce normal colonies in methylcellulose. CD34+ cells were enriched from 6 AML samples (4 subtype A [samples 1, 3, 8, and 9] and 2 other subtype AMLs [samples 14 and 17]) by immunomagnetic positive selection and plated into methylcellulose. The CD34+ enriched cells gave rise to erythroid colonies from all samples (supplemental Figure 2). By contrast, samples depleted of CD34+ cells did not produce erythroid colonies in any of the plates (supplemental Figure 2). The frequency of erythroid colonies varied from 9.5 to 138 colonies per 10^5 cells. The percentage of mutant *NPM* was determined in the cells from the methylcellulose. The mean percentage of *NPM*-mutated cells was significantly lower (P = .003) in the cells derived from the CD34+ -enriched fraction (12.3% ± 17%) than from the CD34-depleted fraction (75.3% ± 36%). This experiment shows that normal progenitors are found in the CD34+ fraction of these leukemias. Given that 4 of these samples had very small CD3+ fractions (< 0.3% of total cells), the data provide additional evidence that the CD3+ cells are normal.

### The CD34+ fraction of subtype A samples gives rise to AML, whereas the CD34+ fraction gives rise to normal hematopoiesis

Although most of the CD34+ cells from subtype A samples contained wild-type *NPM*, the minority that contain mutated *NPM* may be LICs. To test this, we transplanted NOD/SCID/β2m−/− mice with cells from the CD34+, CD34+, or CD34dim (from the R2 region in Figure 2Aii) fractions from 5 subtype A samples. CD34+ cells (derived from cell sorting or immunomagnetic depletion) were transplanted and gave rise to leukemia (Table 3). By contrast sorted CD34+ cells and CD34dim cells failed to initiate leukemia. The CD34+ fraction from 2 samples gave rise to normal multilineage hematopoiesis. The data indicate that LICs are found in the CD34+ fraction of subtype A samples.

### LICs are found within the CD34+CD38− fraction of *NPM* samples but not the CD34+CD38− fraction of subtypes A and B

To determine the expression of CD38 and CD34 on LICs from *NPM*-mutated AML, we transplanted mice with sorted cells from the CD34+CD38−, CD34+CD38+, CD34+CD38+, and CD34+CD38+ fractions (Table 4 and supplemental Tables 1-2). To confirm the nature of the human CD45+ grafts, we performed quantitative PCR on engrafted cells from the majority of experiments. The CD34+CD38− fraction initiated leukemia from all 12 *NPM*-mutated AML samples where this fraction was tested, whereas the CD34+CD38− fraction did not from 6 of 12 samples. For subtypes A and B, the CD34+CD38− fraction gave rise to normal multilineage hematopoiesis (the engrafted cells contained wild-type *NPM*) or no graft. The CD34+CD38− fraction gave rise to leukemia from 5 subtype C samples, though some mice contained both AML and normal human hematopoiesis.

### Serial transplantation

AML cells from successful grafts were transplanted into secondary recipients to assess self-renewal ability. Cells derived from the CD34+CD38− and CD34+CD38− fractions of subtype A samples were capable of initiating leukemia in secondary recipients (Table 5). Cells from one secondary recipient were able to initiate leukemia in multiple tertiary recipients demonstrating self-renewal. Cells from mice transplanted with either CD34+ or CD34− fractions from 3 subtype C samples (samples 17, 24, and 26) were able to initiate leukemia in secondary recipients, indicating that both the CD34+ and CD34− LICs have self-renewal capacity (Table 5). Therefore, the LICs we describe can maintain and propagate leukemia over prolonged periods and are not simply short-lived leukemic progenitors.

### Table 2. Percentage of *NPM*-mutated cells within sorted fractions of AML

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<th>Sample</th>
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<td>B</td>
<td>7.4</td>
<td>45</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>Mean of subtype B</td>
<td></td>
<td>12 ± 4.6*</td>
<td>76 ± 28.2</td>
<td>1.4</td>
<td>98.3 ± 2.0</td>
</tr>
<tr>
<td>20</td>
<td>C</td>
<td>94</td>
<td>98.7</td>
<td>0.3</td>
<td>100</td>
</tr>
</tbody>
</table>

The AML sample number is in the first column. The figures are percentages of cells that contain mutated *NPM* derived from quantitative genomic PCR. ND indicates not done.

*There is no significant difference between the percentage of *NPM* in the CD3+ fraction and the CD3+CD38− fraction (P < .001 for subtypes A and B).
†There is no significant difference between the percentage of *NPM* in the CD3+CD38− fraction (P = .3) or in the CD3−CD38− (P = .1) fractions of subtype A samples.
Frequency of LICs in each fraction

The frequency of LICs in specific fractions was determined by limiting dilution analysis of 4 sorted samples (Table 6). The LIC frequency was not significantly different between the CD34+CD38− and CD34+CD38+ fractions from 2 subtype A samples (P > .3 for each). The frequency of LICs was significantly lower (P < .05 for each) in the CD34+CD38− fraction than the other 3 fractions for one subtype C AML (sample 17). There was no significant difference between the CD34+CD38− fraction and the CD34+CD38+ or the CD34+CD38+ fraction for this sample. When the number of LICs per fraction per 10⁷ mononuclear cells was calculated, the majority of LICs were found to fall within the CD34− fractions as these are much larger than the CD34+ fractions (Table 6). The number of LICs per 10⁷ mononuclear cells was approximately 7-fold higher for the CD34− fraction compared with the CD34+ fraction from an additional subtype C sample with high CD34 expression (sample 25).

Normal CD34+CD38− cells do not contain repopulating cells

As all samples had LICs within the CD34+CD38− fraction (one sample had LICs restricted to this fraction), we tested whether this population of normal hematopoietic tissue has any repopulating capacity in our more immunodeficient mouse model (IVIG-treated NOD/SCID/IL2γ−/− mice); 10⁷ CD3−CD34−CD38− cells from cord blood were transplanted but failed to produce a graft at 20 days (3 mice), 9 weeks (4 mice), and 19 weeks (1 mouse). Similarly, 4.3 × 10⁷ lineage negative CD34−CD38− cord blood cells failed to produce a graft at 9 and 19 weeks, whereas 1.4 × 10⁷ lineage negative CD34−CD38− cells gave rise to multilineage engraftment at both these time points (data not shown).

The immunophenotype of LICs can change after xenotransplant

To assess whether the transplanted cells recapitulate the original leukemia, we examined the expression of myelo-monocytic markers on human CD45− cells present in the bone marrow of mice successfully transplanted with subfractions of AML. The leukemia cells expressed mature myelo-monocytic markers (CD14, CD15, CD36) consistent with the French American British classification (FAB) subtype of the original sample (supplemental Table 3). Myelo-monocytic and monocytic/blast cells (M4 and M5) expressed mature monocytic markers, whereas myeloblastic subtypes (M1 and M2) expressed CD33 but only a small minority of cells expressed the mature myelo-monocytic markers (supplemental Figure 3).

However, we noted some changes in the phenotype of engrafted AML cells. Only one of 13 samples (sample 19) maintained expression of CD34. The majority of engrafted leukemia cells had a CD34+CD38− phenotype (Figure 2). By contrast, a CD34+ population was seen in the normal graft derived from the CD34+CD38− fraction of sample 15 (Figure 2Biv). Given that the CD34+ fraction from some subtype C samples initiated leukemia in primary and secondary recipients and yet engrafted cells did not express CD34,
the data suggest that the CD34+ LICs had lost CD34 expression in the mice. Similarly, LICs from sample 7 were shown to lack CD14 expression in the primary sample but become CD14+ within the mice (Figure 3).

**NPM wild-type AMLs with low CD34 expression have CD34− LICs**

Although the expression of CD34 is significantly higher in NPM wild-type AML, some NPM wild-type samples have low expression of CD34. We sorted 2 such wild-type NPM samples (termed WT1 and WT2) and transplanted the cells into immunodeficient mice. The LICs were restricted to the CD34+ fraction (Tables 3 and 4). The immunophenotype of the graft from WT1 resembled that derived from the subtype A NPM-mutated samples, whereas WT2 comprised mostly CD34−CD38− cells.

**Table 3. Results of transplantsations from CD34−, CD34dim, and CD34+ fractions of AML into immunodeficient mice**

<table>
<thead>
<tr>
<th>AML sample</th>
<th>Subtype</th>
<th>Strain</th>
<th>Time, weeks</th>
<th>MNC dose x 10^7</th>
<th>Mice with AML/mice total</th>
<th>CD34−</th>
<th>CD34+</th>
<th>CD34dim</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>B2m</td>
<td>12</td>
<td>24</td>
<td>2/2 (2.8%)</td>
<td>7.8</td>
<td>5.5</td>
<td>0/3</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>NSG</td>
<td>12</td>
<td>5</td>
<td>0/2</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>B2m</td>
<td>13</td>
<td>5</td>
<td>2/2 (1.2%)</td>
<td>2.5</td>
<td>5.2</td>
<td>0/1</td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>NSG</td>
<td>13</td>
<td>5.4</td>
<td>0/1</td>
<td>4.2</td>
<td>19</td>
<td>0/1†</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
<td>B2m</td>
<td>13</td>
<td>13</td>
<td>4/4 (68%)</td>
<td>8.4</td>
<td>15</td>
<td>0/1</td>
</tr>
<tr>
<td>WT1</td>
<td>A</td>
<td>B2m</td>
<td>9</td>
<td>6</td>
<td>6/6 (100%)</td>
<td>3.2</td>
<td>6</td>
<td>0/1</td>
</tr>
</tbody>
</table>

NA indicates not applicable as these samples lack the CD34dim population; NT, not tested; and WT1, wild-type NPMAML sample 1. The figures in parentheses indicate the mean percentage of AML in the mouse bone marrow.

*Sorted human cells from mouse bone marrow were shown to contain mutated NPM by quantitative PCR.
†Normal multilineage engraftment observed; the DNA from the grafts contained wild-type NPM.
‡The LICs from sample 7 are found in the CD34+ fraction (Table 4).

**Discussion**

LICs were found within the CD34+ fraction of all 15 AMLs (NPM-mutated) that were studied in vivo. Whereas all 6 subtype A samples had LICs restricted to the CD34+ fraction, all subtype C samples had both CD34+ and CD34− LICs. These LICs were capable of initiating leukemia in secondary recipients and so are not simply short-lived leukemic progenitors.

The apparent discrepancy between this and earlier studies is due to our focus on NPM-mutated AML and the improved tools we used (more immunodeficient mice, measures to abrogate the inhibitory effects of anti-CD38 antibody, and improved graft identification). Conventional NOD/SCID mice can only act as a host for some AMLs, notably those with a worse prognosis.

**Table 4. Results of the transplantation of CD34−CD38−, CD34−CD38+, CD34+CD38−, and CD34+CD38+ fractions from AML**

<table>
<thead>
<tr>
<th>AML sample</th>
<th>Subtype</th>
<th>Strain</th>
<th>Time, weeks</th>
<th>CD34−CD38−</th>
<th>CD34+CD38−</th>
<th>CD34−CD38+</th>
<th>CD34+CD38+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>B2m</td>
<td>12</td>
<td>NT*</td>
<td>NT*</td>
<td>NT*</td>
<td>0/2†</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>NSG</td>
<td>12</td>
<td>2/2 (99%)</td>
<td>3/3‡ (49%)</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>B2m</td>
<td>5-9</td>
<td>0/2</td>
<td>3/3 (70%)</td>
<td>NT§</td>
<td>NT§</td>
</tr>
<tr>
<td>9i</td>
<td>A</td>
<td>NSG</td>
<td>17</td>
<td>0/2</td>
<td>1/1 (84%)</td>
<td>0/1</td>
<td>Died</td>
</tr>
<tr>
<td>9II</td>
<td>A</td>
<td>B2m</td>
<td>12-15</td>
<td>0/2</td>
<td>1/1 (0.1%)</td>
<td>NT§</td>
<td>NT§</td>
</tr>
<tr>
<td>10</td>
<td>A</td>
<td>NSG</td>
<td>16</td>
<td>1/1 (0.3%)</td>
<td>2/2 (26%)</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>12</td>
<td>B</td>
<td>NSG</td>
<td>16</td>
<td>0/2</td>
<td>2/2 (0.3%)</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>15</td>
<td>B</td>
<td>NSG</td>
<td>8-15</td>
<td>1/1 (0.3%)</td>
<td>1/1 (2.5%)</td>
<td>4/5 (1.4%)</td>
<td>0/1</td>
</tr>
<tr>
<td>17II</td>
<td>C</td>
<td>B2m</td>
<td>9-12</td>
<td>6/6 (57%)</td>
<td>7/8 (39%)</td>
<td>6/8 (32%)</td>
<td>4/6 (38%)</td>
</tr>
<tr>
<td>17III</td>
<td>C</td>
<td>NSG</td>
<td>12</td>
<td>NT</td>
<td>12</td>
<td>NT</td>
<td>12 (76%)</td>
</tr>
<tr>
<td>18</td>
<td>C</td>
<td>B2m</td>
<td>10</td>
<td>0/1</td>
<td>1/1 (4.4%)</td>
<td>1/1 (3.7%)</td>
<td>1/1 (1.6%)</td>
</tr>
<tr>
<td>19</td>
<td>C</td>
<td>B2m</td>
<td>12</td>
<td>0/2</td>
<td>1/2 (0.1%)</td>
<td>0/2</td>
<td>1/2 (0.3%)</td>
</tr>
<tr>
<td>24</td>
<td>C</td>
<td>B2m</td>
<td>12</td>
<td>1/1 (20%)</td>
<td>2/2 (40%)</td>
<td>2/2 (11%)</td>
<td>(24%)</td>
</tr>
<tr>
<td>25</td>
<td>C</td>
<td>B2m</td>
<td>12</td>
<td>2/2</td>
<td>3/3 (6.7%)</td>
<td>3/3 (26%)</td>
<td>0/1</td>
</tr>
<tr>
<td>26</td>
<td>C</td>
<td>B2m</td>
<td>12</td>
<td>3/3 (73.3%)</td>
<td>3/3 (37.2%)</td>
<td>1/1 (8.9%)</td>
<td>1/1 (0.9%)</td>
</tr>
<tr>
<td>WT2</td>
<td>NA</td>
<td>B2m</td>
<td>7</td>
<td>4/4 (49%)</td>
<td>0/1</td>
<td>0/1</td>
<td>NA</td>
</tr>
</tbody>
</table>

The AML sample is indicated in the first column and the subtype in the second. Some samples were tested in more than one strain of mouse (indicated by the lowercase Roman numeral). NSG indicates that NOD/SCID IL2γ−− mice were used in the experiment; B2m, NOD/SCID IL2γ−− mice; NT, not tested; WT2, wild-type NPM AML sample 2; and NA, not applicable as this sample lacks the CD34−CD38− population. The figures in parentheses indicate the mean percentage of AML in the mouse bone marrow.

*The LICs from sample 1 are restricted to the CD34+ fraction (Table 3).
†Normal multilineage engraftment observed from the CD34−CD38− fraction of one or more mice. The DNA from the grafts contained wild-type NPM.
‡Both CD45−CD33− and CD45−CD19+ populations were observed in some mice transplanted with CD34+CD38− cells. The CD19+ cells contain mostly wild-type NPM, whereas the CD33− fractions contain mostly mutant NPM, consistent with the presence of LICs and normal repopulating cells in the CD34+CD38− fraction. Some of the data from the CD34+CD38− and CD34+CD38+ fractions of samples 15 and 18 have been previously published.9
Consequently, earlier studies may have been biased away from samples with relatively favorable features such as NPM mutation and low CD34 expression (<50% of NPM mutant AMLs engraft NOD/SCID mice). By contrast, the newer strains can act as a host for more than 80% of NPM-mutated AML. There may have been a further bias against subtype A samples relative to subtype C samples, as the subtype A samples have a lower incidence of FLT3 ITD, a poor risk mutation.

Using IVIG to abrogate the effects of anti-CD38 antibody, we noted that LICs were found in the CD34+CD38− fraction and either of the 2 CD38+ fractions from 3 of 6 subtype C samples. It is conceivable that the CD34+ LICs (CD34+CD38+ and/or CD34+CD38−) from some subtype C samples may have been eliminated by the anti-CD38 antibody in earlier studies, leading to the impression that only CD34+CD38− LICs exist. Consistent with this explanation, 2 studies in which anti-CD38 antibody was not used showed LICs in both CD34+ and CD34− fractions from some samples.

Lastly, the methodology used in some earlier studies could not always determine whether grafts were leukemic or normal (from normal CD34+ cells within the leukemia sample). Most early studies did not use immunophenotyping to identify the lineage of engrafted human CD45+ cells and so could not readily discriminate between normal multilineage engraftment and unilineage AML. Cytogenetic and molecular techniques would not have added much, as most NPM-mutated AMLs have a normal karyotype (and NPM mutation was not identified until later). Therefore, some grafts from the CD34+ fractions of AML may have been normal but misclassified as leukemic.

More than one fraction initiated AML from 10 samples (Table 4). This probably reflects changes in expression of CD34 and CD38 on LICs under different conditions or at different times. Mouse hematopoietic stem cells (HSCs) show variation in the expression of these markers after exposure to chemotherapy or after mobilization by granulocyte colony-stimulating factor. Similarly CD34 is expressed on HSCs from young mice but is lost as mice age. The CD34+ fraction from sample 17 was able to initiate AML in primary and secondary recipients, yet the engrafted cells lacked CD34. Thus the LICs were initially CD34+ but became CD34− within the mouse bone marrow environment. The mice appear to push the LIC phenotype toward a more differentiated one with increased CD38.

---

### Table 5. Details of secondary transplants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction</th>
<th>Secondary recipients*</th>
<th>Tertiary recipients*</th>
<th>Time in vivo, weeks†</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>CD34−CD38−</td>
<td>4/4</td>
<td>5/5</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>CD34−CD38−</td>
<td>2/2</td>
<td>NT</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>CD34−CD38−</td>
<td>5/5</td>
<td>5/5</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>CD34−CD38−</td>
<td>3/3</td>
<td>NT</td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>CD34−CD38−</td>
<td>1/1</td>
<td>NT</td>
<td>34</td>
</tr>
<tr>
<td>17</td>
<td>CD34−CD38−</td>
<td>3/3</td>
<td>NT</td>
<td>24</td>
</tr>
<tr>
<td>17</td>
<td>CD34−CD38−</td>
<td>3/3</td>
<td>NT</td>
<td>24</td>
</tr>
<tr>
<td>17</td>
<td>CD34−CD38−</td>
<td>1/1</td>
<td>NT</td>
<td>25</td>
</tr>
<tr>
<td>26</td>
<td>CD34−CD38−</td>
<td>3/3</td>
<td>NT</td>
<td>24</td>
</tr>
<tr>
<td>26</td>
<td>CD34−CD38−</td>
<td>4/4</td>
<td>NT</td>
<td>24</td>
</tr>
<tr>
<td>26</td>
<td>CD34−CD38−</td>
<td>4/4</td>
<td>NT</td>
<td>24</td>
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<tr>
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<td>CD34−CD38−</td>
<td>0/1</td>
<td>NT</td>
<td>24</td>
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<td>CD34−CD38−</td>
<td>1/1</td>
<td>NT</td>
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</tr>
<tr>
<td>24</td>
<td>CD34−CD38−</td>
<td>1/1</td>
<td>NT</td>
<td>24</td>
</tr>
</tbody>
</table>

NT indicates not tested.

*Data for secondary and tertiary recipients are reported as mice with AML/mice transplanted.
†Time in vivo refers to the time from transplantation in the primary recipient to the termination of the final recipient. Sample 7 is very aggressive clinically and mice became sick due to leukemia 3 to 5 weeks after transplantation, hence the relatively short duration.

---

### Table 6. LIC frequency in sorted fractions of AML

<table>
<thead>
<tr>
<th>AML</th>
<th>Fraction</th>
<th>10⁵ cells</th>
<th>10⁴ cells</th>
<th>10³ cells</th>
<th>2 x 10⁴ cells</th>
<th>5 x 10⁴ cells</th>
<th>10⁵ cells</th>
<th>5 x 10⁴ cells</th>
<th>10⁵ cells</th>
<th>3 x 10⁴ cells</th>
<th>10⁵ cells</th>
<th>3 x 10⁴ cells</th>
<th>LIC frequency (95% confidence interval)</th>
<th>LICS per 10⁶ MNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>CD34−CD38−</td>
<td>2/4</td>
<td>3/4</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1 in 458</td>
<td>(153-1369)</td>
<td>432</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CD34−CD38−</td>
<td>0/4</td>
<td>4/4</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1 in 873</td>
<td>(275-2768)</td>
<td>918</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>CD34−CD38−</td>
<td>0/3</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>3/3</td>
<td>1 in 245</td>
<td>454 (85 666-701 648)</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>CD34−CD38−</td>
<td>0/4</td>
<td>2/5</td>
<td>2/2</td>
<td>2/2</td>
<td>3/3</td>
<td>1 in 231</td>
<td>326 (83 726-639 131)</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>CD34−CD38−</td>
<td>0/1</td>
<td>1/3</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>1 in 2164</td>
<td>(538-8698)</td>
<td>228</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>CD34−CD38−</td>
<td>2/3</td>
<td>6/8</td>
<td>1/3</td>
<td>1/3</td>
<td>1/3</td>
<td>1 in 540</td>
<td>236 (136-1336)</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>CD34−CD38−</td>
<td>3/5</td>
<td>2/2</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1 in 1091</td>
<td>(339-3508)</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>CD34</td>
<td>0/1</td>
<td>0/2</td>
<td>0/3</td>
<td>2/2</td>
<td>1 in 3</td>
<td>587 (885 468-12 504 816)</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>CD34</td>
<td>0/3</td>
<td>1/2</td>
<td>2/3</td>
<td>2/2</td>
<td>1 in 878</td>
<td>831 (321 224-2 404 374)</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CD34 is not a stable feature of the LICs from most subtype B and leukemic cells within the mice suggests that the expression of CD34 may derive from CD34+ cells from the original LICs through acquisition of additional hits. The evolution of new generations of LICs has types may have evolved from the original LICs through acquisition of mixed mutated nucleophosmin is stable in primary AMLs, and this phenotype cannot be blindly relied upon to prospectively identify LICs. Moreover, we have demonstrated that LICs can change their phenotype, and this may well explain the observation that LICs are found in multiple fractions from some AMLs. This property will mean that LICs will be harder to completely eliminate using targeted therapies such as monoclonal antibodies.

In conclusion, we have demonstrated that the phenotype of LICs from NPM-mutated AMLs is different to that previously reported for unselected AMLs. In some the LICs were exclusively CD34+, whereas other samples had both CD34- and CD34+ LICs. The study reinforces the idea that there is greater heterogeneity in the phenotype of LICs than previous studies indicated. LICs are not found in the CD34+CD38- fraction in approximately half of NPM-mutated AMLs, and this phenotype cannot be blindly relied upon to prospectively identify LICs. Moreover, we have demonstrated that LICs can change their phenotype, and this may well explain the observation that LICs are found in multiple fractions from some AMLs. This property will mean that LICs will be harder to completely eliminate using targeted therapies such as monoclonal antibodies.

Acknowledgments

We are indebted to patients who gave samples. We thank Dr. Michael Jenner for providing diagnostic information.

D.C.T. is supported by a Medical Research Council Clinician Scientist Fellowship. This work was supported by Cancer Research UK and a European Commission Grant (LSHC-CT-206-037632; D.B.) and by funding from National Cancer Institute (P01 CA95426; J.G.G.).

Authorship

Contributions: D.C.T. designed and performed research, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript; J.V. performed research and analyzed and interpreted data; F.M.-M., E.G., K.S. and T.L. performed research; D.L. provided vital data; H.O. and J.C. provided vital materials; S.G.A. contributed vital materials; T.A.L. was responsible for the Tissue Bank and clinical database; J.G.G. interpreted data and wrote the manuscript; and D.B. designed research and analyzed, interpreted data, and wrote the manuscript.

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

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