Leukemia stem cell diversity in CD34-negative versus CD34-positive acute myeloid leukemia. Combining CD34/CD38-defined with SP-defined stem cell populations

B. Moshaver
A. Kelder
G.J. Ossenkoppele
A.H. Westra
S. Zweegman
G.J. Schuurhuis

Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands

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Abstract

Background: The concept of leukemia stem cells (LSCs) originating from a transformed CD34+CD38- hematopoietic stem cell (HSC), has been challenged by the evidence for a more mature progenitor-like cell with acquired stem cell properties as a source of acute myeloid leukemia (AML). In addition, LSCs have been found to originate from side population (SP) cells, being characterized by high Hoechst 33342 efflux and the presence of cells other than CD34+CD38-. Whether these differently defined LSCs reflect different biological LSC behavior, thereby affecting clinical outcome is currently unknown. This study compares the differently defined stem cell compartments in two groups of AML patients: CD34-negative and CD34-positive AMLs.

Methods: Primary AML cells were investigated using flow cytometry, clonogenic assays, and fluorescence in situ hybridization (FISH) to assess the relationship between CD34+CD38- and SP defined stem cell compartments. Aberrant immunophenotypic marker expression was used to designate the nature of cells (normal/malignant).

Results: Truly CD34-negative AML were found to be characterized by a very small (usually < 1%) cytogenetically normal CD34-positive population. The SP compartment contained essentially normal CD34+CD38- and CD34-CD38-normal cells. The CD34-38+ compartment was most prominent in SP and essentially malignant. In non-SP (NSP) very few normal cells were found and CD34-CD38- and CD34-CD38+ were most prominent and malignant. In contrast, in CD34-positive AML both SP and NSP compartment contained essentially malignant cells with all four combinations of CD34/CD38. Nevertheless, normal cells with all CD34/CD38 combinations were present as well. Clonogenic assays showed that the stem cell activity of both normal and leukemia cells in the NSP fraction is much lower compared to the SP fraction, both in CD34-negative and in CD34-positive AML.

Conclusions: The obtained data indicate that in CD34-negative AML the biologically important LSCs are CD34-38+ SP cells, whereas in CD34-positive AML the biologically important LSCs are SP cells with all four combinations of CD34/CD38. These differences suggest that both types of AML are separate entities underlying differences in prognosis and warranting different therapeutic approaches.

Introduction

The exact phenotype of the leukemia stem cell (LSC) is far from elucidated yet. Although the putative LSC has long been considered to be harbored in the CD34+38- sub-fraction (1,2), this has recently been challenged by the finding that leukemia may also originate from CD34-negative stem cells (3,4). Amongst others, these cells have been proposed to be present in the so-called side population (SP) cells, i.e. a specific subpopulation of cells with high MDR efflux pump activity (5). These were indeed shown to have leukemia initiating capacity in a NOD/SCID mouse model (6,7). SP cells appeared to be largely CD34-negative with varying levels of CD38 expression (6). The frequency of SP cells is in general considerably lower than the CD34+CD38- cells (8). Whether combination of immunophenotypical and functional characteristics would allow either to narrow down the size of the LSC compartment and/or even to define biological differences of LSCs leading to different clinical outcome, is currently unknown. However, it can be anticipated that investigating both immunophenotypical and functional stem cell characteristics may allow defining the LSC in such a more specific way. We have recently shown a low-frequency (median 0.0016%) aberrant marker-positive sub-fration of the SP compartment as a likely candidate to be enriched for LSCs (8). In this respect investigating LSC separately in both CD34-negative leukemia and CD34-positive leukemia is of special interest, as in CD34-negative leukemia only a small CD34-positive cell fraction is found and this fraction likely contains only normal cells (9), suggesting that the tiny CD34+CD38- subpopulation herein may well be normal too. If so, CD34-negative leukemia by definition must contain CD34-negative AML stem cells, while CD34-positive AML may theoretically contain both CD34-negative and CD34-positive LSC. Since details on the clonogenic capacity of SP and non-SP (NSP) cells in CD34-negative and CD34-positive leukemia are lacking, the role of SP herein is not clear. Such investigations are hampered by the fact that CD34+CD38- and SP phenotypes are a characteristic of both LSC and HSC, both being present in bone marrow samples of patients with AML. Thus far this allowed only retrospective analysis, showing for example with FISH analysis, the presence of mixtures of normal and leukemia cells in the presumed stem cell compartments. Prospective analysis of normal and leukemia cells is now possible by the possibility to discriminate between leukemia and normal stem cells using aberrant cell surface antigen expression on LSC (10,11). Recently, we showed that in addition to aberrant immunophenotypic cell surface marker expression flowcytometrically...
Immunophenotyping and Cell Sorting

Primary AML cells (1 X 10^6 cells per ml) were stained with 5 µg/ml Hoechst 33342 dye (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) in PBS and incubated at 37°C for 2 hours according to Goodell et al. (10). After Hoechst staining, cells were washed and re-suspended in 100 µl of cold (4°C) Hanks’ balanced salt solution (HBSS; Cambrex, Verviers, Belgium, http://www.cambrex.com) with 2% fetal calf serum (FCS) and incubated for 30 minutes on ice with combinations of fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), allophycocyanin (APC)-labeled monoclonal antibodies (MoAbs). Anti-CD34 FITC, anti-CD48 FITC, anti-CD19 PE, anti-CD56 PE, anti-CLL-1 PE, and anti-CD38 APC MoAbs were all from BD Biosciences (San Jose, CA, http://www.bdbiosciences.com) except anti-CLL-1 PE and its PE-labeled isotype control, which were obtained and used as previously described (10). CD48 MoAb was used to exclude lymphocyte cells from the SP population (8). The LSCs were defined using aberrant marker expression as previously described for the CD34+CD38- stem cell compartment (11). We used CD19, and CD56, which are frequently used in AML MRD detection using leukemia-associated phenotype. After antibody staining, cells were washed with cold HBSS + (HBSS + 2% FCS), re-suspended in 1 ml of cold HBSS + and stained for 5 minutes with 2 µg/ml propidium iodide (PI, Sigma-Aldrich), enabling exclusion of dead cells. Cells were kept on ice until FACS analysis. Data acquisition was performed using a FACSCanto II (with red, blue and violet solid-state lasers) from BD Biosciences; analysis was performed using CellQuest and FACSDiva software (BD Biosciences). A minimum of 20 x 10^6 cells were used in all experiments. The Hoechst dye was excited with a 405-nm violet (FACSCanto II) laser and detected with 450/BP50 optical filter. Regions were set to detect the viable SP cells as shown in Figure 1. Cells were sorted using a FACSAria (with red, blue, and violet solid-state lasers; BD Biosciences). Cells were kept on ice during the whole procedure. For further culturing, cells were sorted directly into cold culture medium. Purity of sorted populations was >98%.

LSC and HSC Scatter (Comparison with Lymphocytes)

The relative position of the populations of interest in the FSC and SSC was established in an objective way: “high” and “low” was defined by determining the ratio of the median FSC or SSC value of the population of interest by the median value of the lymphocytes present in the same sample. Previous work defined FSC\textsuperscript{high} as a ratio > 1.3 and SSC\textsuperscript{high} as a ratio > 1.4 (Terwijn M, et al. submitted). In this paper, whenever primary gating was performed on scatter characteristics.

Materials and methods

Leukemic Bone Marrow Cells

Bone marrow (BM) samples were collected at diagnosis after informed consent from 11 AML patients (4 females, 7 males) with a median age of 48 years (range 23-70) and were used for a more detailed investigation. Mononuclear cells were isolated by Ficoll gradient (1.077 g/ml; Amersham Biosciences, Freiburg, Germany, http://www.amersham.com) and washed with phosphate-buffered saline (PBS; ICN Biomedicals, Aurora, OH, http://www.icn.biomed.com). Red blood cells were lysed afterward by 10 minutes of incubation on ice, using 10 ml of a solution containing 155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM Na2 EDTA, pH 7.4, added directly to the cell pellet. After washing, cells were frozen in RPMI (Gibco, Paisley, U.K., http://www.invitrogen.com) with 20% heat-inactivated fetal bovine serum (FBS; Greiner Bio-One, Alphen a/d Rijn, The Netherlands, http://www.gbo.com/en) and 10% dimethyl sulfoxide (Riedel-de Haen, Seelze, Germany, http://www.riedeldehaen.de), in isopropanol-filled containers at -80°C and subsequently stored in liquid nitrogen. When needed for analysis, cells were thawed and suspended in pre-warmed RPMI with 40% FBS at 37°C. Cells were washed and allowed to recover for 45 minutes in the same medium at 37°C. Cells were washed again and re-suspended in PBS with 0.1% bovine serum albumin (ICN Biomedicals, Aurora, OH).
normal stem cells: multiplication factor ≥ 8 means AML stem cells (FSC<sup>high</sup>/SSC<sup>high</sup>), while multiplication factor < 8 means normal stem cells (FSC<sup>low</sup>/SSC<sup>low</sup>). It should be noted that SP cells with very high SSC are excluded from the analyses as these are non-clonogenic (8).

**Definition of CD34-Negative and CD34-Positive AML**

Previously we showed that part of AML cases is characterized by the presence of a small but distinct population (usually < 1%) of CD34-positive cells that were normal using functional assays and FISH analyses (9). We used this definition to study these CD34-negative patients separately from CD34-positive patients.

**Suspension Culture of AML SP Cells**

The suspension culture was performed essentially as previously described (8). The sorted SP subpopulations (3,500–5,500 cells) were mixed with 1 X 10<sup>5</sup> NSP cells and re-suspended in 250 µl per well of CellGro medium (Cellgenix, Vancouver, BC, Canada, http://www.cellgenix.com) containing 20 ng/ml interleukin (IL)-3, 100 ng/ml Flt-3 ligand, and 100 ng/ml stem cell factor (SCF) (all from Peprotech, Basel, Switzerland, http://www.peprotech.com) prior to plating in 96-well round-bottomed plates (Greiner Bio-One). In addition, 1 X 10<sup>5</sup> and 1 X 10<sup>6</sup> NSP cells were plated as control for the mixed (SP + NSP) cultures. Suspension cultures were incubated at 37°C in 5% CO₂ and received weekly half-medium changes. Usually in this assay these weekly half-medium changes are accompanied by demi-population of cells; however, since the numbers of SP cells were very low, we chose to harvest all cells at one time point only (5 weeks). Subsequently, all harvested cells were cultured in a 14-day colony-forming unit (CFU) assay (see below).

**CFU Assay**

Assays for leukemia CFUs were performed by plating cells in methylcellulose medium (H4434; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). Cultures were scored after 14 days for the presence of clusters (4–20 cells) and colonies (more than 20 cells). The number of colonies from the sorted SP + NSP or NSP cells was calculated as previously described (12). Final clonogenic output was normalized to either one million SP or one million NSP input cells, i.e. the cells put in suspension culture immediately following sorting for SP and NSP cells at the start of the (5 + 2 weeks) experiment. Alternatively, clonogenic output was normalized to one million blast cells (containing SP and NSP cells at original ratio’s), respectively.
Fluorescence In Situ Hybridization (FISH) Analysis of FACS-Sorted SP Cells
For interphase FISH, the FACS-sorted SP cells were washed three times with 3 ml of 3:1 methanol/acetic acid fixative and suspended in 100 μl of fixative. Subsequently, one droplet was gently placed on an object slide and air-dried. Dual-color (spectrum green and spectrum orange fluorophores) labeled LSI DNA probes (Vysis, Downers Grove, IL, http://www.vysis.com) were applied to the de-natured cells and incubated as previously described (9). The following probes were used: the LSI AML1/ETO dual color for t(8;21) and the LSI TEL/AML1 ES dual color for del 12(p13). Hybridization and deletion signals were scored in 50 interphase nuclei with an Axioscop 20 (Carl Zeiss, Jena, Germany, http://www.zeiss.com) fluorescence microscope with three single-band-pass filters and one triple-band-pass filter. Nuclei were scored positive for the fusion gene, when a green spot and an orange spot were less than one spot diameter apart. Nuclei were scored positive for deletion 12, when one green spot was absent. The images were captured with a digital camera using CytoVision 4.1 software (Applied Imaging Corp., Newcastle, U.K., http://www.appliedimaging.com).

Results
The Nature of Cell Surface Marker-Positive FSC<sup>high</sup> SSC<sup>high</sup> and Cell Surface Marker-Negative FSC<sup>low</sup>/SSC<sup>low</sup> SP Cells in CD34-Positive AML
Firstly, we studied whether the finding that CD34<sup>+</sup>CD38<sup>-</sup> LSC are FSC<sup>high</sup>/SSC<sup>high</sup>, while HSC CD34<sup>+</sup>CD38<sup>-</sup> are FSC<sup>low</sup>/SSC<sup>low</sup>, could be confirmed for SP stem cells as well. Table 1 gives an example, by using FISH analysis, that indeed myeloid FSC<sup>high</sup>/SSC<sup>high</sup> SP and NSP cells are malignant and myeloid FSC<sup>low</sup>/SSC<sup>low</sup> SP and NSP cells are normal.

Next we studied the interrelationship between SP and CD34<sup>+</sup>CD38<sup>-</sup> compartments separately in CD34-negative and CD34-positive patients (for definition see Materials and Methods). In addition, to further refine the definition of LSC and HSC in these groups, other compartments (CD34<sup>+</sup>CD38<sup>-</sup>, CD34<sup>-</sup>CD38<sup>-</sup>, CD34<sup>-</sup>CD38<sup>+</sup>, CD34<sup>+</sup>CD38<sup>-</sup>) were studied as well. Figure 1 shows the gating strategy.

CD34-Negative AML
Four CD34/CD38 defined subpopulations in five CD34-negative AML patients were studied. The relative frequencies in SP and NSP AML and normal compartments are shown in Figures 2A and B.

<table>
<thead>
<tr>
<th>P</th>
<th>Time point</th>
<th>Cytogenetic aberration</th>
<th>Surface marker-&lt;br&gt;FSC&lt;sup&gt;high&lt;/sup&gt;/SSC&lt;sup&gt;high&lt;/sup&gt;</th>
<th>Surface marker-&lt;br&gt;FSC&lt;sup&gt;low&lt;/sup&gt;/SSC&lt;sup&gt;low&lt;/sup&gt;</th>
<th>Surface marker-&lt;br&gt;FSC&lt;sup&gt;high&lt;/sup&gt;/SSC&lt;sup&gt;high&lt;/sup&gt;</th>
<th>Surface marker-&lt;br&gt;FSC&lt;sup&gt;low&lt;/sup&gt;/SSC&lt;sup&gt;low&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Directly after sort§</td>
<td>t(8;21)</td>
<td>86 14</td>
<td>73 21</td>
<td>78 14</td>
<td>ND ND</td>
</tr>
<tr>
<td>1</td>
<td>After 5 weeks liquid culture#</td>
<td>del(12)</td>
<td>78 14</td>
<td>ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Directly after sort§</td>
<td>t(8;21)</td>
<td>86 14</td>
<td>73 21</td>
<td>80 14</td>
<td>ND ND</td>
</tr>
<tr>
<td># counted on 35-38 cells.</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

In the sorts lymphoid CD48-positive populations were excluded (8). Since the SP population makes up only a small fraction of the total blast compartment (1.6% and 0.5%), for our purposes this NSP population can be considered as the whole blast cell compartment.

CD34<sup>-</sup>CD38<sup>-</sup> Population
Figure 3 shows an example of CD34-negative AML with a small aberrant cell surface marker-negative FSC<sup>low</sup>/SSC<sup>low</sup> normal CD34<sup>-</sup>CD38<sup>-</sup> population within the SP compartment, but with no aberrant marker-positive CD34<sup>+</sup>CD38<sup>-</sup> cells present. Figure 4 summarizes the results of the five CD34-negative AML cases and reveals that malignant CD34<sup>-</sup>CD38<sup>-</sup> cells (aberrant cell surface marker-positive and FSC<sup>high</sup>/SSC<sup>high</sup>) were completely absent in SP cells, and absent or very low in the NSP cells. Normal CD34<sup>-</sup>CD38<sup>-</sup> SP cells were found in 3/5 cases while two remaining cases had no detectable CD34<sup>-</sup>CD38<sup>-</sup> population (Figure 4A),
In the latter two cases HSC were present in the NSP population be it at low frequencies (Figure 4B). In case 3, HSC were present in both SP and NSP. Overall, there is thus no common LSC that is defined both by SP and CD34+CD38- characteristics. With the complete absence in SP compartment of AML CD34+CD38- stem cells and also in 3/5 cases within NSP compartments, for CD34-negative AML there should be (an)other candidate LSC compartment(s) outside the CD34+CD38- compartment, i.e. either CD34-negative cells or CD34+CD38+.

Cells were stained for SP, CD34, CD38, CLL-1, as outlined in Materials and Methods. A-E: Example of CD34-negative AML SP population with a small CD34+CD38- population and with different marker expression and FSC/SSC patterns (patient 1 of the CD34-negative AML group). Viable cells were gated as described in Figure 1. Subsequent gating steps are described below, but can be read from the figure as well. (A): Hoechst red versus Hoechst blue identified the viable side population cells (region R2). SP cells from region R2 of plot A were displayed on plot B of CLL-1 versus SSC. (B): Aberrant CLL-1 expression on SP cells. Note that the CLL-1-positive population (red) has slightly higher SSC than the CLL-1-negative population (green). Regions R3 (CLL1-negative) and R4 (CLL1-positive) were used in plot C. (C): Location of CLL1-negative (R3) and CLL1-positive (R4) SP cells on plot C of FSC versus SSC. Note that CLL1-negative (green) cells have lower FSC and SSC than CLL1-positive (red) cells (factor 1.8 and 2.1, respectively). (D): Location of CLL1-positive FSClow/SSC low SP cells from region R4 of plot B on plot D of CD34 versus CD38. There is no CD34+CD38- SP population present within this population (region R7). (E): Location of CLL1-negative FSCmed/SSCmed SP population from region R3 of plot B on plot E of CD34 versus CD38. There is a small but distinct CD34+CD38- SP population present within this population (region R8). This CD34+CD38- population is of normal origin (aberrant cell surface marker-negative and FSCmed/SSCmed). So it can be concluded that the only CD34+CD38- population present is of normal origin (CLL1-negative with FSCmed/SSCmed).
present in 5/5 cases but contained only normal CD34-CD38- SP cells, except for patient 4 who had an additional small malignant population (7.5% of total SP cells). The corresponding NSP populations contained malignant cells in 5/5 cases, be it at low frequencies, with a normal component present in 3/5 cases. So, the CD34-CD38- compartment may contain LSCs, but these are predominantly present in the NSP compartment.

CD34-38+ Population

Table 3 shows the results for the CD34-CD38+ fraction. In contrast to the CD34+CD38- and CD34-CD38- SP fraction, this fraction contained almost exclusively malignant cells, while the NSP population harbored predominantly leukemia, but also low percentages of normal cells. CD34-CD38+ cells represent the largest part of both SP and NSP compartments (Figure 2). The abundance of CD34-CD38+ cells within the SP compartment can be seen in the example shown in Figure 3 (panels D and E).
Table 3 Percentages of normal and malignant CD34-CD38+ cells within SP and NSP populations of CD34-negative AML patients

<table>
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<tr>
<th>Patient</th>
<th>%SP</th>
<th>% of SP</th>
<th>Surface marker</th>
<th>% of NSP</th>
<th>Surface marker</th>
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<tr>
<td>1</td>
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<td>P (A)</td>
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<td>N (N)</td>
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<td>N (N)</td>
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<td>N (N)</td>
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</table>

P: Positive for aberrant cell surface marker
(A): AML
N: Negative for aberrant cell surface marker
(N): Normal
0 and -: Not present

Table 4 Percentages of normal and malignant CD34+CD38+ cells within SP and NSP populations of CD34-negative AML patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>%SP</th>
<th>% of SP</th>
<th>Surface marker</th>
<th>% of NSP</th>
<th>Surface marker</th>
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<td>-</td>
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<tr>
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<td>-</td>
<td>0.52</td>
<td>P (A)</td>
</tr>
</tbody>
</table>

P: Positive for aberrant cell surface marker
(A): AML
N: Negative for aberrant cell surface marker
(N): Normal
0 and -: Not present

CD34+38+ Population
Table 4 shows the results for CD34+CD38+ fraction. In 4/5 cases the SP fraction did not contain CD34+CD38+ cells, with very low frequencies found in the NSP fraction.

CD34-positive AML
We next investigated six CD34-positive AML patients (for definition, see Materials and Methods), and found profound differences compared with CD34-negative AML patients using the same aberrant cell surface marker/scatter criteria. Figures 2C and 2D summarize the frequencies of the four CD34/CD38 defined AML and normal subpopulations within SP and NSP compartments. In CD34-positive AML, compared to CD34-negative AML, a more balanced distribution between LSC and HSC frequencies can be seen in both SP and NSP.

CD34+38- Population
Figure 5 shows an example of CD34-positive AML with an aberrant cell surface marker-positive FSC<sup>high</sup>/SSC<sup>high</sup> malignant population that is also in part CD34+CD38-. There are no CD34+CD38- cells present in the aberrant cell surface marker-negative FSC<sup>low</sup>/SSC<sup>low</sup> populations. Figure 6 summarizes the results of the 6 CD34-positive patients. In contrast to CD34-negative AML, both SP and NSP cells now contained malignant CD34+CD38- cells in all cases, whereas normal stem cells were found in only 2/6 cases in SP and 1/6 cases in NSP (Figure 6A). Clearly, and in contrast to CD34-negative AML, frequency of normal stem cells seems markedly suppressed. Therefore, unless the HSC frequency in CD34+CD38- is extremely low and below detection limit (< 0.001%), there might be (an)other candidate HSC compartment(s) outside the CD34+CD38- compartment (see below).

The striking difference between CD34-negative and CD34-positive AML is that in CD34-negative leukemia, the normal HSC predominates in the CD34+CD38- cell fraction, in both SP and NSP, while in the same compartment in CD34-positive...
Cells were stained for SP, CD34, CD38, CD66, CLL-1 as outlined in Materials and Methods. A-E: Example of a CD34-positive AML SP population with a small AML CD34+CD38- population and with different surface marker expression and FSC/SSC patterns (patient 1 of the CD34-positive AML group). Viable cells were gated as described in Figure 1. Subsequent gating steps are described below, but can be read from the figure as well. (A): Hoechst red versus Hoechst blue identified the viable SP cells (region R2). SP cells from region R2 of plot A were displayed on plot B with CD19 versus SSC. (B): Aberrant CD19 expression on SP cells. Regions R3 (CD19-negative) and R4 (CD19-positive) were used in plot C. Location of CD19-negative (R3) and CD19-positive (R4) SP cells of plot B on plot C with FSC versus SSC (region R5 and R6, respectively). Note that CD19-negative (green) cells and CD19-positive (red) cells are FSClow/SSC low and FSC high /SSC high, respectively (see also legend Figure 1). (D): Location of CD19-positive FSC low/SSC low SP cells from region R4 of plot B on plot D with CD34 versus CD38. There thus is a CD34+CD38-CD19+ population present within SP population (region R7). This CD34+CD38- population is of malignant origin (aberrant cell surface marker-positive and FSC low/SSC low). (E): Location of CD19-negative FSC low/SSC low SP population from region R3 of plot B on plot E with CD34 versus CD38. There is no normal CD34+CD38- population present within SP population (region R8). So it can be concluded that the only CD34+CD38- population present is of malignant origin (CD19-positive with FSC low/SSC low).
leukemia initiating ability. However, since in CD34-negative AML LSCs were shown to be present in different compartments, we investigated the properties of other CD34/CD38 defined putative stem cell compartments in these CD34-positive AML patients as well.

CD34-CD38- Population
Table 5 shows a summary of the results in the CD34-CD38- population. The CD34-CD38- compartment contained malignant cells in 4/6 cases in SP and in 5/6 cases in NSP. A normal (aberrant cell surface marker-negative/FSClow/SSClow) CD34-CD38- population was present in all 6 cases both in the SP and NSP compartment. Since in 4/6 cases normal CD34+CD38- presumed stem cells were missing in the total CD34+CD38- compartments (including both SP and NSP, Figure 6), the CD34-CD38- compartment may represent an alternative source of HSC.

Table 5 Percentages of normal and malignant CD34-CD38- cells within SP and NSP populations of CD34-positive AML patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>%SP</th>
<th>% of SP</th>
<th>Surface marker</th>
<th>% of NSP</th>
<th>Surface marker</th>
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<td>35</td>
<td>N (N)</td>
<td>2.80</td>
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P: Positive for aberrant cell surface marker
(A): AML
N: Negative for aberrant cell surface marker
(N): Normal
0 and - : Not present

Table 6 Percentages of normal and malignant CD34-CD38+ cells within SP and NSP populations of CD34-positive AML patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>%SP</th>
<th>% of SP</th>
<th>Surface marker</th>
<th>% of NSP</th>
<th>Surface marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.60</td>
<td>8.5</td>
<td>N (N)</td>
<td>5</td>
<td>N (N)</td>
</tr>
<tr>
<td>2</td>
<td>3.630</td>
<td>30.8</td>
<td>N (N)</td>
<td>13.8</td>
<td>N (N)</td>
</tr>
<tr>
<td>3</td>
<td>0.500</td>
<td>2.7</td>
<td>N (N)</td>
<td>6.5</td>
<td>P (A)</td>
</tr>
<tr>
<td>4</td>
<td>0.030</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.700</td>
<td>0.90</td>
<td>N (N)</td>
<td>17.4</td>
<td>N (N)</td>
</tr>
<tr>
<td>6</td>
<td>1.500</td>
<td>2.3</td>
<td>N (N)</td>
<td>6.9</td>
<td>N (N)</td>
</tr>
</tbody>
</table>

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CD34-CD38+ and CD34+CD38+ Populations
Tables 6 and 7 shows that both SP and NSP contain mixtures of normal and AML cells with generally higher percentages of AML compared to normal.

Clonogenicity of SP and NSP LSC and HSC
In order to estimate the relative contribution of the SP and NSP fractions to stem cell activity, the clonogenic ability of presumed normal and AML SP and NSP cells was assessed. This was done in a prospective way: both aberrant cell surface marker-positive FSClow/SSClow and aberrant cell surface marker-negative FSClow/SSClow SP and NSP cells from patients 4 and 5 (CD34-negative) and patients 1, 3 and 6 (CD34-positive) were sorted and subsequently cultured in a suspension culture assay. After 5 weeks, all cells were harvested and were placed into methylcellulose for the detection of day 14 colonies. In order to prove that
aberrant cell surface marker-positive clonogenic cells were indeed malignant and aberrant cell surface marker-negative cells were normal, after 5 weeks of culturing. FISH analysis was performed on the colonies originating from aberrant cell surface marker-positive or aberrant cell surface marker-negative SP cells from two CD34-positive patients (patients 1 and 3 with t(8;21) and del(12), respectively). Indeed, the majority of the colonies originating from aberrant cell surface marker-positive SP cells were found to be malignant, whereas no clonogenic NSP cells could be assessed (Figure 7 and Table 1).

From Figure 7 it can also be deduced that both normal and AML SP compartments contain much higher number of colonies, normalized to number of input cells, as compared to NSP cells. SP cells are thus more potent in generating colonies originating from primitive cells, than NSP cells. Even when correcting for the low SP percentages (<1%, see Figures 4C and 6C), the total number of colonies originating from the SP compartment was still higher than from the NSP compartment (compare Figure 7A with Figure 7C for CD34-negative-AML and Figure 7B with Figure 7D for CD34-positive AML). In addition, the LSC in the SP compartment seems more potent than the HSC in the similar compartment.
With the results of the previous paragraphs it can now be stated that the malignant stem cells in CD34-negative AML most likely originate from a CD34+CD38+ cell population contained in the SP fraction, while the HSC may be derived from CD34+CD38− or CD34-CD38- population contained in the SP fraction. For CD34-positive AML cases the malignant stem cells mainly originate from the SP fraction, but, in contrast to CD34-negative AML, may be contained within all four CD34/CD38 defined subpopulations. With the consistent presence of normal CD34+CD38- and CD34-CD38- cells in all CD34-positive patients, CD34+CD38- SP and CD34-CD38- SP compartments likely contain the HSCs.

**Discussion**

In order to narrow down the putative stem cell compartment in AML, in the present paper we primarily aimed to assess the relationship between two possible, differently defined, stem cell compartments in AML. The first is immunophenotypically defined as CD34+CD38-, the second is functionally defined by increased efflux of Hoechst 33342 dye, the so called side population (SP). As both characteristics apply to normal hematopoietic stem cells as well, we used our previously described parameters that enable to discriminate between HSCs and LSCs both in SP defined and in CD34+/CD38- defined stem cell compartments (8,10,11, Terwijn M, Blood 114:165, 2009).

We found that in CD34-negative AML the LSC most likely originates from a CD34-CD38+ cell population contained in the SP fraction, while the HSC may be derived from CD34+CD38- or CD34-CD38- compartment in the SP fraction. For CD34-positive AML cases the malignant stem cells too originate from the SP fraction, but in contrast to CD34-negative AML, may be contained in all four CD34/CD38 defined subpopulations. For HSC again the CD34+CD38- and CD34-CD38- SP compartments are the most likely candidates.

This is in agreement with the recently published results of Taussig and colleagues who investigated the engraftment potential of NPM-1 mutated AML (13). Although the presence of NPM-1-mutations does not necessarily imply CD34-negativity, as is clear from the authors study population, it is nevertheless paralleled by decreased CD34 expression. According to our definitions (9), CD34-negative cases in general have <1% CD34. Since the group of Taussig provided CD34 percentages (in their Table 1), we thus were able to quite reliably identify in their AML population the truly CD34-negative cases (13). Two other important differences between our study and the study of Taussig are, firstly, that in the latter study normal and malignant cells were not prospectively defined (13). In engraftment studies therefore malignant or normal clones may be out-competed by other(s), resulting in under- or over-estimation of their stem cell character. Secondly, in that study the SP definition was not taken into account. The latter is important since we have shown that the SP population is extremely potent in producing both normal and leukemia colonies (Figure 7A).

In the CD34-negative type of AML, the CD34-negative NPM1-mutated CD34+38- cells were found to be normal as reflected by multi-lineage engraftment (13). Accordingly, we found only normal CD34+CD38- in the highly clonogenic SP compartment, as defined by the lack of aberrant cell surface markers and a FSClow/SSCw pattern. Although we detected CD34+CD38- leukemia cells, it was only in two patients and importantly, only in the less clonogenic NSP fraction. The high clonogenic ability for HSC, along with the relative lack of clonogenic ability for LSC, might explain a lack of leukemic engraftment for CD34+CD38- cells in the study of Taussig (13). Apart from the CD34+CD38- fraction, the CD34-CD38- fraction, both being present in SP and NSP fractions, are normal HSC candidates in CD34-negative AML. The finding that no colonies are formed from the NSP fraction (Figure 7A) and the fact that only CD34+CD38- cells show multi-lineage engraftment (13), together with our SP data, suggests that the most potent HSC reside in CD34+CD38- SP fraction, although a role for CD34-CD38- remains possible.

Leukemia engraftment in the Taussig study of these CD34-negative cases originated from the CD34-CD38- and especially from the CD34-CD38+ population (13). In agreement with the latter observation, we found that the CD34-CD38+ population, which made up the vast majority of both SP and NSP population, were almost all malignant (Figures 4A and 4B, and Table 3). Taking into account the high clonogenic potency of SP cells, these observations strongly suggests that the CD34-CD38+ primitive AML cells that show leukemia engraftment, originate from the SP cells. In addition, we mainly recovered only a low percentage of malignant CD34+38- cells from the NSP compartment, while in 4/5 cases no leukemia cells were present in the SP fraction. This correlates with the absence of engraftment of CD34+38- cells in the experiments of Taussig (13). There is one discrepancy: CD34-CD38- cells were able to engraft, while we found these cells present mainly in the NSP population, at a low frequency (Table 2). Maybe these cells account for the low residual AML stem cell activity found in the NSP fraction in the clonogenic assay performed for the two CD34-negative patients (Figure 7A).
The picture was completely different for **CD34-positive** type of AML: we found that both SP and NSP cells contained leukemia CD34+CD38- cells in all 6/6 cases, while in some cases also normal CD34+CD38- cells were present (Figure 6). In good agreement with this, in the experiments of Taussig, leukemia engraftment was prevalent, while in some cases normal engraftment occurred (13). The latter may occur with a more advantageous distribution of HSC versus LSC SP (e.g. in our patient 6, Figure 6 and Table 5), which is compatible with the observation of leukemia engraftment (13). One should keep in mind that the absence of either multi-lineage or leukemia engraftment in leukemia BM populations in which leukemic and normal cells have not been prospectively identified and separated, is always difficult to interpret: in the in vivo experiments normal and leukemia cells co-exist and engraftment either one population may out-compete the other.

The CD34-CD38+ population in the CD34-positive leukemias contained leukemia cells as well as normal cells in most cases in SP and NSP (Table 6), but similar to CD34-CD38- population in CD34-negative leukemia, again only leukemia engraftment was seen with no normal engraftment (13). Lastly, the CD34+CD38+ population contained mainly malignant cells in part of the cases in both SP and NSP, which is in agreement with the observation that these populations give rise to leukemia engraftment (Table 7).

The absence of normal engraftment in populations other than CD34+CD38-, led Taussig and colleagues to conclude that only the CD34+CD38- compartment provides normal stem cells. It can, however, not be excluded that other compartments contribute as well: in CD34-negative AML, the CD34+CD38- compartment is for a large part or even completely normal, allowing multi-lineage engraftment. However, the other CD34/CD38 defined populations in CD34-negative AML as well as in CD34-positive AML usually contain mixtures of normal and AML stem cell candidates. Given the fact that the latter constitute the majority, the interpretation of the in vivo experiments with no purified normal and AML cells being used, is quite difficult when it comes to the inherent potency of part of the CD34-CD38- cells to result in normal engraftment. Nevertheless, the fact that only the CD34+CD38- fraction and not the CD34-CD38- cells, is able to engraft in a multi-lineage fashion in CD34-positive AML (13), the latter despite the probably low frequencies of the normal CD34+CD38- cells herein (Figure 4), suggests that the most potent normal stem cells are present within the CD34+CD38- population. From our results, in combination with experiments performed by Taussig and colleagues (13) the hypothesis can be raised that CD34-negative AML, which may include a substantial percentage of NPM-1 mutated cases and also other cytogenetic and molecular aberrancies and normal cytogenetic cases, forms a separate class of AML cases with CD34-negative leukemia initiating cells. These patients have relatively good prognosis as we established in a series of 394 AML patients with 85 CD34-negative cases (unpublished). It is likely that this is caused at least in part by the fact that CD34-negative cells are therapy-sensitive and less leukaemogenic compared to CD34+CD38-LSCs in CD34-positive AML. This is supported by the following observations: a) CD34-negative blast cells very often have low levels of multidrug resistance and are thereby more sensitive to MDR drugs than CD34-positive blast cells (14,15); b) patients with poor prognosis usually are CD34-positive and it is this class of AML that results in leukemic engraftment in NOD/SCID mouse that are not completely immune-compromised (16). We found that leukemic cells within the SP compartment have a higher clonogenic potential as compared to similarly phenotypically defined cells obtained from the NSP compartment. Combination of immunophenotypical and functional characterization of stem cells may therefore be useful in narrowing down the stem cell compartment and to define the biologically most important sub-fraction of LSCs. However, there has been discussion on the value of the SP definition of stem cells. Firstly, SP cells may define therapy resistant cells which not necessarily coincide with stem cell character (17,18). However, the mere finding that SP cells form primitive colonies to a much larger extent than NSP cells, suggests that both properties are true. Secondly, the use of Hoechst may result in toxicity for stem cells present in the NSP population, thereby masking the leukemogenic and clonogenic ability of these cells (19). We and others, however, have found no evidence for such in clonogenic assays (5,6,8).

In recent models with extreme immune suppression, not only the levels of engraftment are higher but also the percentage of cases in which engraftment can occur, is very high (22). This strongly indicates that the less leukemogenic stem cells did not give rise to engraftment in earlier NOD/SCID mouse models, but now, in severely immune-compromised mice, get a chance to engraft. It would be highly informative to reproduce the mouse experiments which showed engraftment of CD34+CD38-, CD34+CD38+ and CD34-CD38+ cells (17,21), but now firstly, using our definitions of CD34-positivity, secondly, prospectively separate normal from leukemia stem cells and thirdly, taking the SP compartment into account. Lastly, in mixing experiments it can subsequently be established which clone is the most leukemogenic and therapy resistant. The co-existence of different leukemia initiating compartments, with a potency to survive serial transplantations, fits quite well in the observations of Dick’s group that engraftment...
in AML reveals clonal diversity with some clones persisting and others dying out (12). Our observations imply that the choice of in vivo models is crucial and should consider the existence of two clearly different subgroups in AML based on CD34 expression patterns. This holds true also for testing new LSC directed drugs: when aiming at eradication of CD34-negative AML one might best use the most immune-compromised mice and focus on CD34-CD38+ cells, but when aiming at eradication of CD34-positive AML, the less restricted model may be most appropriate with a focus on CD34+CD38− cells. It would be worth considering whether further sub-division of CD34-positive AML (positivity roughly ranging from 1% to 100%) as to leukemogenic ability in different mouse models, is worthwhile.

In the present paper we have thus shown that using flowcytometry to prospectively separate putative AML stem cells from putative normal stem cells, together with the separate characterization of SP versus NSP cells has allowed us to define putative LSC and HSC in two classes of AML patients that are defined by using a new definition of CD34-positive and CD34-negative AML.

These subpopulations defined by aberrant markers, Hoechst and CD34/CD38 should be studied in dependence of their potency to give leukemia engraftment in mice that differ in the level to which animal are immune-compromised. If this is a factor of importance in patients, one might ultimately relate a particular LSC in mice that differ in the level to which animal are immune-compromised. If this is a factor of importance in patients, one might ultimately relate a particular LSC compartment to patient’s immune status. Lastly, using this stem concept, the possibility should be explored to purify LSC and HSC compartments and define other discrimination properties that may well result in discovery of new and highly worthwhile.

Acknowledgments

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Literature cited