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Research paper

Relationship between CD34/CD38 and side population (SP) defined leukemia stem cell compartments in acute myeloid leukemia

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ABSTRACT

Leukemic stem cells (LSCs), defined by CD34/CD38 expression, are believed to be essential for leukemia initiation and therapy resistance in acute myeloid leukemia. In addition, the side population (SP), characterized by high Hoechst 33342 efflux, reflecting therapy resistance, has leukemia initiating ability. The purpose of this study is, in both CD34-positive and CD34-negative AML, to integrate both types of LSC compartment into a new more restricted definition. Different CD34/CD38/SP defined putative LSC and normal hematopoietic compartments, with neoplastic or normal nature, respectively, were thus identified after cell sorting, and confirmed by FISH/PCR. Stem cell activity was assessed in the long-term liquid culture stem cell assay. SP fractions harbored the strongest functional stem cell activity in both normal and neoplastic cells in both CD34-positive and CD34-negative AML. Overall, inclusion of SP fraction decreased the size of the putative CD34/CD38 defined LSC compartment by a factor > 500. For example, for the important CD34 + CD38- LSC compartment, the median SP/CD34 + CD38- frequency was 5.1 per million WBC (CD34-positive AML), and median SP/CD34-CD38 + frequency (CD34-negative AML) was 1796 per million WBC. Improved detection of LSC may enable identification of therapy resistant clones, and thereby identification of novel LSC specific, HSC sparing, therapies.

1. Introduction

Acute myeloid leukemias are thought to originate from leukemia initiating cells which have stem cell properties and often are referred to as leukemic stem cells (LSC). LSCs may be present in both CD34 + CD38- [1] and CD34 + CD38 + or CD34-negative cellular compartments [2–4]. CD34 + CD38- compartment is probably the most important compartment in CD34 positive AML since it is this fraction that contains the in vitro and in vivo most therapy resistant [5,6] and leukemogenic [7,8] cells. Above that, CD34 + CD38- frequencies have prognostic impact both at diagnosis and at follow up [9,10] In addition to CD34/CD38 defined LSC, leukemia initiating cells have been shown in NOD/SCID mice to be present in the so-called side population (SP) cells, i.e. a subpopulation of cells with high multi-drug resistant (MDR) efflux pump activity [11], which thereby provides a direct link with therapy resistance [11,12]. SP may contain each of the CD34/CD38 defined compartments [12] and it is therefore of great interest if the combination of immunophenotypical and functional characteristics would allow to narrow down the size of the LSC compartment, thereby identifying the most potent and most therapy resistant LSCs in both CD34-positive and CD34-negative AML. In earlier work we have shown a low-frequency (median 0.0016%) sub-fraction of the SP compartment which has aberrant (leukemia associated) marker expression and is therefore a likely candidate population to be enriched for LSCs [13]. However, in that study aberrant cells have not been characterized in terms of CD34 and CD38 expression. We have provided methods to prospectively identify both the neoplastic and normal cells for all CD34/CD38- defined compartments [9,13–17], using aberrant marker expression [9,13–17] and aberrant light scatter aberrancies [9].

Commonly, the percentage of CD34 + cells is used to determine if an AML patient is CD34-positve or CD34-negative. We have shown that truly CD34-negative AML, i.e. AML with no neoplastic CD34 + cells present, can be ascertained using immunophenotypical, functional and molecular characteristics [18,19]. CD34-negative leukemia therefore,

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by definition, contain only CD34 negative LSCs, while in CD34 positive AML all compartments may contain LSCs. The role of SP herein is unknown. As CD34 expression is thought to go along with apoptosis and multi-drug resistance [20,21], in the present study we established the relationship of the frequency of the total CD34 fraction with the distribution over CD34/CD38 defined putative LSC fractions. For the earlier-mentioned reasons, in CD34 positive AML, for the SP studies, we specifically focused on the CD34 + CD38- compartment but, for completeness, the other three CD34/CD38 defined compartments are shortly discussed as well. In CD34 negative AML, the importance of the latter compartments are emphasized. In addition, also the CD34 + CD38- normal hematopoietic (HSC) fraction was similarly studied as well. Above that, the behavior of these fractions in functional studies was explored. The impact of these findings on therapy resistance and prognosis will be discussed.

2. Materials and methods

2.1. Patients and leukemic bone marrow cells

Patients in the HOVON (Hemato-Oncology foundation for adults in Netherlands) studies were treated with cytarabin/anthracycline regimens. These studies were centrally reviewed and approved by Medical Ethical Review Committee (METC), Erasmus Medical Center, Rotterdam for all participating centers for the total study (clinical plus side studies). Protocol numbers and approval numbers are: H42a (2000-220); H81 (2006-215); H92 (2008-216); H102 (2009-293). In addition, the VU Amsterdam METC review board approved these centrally approved studies for local feasibility with HOVON/METC numbers: H42a (2001/50); H81 (2006/215; same as central review since VUmc was central center); H92 (2008/292); H102 (2010/56). In all studies, patients had to provide their written informed consent before entrance into the study. Patient details are listed in Supplementary Table 1; 13/17 patients were < 60 years, and 4/17 were > 70 years. Cell preparation was performed according to standard procedures for Ficoll gradient purification, lysis and freeze/thawing. After freeze-thawing both immunophenotypic and functional characteristics are recovered [22] Details on this are in Supplementary Text ("Patients and leukemic bone marrow cells").

2.2. Hoechst staining, immunophenotyping and cell sorting

Primary AML cells (1×10^6 cells per ml) were stained with 5g/ml Hoechst 33,342 dye (Molecular Probes, Eugene, OR) in PBS and incubated at 37 °C for 2 h, essentially according to published data [11,13]. After Hoechst staining, cells were stained with antibodies according to standard procedures (details in Supplementary Material and Methods). Antibodies were against CD34, CD45, CD38, CD19, CD33, CD56, CD123, CLEC12A (CLL-1). FACS analysis is shown in Suppl. Fig. 1. Details, including cell sorting procedures, are outlined in Supplementary Material and Methods. In addition, the comparison of marker expression between populations with different degrees of maturation (e.g blast cells versus CD34 + CD38- cells versus SP cells) has been outlined in paragraph "Rationale for choice of markers" in the Supplementary Text.

2.3. The use of scatter properties to define LSC and HSC

Since aberrant marker expression does not always lead to clear conclusions as to the neoplastic/normal population character, for the analyses of the CD34/CD38 defined compartments, forward scatter (FSC) and sideward scatter (SSC) values are included, since these have already been shown to help to define neoplastic and normal CD34 + CD38- cells present in the same bone marrow [9]. Details are in the text and in Supplementary Text and Supplementary Figures and Tables.

2.4. 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 of CD34-positive cells that, based on functional assays, was normal, and PCR and FISH analyses [18,19]. Although these CD34-negative patients have low percentages of CD34-positive cells (usually < 1%) not all patients with a low percentage of CD34-positive cells are CD34-negative, as true negativity is defined by the complete absence of neoplastic CD34+ cells. In the present study we defined normal vs. neoplastic cells by using immunophenotypic and scatter aberrancies described above, in part of the cases supplemented by PCR analyses (for NPM1 mutations and FLT3-ITDs). We selected 14 CD34-positive defined patients with different CD34 expression and 3 CD34-negative patients (which about reflects their incidence in our large AML patient cohorts).

2.5. Suspension culture of AML SP and non-SP cells

During liquid culture [2], putative CFU capacity of progenitors diminishes and primitive (stem) cells survive and progress to progenitors within at least 5 weeks of culture. The CFU potency can then be assessed in a subsequent two weeks CFU assay. Outcome of liquid culture stem cell assay parallels in vivo engraftment data [2]. The suspension culture now used for purified SP and Non-SP (NSP) cells was performed essentially as previously described earlier [9]. Details are Supplementary Text.

2.6. CFU assay

Assays for leukemia CFUs were performed by plating all the cells, harvested from the suspension culture, in methylcellulose medium (H4434; StemCell Technologies, Vancouver, BC, Canada). 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 and NSP cells was calculated as previously described for CD34 + CD38- [13]. 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. In addition, the total contribution of SP and NSP to the clonogenic output of blast cells was calculated from the just described clonogenic output of purified SP and NSP cells, and corrected for the percentages of SP and NSP cells within these input blasts.

2.7. Fluorescence in situ hybridization (FISH) analysis of FACS-sorted SP and NSP cells

For interphase FISH, the FACS-sorted SP and NSP cells were prepared as described [18] using the probes LSI AML1/ETO dual color for t [8,21] and the LSI TEL/AML1 ES dual color for del 12(p13). Details are in Supplementary Material and Methods.

2.8. PCR analysis of FACS-sorted SP and NSP cells

Genomic DNA was isolated from sorted cell fractions with Nucleospin Tissue XS (Bioke, Leiden, The Netherlands). Fms-like tyrosine kinase 3 internal tandem duplications (FLT3/ITD) were analyzed by PCR amplification, as we described previously [23]. For details see Supplementary Material and Methods.

3. Results

3.1. Stem cell function of SP and NSP cells

SP and NSP neoplastic and normal cell fractions, purified by marker expression and, where appropriate, light scatter properties, were

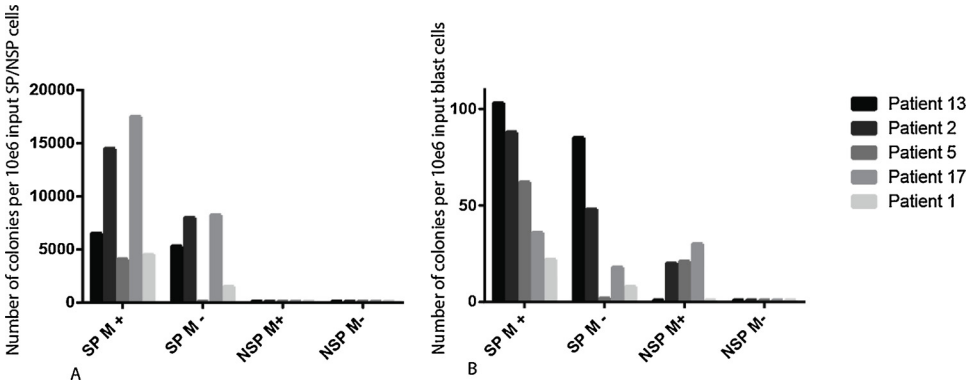

Fig. 1. Long-term clonogenic capacity of sorted SP and NSP subpopulations of four CD34-positive patients and one CD34-negative AML patient. Cells were identified and sorted as outlined earlier. Both aberrant surface marker-positive (M+) and aberrant surface marker-negative (M-) SP and NSP cells from patient 1, 2, 5 and 13 (CD34-positive) and patient 17 (CD34-negative) were sorted and subsequently cultured in a suspension culture assay (details in Material and Methods). After 5 weeks, all cells were harvested and were placed into methylcellulose for the detection of day 14 colonies (details in Materials and Methods). Numbers of colonies are depicted on the y-axis. **1A**: number normalized to 1×10^6 input SP cells and 1×10^6 input NSP cells, respectively. **1B**: absolute number of colonies in 10^6 input blast cells calculated from A., using the frequencies of SP and NSP cells in the input total blast population.

subjected to liquid culture assay with subsequent clonogenic assay, to assess the initial presence of cells with stem cell properties (see Materials and Methods). Cell fractions were sorted from four CD34+ and one CD34-negative patient. When normalized to numbers of input SP and NSP cells, SP cells in 4 CD34 positive cases positive (#1, #2, #5 and #13) and 1 CD34 negative case (#17) were much more potent than NSP cells in generating colonies that originate from the most primitive cells in the AML. This was true for both the neoplastic SP fraction (SP marker positive, SP M+), and the normal SP fraction (SP M-) (Fig. 1A). Although the frequencies of SP cells in the input blast population are much lower (< 1% of total number of blast cells, see patient 1, 2, 5, 13) than in NSP fraction, even the absolute numbers of colonies originating from the input total blast compartment were still higher for SP than NSP (Fig. 1B). In two earlier described CD34-positive patients [13], FISH analysis [patient #1 had del [12]; patient #2 had t(8;21)] showed that, after liquid culture and subsequent clonogenic assay, colonies originating from surface sorted marker-positive SP cells were in vast majority neoplastic in contrast to marker negative cells (Suppl. Table 2).

3.2. Interrelationship between SP and CD34/CD38 defined compartments

Next we investigated distribution of all four CD34/CD38 defined neoplastic and normal sub-compartments in SP and NSP. The results for the CD34 + CD38- compartment are in the main text; the results for the other three compartments are briefly described in the main text and in more detail in the Supplementary text and in the Supplementary Figures /Tables. The gating strategy identifying SP and NSP singlet $CD45^{dim}$ blast cells (Suppl. Fig. 1) preceded the identification of all subpopulations studied. Results of the CD34-positive and CD34-negative AML cases are described separately below. We present the data/analyses in dependence of CD34 percentage since, as outlined in the Introduction section, this may well be relevant for therapy resistance. In CD34 + CD38- cells there is high specificity for aberrant immunophenotypes [7,9]. Throughout the paper, in the tables on the CD34 + CD38- compartment, the terminology "A" refers to a compartment that contains only AML cells, while the term "N" means that the cells are all normal (in all CD34/CD38 compartments). One should realize that immunophenotypically defined aberrancies are not completely specific for AML cells in the CD34 + CD38+, CD34-CD38+ and CD34-CD38- compartments, and therefore in these cases "A" refers to the presence of AML cells, but it cannot be excluded that part is normal. Similar to CD34 + CD38- compartment, "N" refers to presence of normal cells only.

3.3. CD34-positive AML

3.3.1. FSC and SSC to help to define LSC and HSC

To define the nature of a sub-population, primarily marker expression was used. For CD34 + CD38- population, light scatter properties are also helpful, with LSC often having higher FSC/SSC than HSC [9] (Fig. 2; see also Suppl. Material and Methods).

In the current study again we found for CD34 + CD38- cells, that FSC/SSC in the neoplastic compartment was lower compared to HSC; this was found in both SP and NSP compartments (Fig. 2). Remarkably, in SP compartment in both the neoplastic CD34 + CD38- population, as well as in CD34 + CD38- HSC, FSC/SSC was lower compared to their NSP counterparts (Fig. 2). In the other 3 CD34/CD38 compartments similar observations were made (Suppl. Fig. 2), thus providing another parameter (FSC/SSC) to discriminate normal and neoplastic sub-populations.

These data show that FSC/SSC are helpful in fine-tuning neoplastic and normal character that was primarily based on marker expression. Also, it suggests that neoplastic and normal SP cells may be less differentiated compared to their corresponding NSP cells, while neoplastic SP and NSP cells may be more differentiated compared to their normal counterparts.

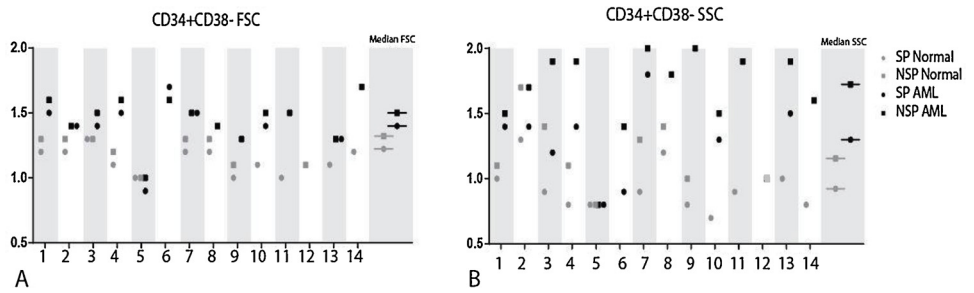

Fig. 2. FSC and SSC in neoplastic and in normal and CD34 + CD38- defined populations in SP and NSP fraction in CD34-positive AML. Neoplastic and normal cells were primarily identified using aberrant marker expression [14–16]. All data concern CD34 + CD38- cells. A. FSC; B. SSC. Black symbols are for neoplastic CD34 + CD38- cells, grey symbols are for normal CD34 + CD38- cells. Circles are for SP and rectangles for NSP. Note that normal SP and NSP cells (grey symbols) overall have lower FSC (3 A) and SSC (3B) compared to their neoplastic SP and NSP equivalents (black symbols). Overall, SP cells (grey and black circles) have lower FSC and SSC than corresponding NSP cells (gray and black rectangles). Data for the other CD34/CD38 defined populations are in Suppl. Fig. 2.

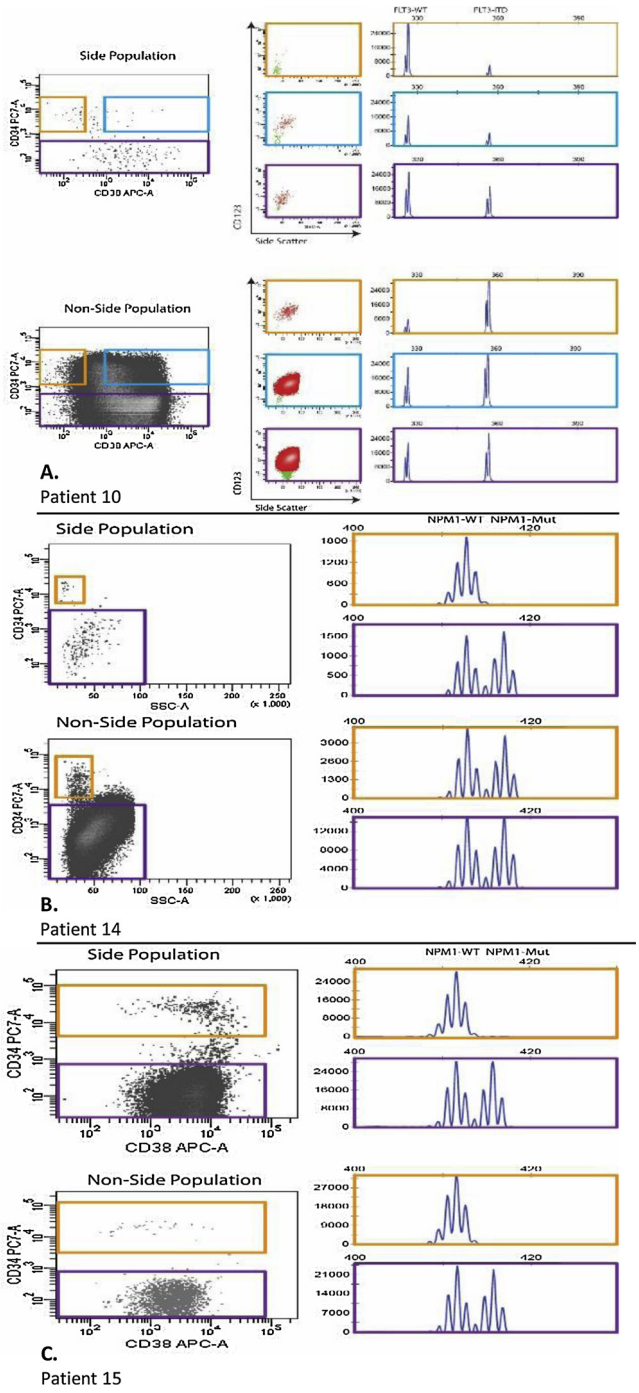

Fig. 3. Immunophenotypic and/or molecular characterization of CD34/CD38 defined SP and NSP subpopulations in CD34-positive (A, B) and CD34-negative (C) patients.

SP and NSP cells were gated as described in Suppl. Fig. 1**A.** CD34-positive AML (patient 10). Within SP (upper part), both CD34 + CD38- and CD34 + CD38+ and CD34-CD38- were, at least partly, neoplastic as proven using PCR for FLT3-ITD on sorted sub-fractions (right panels). Colors of the immunophenotype part correspond to colors in the PCR part. The middle panels confirm that also immunophenotypically the fractions contain both neoplastic (CD123 positive; red dots) and normal (CD123 negative; green dots) cells. A similar conclusion can be drawn for NSP (second set of pictures), although the contribution of neoplastic cells to the CD34 + CD38- NSP compartment is much larger than for SP, as reflected by immunophenotype (middle panel) and PCR (right panel).

B. CD34-positive AML (patient 14). Within SP (upper panels) the very small sorted CD34+ subpopulation was normal (right, first panel: NPM1 negative), while the CD34- SP population was neoplastic (right, second panel: NPM1 positive). Colors in left (immunophenotype) and right (PCR) panel correspond. In contrast, in the sorted NSP populations (lower panels) both CD34+ and CD34- cells contained neoplastic cells (third and fourth panels on the right). In the absence of an immunophenotypic marker in this case the "immunophenotypic" evidence for neoplastic or normal character was based on lower FSC/SSC of the normal compartment. This is illustrated for SSC in the CD34/SSC in the left upper SP panel.

C. CD34-negative AML (patient 15). In contrast to CD34-positive AML, CD34+ populations were normal in both SP (right upper panel) and NSP (right third panel), whereas CD34- cells were neoplastic in both SP (right second panel) and NSP compartments (right fourth panel). In the absence of an immunophenotypic marker in this case the "immunophenotypic" evidence for neoplastic or normal character was based on FSC/SSC of the normal compartment (described later for the 4 CD34 negative cases in Suppl. Fig. 5).

3.3.2. The nature of subpopulations determined by PCR and immunophenotyping

In previous studies we have extensively shown the relation between aberrancies in marker expression, scatter properties, ALDH activity and CD34/CD45 expression on the one side and LSC versus HSC molecular character on the other [9,13,24,25]

In the present paper, in five cases we performed molecular confirmation for the immunophenotypic sub-population make up. An example of molecular make up of SP and NSP fractions, sorted based on marker expression, is shown in Fig. 3A. In the absence of aberrant marker expression in this patient we relied on scatter properties (FSC/SSC), as described for Fig. 2 above, and level of CD34 and or CD45 expression [9]. Such an example in another CD34-positive patient is shown in Fig. 3B. In 49 out of the 55 evaluable sub-populations present in these 5 CD34 positive patients (defined by CD34/CD38-, SP/NSP, neoplastic/normal), concordance was found with the molecular assessments (reported in detail below). We then studied nine other CD34-positive cases, where, in the absence of molecular aberrancies, we relied on immunophenotyping (marker expression and scatter characteristics) to describe the nature of SP and NSP compartments. Details of neoplastic/normal character will be described below for all CD34/CD38/SP defined compartments.

3.3.2.1. CD34+CD38-. Table 1 summarizes the nature of CD34 + CD38- compartment in the SP and NSP fraction, as a function of CD34 percentage, and as defined by immunophenotyping and in part of the cases with PCR too. Fig. 4 shows that the contribution of neoplastic CD34 + CD38- cells (black bars) to both the total SP compartment (Fig. 4A) and the NSP compartment (Fig. 4B) overall decreased with decreasing CD34+ percentage. Concomitantly,

Table 1
Summary of nature of SP and NSP CD34 + CD38- cells in CD34-positive AML.

Pt	34%	SP		NSP		Conclusion	
		Mol.	Surface	Mol.	Surface	SP	NSP
1	98	–	A	–	A	A	A
2	93	–	A	–	A	A	A
3	89	–	A	–	A	A	A
4	60	A	A	A	A	A	A
5	60	–	A	–	A	A	A
6	55	–	A	–	A	A	A
7	37	N	A	A	A	N	A
8	33	–	N	–	A	N	A
9	10	–	N	–	A	N	A
10	10	A	A	A	A	A	A
11	1.8	–	N	–	A	N	A
12	1.1	–	NP	–	N	NP	N
13	0.7	A	A	A	A	A	A
14	0.1	N	N	A	A	N	A

N, all cells have normal molecular phenotype (column "Mol.") or immunophenotype (column "Surface"), or both. A, at least part of the cells have aberrant molecular or immunophenotypic aberrancies. NP, population not present.

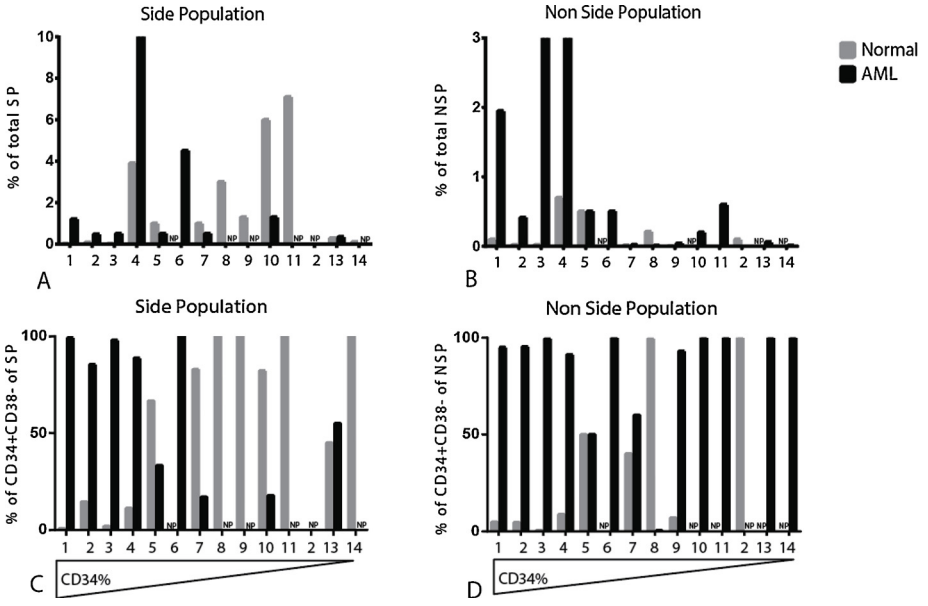

Fig. 4. Frequencies of neoplastic and normal CD34 + CD38- populations in SP and NSP in CD34-positive AML.
A,B. As percentage of total SP (A) and total NSP (B) compartment. The cases are arranged according to decreasing total CD34+ percentage of the AML. C,D. As percentage of the CD34 + CD38- compartment within the SP (C) and NSP (D) compartment; the neoplastic and normal fractions together thus make up 100% of the total CD34 + CD38- SP or the total CD34 + CD38+ NSP compartment. The cases are arranged according to decreasing total CD34+ percentage of the AML.

however, the normal CD34 + CD38- cells (hematopoietic stem cells, HSCs, grey bars), as percentage of total SP, increased (Fig. 4A). The latter was not seen in the NSP fraction (Fig. 4B). The enrichment of HSCs in SP compartment, as seen with decreasing percentage of CD34, is best illustrated in Fig. 4C, where HSC is shown as percentage of the CD34 + CD38- part of the SP compartment. Such was not seen in the NSP compartment (Fig. 4D).

3.3.2.2. CD34 + CD38+, CD34-CD38+ and CD34-CD38-. For the CD34 + CD38+ compartment, similar to CD34 + CD38- compartment, the contribution of neoplastic cells decreased with decreasing CD34 percentage, however with no enrichment of normal cells (see Suppl. Text "Detailed analysis of CD34 + CD38+, CD34-CD38+ and CD34-CD38- compartments in CD34 positive AML" and corresponding Suppl. Tables and Suppl. Figures referred to in that text).

In the CD34-CD38+ compartment, as intuitively expected, the contribution of neoplastic cells increased with decreasing CD34 percentage. No clear trends were seen for the CD34-CD38- compartment (see the same paragraph in Suppl. Text).

3.4. CD34-negative AML

Similar to CD34 positive AML FSC/SSC helps to discriminate neoplastic and normal cells SP and NSP cells (see Supplementary paragraph "CD34 negative AML" and corresponding Suppl. Fig. 5 on this).

By immunophenotyping, we studied 3 CD34-negative cases, where in two cases (example in Fig. 3C) molecular aberrancies could be studied too. The normal character of all CD34+ cells in CD34 negative AML, as defined by immunophenotyping, was molecularly confirmed for 7 additional cases (not shown here; (19)). In agreement with the definition of CD34 negativity, both the CD34 + CD38- compartment (Table 2, Suppl. Fig. 6A and B) and the CD34 + CD38+ compartment (Suppl. Table 4, Suppl. Fig. 6 C,D)was normal in SP and NSP. CD34-CD38+ and CD34-CD38- cells are present in both SP and NSP in all 3 cases (Suppl. Table 4 and Suppl. Fig. 6 E,F and Fig. 6 G,H), where in the CD34-CD38+ compartment the SP and NSP compartments contain almost exclusively neoplastic cells (Suppl. Fig. 6 E,F).

Table 2
Summary of nature of SP and NSP CD34 + CD38- cells in CD34-negative AML.

Pt	34%	SP		NSP		Conclusion	
		Mol.	Surface	Mol.	Surface	SP	NSP
15	0.1	N	N	N	N	N	N
16	0.1	N	N	N	N	N	N
17	0.2	NP	NP	–	N	NP	N

N, all cells have normal molecular phenotype (column "Mol.") or immunophenotype. (column "Surface"), or both; NP, population not present.

3.5. Frequencies of putative the most primitive neoplastic and normal populations in CD34 positive and CD34 negative AML

The SP phenotype, in cooperation with the CD34/CD38 defined immunophenotypes, may prospectively identify a sub-compartment enriched for leukemia initiating, therapy resistant, cells. In CD34 positive AML, it is the CD34 + CD38- population that shows the most pronounced differences between neoplastic and normal cells in distribution over SP and NSP populations.

The frequency of neoplastic SP fraction in CD34-positive AML was median 5.1 in 10^6 (range 0–1,935 in 10^6) blast cells. In CD34 + CD38- NSP this was much higher (median 3028 in 10^6 blast cells) The introduction of SP thus narrows down the neoplastic CD34 + CD38- compartment with a median factor 595 compared to the whole neoplastic CD34 + CD38- population (Suppl. Table 5, legends). For the CD34 + CD38- HSC the median frequency was 60 in 10^6 cells for SP and 199 in 10^6 cells for NSP, with a median enrichment factor in SP of 4.3 (Suppl. Table 5, legends).

In CD34-negative AML, the only consistently present neoplastic population had the CD34-CD38+ immunophenotype, the compartment that likely contains the (CD34 negative) LSC. The frequency of neoplastic putative LSC containing CD34-CD38+ SP fraction was median 1796 in 10^6 cells. For NSP this was median 976,023 in 10^6 cells. This indicates that incorporation of the SP fraction narrows the CD34-CD38+ LSC compartment by a factor of 544 (Suppl. Table 6, upper part), while the HSC compartment was reduced by a factor of only 6.6 (Suppl. Table 6, lower part).

4. Discussion

For AML, different types of stem cell compartments have been described: one is immunophenotypically defined, and was originally restricted to the CD34 + CD38- type, but later, using more immune-compromised mice models, extended to CD34 + CD38+ and CD34 negative cases [3,4,8]. Another important stem cell compartment is

functionally defined by increased efflux of Hoechst 33,342 dye, the so called side population (SP). The present paper characterizes the immunophenotypic and functional compartments and defines the size of the cellular compartments that likely contain the leukemia initiating and therapy resistant cell, also in dependence of CD34 status of the AML.

A common characteristic for the stem cell compartments currently described in literature, is that the size of the compartments by far exceeds the frequency of true leukemia initiating cells, as these are defined e.g. by limiting dilution in vitro and in vivo, and which are in the order of 1:10,000 to 1:1.000,000 [6] while CD34 + CD38- and SP defined frequencies as in our studies are in the order of 1:10,000 up to 1:10. Narrowing down the putative stem cell compartment using easily assessable parameters would allow more accurate estimations of frequencies relevant to predict clinical outcome. Moreover, it would also guarantee characterization of more relevant parts of the total leukemia stem cell containing compartment, performed with the intention to find new therapeutic targets. We used our previously described parameters that enable to discriminate between HSCs and primitive neoplastic cells [9,13–15]. In the present paper, the introduction of SP was found to narrow down the sizes of relevant compartments. In line with findings by others in CD34 + CD38- cells [6,8], our results suggest that in CD34-positive AML, the potent leukemia initiating cells likely is present in the CD34 + CD38- SP compartment, leading to a new calculation of the primitive neoplastic cell frequency, ie median 5.1×10^{-6}. In CD34-negative AML, it is the CD34-CD38+ that likely contains the leukemia initiating cells [4], which is not very helpful since these make up the vast majority (> 99%) of the blast cells. We now suggest that it is likely the > 500-fold less frequent SP part of CD34-CD38+ compartment which contains the most potent leukemia initiating cells. In line with these hypotheses, for both CD34 positive and CD34 negative AML, these functionally (SP) defined compartments have over 500 times higher stem cell activity in liquid culture assay compared to the NSP compartment both in CD34-positive and in a CD34-negative AML. It should be stressed here that the final proof for these hypotheses should come from experiments in which leukemia/normal engraftment and therapy resistance is shown in the SP/CD34/CD38 defined sub-populations. Because of the very low frequencies of these sub-populations we were not already able to perform successful engraftment experiments (results not shown).

CD34 percentage has been reported as a strong prognostic parameter, probably due to its relation with apoptosis and multidrug resistance [20,21,26,27]. A striking observation was that, with decreased CD34 percentage, the contribution of neoplastic CD34 + CD38- cells was overruled by the normal CD34 + CD38- HSC compartment. CD34 negative AML are at the very end of the spectrum by harboring only normal CD34 + CD38- and CD34 + CD38+ cells, with CD34-CD38+ as the most likely candidate to contain the leukemia initiating cells.

Data on leukemia engraftment of CD34-positive AML described by Taussig et al, are in concordance with our results [4]. In some cases normal engraftment was seen, which may occur if the frequency of HSC SP increases at the cost of LSC SP frequency, as seen by us at the lower percentages of CD34. In addition, these authors found an absence of normal engraftment in populations other than CD34 + CD38- and concluded that only the CD34 + CD38- compartment provides normal stem cells. More recent findings by the same group suggest that there may be CD34-negative HSC as well [28]. Nevertheless, the fact that only the CD34 + CD38- fraction and not the CD34-CD38-, is able to engraft in a multi-lineage fashion in CD34-negative AML [4], suggests that the most potent normal stem cells are present within the CD34 + CD38- SP population despite the probably low frequencies (see Suppl. Table 5).

According to our definitions [18,19], CD34-negative cases in general have < 1% CD34 expression. CD34-negative patients have relatively good prognosis as we established in a series of 706 AML patients with 158 CD34-negative cases [19]. This good prognostic impact is likely caused, at least in part, by the fact that, compared to CD34 + CD38- LSC in CD34 positive AML, CD34-negative LSCs are not only more therapy-sensitive, but also less immunogenic [6], and less leukemogenic [5] in the bone marrow niche. The possibility that the CD34 negative character of AML at diagnosis changes at follow up resulting in CD34 positive AML, possibly explaining subsequent increases in leukemogenicity and therapy resistance, is ruled out by the observation that truly CD34 negative cases at diagnosis (usually with CD34 percentages of < 1%, all representing normal CD34 positive cells) remain CD34 negative at relapse (comparison of diagnosis and relapse in 44 CD34 negative cases [29]. In contrast, increases of CD34 expression in CD34 positive AML are commonly found. Our results in CD34-negative AML are also in agreement with Taussig and colleagues who investigated the engraftment potential of NPM-1 mutated AML [4], although the presence of NPM-1 mutations does not necessarily imply CD34-negativity [19]. Two important differences between our study and the study of Taussig are that they did not prospectively define normal and neoplastic cells [4]. 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. In addition, the SP compartment was not taken into account. In addition, in a recent update of our database of a finished clinical study, the number of CD34 negative cases using our own definition [19] was 9% on a total of 900 cases (n = 81). Of these 79.5% was NPM1 mutated (62/78 evaluable cases), 0% MLL rearranged (0/70 t [8,21] and 0/71 in.(16)) (no PML-RAR included in the study). 18.8% (13/69 evaluable patients) had FLT3 ITD, and these FLT3ITD patients had all low allelic ration (< 0.6) and were all also NPM1 mutated. Clearly and in agreement with a thorough study of Gerber and colleagues [30], most of the CD34 negative cases are thus associated with NPM1 mutations. We also show that the functional/immunophenotypical model in the present paper covers most cytogenetic sub-types in the whole (CD34+ and CD34-) AML population.

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 [31,32]. However, the mere finding that SP cells contain leukemia initiating cells [13,33] and in our present study form primitive colonies to a much larger extent than NSP cells, are in favor of the idea that both therapy resistance and leukemia initiating ability go alongside in SP cells. The prognostic impact of SP frequency in AML is still largely unknown. Recently, response to induction therapy was reported to be predicted by the frequency of CD34 + CD38 low/neg blast cells but not by SP frequency [31]. However, this observation may be completely obscured by the highly heterogeneous character of the SP population. Firstly, only a minority of cells having an immature immunophenotype ([13]; this paper), which had very high stem cell activity compare to the more mature immunophenotype present in SP [13]. Secondly, marker expression and/or scatter properties are necessary to discriminate LSC from HSC, which are highly variable within the primitive population ([13] and this paper).

In conclusion, our newly defined stem cell concept including both CD34/CD38 immunophenotypes and the functional SP compartment, will enable to better discriminate putative LSC and HSC compartments which will facilitate the discovery of new and highly AML- stem cell specific targets. In relation to this, in the molecularly and immunopenotypically very heterogeneous diagnosis AML [34], such approach may also lead to prospective identification of sub-populations containing particular clones that survive therapy and become abundant at relapse. Such we have previously shown for FLT3 mutations present at low frequencies in diagnosis CD34 + CD38- neoplastic sub-population [23].

Further studies are warranted to determine the potency of the subpopulations (defined by aberrant markers, light scatter characteristics, Hoechst and CD34/CD38) to give leukemia engraftment in mouse models, and, moreover, to study therapy resistance and outgrowth to

relapse in relevant model systems and to compare with the fate of these populations in the patient.

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