Identification and characterization of acute myeloid leukemia stem cells

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Identification of a small subpopulation of candidate leukemia initiating cells in the side population (SP) of patients with acute myeloid leukemia

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Abstract

In acute myeloid leukemia (AML), apart from the $CD34^{+}CD38^{-}$ compartment, the side population (SP) compartment contains leukemic stem cells (LSC). We have previously shown that $CD34^{+}CD38^{-}$ LSC can be identified using stem cell associated cell surface markers including CLL-1 and lineage markers such as CD7, CD19 and CD56. A similar study was performed for AML SP in order to further characterize the SP cells with the aim of narrowing down the putatively very low stem cell fraction. FACS analysis of 48 bone marrow and peripheral blood samples at diagnosis showed SP cells in 41 of 48 cases which were all partly or completely positive for the markers, including CD123. SP cells in normal bone marrow (NBM) were completely negative for markers, except CD123. Further analysis revealed that the SP fraction contains different subpopulations: 1. Three small lymphoid subpopulations (with T-, B- or NK- cell markers); 2. A differentiated myeloid population with high forward and sideward scatter (FSC^{high}/SSC^{high}), high CD38 expression and usually with aberrant marker expression; 3. A more primitive FSC^{low}/SSC^{low}, $CD38^{low}$, marker negative myeloid fraction; 4. A more primitive FSC^{low}/SSC^{low}, $CD38^{low}$, marker positive myeloid fraction. NBM contained the first three populations, although the aberrant markers were absent in the population 2. Suspension culture assay showed that FSC^{low}/SSC^{low} SP cells were highly enriched for primitive cells. FISH analyses showed that cytogenetically abnormal colonies originated from sorted marker positive cells, while the cytogenetically normal colonies originated from sorted marker negative cells. In conclusion, AML SP cells could be discriminated from normal SP cells at diagnosis based on expression of CLL-1 and lineage markers. This reveals the presence of a low frequency (median 0.0016 %) SP subfraction as a likely candidate to be enriched for leukemia stem cells.

Introduction

Acute myeloid leukemia (AML) is generally regarded as a disease likely to originate from the hematopoietic stem cell (HSC) [1]. There is an increasing body of evidence pointing towards the importance of the leukemic stem cell (LSC) for the occurrence of minimal residual disease (MRD) and relapse. This might well be explained by properties LSCs share with normal stem cells, such as relative quiescence and resistance to apoptosis. Accordingly, we have previously described that a high stem cell frequency in AML predicts MRD cell frequencies after chemotherapeutic treatment, resulting in poor prognosis [2]. In order to both determine the number of remaining LSCs after therapy for prognostic purposes, as well as to apply targeted therapy, immunophenotypic molecular and functional characterization of LSCs is of utmost importance. However, there is a need for new markers, preferably on the cell surface, to discriminate between normal $CD34^{+}CD38^{-}$ cells and malignant $CD34^{+}CD38^{-}$ cells, as there is considerable overlap in expression of currently available markers. Recently, we found that C-type lectin-like molecule-1 (CLL-1) and leukemia-associated lineage markers provide the opportunity to discriminate between HSC and LSC, as they were found to be solely expressed on leukemic $CD34^{+}CD38^{-}$ cells [3, 4] . In addition, new definitions of LSCs should be generated, as not all AML cases have one or more of the aberrant markers present and moreover, not all LSCs may have the $CD34^{+}CD38^{-}$ immunophenotype, e.g. in true CD34-negative AML. Moreover, the real number of stem cells is likely to be much lower than present in the so-called stem cell compartment [1, 5], suggesting that the definition of the stem cell compartment should be refined. An alternative stem cell compartment may be the so-called side population (SP). SP cells are defined by their ability to efficiently efflux Hoechst 33342 dye. In NBM, the SP was indeed found to be enriched for stem cells [6, 7]. Accordingly in AML, the SP compartment is able to initiate leukemia in NOD/SCID mice, whereas the non-SP (NSP) compartment is not [8]. It is tempting to speculate that the frequency of real LSC within the SP compartment is higher than within the $CD34^{+}CD38^{-}$ compartment as the SP frequency is much lower than that of the $CD34^{+}CD38^{-}$ cells [8]. Since the SP compartment has previously been shown by FISH analysis to contain both malignant and normal cells [9], we sought for characteristics /markers with the ability to discriminate between these malignant and normal SP cells. These would allow the primitive AML SP cells to be traced at diagnosis and during/after treatment. Moreover, both normal and AML SP cells could then be studied separately for therapeutic target

finding. This would provide functional and molecular biological differences between AML and normal stem cells under the most clinically relevant conditions: both types of stem cells present in the same bone marrow. Lastly, it would enable the definition of AML SP stem cells to be finetuned.

Materials & methods

Leukemic and normal bone marrow cells

BM samples were collected at diagnosis after informed consent from 48 AML patients (20 females, 28 males) with a median age of 49 years (range 19-75). In four cases, BM was not available at diagnosis and peripheral blood was used. NBM was obtained after informed consent from patients undergoing cardiac surgery. The majority of the samples were analyzed immediately. Mononuclear cells were isolated by Ficoll gradient (1.077 g/ml, Amersham Biosciences, Freiburg, Germany). Red blood cells were lysed afterwards by 10 minutes incubation on ice, using 10 ml of a solution containing 155 mM NH_4Cl, 10mM $KHCO_3$, and 0.1 mM Na_2EDTA pH 7.4 added directly to the cell pellet. After washing, cells were frozen in RPMI (Gibco, Paisley, UK) with 20% heat-inactivated fetal bovine serum (FBS, Greiner, Alphen a/d Rijn, The Netherlands) and 10% dimethylsulfoxide (Riedel-de Haen, Seelze, Germany) in isopropanol-filled containers 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 enabled to recover for 45 min in the same medium at 37°C. Cells were washed again and re-suspended in PBS with 0.1% bovine serum albumin (BSA, ICN Biomedicals, Aurora, OH, USA).

Flow cytometry and cell sorting

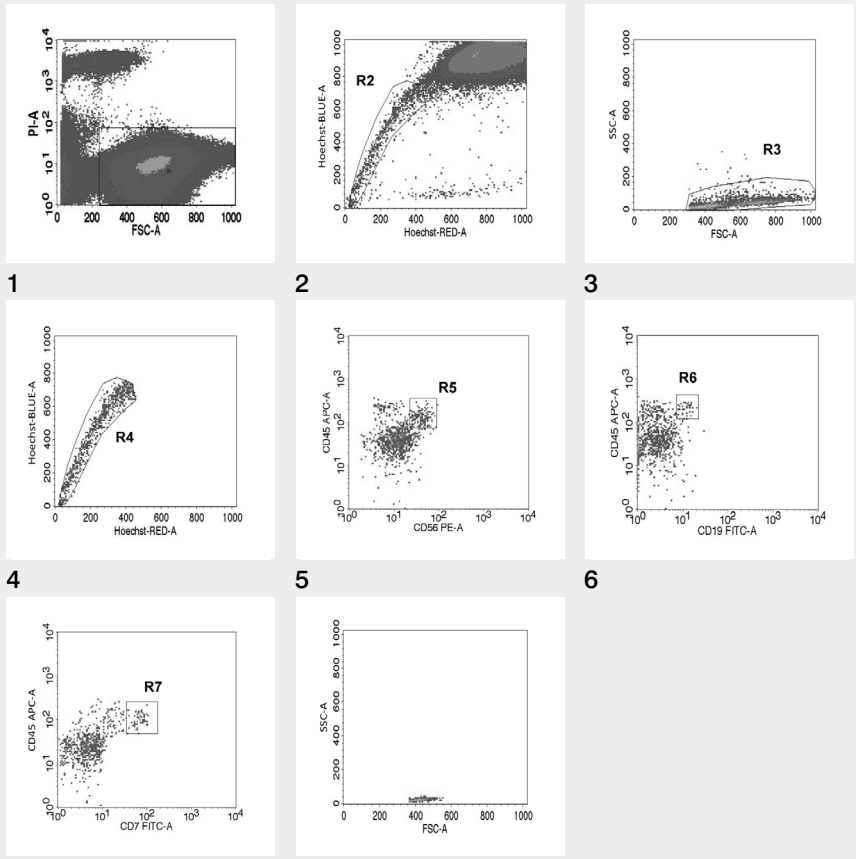
Primary AML cells ($1x10^6$/ml) were stained with 5 μg/ml of Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA) with or without BCRP inhibitor KO143 (200 nM, Sigma-Aldrich Chemie, Steinheim, Germany) and incubated at 37°C for 2 hours according to Goodell et al. [6]. After Hoechst staining, cells were washed and re-suspended into 100 μl of cold (4°C) Hanks' balanced salt solution (HBSS, Cambrex Bio Science, Belgium) + 2% fetal calf serum (FCS) and incubated for 30 minutes on ice with combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) labelled monoclonal antibody (MoAbs). Anti-CD45 APC, Anti-CD45 PE, anti-CD38 APC, anti-CD34 FITC, anti-CD7 PE, anti-CD19 PE,

anti-CD56 PE, anti-CD48 PE [10] and anti-CD123 PE MoAbs were all from Becton Dickinson Biosciences (BD Biosciences, San Jose, CA, USA). In order to define the leukemic stem cells, we used CD7, CD19 and CD56, which are frequently used in AML MRD detection using leukemia associated phenotype (LAP); anti-CLL-1 and isotype control were used as previously described [4, 11]. After antibody staining, cells were washed with cold HBSS+ (HBSS + 2% FCS), re-suspended in one ml cold HBSS+ and stained for 5 minutes with 2 μg/ml propidium iodide (PI; Sigma St. Louis, MO, USA) enabling exclusion of dead cells. Cells were kept on ice until FACS analysis. Data acquisition was performed using either a FACS Vantage (equipped with red, blue and ultra violet lasers) or a FACSCanto II (with red, blue and violet solid-state lasers), both from BD Biosciences; analysis was performed using Cell Quest and FACSDiva software (BD Biosciences). The Hoechst dye was excited with 350 nm UV (FACS Vantage) or 405 nm violet (FACSCanto II) laser and detected with 450/BP20 and 450/BP50 optical filters, respectively. Gates were set to detect the viable SP cells as shown in figure 1A. 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%.

Suspension culture of AML SP cells

The suspension culture was performed essentially as has been previously described [12]. The sorted SP (sub)populations (3,000-5,000 cells) were mixed with $1x10^5$ NSP cells and re-suspended in 250 μl/well CellGro medium (Cellgenix, Vancouver, Canada) containing 20 ng/ml IL-3, 100 ng/ml Flt-3 ligand and 100 ng/ml SCF (all from Pepro Tech, Basel, Switserland) prior to plating in 96-well round-bottom plates (Greiner, Frickenhausen, Germany). Additionally, $1x10^5$ and $1x10^6$ non-SP (NSP) cells were plated as control for the mixed SP+NSP population. Suspension cultures were incubated at 37°C in 5% CO_2 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, i.e. 5 weeks. Subsequently, all harvested cells were cultured in a 14 day colony forming unit (CFU) assay.

Figure 1A SP cell immunophenotyping: Gating strategy and SP subpopulations



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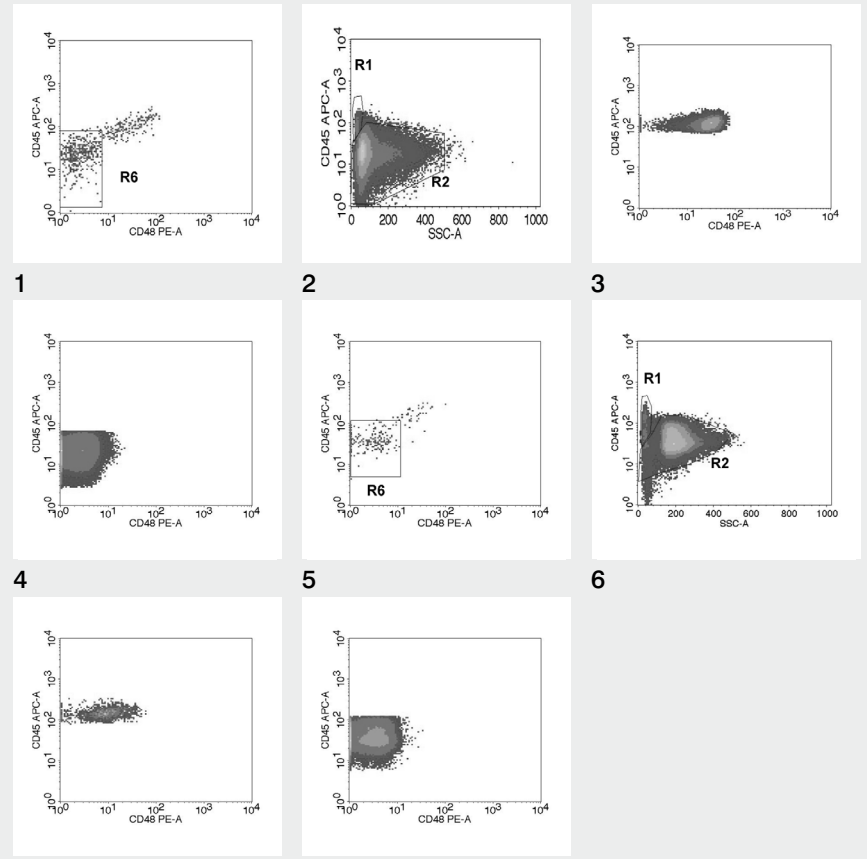
Cells were stained for SP, CD45, lineage markers (CD7, CD19, CD56) and CD48, as outlined in materials and methods. **A1-4**: One representative example of AML SP immunophenotyping with different marker expression patterns and populations with different FSC and SSC. **A1**: FSC vs. PI of an AML sample (R1). Gating on PI-negative cells identifies viable cells (R1). Gate R1 is used in 2. **A2**: Hoechst red vs. Hoechst blue identifies the viable side population cells (R2). Gates R1+R2 are used in 3. **A3**: FSC/SSC is used to select a cluster or clusters of cells (R3). Non specific events are excluded here. Note the presence of two clusters differing in SSC but especially in FSC (1A3). The large cluster is located in the whole blast region (not shown). Gates R1+R2+R3 are used in 4. **A4**: This shows the final population of viable SP cells containing as few as possible non specific events (R4). **A5-8**: Representative example of lymphoid SP subpopulations. Three small lymphoid subpopulations were detected within SP cells. **A5**: Gate R5 shows CD45^{high}/CD56+ SP cells. **A6**: Gate R6 shows CD45^{high}/CD19+ SP cells. **A7**: Gate R7 shows CD45^{high}/CD7+ SP cells. The CD45^{high} cells with low expression of CD7 are the CD56+ cells from A5. **A8**: Location of cells from gates R5+R6+R7 in FSC/SSC.

Figure 1B SP cell immunophenotyping: Gating strategy and SP subpopulations



1 2 3

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Cells were stained for SP, CD45, lineage markers (CD7, CD19, CD56) and CD48, as outlined in materials and methods.
B1-4: one representative example of CD48 expression on SP and non-SP subpopulations of an AML patient. **B1**: Gate R6 shows CD48-/CD45^{dim} myeloid SP population. Lymphocytes (CD45^{high}) are CD48+. **B2**: CD45^{high} Lymphocytes (R1) and CD45^{dim} myeloid cells (R2) from non-SP population are selected and used in 3 and 4. **B3**: Lymphocytes (CD45^{high}) from non-SP population are essentially CD48 positive. **B4**: Myeloid cells (CD45^{dim}) from non-SP population are CD48 negative.
B5-8: one representative example of CD48 expression on SP and non-SP subpopulations of a NBM. The results were similar to B1-4, i.e. the lymphoid population is CD48 positive and the myeloid population is CD48 negative.

CFU assay

Assays for leukemic CFUs were performed by plating cells in methylcellulose medium (H4434, Stemcell Technologies, VA, CA). 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 expressed as number of colonies per million input cells, i.e. cells immediately following sorting at the start of the (5+2 weeks) experiment.

FISH analysis of FACS sorted SP cells

For interphase FISH, the FACS sorted SP populations were washed three times with 3 ml of 3:1 methanol/acetic acid fixative and suspended in 100 μl fixative. Subsequently, one droplet was gently placed onto an object slide and air-dried. Dual-color (spectrum green and spectrum orange fluorophores) labelled LSI DNA probes (Vysis Inc, Downers Grove, IL, USA) were applied to the denaturated cells and incubated as previously described [13]. 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 GmbH, Jena, Germany) 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 and orange spot were less than one spot diameter apart. Nuclei were scored positive for the deletion 12, when one green spot was absent. The images were captured with a digital camera using CytoVision 4.1 software (Applied Imaging Corporation, Newcastle, UK).

Statistical analysis

Statistical analysis was performed using SPSS 9.0 software package (SPSS, Chicago, IL, USA). The Wilcoxon signed-rank test was used to determine differences between paired samples. Statistical significance was evaluated at $p<0.05$. Average values were expressed as mean ± standard error of the mean (SEM).

Results

In order to distinguish AML SP cells from normal SP cells at diagnosis, we attempted to find leukemia stem cell associated immunophenotypic markers, i.e. those not staining the normal SP stem cells. We used CLL-1 and IL-3 receptor α-chain CD123, previously reported to be leukemic stem cell markers [4, 14] and the lineage markers CD7, CD19 and CD56, used to define blast cell aberrancies suitable for immunophenotypic MRD detection [15] and staining of AML $CD34^+CD38^-$ LSC [3].

Relationship between CD34 and CD38 expression and the SP phenotype

To define the immunophenotype of AML SP cells in relation to NBM SP cells, 44 bone marrow samples and four PB samples of AML diagnosis patients were investigated. SP cells were detectable in 41/48 AML patients (85%) with a median frequency of 0.07% (expressed as % of whole blast cells, range 0.002%-7.6%). In all individual cases with both $CD34^+CD38^-$ and SP stem cell compartments present (n=36), the $CD34^+CD38^-$ compartment had a higher frequency than the SP in this subset of samples: 0.47% (range 0.01%-26.6%) versus 0.03% (range 0.002-7.6, p=0.002). The median frequency of $CD34^+CD38^-$ cells within the SP compartment was 2.5% (range 0%-49%). SP cells were detected in all 12 NBM samples with a median frequency of 0.12 % (range 0.008%-4.1%).

The SP compartment in AML contains normal lymphocytic cells

During the immunophenotyping of AML SP cells we detected a small SP population with a low forward scatter (FSC) and a slightly lower sideward scatter (SSC); one representative example is shown in figure 1A3. Part of these cells showed expression of the markers CD7 or CD19 or CD56 expression, a characteristic shared by blast cells in part of AML cases [15]. However, as FSC/SSC was lower than for blast cells and similar to normal lymphocytes, further analysis was performed using antibodies against CD45, which enables discrimination between different types of WBC, and CD48 [10], a GPI-anchored protein which is expressed on mature lymphocytes. Figures 1A5-8 identifies three small lymphoid sub-populations (all CD34 negative and CD33 negative) within the SP compartment, which also had low FSC: 1. NK-like cells with $CD45^{high}/CD56^+/CD7^{low}$ expression, with a median frequency of 4% (% of whole SP compartment, range 2-8%, n=8, example in figure 1A5); 2. B-lymphocytes with $CD45^{high}/CD19^+$ phenotype, and a

median frequency of 2% (range 0-6%, n=8, example in figure 1A6) and 3. T-lymphocytes with CD45^{high}/CD56^{-}/CD7^{high} expression, with a median frequency of 7% (range 2-14%, n=8, example in figure 1A7). The latter resembled the previously described CD34-CD7+ SP cells [16]. All three CD45^{high} populations cluster together in the low FSC area (figure 1A8; compare with figure 1A3). Similar to the AML samples, NBM samples also showed these three lymphoid SP subpopulations: NK-like, B- and T- cells with a median frequency of 5%, 2% and 8%, respectively (n=3). Therefore, it is highly likely that the AML SP compartment contains a subset of normal lymphocyte cells. In the malignant samples all three lymphoid SP subpopulations (CD45^{high}, FSC^{low}) were CD48 positive (figure 1B1). In contrast, myeloid SP cells (CD45^{dim}) were CD48 negative (figure 1B1, R6). For comparison, the CD45^{high} lymphoid cells within the non-SP population (figure 1B2, R2) were also CD48 positive (figure 1B3) while the myeloid cells were not (figure 1B4). The NBM samples showed similar results (figures 1B5-8). Thus, staining with CD48 enables the non-myeloid cells to be excluded from further analyses of stem cell activity in the myeloid SP sub-compartment.

Leukemic cells with aberrant marker expression are present in the SP compartment

Marker expression on the CD45dim AML SP cells was determined after exclusion of the CD45^{high} lymphocytic cells. In 39/41 cases with SP present, the SP cells were partly or completely positive (defined as ≥10% expression) for CLL-1 (in all 41 samples: median 53%, range 2-100%) and in 27/41 cases partly or completely positive for CD123 (median 30%, range 0-100%; n=41). In 25/41 cases SP cells were partly or completely positive for either one or more of the three lineage markers, previously shown to mark AML CD34^{+}CD38^{-} stem cells [3], i.e. CD7, CD19 and CD56. Median expression for CD7 was 35% (range 18-80%; n=13), for CD19 55% (range 20-95%; n=5) and for CD56 50% (range 25-100%; n=17). As CLL-1 and aberrant lineage marker expression have never been found on normal CD34^{+}CD38^{-} previously by our laboratory [3, 4], this strongly suggest that malignant myeloid cells in the SP cell compartment in AML at diagnosis can be identified using aberrant expression of markers. This was confirmed by comparison with surface marker expression on SP cells in 12 normal bone marrow samples. These SP cells were negative for CLL-1 (median 0%, range 0-4%), CD7 (median 0%, range 0-3%), CD19 (median 0%, range 0-3%) and CD56 (median 0%, range 0-4%), although not for CD123 (median 27%, range 1-82%). Therefore, both CLL-1 and lineage markers, but not CD123, are suitable to discriminate between

malignant and normal SP stem cells at diagnosis. FISH analysis for three t(8;21) and one del(12) AML patients (in table 1: patients 2, 10, 41 and 22, respectively) showed that the majority of cells were indeed malignant (88%, 76%, 78% and 80%, respectively). However, both marker patterns and FISH analyses indicate that, even after correction for the lymphoid compartment (not shown), part of the myeloid SP cells should still be of normal origin.

Table 1 Marker expression on myeloid, scatter defined SP subpopulations in AML patients

	As % of LSSC and HSSC SP							
	CLL-1		CD7		CD19		CD56	
Patient	LSSC	HSSC	LSSC	HSSC	LSSC	HSSC	LSSC	HSSC
1	46	78	-*	-	-	-	-	-
2	25	#	-	#	95	#	85	#
3	72	80	-	-	-	-	92	90
4	43	89	-	-	-	-	-	-
5	57	75	-	-	-	-	60	58
6	8	54	36	24	-	-	26	43
7	20	93	78	37	-	-	-	-
8	100	100	-	-	-	-	100	100
9	57	87	-	-	11	5	-	-
10	22	62	26	3	16	6	22	65
11	6	48	57	23	-	-	-	-
12	46	#	-	#	-	#	39	#
13	34	86	37	2	-	-	21	75
14	50	86	41	5	-	-	-	-
15	20	60	-	-	-	-	28	70
16	33	75	-	-	52	33	-	-
17	18	45	-	-	-	-	-	-
18	10	84	-	-	-	-	-	-
19	77	100	50	42	-	-	-	-
20	3	20	-	-	-	-	-	-
21	53	63	29	7	-	-	-	-
22	2	15	-	-	-	-	43	21

Table 1 Continued

	As % of LSSC and HSSC SP							
	CLL-1		CD7		CD19		CD56	
Patient	LSSC	HSSC	LSSC	HSSC	LSSC	HSSC	LSSC	HSSC
23	65	77	-	-	-	-	-	-
24	62	83	-	-	-	-	-	-
25	48	83	-	-	-	-	-	-
26	10	75	35	4	-	-	-	-
27	15	78	21	11	-	-	-	-
28	78	87	-	-	-	-	-	-
29	50	99	-	-	-	-	-	-
30	20	72	25	5	-	-	-	-
31	34	95	40	0	-	-	-	-
32	1	0	-	-	-	-	-	-
33	28	57	-	-	-	-	36	38
34	50	48	-	-	-	-	-	-
35	23	92	-	-	-	-	-	49
36	86	99	-	-	-	-	-	-
37	34	54	-	-	-	-	-	-
38	20	65	-	-	-	-	54	29
39	63	92	45	20	-	-	-	-
40	66	92	-	-	60	29	-	-
41	30	99	-	-	56	22	19	35
Median	34 (1-100) † ‡	78 (0-100) †	37 (21-78) † ‡	7 (2-42) †	54 (11-95) † ‡	22 (5-33) †	39 (19-100) † ‡	53 (21-100) †
NBM	CLL-1		CD7		CD19		CD56	
	LSSC	HSSC	LSSC	HSSC	LSSC	HSSC	LSSC	HSSC
Median	0 (0-0)	0 (0-4)	0 (0-3)	0 (0-0)	0 (0-3)	0 (0-0)	0 (0-0)	0 (0-4)

-*: Not measured
#: HSSC population not present
NBM: normal bone marrow (n=12, for all markers).
† Differences in intra-patient marker expression between HSSC and LSSC (HSSC>LSSC for CLL-1 and CD56; HSSC<LSSC for CD7 and CD19) were significant for CLL-1 (p=0.0001), CD7 (p=0.001) and CD19 (p=0.04); but not for CD56.
‡ No significant differences in inter-patient marker expression on LSSC cells between CLL-1, CD7, CD19 and CD56.

The myeloid SP compartment is heterogeneous in scatter properties and CD34 and CD38 expression

On closer inspection, in 39/41 samples heterogeneity for sideward scatter was observed in the myeloid SP compartment (example in figure 2A1), showing two different subpopulations: 1. High sideward scatter (HSSC) cells with a median frequency of 56 % (% of whole SP compartment, range 4%-91%); 2. Low sideward scatter (LSSC) myeloid SP cells with a median frequency of 44 % (% of whole SP compartment, range 9%-96%). In 2/41 cases only the LSSC SP cells presented as a separate population (example previously shown in figure 1A3).

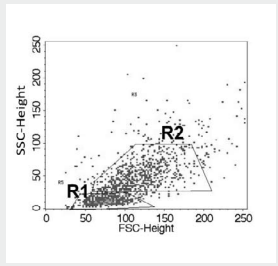
Backgating of HSSC and LSSC cells in Hoechst bivariate plots showed their presence throughout the SP population with no significant difference in Hoechst staining between LSSC and HSSC SP cells (figures 2B1-3). HSSC and LSSC populations were also seen in 9/12 NBM SP cells (one representative example is shown in figure 2C1), with only LSSC SP present in the remaining 3 cases (not shown).

HSSC AML SP cells had high CD38 expression with a median frequency of 84% (range 0%-100%, n=39, example in figure 2A4). LSSC SP cells had significantly (p=0.04) lower CD38 expression (example figure 2A4) with a median frequency of 43% (range 1-100%, n=41, p=0.04). The median CD34 expression on HSSC and LSSC SP cells was 19% (range 0-99%) and 41% (range 1-99%), respectively. Apparently, compared to HSSC cells, LSSC cells have characteristics indicative of their primitive nature: higher CD34 expression, lower CD38 expression and lower SSC.

Combining scatter properties and marker expression defines the presence of at least three different myeloid SP subpopulations

Subsequently, aberrant marker expression was studied separately in HSSC and LSSC (data provided per patient in table 1). CD123 was not included since, as reported earlier, it is expressed on normal bone marrow SP cells as well. HSSC showed heterogeneous expression patterns for the markers CD7, CD19, CD56 and CLL-1 (e.g. patient 10: 62% CLL-1$^+$, 3% CD7$^+$, 6% CD19$^+$, and 65% CD56$^+$). However, the HSSC population usually showed homogeneous expression (either high or low or absent) for any of these markers. In contrast, within the LSSC SP cells in general, two clearly discernable myeloid subpopulations could be identified: one negative for aberrant markers and the other with aberrant markers present. As a particular example, figure 2A2 shows CD56 expression on the majority of HSSC SP cells, but only on part of the LSSC fraction. In general, both

Figure 2A SP subpopulations in AML



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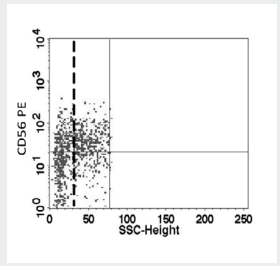

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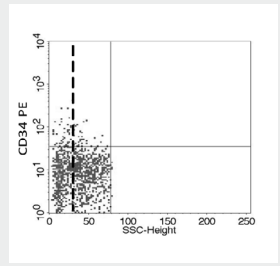

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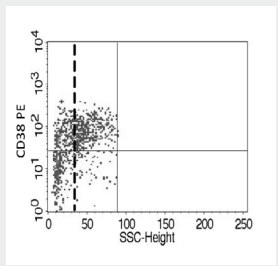

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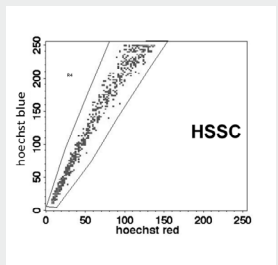
Figure 2B Hoechst staining patterns of HSSC and LSSC AML SP subpopulations



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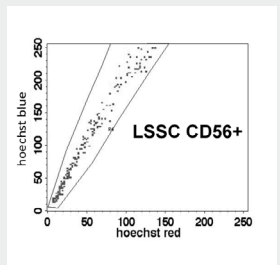

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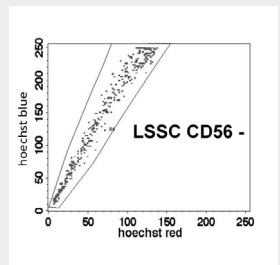

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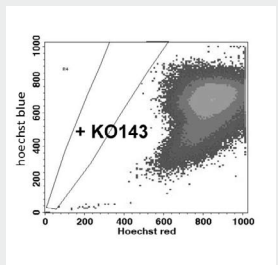
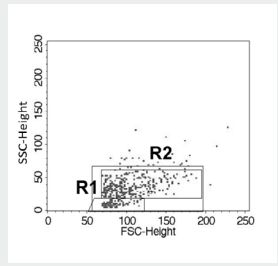


4

Figure 2C SP subpopulations in NBM



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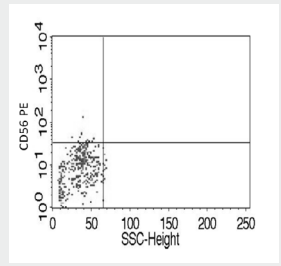

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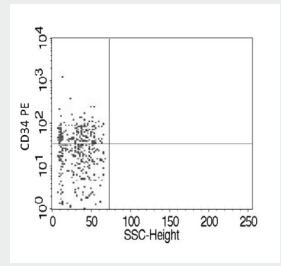

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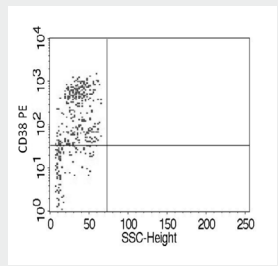


4

Cells were stained with Hoechst 33342 and with antibodies for expression CD34, CD38 and lineage marker CD56, as outlined in materials and methods.

A1-4: One representative example of two SP subpopulations with different sideward scatter: Low sideward scatter (R4: LSSC) and high sideward scatter (R2: HSSC). Dashed line separates HSSC and LSSC. In this case the HSSC population was also characterized by high FSC. The LSSC population was partly $CD56^+$ while the HSSC population was largely $CD56^+$. Both the HSSC and LSSC fractions were largely CD34 negative (figure 2A3) while especially the HSSC populations was largely $CD38^+$ (figure 2A4) in contrast to the LSSC.

B1-4: Hoechst staining patterns of HSSC and LSSC SP subpopulations of the same patient shown in A. HSSC and LSSC showed no significance difference in Hoechst staining: LSSC had a MFI of 70 and 141 for Hoechst red and blue, respectively; and HSSC had a MFI of 75 and 161 for Hoechst red and blue, respectively (three other samples analyzed showed the similar result) The SP cells were ablated when BCRP1 inhibitor KO143 (200 nM) was included during the Hoechst incubation (B4).

C1-4: One representative example of a NBM with two SP populations with different sideward scatter. CD34 and CD38 expression (figure 2C3 and 2C4, respectively) are typical for NBM. As illustrated in Figure 2C2 (and summarized in table 1), CD56 is absent on both SP subpopulations (figure 2C2). Gating on LSSC and HSSC is slightly different from AML (A1), since increasing the R1 region to the size of R1 in figure 1A1 would result in R1 including 2 populations with clearly different scatter properties. In the absence of further information on other properties, this is dissuaded.

HSSC and LSSC compartments showed heterogeneous expression patterns of CLL-1 and aberrant markers with often large intra-patient differences in expression between both compartments (see e.g. patient 6, 10, 11, 15, 27, 35, 38 and 41 in table 1). Overall, compared to LSSC, marker expression was higher on HSSC for CLL-1 and CD56, but lower for CD7 and CD19. No specific localization of the marker positive or marker negative subpopulation was seen in the Hoechst plot (figure 2B1-3).

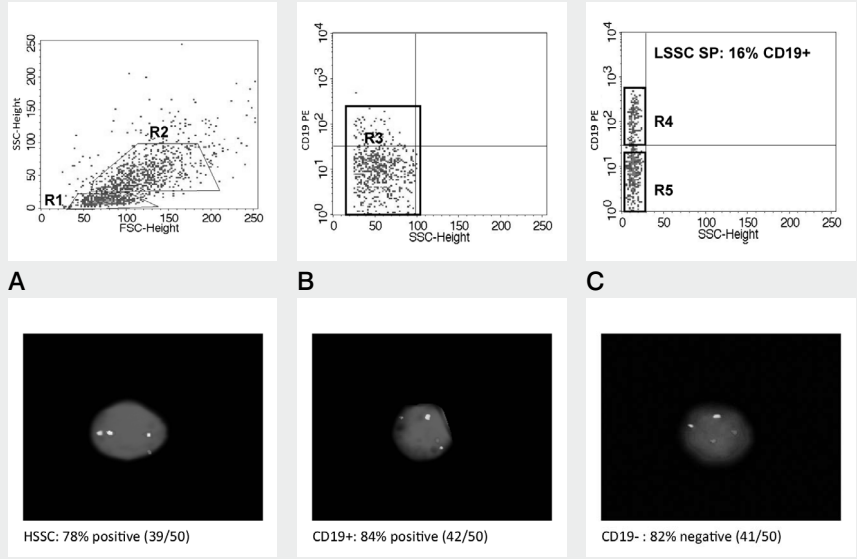
In order to confirm the putative malignant characteristics of the three identified myeloid SP sub-populations, we sorted these for two cases and performed a FISH analysis. Both in patient 10 (figure 3) and patient 41 (not shown), sorted CD19 positive LSSC SP cells were largely positive for t(8;21), while sorted CD19 negative LSSC SP cells were largely FISH negative (82% and 86%, respectively). However, the HSSC SP cells, although t(8:21) positive, were largely CD19 negative, thereby explaining the observed discrepancies between marker expression and FISH analysis when studying the whole myeloid SP compartment. In these particular cases, the use of other markers (CLL-1 and CD56 for patient 10, CLL-1 for patient 41) confirmed the malignancy of these CD19 negative cells. The discrepancies either between FISH analysis and marker expression and/or between expression of two or more different markers used is mainly caused by the contribution of HSSC, as there is no statistically significant difference between expression of CLL-1, CD7, CD19 and CD56 on the LSCC SP (table 1 summary in the row just below patient 41).

Apart from difference in SSC, FSC was also lower in 36 patients in the LSSC population (example in figure 3). Both the differences in SSC, FSC, CD34 and CD38 expression strongly suggest that the LSSC population is likely to be more primitive than the HSSC population and contains both marker positive AML stem cells and marker negative normal stem cells. Clonogenic assays were performed in order to support this hypothesis.

The LSSC SP subfraction is enriched with primitive cells

Both LSSC ($CD38^{low}$: range of expression 22%-35%) and HSSC ($CD38^{high}$: range of expression 62%-85%) SP cells for seven AML patients (five CD34 positive: patients 9, 10, 22, 35, 41 and two CD34 negative: patients 37 and 40) 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. The HSSC SP cells had a very low clonogenic capacity (median of 400 per million input cells, range 250-650), whilst the LSSC SP cells had a very high

Figure 3 FISH analysis of HSSC and LSSC SP cells in a t(8;21) AML patient



A: FSC/SSC plot of the SP cells from a t(8;21) AML patient (patient 10) with two subpopulations (HSSC and LSSC). Figure 3A is the same as figure 2A.
B: HSSC SP cells (largely CD19 negative, R3) were sorted and investigated by FISH analysis in D.
C: $CD19^{+}$ cells (R4) and $CD19^{-}$ cells (R5) present within the LSSC SP cells were sorted and investigated by FISH analysis in E and F, respectively.
D-F: between brackets are the numbers of cells/percentage positivity scored for the different SP subfractions.

clonogenic capacity (median of 19,375 per million cells, range 7,253-24,645; figure 4). The results show that LSSC SP cells with the low CD38 expression have a more primitive character in functional assay.

To further discriminate within the LSSC population between the clonogenic ability of presumed normal and AML cells, both marker positive and marker negative SP LSSC cells from patient 37 (CD34 negative) and patient 41 (CD34 positive) were used in the functional assay. All populations formed high numbers of colonies: for patient 37 the data were 14,645 colonies/10^6 for marker positive and 8,010 colonies/10^6 for marker negative populations. For patient 41 these figures were 6,460 colonies/10^6 and 5,270 colonies/10^6, respectively (not shown). FISH analysis

performed on week 5 of the colonies originating from marker positive or marker negative LSSC cells of two patients (patients 22 and 41 with del(12) and t(8;21), respectively), showed that the majority of the colonies originating from marker positive LSSC cells were indeed malignant (80% and 78%, respectively; counted on 45 and 50 cells, respectively). The colonies originating from marker negative LSSC cells of both patients were predominantly of normal origin (both 86% FISH negative; counted on 35 and 38 cells, respectively). No colonies were formed in the controls starting either with 100,000 or a million NSP cells (not shown).

To control for the possible negative effect of Hoechst exposure on the function of NSP population, sorted SP populations for four patients (patient 22, 37, 40 and 41) were re-incubated with Hoechst (2 hours at 37°C) in the absence or presence of the inhibitor and subsequently put into CFU-assay. After 14 days both populations showed similar numbers of colonies for all four patients: a median of 52 (range 40-58) and 47 (range 37-51) colonies per million in the absence or presence of the inhibitor, respectively. These results show that increasing Hoechst binding in SP cells by allowing these to become non-SP cells had no impact on the clonogenic capacity at concentrations used for this studies.

Taken together, the median frequency of total SP cells was 0.03 % (n=25), when using the markers with highest expression, whereas the frequency of marker positive LSSC SP cells was only 0.0016 % of WBC (range 0.0002-0.0056%

Discussion

AML is regarded to originate, at least in some cases, in the hematopoietic stem cell compartment [1]. The lack of durable response in a high percentage of AML patients suggests that current treatments do not effectively target LSCs. Therefore, LSCs have to be identified in order to prove their persistence after current treatments, whilst their characterization might pave the way to identify new therapeutic targets.

In CD34-positive AML the stem cell has been recognized as CD38 negative [3]. However, not all stem cells can be defined as $CD34^+CD38^-$. At diagnosis, 5% of AML patients with a $CD34^+$ phenotype lack a clearly detectable $CD34^+CD38^-$ compartment (< 0.01%) and about 20% are CD34 negative and thereby, by definition, $CD34^+CD38^-$ negative [2]. An alternative stem cell compartment for these cases is offered by the side population. This population is highly enriched for HSCs [6] in NBM. SP cells are also present in bone marrow of AML patients,

and are capable of initiating leukemia after transplantation into NOD/SCID mice, suggesting that these cells contain candidate LSCs [8].

In the present study we reported that the frequency of SP cells is far lower (factor about 16) than that of $CD34^+CD38^-$ cells. However, using flow cytometry, we have found that the SP fraction was still highly heterogeneous and contained different subpopulations: 1. Three small normal lymphoid subpopulations (T-, B- and NK-like cells) with low forward scatter and side scatter (FSC/SSC) properties, high CD45 expression and CD48 expression; 2. A differentiated (high FSC/SSC, high CD38, low CD34) myeloid population with or without aberrant leukemic marker expression; 3. A primitive low-frequency myeloid fraction (low FSC/SSC, low CD38, high CD34), negative for aberrant leukemic markers and likely enriched for primitive normal cells; 4. A primitive low-frequency myeloid fraction (low FSC/SSC, low CD38, high CD34) with aberrant leukemic markers present and likely enriched for primitive AML stem cells. NBM showed the first three populations, however these were always with marker expression absent in population 2. These results strongly suggest that the majority of SP cells are malignant, which was indeed confirmed by FISH analysis in four of our patients and has also been shown by others [8, 9]. Suspension culture followed by CFU assays showed that low FSC and SSC SP cells with low CD38 expression have a more primitive character in both CD34 positive and CD34 negative AML patients.

Heterogeneity in terms of malignancy of SP cells has been previously described [9], suggesting that normal stem cells are present and probably constitute the $CD34^+CD38^-$ SP compartment. Although the relationship between the SP compartment and the $CD34^+CD38^-$ compartment was not the focus of the present paper, we were able to confirm that observation, although only in CD34 negative AML, as defined by CD34 expression lower than 1% [13]. In contrast, in CD34 positive AML, the $CD34^+CD38^-$ population consisted of both a malignant and a normal compartment (unpublished data).

Identification of malignant stem cell subpopulations among normal stem cells has thus become possible using aberrant marker expression. Both the earlier defined CLL-1 antigen [4] and lineage markers [3] were now found to characterize the AML SP compartment. There were, however, large differences between patients in expression of the markers used. Moreover, even for a given patient, the expression pattern of the markers showed large differences on many occasions. Evidently, one or more particular markers may not cover the whole AML part of the SP compartment, which in turn may well result in under-estimation of the AML component. However, this problem may be circumvented due to the following

peculiar characteristics: whereas CLL-1 expression was significantly higher in the HSSC fraction, CD7 and CD19 were higher in the LSSC fraction (table 1). As a result, even in the absence of CD7 and/CD19 expression, the HSSC population may still be malignant as illustrated for patient 10 in figure 3 and table 1. Most importantly, the median expression of the four markers did not differ significantly in the most relevant compartment i.e., the LSSC myeloid compartment. On the other hand, there were still differences in marker expression in the LSSC fraction within individual patients: e.g. in patient 6 (lower CLL-1 expression compared to CD7 and CD56) or patient 39 (higher CLL-1 expression compared to CD7). Careful examination of such cases shows that this may reflect an old dilemma in flow cytometry with regard to the definition of positivity: a small shift of a whole population is likely to indicate that all cells are positive, but with low intensity of expression. However, using expression as the percentage positivity compared to isotype controls, results in percentages far lower than 100%. It is therefore quite likely that if there is good congruence between the markers used, the marker of choice should then be the one which allows best discrimination between putative normal and putative AML cells.

When the marker with the best discriminatory power on LSSC SP cells was used for each individual case of AML, the range of AML LSSC SP cells found was 0.0002%-0.0056% of WBC (median 0.0016). This is now closer to the real AML stem cell frequency, which may be as low as 0.0001%, as estimated using limiting dilution experiments [1, 17]. The future challenge would be to study the interrelationship between the $CD34^{+}CD38^{-}$ and SP stem cell compartment in CD34 positive AML and to identify the AML stem cell compartment in truly CD34 negative AML.

Interestingly, we could distinguish between the lymphoid and myeloid compartments within the SP and non-SP populations by using CD48. As the lymphocytes have low FSC and SSC and may thereby interfere with a correct analysis of malignant, and especially normal LSSC SP cells, it is important to be able to distinguish these from myeloid stem cells. This would facilitate the study of stem cell compartment: CD48 fulfils this demand. CD48 is a glycosylphosphatidylinositol-anchored protein belonging to the CD2 subfamily [18]. It is a low-affinity ligand for CD2 and is implicated as an important co-stimulatory molecule in lymphocyte activation. CD48 has also been used in other studies together with other SLAM family markers CD150 and CD244 to identify stem cells and progenitors in mice [19]. In addition to hematopoietic malignancies, SP cells have been identified in various tumors [20]. It will be interesting to establish whether the SP compartment in these tumors has heterogeneity similar to that detected in this study for AML.

Such information might be a valuable tool to study and target these cells. In this respect we have already been able to identify LSSC and HSSC SP cells in glioblastoma and CML SP cells (unpublished data).

Similar to the characterization of the leukemic blasts by using aberrant lineage markers [15, 21], the identification of immunophenotypical characteristics specific for the malignant primitive SP cells at diagnosis would offer opportunities to study primitive AML SP cells under conditions of minimal residual disease after chemotherapy. This might not only identify patients at risk for relapse, thereby improving the clinical significance of MRD cell studies [14], but also enable characterization of cells that probably represent the most relevant target cell population to design new therapies [3, 4]. This very low frequency SP sub-population is a likely candidate to be enriched for leukemia-initiating cells, although the leukemia initiating ability of the identified primitive SP compartment will need proof in animal models.

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